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FACULTAD DE VETERINARIA
Departamento de Sanidad Animal

TESIS DOCTORAL

Antileishmanial activity of Allicin: mechanism of action, "in vivo" efficacy and value in combined therapy with Amphotericin B

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PRESENTADA POR

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Director

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DEPARTAMENTO DE SANIDAD ANIMAL

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Dña. María Jesús Corral Caridad

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José María Alunda Rodríguez, Doctor en Biología y Catedrático de Parasitología y Enfermedades Parasitarias, perteneciente al Departamento de Sanidad Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid,

CERTIFICA:

Que la memoria presentada por la Licenciada en Veterinaria y Máster Universitario de Investigación en Ciencias Veterinarias, Dña. María Jesús Corral Caridad, titulada:

> "Antileishmanial activity of Allicin: mechanism of action, *in vivo* efficacy and value in combined therapy with Amphotericin B"

para optar al grado de Doctor ha sido realizada bajo mi dirección en las instalaciones del Departamento de Sanidad Animal de la Universidad Complutense. El trabajo presentado contiene mejoras metodológicas de interés en el estudio de moléculas con potencial leishmanicida/leishmaniostático, actividad de alicina y su combinación con Anfotericina B (AmB) sobre promastigotes y amastigotes intracelulares de *Leishmania*, exploración del mecanismo de acción de alicina, y un estudio de la eficacia de esta molécula, sola y en combinación con AmB, en el control de la leishmaniosis experimental en criceto. La extensión del trabajo, el conocimiento de la temática objeto del estudio, los resultados obtenidos y la discusión de los mismos por parte de Dña. María Jesús Corral Caridad cumplen satisfactoriamente los requisitos para optar a dicho Grado.

Lo que firmo en Madrid a 20 de noviembre de dos mil catorce

Dr. J. Mª Alunda Rodríguez

A mi madre

Cierro los ojos y te veo. Ojalá, desde donde estés, puedas verme tú también y te sientas orgulloso de mi, Papá.

Te echo de menos.
Hoy un poco más que todos los días.

“At times our own light goes out and is rekindled by a spark from another person. Each of us has cause to think with deep gratitude of those who have lighted the flame within us.”

Albert Schweitzer

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Agradecimientos

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Foreword

Foreword

Leishmaniases are vectorial parasitic infections caused by *Leishmania* species (Protista), affecting vertebrates particularly humans and dogs and present in all inhabited continents. Disease conditions are very variable ranging from self-limiting, although disfiguring, cutaneous infections to fatal visceral leishmaniasis. The human disease, in all its presentations, is a heavy burden for both patients and health systems if available. Visceral leishmaniasis, caused mainly by anthroponotic *L.donovani* and zoonotic *L.infantum* (=*L.chagasi*), is the second most lethal parasitic disease actually being responsible for over 40,000 deaths per year. The latter species is also the causative agent of canine leishmaniasis, a first order veterinary pathology in the Mediterranean basin and South America. Conservative estimations of *L.infantum* prevalence in Spain lead to more than 250,000 dogs presently infected with this species and more than probably millions of dogs are infected in Brazil. In addition to its relevance in veterinary clinics these infections are of public health concern since dogs are considered the main reservoir of human infection.

Despite the financial effort made by international and national funding agencies, campaigns by health authorities and the research work done by laboratories around the world improvement of prevalence and incidence rates of the disease, especially in those regions where the processes are more severe, has been modest. Moreover, war- and famine-related human migrations, opening of rural areas for urban development, absence of reliable national health services in many endemic countries besides climatic changes, global economic crisis and emergence of new epidemiological patterns have contributed to the actual extension of the disease. In spite of the recent Madrid outbreak, the levels of human infection in Spain are low and linked, for the most part, to inmunocompromissed patients (e.g. HIV+, solid organs transplant recipients). By its part, in the veterinary arena, no actual evidence of reduction in incidence or prevalence of the infection is presently available.

Given the difficulties of vector control and the impracticality –and probably unusefulness- of reservoirs control (i.e. dog culling) in the case of zoonotic visceral leishmaniasis caused by *L.infantum*, control relies on the use of antileishmanial drugs. Nonetheless, chemotherapeutic arsenal is very limited and the majority of the currently used drugs have serious shortcomings including toxicity at the required doses, easy selection of resistance when used in monotherapy, teratogenicity and high price of the most effective and less toxic presentations. To make matters

worse there is an increasing number of reports on *Leishmania* resistance to the first line drugs (i.e. pentavalent antimonials). Unfortunately no new affordable and short-course treatments of null or reduced toxicity for the treatment of leishmaniasis are foreseen either from the pharmaceutical industry pipelines or research laboratories.

Among the strategies followed to identify leads that, eventually, could progress along the antileishmanial drug development, namely combinatorial chemistry, lower toxicity presentations, "piggy backing" strategy –new indications for old drugs-, discovery of new chemical entities, the exploration of the potential value of molecules from or derived from natural sources has been comparatively less developed. The investigation on natural products, once the drug store, is hampered by the complexity of the mixtures and their difficulties to fit well into the majority of drug screening methods. However, current technology improvements in analysis and separation of active molecules, and bioguided development could overcome some of the inconveniencies of these natural products thus allowing the study of more complex molecular scaffolds as potential antileishmanial drugs. Unstable Diallyl thiosulfinate (=allicin) and related compounds, present in plants belonging to the family Alliaceae, have shown antiproliferative activity on transformed mammalian cells and some Protista. Availability of a stabilised preparation of this molecule prompted us to evaluate its potential antileishmanial activity, alone or in combination, *in vitro*, *ex vivo* and *in vivo* and to determine the underlying mechanism of action.

CHAPTER 1

Literature review

and

Objectives

1.1. History of the disease

Leishmaniasis is a group of parasitic vectorial chronic diseases affecting man and other vertebrates caused by Protista from the Genus *Leishmania* (Kinetoplastida, Trypanosomatidae). The disease is present in all areas where the sandfly vectors (*Phlebotomus* and *Lutzomyia*, Psychodidae) are present and clinical courses range from self-limiting to fatal unless treated infections. Wide geographical distribution, high prevalence and incidence of the infection suggest that the relationship between man and *Leishmania* was probably established very early during human evolution.

Paleopathological studies have shown that mummies, skeletons and bone marrow samples from Upper Nubia and Egypt dating back 4,000 years BC allowed the amplification of a 120-pb fragment of kinetoplastid mitochondrial DNA from *Leishmania* and showed the high prevalence of the infection (12.9% of positive examined mummies) (Zink et al., 2006). Extraordinary climatic conditions besides the expertise of the Egyptian embalmers were not present in the other ancient Middle East and Mediterranean empires. However, there are indirect references from medical texts, particularly related to the observed lesions. Insofar the first historical reference to leishmaniasis comes from a series of medical writings in cuneiform tablets from the library in Nineveh of Ashurbanipal (668 BC – c. 627 BC), the last great king of the Assyrian empire. These texts, dated around the VIIth century BC but probably corresponding to over 1500 years BC, describe cutaneous processes compatible with the disease. In the Old Testament there are also references not only to the cutaneous leishmaniasis (CL) in the Egyptian population - corresponding to the Exodus (Oumeish, 1999)- but also in the Deuteronomy, written ca. VIth BC century, using the Jewish word ʿ*afolim* – leishmaniasis (Deut. 28:27).

Not many notices on leishmaniasis from Greece and the Roman Empire are known except for the description of a transmittable cutaneous disease, reported by Pliny the Younger, common among the wealthy people and apparently brought from Asia (Retief & Cilliers, 2000). However, these lesions could be ascribed to other disease conditions. This is most surprising given the geographical extension of the Roman Empire to places where previous leishmanial infections had been reported (see above).

From Xth century onwards there are many reports on the so-called "oriental sore" in the Old World. Recently *L.infantum* infection has been diagnosed in bone samples from Eleonora di Toledo (1522-1562 AD) (Figure 1), wife of Cosimo I de Médici (Nerlich et al., 2012). Whether the infection was acquired in Italy or in Spain is not known but this finding supports that *Leishmania* infection was present in Southern Europe.

Figure 1. Portrait of Eleonora di Toledo with his son Giovanni de Médici. Painted by Agnolo Bronzino (1503-1572). Location: Uffici Gallery, Florence, Italy.

Figure 2. Peruvian pottery (huaco) showing facial mutilations thought to represent mucosal leishmaniasis. (From Lainson & Shaw, 2005).

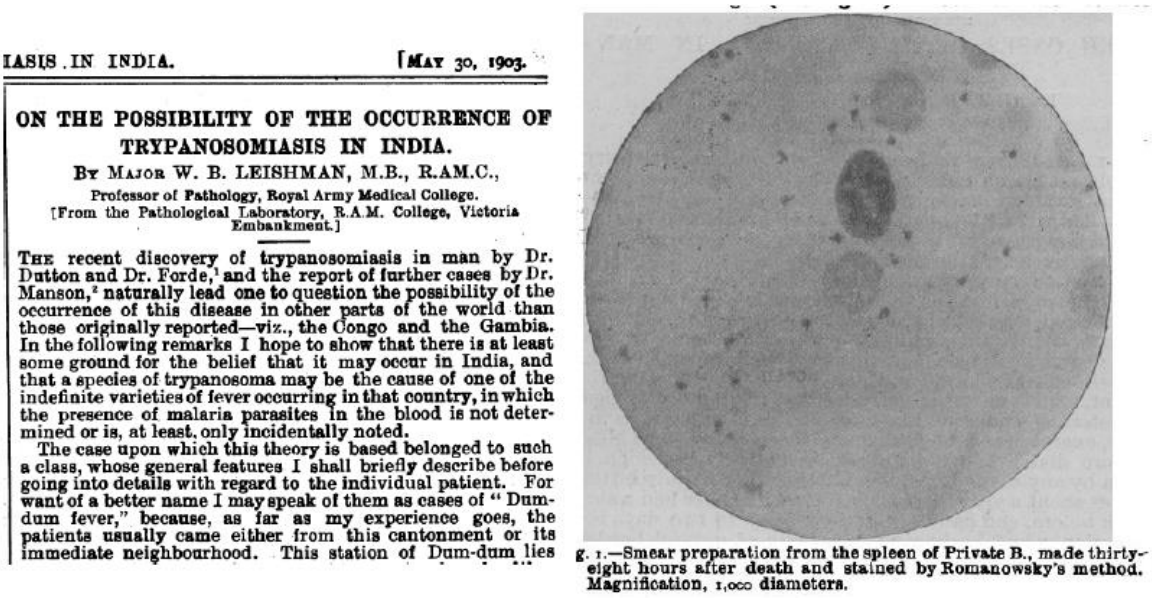
Similarly, in the Americas, facial lesions corresponding to CL have been extensively represented in pre-Inca pottery (Figure 2) (Lainson & Shaw, 2005). Texts from the 15th and 16th centuries, and then during the Spanish colonization, mention the risk run by seasonal agricultural workers who returned from the Andes with skin ulcers which, in those times were attributed to "valley sickness" or "Andean sickness" (Bari, 2006).

Medical services from the colonial armed forces, particularly British and French, recognized this "new" disease. Thus, the first clinical description by western standards of CL was done by Alexander Russell, in 1756, calling the clinical signs and lesions observed on a Turkish patient from Aleppo (presently known as Halab, Syria), "Aleppo boil" (Choi & Lerner, 2001; Cox, 2002). Using his words *"After it is cicatrized, it leaves an ugly scar, which remains through life, and for many months has a livid color. When they are not irritated, they seldom give much pain"*. Besides this name many other regional synonyms of the diseases were and still are currently used (e.g. Jericho boil, Algerian boil). By its part, in 1824, VL is described in Jessore, India, as a feverish process with splenomegaly, caquexia, anemia and skin darkening. The clinical condition received the names of "kala-azar", "black fever" or "Dum-Dum fever" among others. Before that date the disease was misdiagnosed as quinine-refractory cases of malaria (Gibson, 1983).

The first observation within macrophages of the parasite causing Old World CL is attributed to the American pathologist James Homer Wright, in 1903, after the observation of protozoa in a case of tropical ulcer (Delhi sore) (*J. Med. Res.* 10:472–482). However, it seems clear that the Scottish Surgeon Major David Douglas Cunningham (1843–1914) also observed it in a patient in Calcutta, India in 1885. Nevertheless he did not recognize the nature of the

aetiological agent and described it as of fungal origin. Shortly afterwards, in 1898, the Russian sergeant D.F. Borovsky accurately described in Tashkent, Uzbekistan (then Russia), the morphology of the causative agent including the kinetoplast and identified it as a protozoan but he did not name it (Bari, 2006).

In the case of VL, uncertainty about the relationship between the causative agent and the clinical disease continued fueled by the belief of Ronald Ross of being just an atypical presentation of malaria. Scientific communication at the end of the XIXth century and the first years of the XXth century was quite limited and more than probably these findings in CL were not known by the Scottish army doctor, William Leishman and the Professor of Physiology at Madras University, Charles Donovan. These two authors published in 1903, in the same journal (*British Med. J.*) and two months apart, the identification of a parasite in spleen samples from patients with kala-azar. In his contribution Leishman suggested the relationship of the described agent to the better known trypanosomes (Figure 3). Ronald Ross, at the end of the same year named the new agent as *Leishmania donovani* as homage to the two discoverers (Gibson, 1983).



IASIS IN INDIA. [MAY 30, 1903.

ON THE POSSIBILITY OF THE OCCURRENCE OF TRYPANOSOMIASIS IN INDIA.

BY MAJOR W. B. LEISHMAN, M.B., R.A.M.C.,

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THE recent discovery of trypanosomiasis in man by Dr. Dutton and Dr. Forde,[1] and the report of further cases by Dr. Manson,[2] naturally lead one to question the possibility of the occurrence of this disease in other parts of the world than those originally reported—viz., the Congo and the Gambia. In the following remarks I hope to show that there is at least some ground for the belief that it may occur in India, and that a species of trypanosoma may be the cause of one of the indefinite varieties of fever occurring in that country, in which the presence of malaria parasites in the blood is not determined or is, at least, only incidentally noted.

The case upon which this theory is based belonged to such a class, whose general features I shall briefly describe before going into details with regard to the individual patient. For want of a better name I may speak of them as cases of "Dum-dum fever," because, as far as my experience goes, the patients usually came either from this cantonment or its immediate neighbourhood. This station of Dum-dum lies

g. 1.—Smear preparation from the spleen of Private B., made thirty-eight hours after death and stained by Romanowsky's method. Magnification, 1,000 diameters.

Figure 3. Reproduction of the original publication by W. Leishman (1903) in the *British Med. J.* showing the presence of amastigotes of *Leishmania* in spleen smears from a deceased patient with "Dum-dum fever".

The next year in North Africa, Cathoire and Alphonse Laveran identified "Leishman bodies" in children with splenic anemia. This parasite was named *L.infantum* in 1908 by Nicolle who was also the first to culture it in medium NNN (Nicolle, Novy, McNeal) and to identify the dog as the main reservoir for this species. In the first half of the XXth century there were identifications and descriptions of *Leishmania* all over the world. Thus, in 1912, *Leishmania* was identified in Brazil from mucocutaneous lesions (WHO, 2010). The same year the Italian-born Spanish parasitologist Pittaluga reported the presence of VL in Spain. In India, in 1922, Bramachari described the Post Kala-azar Dermal Leishmaniasis (PKDL). The life cycle of the parasite with the involvement of an insect (Psychodidae, *Pheblotomus*) took some more years up to the works from Adler and Ber in Palestine and the demonstration of the transmission of *L.donovani* in India, carried out in 1942 by Colonel Shortt and colleagues (Sewaminath and Anderson) (WHO, 2010). Along the rest of the past century and in present days, investigational effort has been focused on a variety of fields, including the development of research tools to unravel intimate aspects of parasite biology, transmission, species involved, epidemiological patterns, clinical course and its relationship to the aetiological agents involved with the final aim of controlling the extension of the disease and its severity.

1.2. Parasite and vector

Leishmania is a Protista (Kinetoplastida) with heteroxenous life cycle and two morphologies. The extracellular phase, the promastigote, is present in the digestive tract of the insect vector. It is elongated (10-20 µm long x 4-7 µm wide) and motility is achieved by a ca. 20 µm single anterior flagellum emerging from a flagellar pocket. Intracellular organelles, characteristic of kinetoplastids, include a single nucleus with central nucleolus; glycosomes, where glycolysis is performed; acidocalcisomes for intracellular calcium storage and also related to osmorregulation and pH buffering; megasomes, linked to lysosomes (Vannier-Santos et al., 2002) and Golgi body-endoplasmic reticulum (de Souza da & da Cunha-e-Silva, 2003). Besides these organelles the most striking feature of these protists is the presence of a single mitochondrion representing ca. 12% of the total volume of the cell intimately related to a singular structure, the kinetoplast, related to the flagellum and containing its own DNA, kDNA (Figure 8A) (Monzote-Fidalgo & Gille, 2011). The parasitic phase present in vertebrates, the amastigote, is ovoid, presenting a diameter of ca. 2-4 µm; it is sessile and devoid of flagellum. This phase presents the same intracellular organelles as promastigotes although Golgi body and endoplasmic reticulum are reduced. In addition electron microscopy shows that a vestigial flagellar pocket is present (Figure 8B).

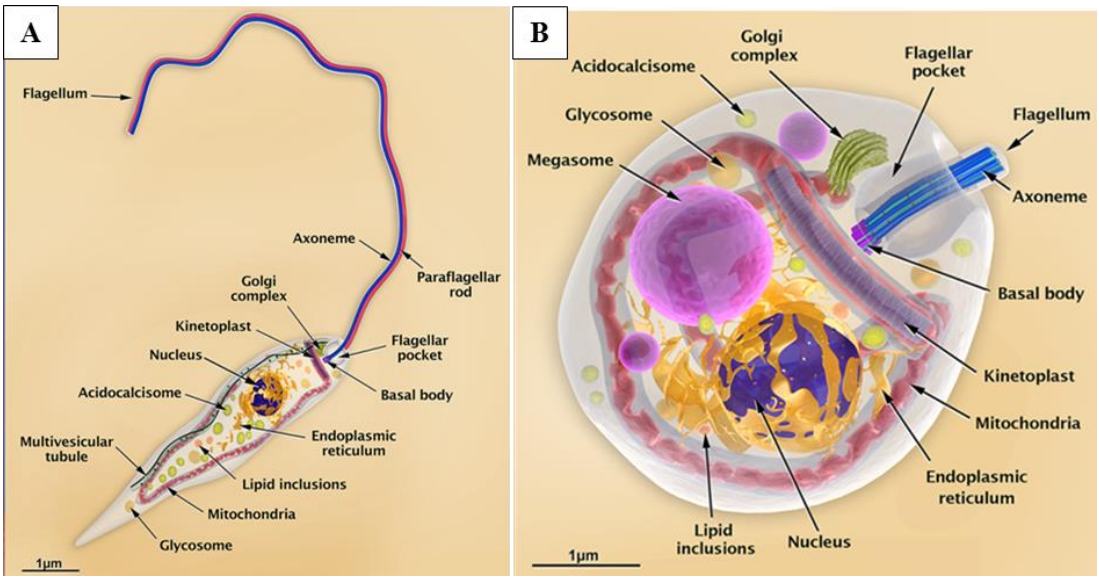

Figure 8. Ultrastructure of *Leishmania* spp (**A**) promastigote (**B)** amastigote. From Teixeira et al., 2013.

Transmission of *Leishmania* species is carried out through the bite of female sandflies from some species of the Genera *Phlebotomus* (Eurasia and Africa) and *Lutzomyia* (Americas) (Diptera, Psychodidae). Sandflies have a worldwide distribution although they are more frequent in arid zones of the Old World and forests in the New World. Some of the species from Eurasia and Africa breed in periurban areas and even inside households. On the contrary, transmission in the Americas is related to people working or living in or close to forests where *Lutzomyia* species are present.

Sandflies have a very limited flight capacity (< 2.5 Km) and measure ~ 3 mm. Head, thorax, abdomen and appendages are covered with hairs, the palps extend beyond the proboscis and are almost folded in two. The wings are folded above the body and the legs are long and slender. They are more active at dawn and dusk. Males are phytophagous whereas females are anautogenous requiring blood-meals for maturation and egg development (Killick-Kendrick, 1999). Transmission ability of *Leishmania* by sandflies is related to the concordance-discordance of the gonotrophic cycle and the development in the midgut of these insects. As far as it is known, in Spain only two species, *Ph.perniciosus* and *Ph.ariasi* are proven vectors.

1.3. Life cycle of *Leishmania* species

Female sandflies, when they take a blood-meal from an infected host, take also *Leishmania*-infected macrophages (Mφ). Amastigotes, within a few hours after ingestion, are engulfed by a peritrophic membrane in the mid-gut where they transform into rapidly-dividing procyclic promastigotes. Two to five days later they acquire a characteristic slender form, nectomones and, subsequently, leptomones. Once digestion has finished, the peritrophic membrane is broken down and the free promastigotes adhere through the flagellum to the villi of epithelial cells of the insect's intestine. This binding is critical for *Leishmania* survival since otherwise they would be expelled by defecation. The binding is achieved by the interaction of the lipophosphoglycan (LPG) layer from the leishmanial surface with galectins present in the epithelial cells from the host. Actually host-specificity is related to the compatibility of this binding (Kamhawi et al., 2004).

When promastigotes detach from the epithelium they migrate towards the stomodeal valve in the upper intestine. At this moment, the promastigotes are contained in a proteophosphoglycan matrix, the promastigotes secretory gel, obstructing the valve. The obstruction plays a fundamental role in the transmission since it facilitates regurgitation. Promastigotes undergo several morphological changes in a process termed metacyclogenesis until they reach the infective or metcyclic stage which migrates to the proboscis. When the infected sandfly takes the next blood-meal, metacyclics are regurgitated and enter a new vertebrate host (Warburg & Sclein, 1986; Sacks, 1989).

Metacyclic promastigotes are engulfed by cells from the mononuclear phagocytic system (MPS), including dendritic cells, fibroblasts, neutrophils, Langerhans cells and, particularly, macrophages (Mφ). In the cytosol they are surrounded by a parasitophorous vacuole where the promastigotes transform to amastigotes. Lysosomes fuse with the parasitophorous vacuole resulting in the formation of the phagolysosome. In spite of the extreme conditions within this vacuolar environment *Leishmania* amastigotes are able to survive and multiply. Intracellular proliferation of amastigotes eventually results in lysis of the host cell and the consequent amastigote release to the extracellular milieu or the blood. The free parasites can either be phagocytized invading new host cells or ingested by a sandfly thus continuing the transmission to a new host (Chang et al., 1990; Teixeira et al., 2013). *L. infantum* life cycle is illustrated in Figure 9.

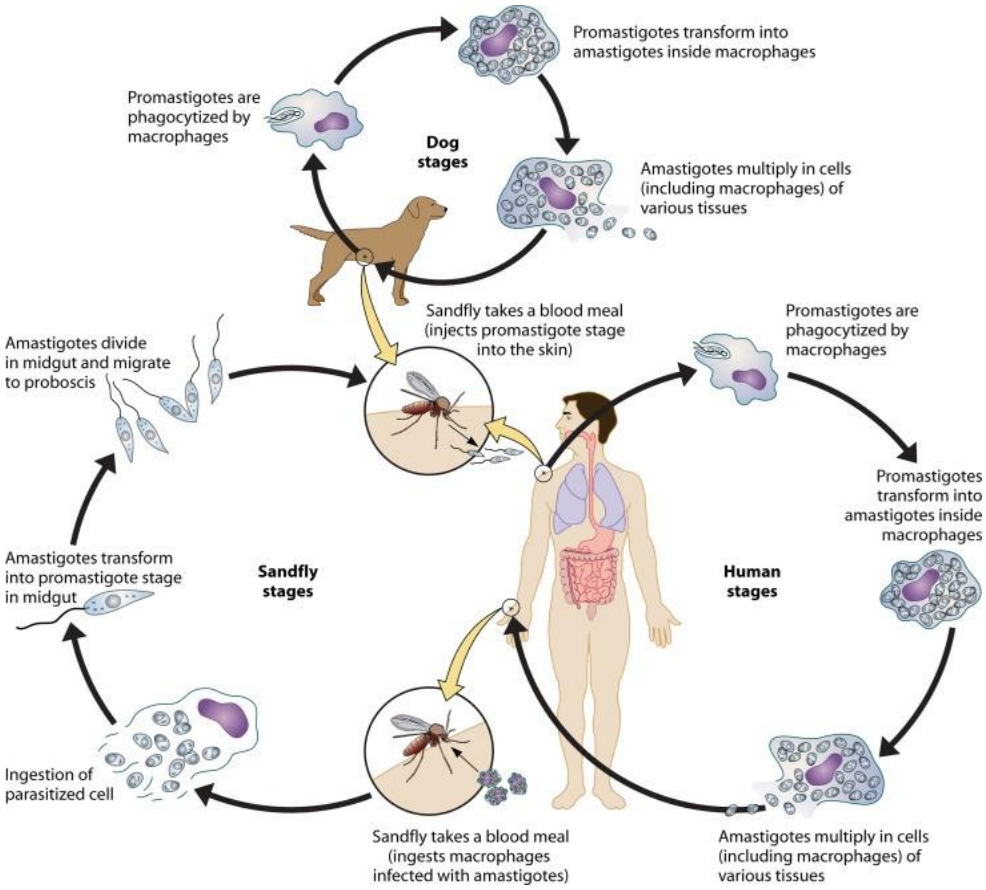

Figure 9. The life cycle of *Leishmania infantum.* The sandfly feeds on a susceptible host and injects metacyclic infective promastigotes during the blood-meal. Promastigotes are phagocytized mainly by macrophages (Mφ) where they transform into amastigotes within the parasitophorous vacuole. Amastigotes actively multiply, eventually rupturing the infected cell releasing amastigotes into blood and tissues. The parasite continues to infect phagocytic cells in the host. Sandflies become infected through feeding on a parasitized host. Ingested amastigotes convert to promastigotes within the sandfly midgut. Promastigotes migrate from the midgut and transform into highly infectious metacyclic promastigotes. From Esch & Petersen, 2013.

1.4. Epidemiology and distribution of leishmaniasis

The disease is endemic in 98 countries and is present in all inhabited continents although in Australia -with autochthonous cases of cutaneous leishmaniasis in kangaroos, wallaroos and wallabies (Rose et al., 2004; Dougall et al., 2009)- no human cases have been yet reported (Alvar et al., 2012). Infection is more frequent in developing countries, where 90% of all the human cases take place. Available data for morbidity rates of leishmaniasis are surely biased, on the basis of the complex interaction between infection and disease (*vide infra*), and higher prevalence in areas of the world with public health and information systems well behind

Western standards. Therefore, the WHO (2010) estimations of 350 million people living in areas at risk and 12 million people currently infected surely underestimate the real prevalence and incidence of the infections. Particularly those caused by visceralizing species, as most of the people infected with these species never develop any sign of leishmaniasis. It is estimated that only one out of ten infected people with *L.donovani* and one out of 100 infected with *L.infantum* develop the disease (Dujardin & Decuypere, 2013). In addition, 90% of all human cases of VL are concentrated in just six countries: India, Bangladesh, Sudan, South Sudan, Brazil and Ethiopia; while Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, Sudan, Costa Rica and Peru account for the 75% of the CL cases (Alvar et al., 2012). Most of these countries lack programs of surveillance or notification (e.g. Mongolia), they have no continuity of data reporting and the official figures are not accurate under-declaring the number of cases. In this sense, Alvar et al. (2012) indicated that the actual number of cases of CL in some countries such as Jordan or Guatemala was from 40 to 47 times higher than those officially recognized. Furthermore, focal distribution of leishmaniasis biases the extrapolation of many estimates.

On these grounds, the annual incidence of VL could be in the range of 200,000-400,000 and of 700,000 to 1.2 million human cases for CL. Perhaps the extension of the infection is even higher since recent estimates based on databases and statistical modeling rise the current human population to 1.71 billion and 1.69 billion individuals living in areas suitable for CL and VL respectively (Piggot et al., 2014). VL reported and predicted distribution worldwide is represented in Figure 10.

Mortality figures should also be carefully taken since only people with access to medical services and follow-up are included. Alvar et al. (2012) estimate that VL causes from 20,000 to 40,000 annual casualties, a similar figure to WHO data (i.e. 50,000 fatal cases). In spite of limitations, these numbers place leishmaniasis as the second most lethal parasitic disease (after malaria) and the ninth within the context of transmittable diseases (WHO, 2010).

Geographical distribution and extension of the disease is related mainly to the presence and population dynamics of sandfly vectors and the availability of reservoirs since most of *Leishmania* species pathogen for humans have a certain degree of zoonotical transmission (Esch & Petersen, 2013). Other transmission ways, although present, are less relevant in epidemiological terms. These include the venereal and vertical (congenital) transmission, blood transfusion in humans and dogs, syringe contamination in intravenous drug addicts (Killick-Kendrick, 1999; Tabar et al., 2008).

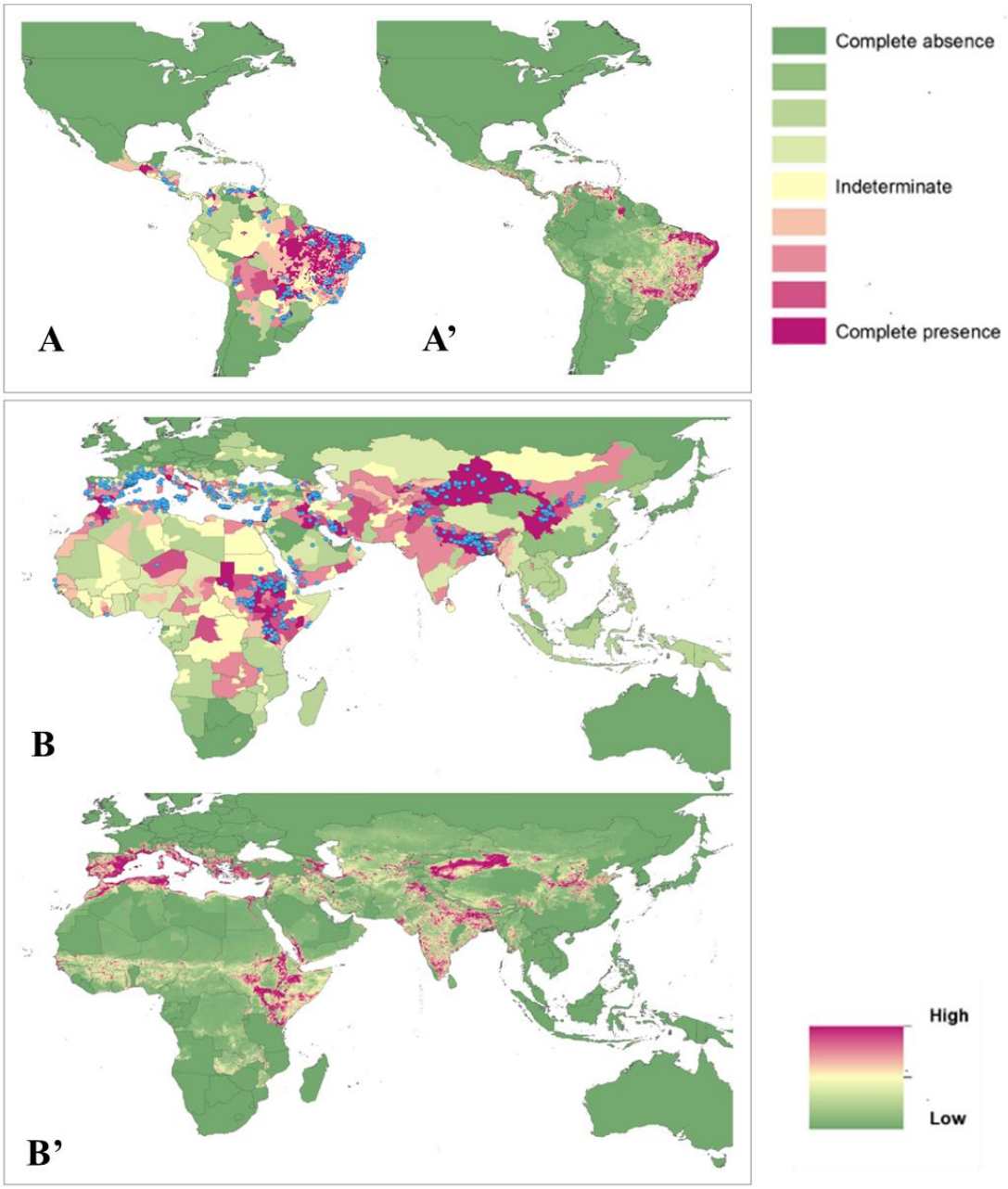

Figure 10. Reported and predicted distribution of visceral leishmaniasis in the New World (**A and A'**) and in the Old World (**B and B'**). (**A and B**): Evidence consensus for presence of the disease ranging from green (complete consensus on the absence: −100%) to purple (complete consensus on the presence of disease: +100%). The blue spots indicate occurrence points or centroids of occurrences within small polygons. (**A' and B'**) Predicted risk of visceral leishmaniasis from green (low probability of presence) to purple (high probability of presence). Adapted from Piggot et al., 2014.

Due to the dynamic nature of diseases, particularly those of complex epidemiology such as leishmaniasis, the appearance of the disease in previously thought free areas is frequent (Desjeux, 2001) and both human and canine cases have been reported in "non-typical" regions (e.g. Central Europe, USA and Canada) (Gaskin et al., 2002; Teske et al., 2002; Maroli et al., 2008; Petersen, 2009; Lobsiger et al., 2010; Tánczos et al., 2012; Clarke et al., 2013). This apparent expansion and the fluctuations in endemic areas have been related to socioeconomic (e.g. poverty, deficient infrastructures, lack of sanitation), nutritional (e.g. access to water supply, malnutrition), wars, migrations, movement of pets (e.g. holidays) and people (e.g. workers, travelers, urbanization), the climatic change and the subsequent expansion of the geographical distribution of vector populations (Desjeux, 2004; Chappuis et al., 2007; WHO, 2010; Gradoni, 2013; Ready, 2014).

Other factors facilitating the expansion of the infection by *Leishmania* are related to the environmental modification carried out by the human population (e.g. deforestation, engineering interventions, agricultural practices); generation of new niches (e.g. HIV-induced immune suppression, iatrogenic immune suppression in recipients of solid organ transplants); reduction of public health investments in low-income countries as a consequence of economic crisis; unavailability of medical services and chemotherapy, besides the therapeutic failures and the reported appearance of resistant *Leishmania* isolates to standard drugs (Desjeux, 2004; Chappuis et al., 2007; Antinori et al., 2008, Dujardin & Decuypere, 2013). Surely all these factors influence *Leishmania* transmission although their relative importance for a given location and *Leishmania* spp is not known in most cases.

In Spain three epidemiological patterns of leishmaniasis are recognized: 1) endemic, with sporadic cases and the dog acting as the main reservoir, 2) cases associated to immune depressing infections (e.g. HIV), non-infectious diseases eliciting an immune depression (e.g. systemic erythematosus lupus) or patients with iatrogenic immune depression (e.g. recipients of solid organs transplants), and 3) epidemic outbreaks (Suárez-Rodríguez et al., 2012). The number of cases of human leishmaniasis in Spain is low and in the period between the years 1996 and 2011 a total of 1,755 cases have been reported according to the "Red Nacional de Vigilancia Epidemiológica" (RENAVE) (National Net of Epidemiological Surveillance). From 2009 to 2012 an outbreak of leishmaniasis was reported in the southwest area of the Madrid autonomous community. The outbreak was related to *L.infantum* infection [confirmed in 421 cases (94.4% of the patients)] although cutaneous lesions were more frequent (64.1%) than visceral processes (35.9%). The mean age was 44 years, though the age range was wide (from 2 months to 95 years). The majority of the cases took place in immunocompetent individuals as only 15.2% of the cases were somehow related to inmmunosuppresive conditions. No

correlation was found between human and dog infections (Arce et al., 2014). Extensive research on potential alternative animal reservoirs showed that, apparently, hares were involved as reservoirs (Molina et al., 2012). This is the largest outbreak of leishmaniasis reported in Europe and, although not yet completely unraveled (e.g. anthroponotic infection), shows the dynamic nature of the disease and the potential new transmission patterns to be considered.

1.5. Human leishmaniasis

Over 30 species of *Leishmania* have been described and Table 1 shows the pathogenic species for humans and domestic animals.

Table 1. *Leishmania* species pathogenic for humans and domestic animals.

Clinical form	**Subgenus (Old World)**		**Subgenus (New World)**	
	L. (Leishmania)	*L. (Viannia)*	*L. (Leishmania)*	*L. (Viannia)*
Visceral Leishmaniasis	*L. donovani* *L.infantum* *L.tropica*		*L.infantum**	
Cutaneous Leishmaniasis	*L.major* *L.tropica* *L.killicki*[a] *L.aethiopica* *L.infantum*		*L.infantum** *L.mexicana* *L.pifanoi*[a] *L.venezuelensis* *L.garnhami*[a] *L.amazonensis*	*L.braziliensis* *L.guyanensis* *L.panamensis* *L.shawi* *L.naïffi* *L.lainsoni* *L.lindenbergi* *L.peruviana* *L.colombiensis*[a]
Mucocutaneous Leishmaniasis				*L.braziliensis* *L.panamensis* *L.guyanensis*

[a] Discussed status; * *L infantum* (= *L.chagasi*) in America. (Adapted from: Baneth, 2006; WHO, 2010)

1.5.1. Cutaneous leishmaniasis (CL)

1.5.1.1. Localized cutaneous leishmaniasis (LCL)

LCL is worldwide distributed although the clinical presentation varies depending of the geographical area and the particular *Leishmania* species. It is the most common form of leishmaniasis (WHO, 2010). In Africa and Eurasia the species incriminated in this presentation are *L.infantum*, *L.tropica*, *L.major* and *L.aethiopica* whereas in the Americas several species

from both Subgenera (*Leishmania, Viannia*) have been associated with the clinical form (Table 1).

LCL can present a single lesion (New World) or multiple lesions (Old World) at the inoculation site, being more frequent in exposed areas of the body (i.e. face, neck and arms). As a rule, lesions begin, after inoculation, with the appearance of a nodule or papule several days to months after infection by sandflies. Lesions progress to ulceration, formation of scars with hypertrophic edges of purple colour (Figure 4). These lesions tend to be self-healing, not painful and not pruriginous. Spontaneous healing takes months to years depending on the *Leishmania* spp involved and the immune response of the host. On occasion draining lymph nodes can be compromised (Choi & Lerner, 2001; WHO, 2010).

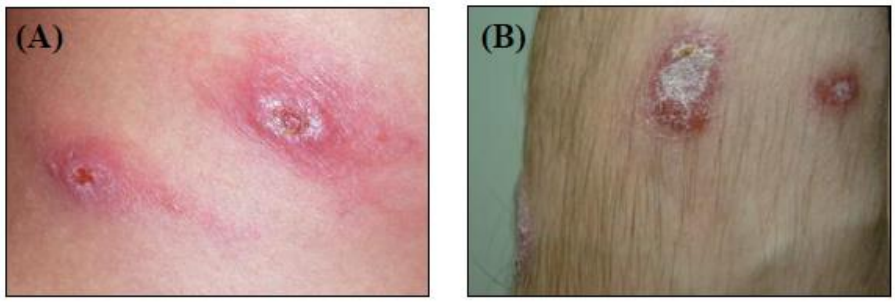

Figure 4. Skin lesions found in localized cutaneous leishmaniasis (LCL). (**A**): Crusts in ulcerated lesions caused by *L.major.* (**B**): Ulcer with hypertrophic margins produced *L.mexicana*. Image from David & Craft, 2009.

1.5.1.2. Disseminated cutaneous leishmaniasis (DCL)

DCL is caused in the Old World by *L.aethiopica* while *L.amazonensis* and *L.mexicana* are the species responsible in the Americas (WHO, 2010). Moreover, in immune compromised patients (e.g. *Leishmania* + HIV coinfection, transplant organ recipients) some other *Leishmania* spp have been incriminated (Couppié et al., 2004).

This clinical form is characterized by the presence of multiple cutaneous lesions in at least two non-adjacent regions of the body (Figure 5A). Although DCL is started with an initial, isolated lesion, patients subsequently develop up to hundreds of lesions of mixed types within a short period of time. Lesions tend to be non-ulcerative and can be papules, nodules or acne-like, with very few or no parasites in the skin tissue. Trunk, members and face are the most

frequently affected areas. Mucosal involvement may be present in ≤ 25 % of the cases (Turetz et al., 2002). Thickening of eyebrows and pinna eventually accompanied by mucosal affection (lip borders and nostrils) can resemble lepromatous leprosy although no nervous system involvement is present in DCL (Choi & Lender, 2001). The disease is chronic and, unlike LCL, not self-healing. Efficacy of chemotherapy is reduced and relapses are frequent (Couppié et al., 2004).

1.5.2. Post Kala-azar Dermal Leishmaniasis (PKDL)

PKDL is a secondary complication of VL caused by *L.donovani*. It has been described in former Sudan (Sudan + South Sudan) and India in the 50% and 5-10% of the treated patients, respectively. Development of this condition takes ~ 6 months after the disappearance of VL in Sudan whereas in India the disease appears after 2-3 years. On occasion PKDL has been found in patients apparently not previously suffering VL (Zijilstra et al. 2003) and also in cases of *L.infantum*-HIV coinfection (Stark et al., 2006). Lesions (nodules, papules and hypo-pigmented or erythematosus maculae) are mainly facial and can spread to other body parts (Figure 5B). Risks factors eliciting PKDL remain unknown and the response to chemotherapy is poor (Zijilstra et al., 2003). African cases tend to be self-healing whereas Indian PKDL generally never disappears (WHO, 2010).

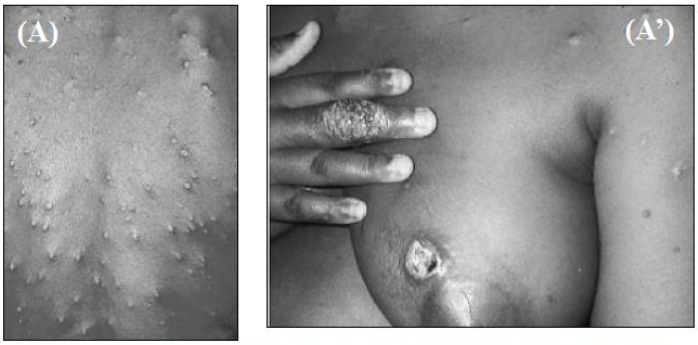

Figure 5A. Skin lesions found in disseminated cutaneous leishmaniasis (DCL). (**A**): Multiple acneiform eruptions in the back. (**A'**): Ulcers in the fingers and breast. Images come from Turetz et al., 2002 (**A**) and Couppié et al., 2004 (**A'**).

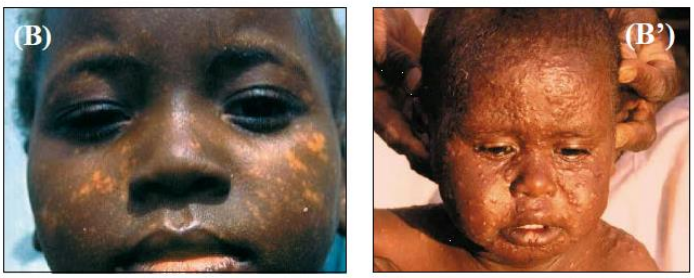

Figure 5B. Post Kala-azar dermal leishmaniasis (PKDL). (**B**): Macular lesions. (**B'**): lesions in the face and trunk. Images taken from Zijilstra et al., 2003.

1.5.3. Muco-Cutaneous Leishmaniasis (MCL)

Aetiological agents belong to the Subgenus *Viannia*, particularly *L.braziliensis* and *L.panamensis*. This clinical form was well known by the autochthonous South Americans before the Spanish conquest. Accordingly, regional names are abundant (e.g. "espundia", "uta", "úlcera del chiclero") in the countries with the highest prevalence such as Brazil, Bolivia and Peru. This clinical form is characterized by disfiguring lesions in oral and nasal mucosae and upper respiratory system (Figure 6). The infection is not self-healing and the secondary bacterial infections are frequent, being pneumonia the most common cause of death (WHO, 2010). It is worth pointing out that these lesions have been also observed in immune compromised patients infected with *L. (Leishmania)*, in particular *L.donovani*, *L.infantum*, *L.tropica* and *L.major* (WHO, 2010). Factors eliciting the development of MCL are not known although both the immunological status of the patient and virulence of species or isolate seem to play a crucial role in the progression of lesions towards mucosae in 1-10% of the infections (David & Craft, 2009).

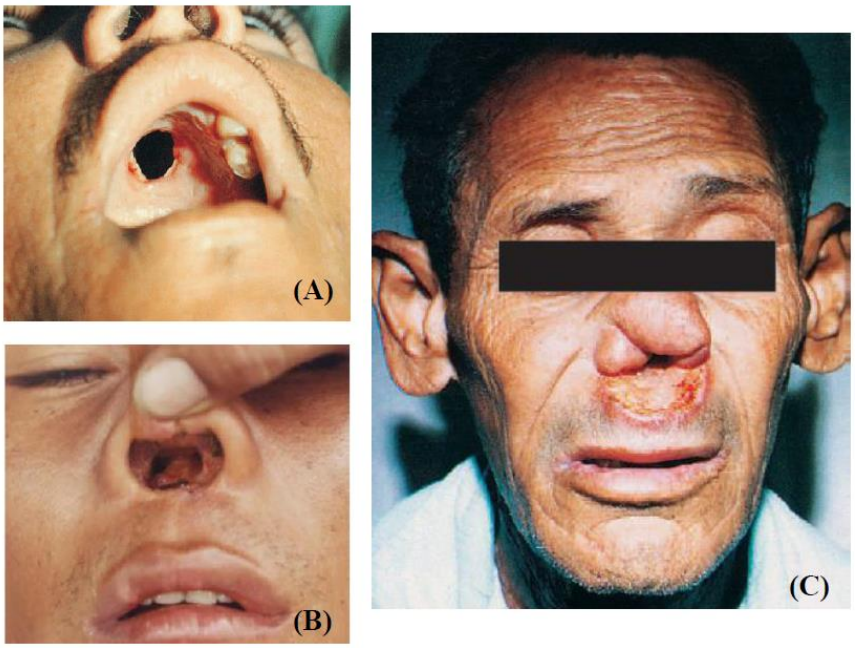

Figure 6. Mucocutaneous leishmaniasis: (**A**) Destruction of the palate due to *L.braziliensis;* (**B**) and (**C**) Active lesion in nasal mucosa (**B**) with perforation of the nasal septum caused by *L.braziliensis*; Images (**A**) and (**C**) from Lainson & Shaw, 2005; and image (**B**) taken from Colvopina et al., 2006.

1.5.4. Visceral leishmaniasis (VL)

VL is caused by *L.donovani* in the Indian subcontinent and East Africa, and by *L.infantum* (=*L.chagasi*) in Europe, North Africa and Central and South America (Chappuis et al., 2007). In addition some cases of VL caused by *L.tropica* have been described (Magill et al., 1993). Infections by *L.donovani* are more frequent in children and young adults although all ages are susceptible. Incubation period is highly variable (10 days to one year or more) and clinical signs and lesions include fever, weight loss, cutaneous paleness and liver and spleen enlargement (Figure 7). In India, *L.donovani* infections characteristically course with darkening of the skin in the face, hands, feet and abdomen. This darkening probably explains the local name of the disease since Kala-azar in Hindi can be translated as "Black fever" (WHO, 2010). Mortality associated to *L.donovani* infections is very high; actually is the second death-causing parasitic disease, after malaria.

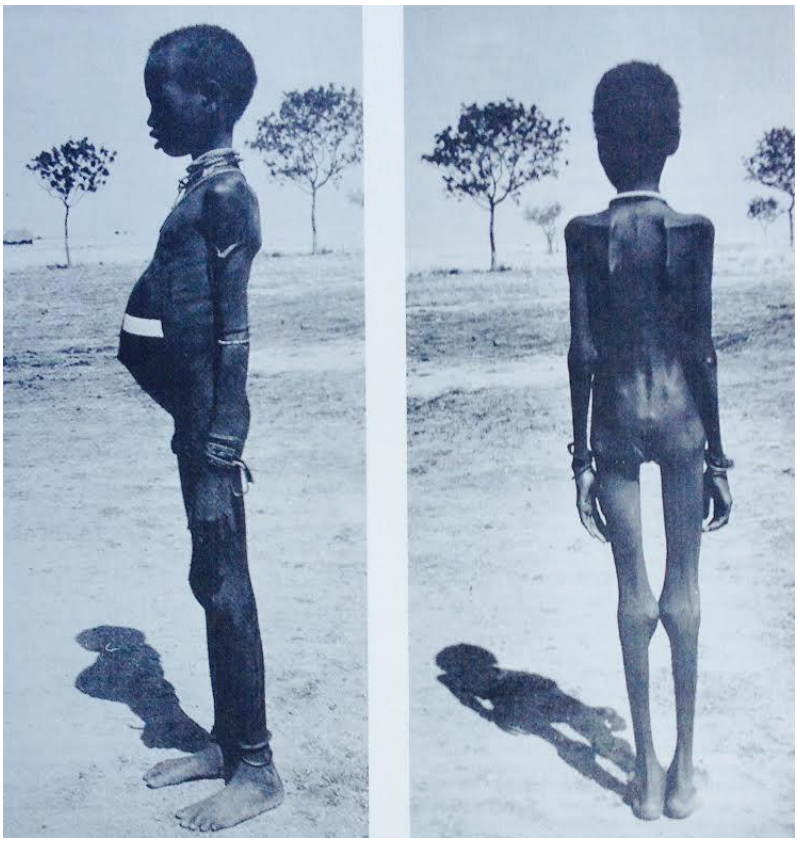

Figure 7. Chronic visceral leishmaniasis by *L.donovani*. Six years old child from Sudan, showing extreme hepatosplenomegaly and emaciation. From Hoogstral & Heyneman, 1969.

1.5.4.1. Anthroponotic and zoonotic visceral leishmaniasis

In previous pages it was stated that VL is the most severe form of leishmaniasis with fatal consequence unless treated (Desjeux et al., 2004). Generally speaking two forms of the disease have been traditionally considered: **Anthroponotic Visceral Leishmaniasis, AVL**, caused by the infections of *L.donovani* and present in the Indian subcontinent and Eastern Africa, and **Zoonotic Visceral Leishmaniasis, ZVL**, provoked by *L.infantum* (=*L.chagasi*) infections in the Mediterranean, including the Iberian peninsula, some parts of Asia (e.g. China) and South America (Chang et al., 1990; WHO, 2010). Transmission of *L.donovani* is considered anthroponotic, humans acting as the main reservoir although the potential role of other animal species has been suggested in India (Singh et al., 2013), Sudan (Dereure et al., 2003) or Nepal (Bhattarai et al., 2010). By its part, *L.infantum* can infect a variety of mammals although dogs are considered the primary reservoir for ZVL. In endemic areas high prevalence of the infection has been reported in red fox (*Vulpes vulpes*) (75%) and other carnivores (e.g. wolves: *Canis lupus*; jackals, *Canis* spp). Moreover, other species (e.g. Crab-eating fox from South America, *Cerdocyon thous*; opossum, *Didelphis* spp; cat, *Felis silvestris catus*; rat, *Rattus rattus*; hare, *Lepus* spp) can be experimentally infected but their role as primary or secondary reservoirs is not yet determined (Quinell & Courtenay, 2009; Domingues-Souza et al., 2014; Millán et al., 2014). ZVL, besides its importance in public health in the distribution area of *L.infantum*, is a first order pathology in a strictly veterinary arena given the prevalence and severity of the disease. Thus it is justified, in our context, to review more extensively the canine leishmaniasis.

1.6. Canine leishmaniasis (CanL)

1.6.1. Distribution and epidemiology of CanL

Dogs (*Canis familiaris*) can be naturally infected by a number of *Leishmania* spp, namely *L.arabica*, *L.major*, *L.tropica*, *L.infantum* and *L.donovani* in Eurasia and Africa, and by *L.amazonensis*, *L.colombiensis*, *L.peruviana*, *L.mexicana*, *L.panamensis* and *L.braziliensis* in America (Campino & Maia, 2013). Their relative role on the basis of the potential of transmission, zoonotic character and severity of the processes is very different. It is considered that *L.infantum* (=*L.chagasi*) is the most relevant species affecting dogs.

CanL has been reported at least in 50 of the 98 countries where human leishmaniasis is present (WHO, 2010). The Mediterranean region and Brazil are the areas with higher incidence and prevalence. There are sporadic cases of CanL in non-endemic regions related to the animal

import or pet movement to recreational endemic areas (Shaw et al., 2009). In the last years an apparent expansion of the disease to previously-free areas in Central Europe has been reported (Teske et al., 2002; Maroli et al., 2008) and the connection between climatic change and the presence of those autochthonous cases has been suggested. More striking have been the North American reports, particularly from the USA (Gaskin et al., 2002; Clarke et al., 2013). CanL, caused by *L.infantum*, was originally diagnosed in some foxhound kennels in North Eastern states, and from 2000-2005 the infection has extended to over 20 states in the USA (Petersen, 2009) and five Canadian provinces (Duprey et al., 2006). Since no recognized vector was known at the time, and other arthropods (fleas, ticks) have not conclusively shown to play any role in the transmission of *Leishmania* (Coutinho et al., 2005; Saridomichelakis, 2009; Dantas-Torres et al., 2010; Colombo et al., 2011) vertical and horizontal transmission have been postulated in the USA and in some European countries (Petersen & Barr, 2009; Naucke & Lorentz, 2012). CanL can be transmitted through blood transfusions (Owens et al., 2001; Tabar et al., 2008) and other sandflies, not previously described as vectors of *Leishmania*, are present in the USA and Canada. Among them, *Lutzomyia youngi* and *Lutzmoyia shannoni* are present in the New World and the latter is a proven vector of the vesicular stomatitis virus. Even though both species can be infected after feeding on infected dogs no actual evidence of their role in the transmission of *L.infantum* has been yet presented (Travi et al., 2002).

It is estimated that over 2.5 million dogs are infected in the endemic areas from Europe and probably millions of dogs and other wild canids in South America (Baneth et al., 2008). This high prevalence is possibly due to the intense infection pressure since animals living in endemic areas can receive up to one bite of an infected sandfly/hour (Saridomichelakis, 2009). In Southern Europe -the most affected area- seroprevalence ranges from 5 to 30% with "hot spots" where this value can reach 40-80% (Franco et al., 2011). Average values in some non-biased surveys carried in Spain were comparable, 5-7% of the total sampled dog population (e.g. Castillo-Hernández et al., 1985; Franco et al., 2011) and some high prevalence areas were also identified in South-eastern Spain with values over 20% (Martín Sánchez et al., 2009) or even > 30% (e.g. Axarquía, Málaga) (Morillas et al., 1996). Moreover, the disease once predominantly rural is becoming more frequent in residential areas around cities (Campino & Maia, 2013).

Estimation of the incidence and prevalence of CanL, in a similar way to that indicated for human leishmaniasis, is challenging by a number of reasons. Among them the presence of asymptomatic carriers (Michel et al., 2011) and the frequent cases of the so-called cryptic infections with varying results depending on the diagnostic method used (Fernández Pérez et al., 2003). In addition to the absence of compulsory notification of CanL some estimates are clearly biased since reports have been based on the clinical cases diagnosed by veterinary services or

practitioners thus producing an unreliable picture of the actual extension of the infections, their incidence and their prevalence (Gradoni, 2013). In the other hand canine census is surely inaccurate in many areas of the world with CanL (e.g. South America) and even in developed countries the actual population of dogs is thought to be considerable higher than the officially reported (Baneth et al., 2008). Biased canine sampled population is not the only source of data inaccuracy in the epidemiology of CanL. Both sensitivity and specificity of diagnostic methods (e.g. IFAT, ELISA, direct observation, PCR) employed in the surveys are variable (Maia & Campino, 2008) and, therefore, the direct comparison of the results reported in studies using different techniques is lacking in significance (Solano-Gallego et al., 2001; Leontides et al., 2002).

1.6.2. Differential canine breed susceptibility

CanL is a chronic and systemic (visceral and cutaneous) parasitic disease with a long incubation period (months to years) (Slappendel, 1988) and affecting dog from both sexes and all breeds (Noli, 1999; Leontides et al., 2002). On occasion a higher prevalence has been reported in some breeds (e.g. Boxer, Cocker, German Shepherd, Rottweiler, Doberman) whereas in other cases prevalence values below the expected have been observed in other breeds, including some local ones (e.g. Ibizan hound, Poodle, Yorkshire terrier) (Ciamarella et al., 1997; Solano-Gallego et al., 2000; França-Silva et al., 2003, Miranda et al., 2008). Insofar no convincing genetic basis for the variable susceptibility has been produced and these differences are surely related to the aptitude and life style of the breeds studied (e.g. guard dog, indoor pet) (Moreno & Alvar, 2002; Miranda et al., 2008; Martín Sánchez et al., 2009; Gálvez et al., 2010). In fact, Pozio et al. (1981) did not observe, in Italy, any relationship between breed and infection.

Another point raised has been the relationship between age and susceptibility. A peak of infection prevalence between 2-3 and 7-8 years age has been reported (Zivicnjak et al., 2005; Gálvez et al., 2010). This apparent age-related finding has been related to the repeated exposure of animals to sandflies (Koutinas et al., 1999; Saridomichelakis, 2009; Martín Sánchez et al., 2009) and not to the differential receptivity. It seems, therefore, that all dog ages are equally sensitive to *L.infantum* (Noli, 1999; Leontides et al., 2002) provided that they have contact with infected sandflies and, actually, a wide sampling (33.937 animals) carried out by França-Silva et al. (2003) could not ascertain any relationship between age of dogs and disease.

1.6.3. Infection and disease in CanL

1.6.3.1. Pathogenesis and immune response in CanL

L.infantum infection does not necessarily lead to CanL. Disease outcome is strongly dependent on the immune response of the hosts and the fate of parasites. Once metacyclic promastigotes are inoculated in the skin of the dogs, and if they overcome the innate immune mechanisms (e.g. complement lysis), they infect different phagocytic cells (macrophages, dendritic cells, Langerhans cells) where they, subsequently, transform into amastigotes. This local infection can be cleared, in which case no further progress of the disease will be seen (self-limited infection). If not cleared infection can be disseminated, transported via infected macrophages, to regional lymph nodes. Amastigotes will survive and multiply within the phagolysosomes, eventually leading to the lysis of the host infected cells. Free amastigotes can then invade new target cells from the mononuclear phagocytic system (MPS). From this point on, the outcome of the infection will vary depending on many factors related to the vector (e.g. repeated infectious bites, intradermal injection of sandfly saliva), the parasite (e.g. virulence) and the host (e.g. genetic background, cell-mediated and humoral immune response, cytokine milieu, concurrent diseases) (Saridomichelakis, 2009).

Dogs can control the infection thus being locally eliminated (i.e. self-limited infection) or restricted to the skin and lymph nodes (i.e. localized generally asymptomatic infection). Parasites can reach by lymphatic and blood systems the target organs, namely bone marrow, spleen, liver, kidneys, ganglions, eyes and digestive tract. Disseminated infection can lead to the appearance of clinical signs and lesions (i.e. symptomatic disseminated infection) or not (asymptomatic disseminated infection) (Solbach & Laskay, 2000; Baneth, 2005). Routinely, infected dogs are classified as susceptible (CanL) characterized by parasite unrestricted multiplication inducing multiple organ pathology through granulomatous inflammation and immune-mediated mechanisms; or resistant (all the remaining outcomes). Resistant phenotypes can effectively kill or control parasite infection remaining clinically normal (Figure 11).

It is generally considered that the wide range of clinical courses found in leishmaniasis are related to the events taking place in the host-*Leishmania* interface (e.g. immune status of the host, virulence of *Leishmania* spp and strain) and the subsequent immune response elicited (Stanley & Engwerda, 2007). For the most part studies have been carried out in experimental models, particularly mice (Kedzierski & Evans, 2014). Experimentally infected resistant mice with *L.major* develop a predominant $CD4^+$ T helper 1 (Th1) phenotype response with IFN-γ production whereas susceptible mice show a dominant $CD4^+$ Th2 response with interleukin IL-

4, IL-5 and IL-3 secretion. This correlation between the polarized response and the outcome of the disease led to the paradigm of the balance between Th1/Th2 being the actual responsible of the disease outcome (Roberts, 2006).

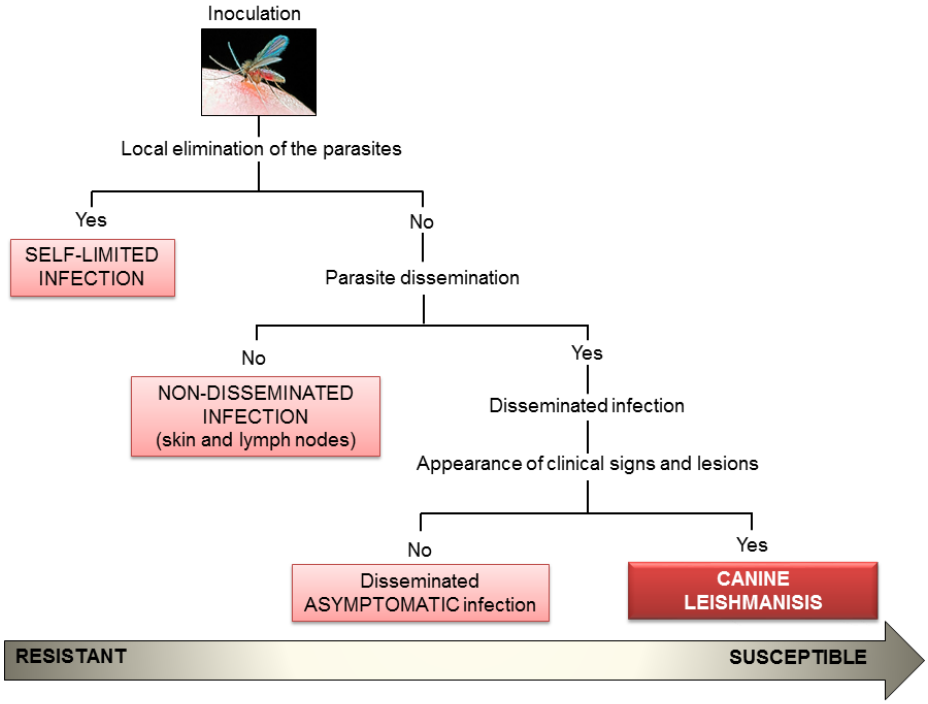

Figure 11. Possible disease outcome after an infection by *L.infantum* (=*L.chagasi*) in the dog. Modified from Saridomichelakis, 2009.

The information on the immune response, after a primary infection, in visceral leishmaniasis and in particular in CanL, is comparatively low (Veras et al., 2010). Generally speaking in leishmaniasis both innate (Mφ, neutrophils, dendritic cells) and adaptive (T cells) response are involved and CD4+ subtype seems to play a crucial role in the final result of the infection. CanL uncontrolled infections are characterized by polyclonal B-cell activation, with high levels of specific and unspecific antibody production, and specific immunosuppression (Mendes Roatt et al., 2014).

With the scarce data available *L.infantum* (=*L.chagasi*) infections in dogs elicit a mixed Th1/Th2 response. Disease progression is associated to a predominant Th2 phenotype, with a low lymphoproliferative response and high levels of IL-4, IL-10, TGF-β whereas resistant animals exhibit a Th1 phenotype, with positive lymphoproliferation and production of Th1

cytokines: IFN-γ, TNF-α and IL-2 (Fernández-Pérez et al., 2003; Reis et al., 2010; Veras et al., 2010; Mendes Roatt et al., 2014). This model of response (Th1/Th2 dichotomy) is more evident in cutaneous leishmaniasis (McMahon-Pratt & Alexander, 2004) and not directly applicable to CanL. As illustrated in Figure 12, probably the actual pattern of immune response in leishmaniasis, particularly in VL, is more complex given the different cell types involved (e.g. Th17, Th9, Tfh2, Treg) (Alexander & Brombacher, 2012), the differential role and importance of signalling molecules along the infection and the limitations of the experimental models used. As a consequence it is anticipated that this dichotomy model should be challenged.

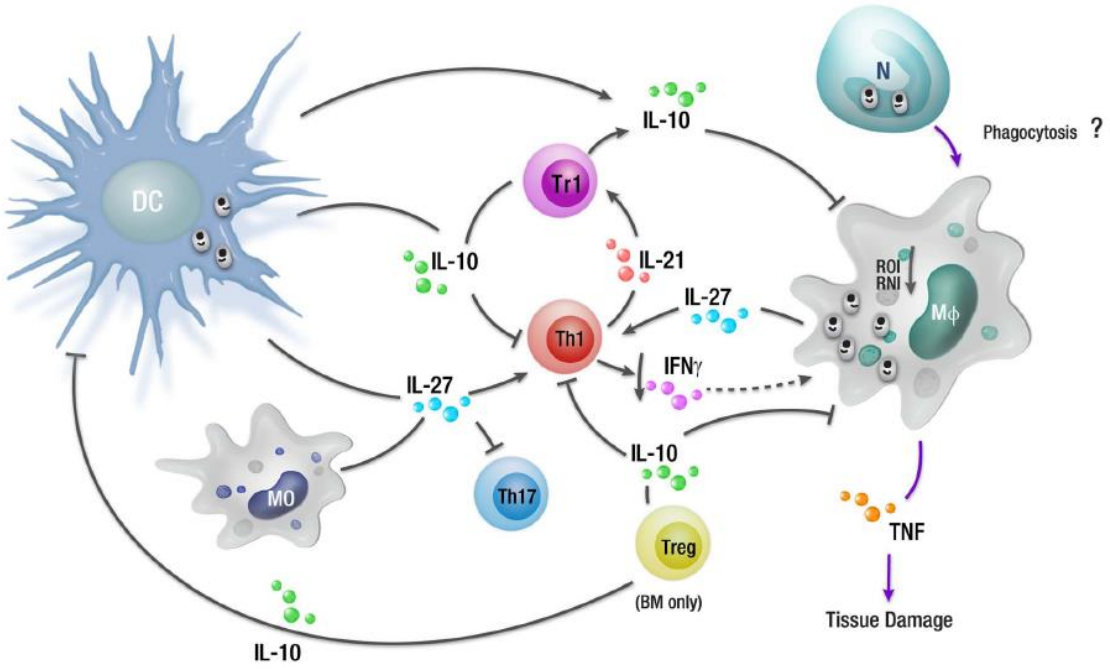

Figure 12. Cellular mechanisms involved in the immune response to a chronic infection by *L.donovani*. A population of regulatory dendritic cells (DCr) in the spleen produces IL-10 this inducing the proliferation of T regulatory cells producing IL-10 (Tr1) besides inhibiting death effector mechanisms in macrophages (monocytes, MO, and MΦ) and other phagocytic cells (including the suppression of ROI and RNI production). IL-27 produced by DCr and MO together with T cell produced IL-21 induce the differentiation of Th1 to Tr1 besides inhibiting Th17. IL-10 produced by Tr1 inhibits antigen presentation thus contributing to a T-cell general dysfunction and diminished IFN-γ production by CD4+. Bone marrow Tregs can also produce IL-10. Image from Faleiro et al., 2014.

1.6.3.2. Clinical signs and lesions in CanL

In CanL almost any organ from infected animals can be affected and no clear cut separation between visceral and cutaneous infections is frequently observed. Actually over 50% of infected dogs exhibit cutaneous lesions (Podaliri Vulpiani et al., 2011). In chronic generalized infections infected animals show lack of appetite, leading to weight loss, and may also present epistaxis, polyuria-polydipsia, irregular fever, neurological and locomotory disturbances and gastrointestinal disorders (e.g. diarrhea, vomiting). Skin lesions, generally not pruriginous, range from nodules, papules or pustules and can be accompanied by exfoliative dermatitis, with or without alopecia and ulcerations. Hyperkeratosis of muzzle and footpads is another common clinical finding. In addition, dogs with CanL can present cachexia, pale mucous and onychogryphosis, generalized lymphadenomegaly, ocular lesions (e.g. blepharitis and conjunctivitis, panophtalmitis and uveitis), muscle atrophy (easily observed in temporal muscles), vascular disturbances (e.g. systemic vasculitis, arterial tromboembolism), liver and, specially, spleen enlargement and bone and muscular disorders (e.g. polyarthritis, polymyositis and osteomyelitis) (Ciamarella et al., 1997; Solano-Gallego et al., 2011). Figure 13 depicts some of the most frequent lesions observed in CanL.

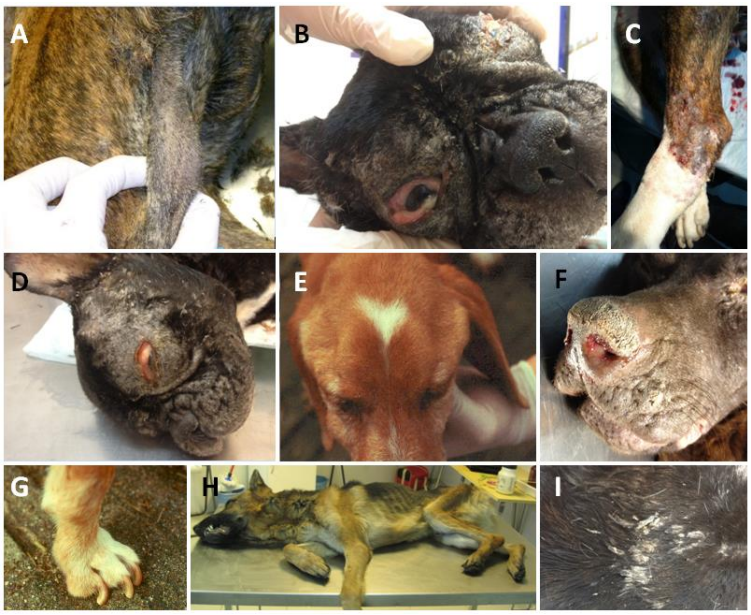

Figure 13. Lesions and clinical signs observed in CanL. (**A**): Lymphadenomegaly, popliteal lymph node; (**B & D**): Ocular lesions [**B**: blepharitis and conjunctivitis; **D:** panophtalmitis]; (**C**): ulcerative pustular dermatitis; (**E**): temporal muscular atrophy; (**F**): Muzzle hyperkeratosis; (**G**): onychogryphosis; (**H**): cachexia; (**I**): exfoliative dermatitis. Image from Corral & Alunda, 2014.

Haematological findings most frequently observed are non-regenerative anemia, leucopenia and hyperproteinemia with hypergammaglobulinemia and hypoalbunemia. Clinical biochemical profile of infected animals shows elevated levels of liver function marker enzymes and urea and creatinine. Proteinuria is a common finding if there is nephropathy (Noli, 1999; Solano-Gallego et al., 2011). *L.infantum* infection in dogs elicits a high production of immunoglobulins unrelated to the parasite. Once surpassed the renal threshold immunocomplexes (IC) are deposited in the kidney inducing glomerulonephritis and interstitial nephritis (Poli et al., 1991; Costa et al., 2000; Clementi et al., 2011) and, finally a renal failure, this dysfunction being actually the ultimate cause of death in most cases (Noli, 1999; Clementi et al., 2011).

1.7. Control of zoonotic visceral leishmaniasis (ZVL)

Control of CanL has the objective, within the wide context of public health, of reducing the prevalence and intensity of dog infections, the main recognized reservoir, with the final aim of lowering the human cases of ZVL (Podaliri Vulpiani et al., 2011). Moreover, CanL caused by *L.infantum* (=*L.chagasi*) is a first order veterinary pathology in many areas of the world particularly the Western Mediterranean and South America. Fossil and molecular evidences suggest that domestic dogs evolved from a population of wolves in Europe, which may have gone extinct. Molecular dating suggests domestication of dogs took place at some point 18,800 to 32,100 years ago (Thalmann et al., 2013). From then up to date men and dogs have been in close relation and therefore dogs have played a fundamental role in many life cycles of human parasites. Similarly, both of them can be infected by *L.infantum* dogs acting as the reservoir. Consequently, any control of ZVL must include measures (e.g. vector control, transmission control, immunoprophylaxis, chemotherapy and chemoprophylaxis, and eventually dog culling) to control dog infections.

1.7.1. Control of vector populations and *Leishmania* transmission

The rationale behind the control of sandfly populations is the reduction or interruption of the transmission of *Leishmania* infection (Alvar et al., 2004). During the last century many government campaigns, particularly in urban and periurban areas, were carried out to control vectorial diseases such as leishmaniasis, filariasis, dengue, malaria. Most control operations involved the modification of vector habitats (environmental management), personal protection measures and, mainly, insecticides due to the affordable cost, rapid response and easy

administration. These measures require to be successful a deep knowledge of the epidemiology, insect fauna biology, ecology of the target territory (WHO, 2010) and the understanding and acceptance of the human population inhabiting the area.

Chemical control of vectors comprises the indoor and outdoor residual insecticide spraying, use of repellents, topical insecticides or insecticide-impregnated materials (Sharma & Singh, 2008). Insecticide spraying is a low cost method and its efficacy is dependent of the type of insecticide, sandfly sensitivity, manner of administration and dosage. Chemicals more widely used were organochlorines (e.g. DDT), organophosphates (e.g. Malathion), carbamates (e.g. Propoxur) and synthetic pyrethroids (e.g. Deltametrin).

In the XIXth century, and even until the 1950s, malaria was endemic in Europe and in the USA (de Zulueta, 1998). Control, and finally eradication, in the continent and the USA was possible through the establishment of different measures (e.g. desiccation of damped areas) although the main role is attributed to DDT (Beard, 2006; Tognotti, 2009). The use of this compound drastically reduced the prevalence of leishmaniasis in many foci and the levels of infection rose again once spraying with DDT was interrupted (Killick-Kendrick, 1999). Toxicity, impact on the environment and risks associated to the use of DDT is still a controversial issue. In fact, despite its proven efficacy, there are many studies describing its toxicity and discouraging its use (Beard, 2006) although this toxicity has been challenged in some retrospective studies (Tognotti, 2009). Moreover resistant populations of insects have been reported (Khishore et al., 2004). Presently WHO (2010) does not completely discourage its use and actually it is employed in hyperendemic areas in India (Sharma & Singh, 2008).

In humans, the use of impregnated bed nets, bed sheets and curtains with insecticides is an effective and low cost method to reduce the transmission rate of *Leishmania* and its value has been confirmed in soldiers in Afghanistan (Reyburn et al., 2000). Nonetheless, individual protection with repellents is also strongly advised.

In the case of CanL some of these approaches are also available. The use of topical insecticides for dogs as strategy to reduce leishmaniasis transmission has gained popularity in the last decades (Foglia Manzillo et al., 2006; Solano-Gallego et al., 2009). The system seems to reduce the CanL prevalence (Ferroglio et al., 2008) and, therefore, the transmission to humans (Gavgani et al., 2002). In Western countries the use of repellents and insecticides in dogs is frequent. Available specialties include collars, spot-on and sprays. Marketed products have a double action acting as repellents for incoming sandflies and lethal for the insects after biting (Podaliri Vulpiani et al., 2011). Delmathrin-impregnated collars reduce by over 80% the number of sandfly bites (Halbig et al., 2000; Reithinger et al., 2001) and there are enough evidences to

recommend their use in dogs in endemic areas (Noli & Auxilia, 2005). They are long-lasting and the effect is maintained at least for six months (Killick-Kendrick et al., 1997). The effect of spot-on formulations with imidacloprid (neonicotinoid insecticide), permethrin (pyrethroid) and combinations is short-lived (ca. 1 month) although their repellent action could be higher (Reithinger et al., 2001; Quinell & Courtenay, 2009; Podaliri Vulpiani et al., 2011).

1.7.2. Control of reservoir population

Control of the canine population includes the availability of an accurate census, recovery of stray dogs and euthanasia. Culling of *L.infantum*-positive dogs is not easily accepted, based on ethical grounds, in Western countries; frequently not practicable and of doubtful efficacy when other sympatric reservoirs are not controlled (e.g. wild infected animals, dogs with "cryptic" undetected infections). Results obtained with this strategy are contradictory (Dietze, 1997; Ashford et al., 1998) and the active selective euthanasia campaign supported by Brazilian health authorities in the 1970s had null or very modest impact on the number of cases of HVL (Moreira, 2004; Costa, 2011). Recently, a randomized community intervention trial carried out in the Brazilian city of Teresina also described the low efficacy of dog culling on HVL incidence (Werneck et al., 2014). Cost-benefit of dog culling has been argued by many authors (*vide supra*) and, although this policy can produce positive results (e.g. China), low success in culling programs has been the rule. The failure has been associated to several factors as: lack of continuity of these programs due to deficient surveillance systems, economic issues, absence of adequately trained professionals; absence of co-implementation of other control strategies; logistic problems that favour late diagnosis (e.g. lack of standardized techniques) and the existence of asymptomatic infectious dogs that guarantee the continuity of the transmission cycle (Costa et al., 2013).

The reported success of the leishmaniasis (AVL, ZVL) control programs in China in the 1950s was probably related to the implementation of combined control measures [non-selective euthanasia of all dogs from the target area, massive chemotherapeutic treatment of humans with antimonials, use of insecticides (DDT and pyrethroids) and strict government control] (Zhi-Biao, 1989). Actually, dog culling alone is not an effective control strategy as it has not eliminated leishmaniasis transmission in any place (Quinell & Courtenay, 2009). Furthermore, ZVL is present in locations were CanL is also prevalent, the association between both diseases is possibly not so tight, and *Leishmania* infections in both hosts (i.e. canids and humans) could have different epidemiological patterns establishing a complex relationship not yet completely elucidated (Costa, 2011).

1.7.3. Immunoprophylactic control of leishmaniasis

The development of a vaccine against leishmaniasis does not seem to be out of reach since a large proportion of infected humans and dogs develop a natural immune response against *Leishmania* thus preventing the outcome of the disease. Furthermore both host species, once the primary infection has been controlled, mount an effective immune response against homologous reinfections (Kumar & Engwerda, 2014) whereas the results with heterologous challenges have been variable and inconsistent (e.g. Porrozzi et al., 2004). Insofar no vaccine for human use against leishmaniasis has been yet registered. There are intrinsic difficulties inherent to the development of an antiparasitic vaccine and actually the number of vaccines for parasitic diseases is very limited (e.g. toxoplasmosis, Bm86, poultry coccidiosis, cattle dictyocaulosis). In addition, the strict present requirements for an ideal antileishmanial vaccine for human use (safe even in immune compromised patients, high efficacy, low number of doses, eliciting sterile immunity thus preventing disease and transmission, low cost, thermal stability to guarantee transport and storage, multispecies) (Ada, 1991) possibly renders the endeavour of achieving a vaccine candidate an almost insurmountable task.

Since the recognition of the disease, well before the identification of the etiological agent, there were attempts of preventing the outcome of the clinical sings and lesions. Within the framework of vaccine development, first generation vaccines include the use of live non-attenuated parasites (i.e. "leishmanization"), heterologous protection using as immunizing agents non-pathogenic species (e.g. *L.tarentolae* to confer protection in BALB/c challenged with *L.donovani*: Breton et al., 2005), inoculation of attenuated parasites without genetic modification, and of dead autoclaved parasites. Unfortunately, elicited protection appears to be negatively correlated to the manipulation of *Leishmania*. Thus, the most effective inoculums are those involving infective promastigotes.

Historically, leishmanization has been an effective immunoprophylactic method against CL. Deliberate inoculation of live parasites in a hidden area (e.g. behind the ears, armpits, gluteus) to protect against lesion development in visible areas (e.g. face, arms) has been practiced for centuries in Middle East endemic areas for cutaneous forms of the disease (Handman, 2001; Dunning, 2009). Saul Adler (1895-1966), from the Hebrew University of Jerusalem observed in Lebanon how mothers exposed the arms of their children to sandfly bites since on experiential store knew that the first self-healing lesion would protect the children in the future against leishmanial challenge (Gavron, 1997). This process, based on the inoculation of alive and virulent parasites, was traditionally implemented in massive vaccination campaigns in the former Soviet Union, Iran and Israel (Greenblatt, 1980; Kellina, 1981; Nadim et al., 1983).

Probably the most impressive use of this immunization took place in the Isfahan (Iran) area during the first years (1982-1986) of the Irak-Iran war (1980-1988) since over two million people, largely military and revolutionary Guard members, were inoculated with 2-3 x 10^5 promastigotes of *L.major*, with a notable success (reduction to 1/6 to 1/8 of the original disease level) (Nadim et al., 1997). Leishmanization is still employed in some areas of Uzbekistan (Khamesipour et al., 2006) and more recently leishmanization with a Sri Lankan naturally attenuated cutaneous isolate of *L.donovani* has been claimed to protect against VL in a mouse model (McCall et al., 2013). Despite the success in most countries this practice has been abandoned by safety concerns (e.g. uncontrolled development of lesions, exacerbation of concurrent dermal diseases such as psoriasis, immune suppression); practical impossibility of standardization, and lack of efficacy and development of delayed type hypersensitivity in turn related to the variability of virulence and infectivity of the inoculated isolates (Handman, 2001). On these grounds the WHO does not further recommend leishmanization (Reithinger et al., 2007).

Pioneer assays with dead parasites carried out in Brazil in the 1940s yielded inconclusive results. Mayrick et al. (1979, 1985) developed two vaccines with autoclaved parasites. The first one, Leishvacin®, included four *Leishmania* species, and the second only *L.amazonensis*. Both were safe and immunogenic but failed to provide any protective response (Dunning, 2009). Given the simple and economic preparation of these autoclaved vaccines several Phase III clinical trials have been performed with and without BCG (Bacille Calmette-Guérin) both against CL (Sharifi et al., 1998; Monemi et al., 1999; Armijos et al., 2004; Vélez et al., 2005) and VL (Antunes et al., 1986; Khalil et al., 2000). Some vaccine candidates achieved a reduction of CL incidence ranging from 18-78% depending on the study (Marzochi et al., 1998; Shariffi et al., 1998; Monemi et al., 1999) although, in general, efficacy was moderate, low or null (Convit et al., 1987; Genaro et al, 1996; Vélez et al., 2000; Handman, 2001; Armijos et al., 2004). In spite of autoclaving *Leishmania* being an economic solution for endemic areas in developing countries, difficulties of standardization and the destruction of many immunogenic proteins during autoclaving (de Luca et al., 1999) possibly could explain the variable results obtained in clinical trials and therefore precluded the extensive use of this immunoprophylactic approach.

Attenuated *Leishmania* could constitute an alternative since virulence was lowered without compromising the immunogenicity of the parasites. Attenuation can be mainly carried out by two mechanisms, i.e. non-directed and directed (genetically engineered) modifications (Färber & Moll, 2013). In our review only non-directed attenuated parasites are considered first generation vaccines. Reduction of *Leishmania* virulence can be achieved by prolonged *in vitro*

subculture (Mitchell et al., 1985); clonal selection (e.g. thermal sensitivity: Gorczynski, 1985); chemical mutagenesis (Kimsey et al., 1993); gamma ray irradiation (Rivier et al., 1993) and pharmacological selection of clones (Daneshvar et al., 2003), among other possible methods.

The search for safe, stable and efficacious anti-*Leishmania* vaccines lead to the attenuation through genetic manipulation (attenuated live vaccines with directed alterations) of wild *Leishmania* stocks and native and recombinant isolated antigens. Both methodologies would constitute second generation vaccines. Directed mutagenesis aims to reduce the virulence while maintaining the majority of antigens present in wild-type *Leishmania* thus inducing immune response and memory (Färber & Moll, 2013). Several approaches have been employed. Among them knockout *Leishmania* mutants apparently induced protection in experimental models (e.g. Souza et al., 1994; Alexander et al., 1998; Veras et al., 1999; Amaral et al., 2002; Uzonna et al., 2004; Silvestre et al., 2007). The introduction of genes (e.g. conferring sensibility to a given drug) within suicide cassettes in the *Leishmania* genome (Ghedin et al., 1998; Davoudi et al., 2003), although appealing, has the potential risk of reactivation or the reversion of the lowered virulence and have not been tested in animal models (Handman, 2001).

Much safer would be the use of purified fractions or antigens. Among them, Leishmune® was the first registered vaccine for CanL. It is presently marketed in Brazil although it is not registered for Europe (Palatnik de Souza, 2012). The vaccine contains fucose-mannan ligand (FML) from *L.donovani* formulated with saponins (Palatnik de Sousa, 1994). A 54 kDa secretory/excretory antigen of *L.infantum*, LiESAp/MDP, apparently elicited a significant protection in field trials with dogs (Lemesre et al., 2005, 2007). More recently, in 2011, Virbac laboratories registered in Europe the vaccine LiESP/QA-21, under the trade name CaniLeish® (EMA/CVMP/296014/2010). This preparation, similar to the former, uses QA-21 as adjuvant (Oliva et al., 2014). The protection elicited by this vaccine seems to be related to the biased development of a long lasting cell response (Moreno et al., 2012; Martín et al., 2014) diminishing the risk of an active infection thus resulting in milder clinical presentations (Palatnik de Sousa, 2008, 2012). A randomised, double-blind, controlled efficacy trial of the LiESP/QA-21 vaccine in naive dogs exposed to two *L.infantum* transmission seasons has been recently published (Oliva et al., 2014). This study corroborated the reduced risk of progression to an active infection or clinical disease after vaccination although it did not prevent *Leishmania* infection.

This vaccine is currently marketed in Spain, Portugal, Greece and Italy. Its use, however, is not exempt of controversy. Besides the high price (100-150 Euros) it requires annual revaccination and given the interference of post-vaccination antibodies with standard IFAT the

use of a rapid serological test (Speed Leish K™) is recommended. Moreover post-vaccination secondary effects have been reported (Coedo, 2013) although other studies described it was well tolerated (Oliva et al., 2014). It should be stated that the complex standardization of the purification process for the massive production of these vaccines surely will preclude their use in human medicine (Fäber & Moll, 2013). In addition, none of the previously mentioned vaccines protects completely from *Leishmania* infection and therefore the immunoprophylaxis of leishmaniasis has not been yet achieved.

Recombinant antigens could solve some of these hurdles since, in principle, they could be produced at low cost, be safe and standardized. The first recombinant protein used as vaccine was gp63 (leishmaniolysin) (Handman, 2001) and subsequently bacterial and viral expression systems to deliver the recombinant products have been developed, among them *Salmonella typhimurium* (Yang et al., 1990); BCG (Connell et al., 1993); *Toxoplasma gondii* (Ramírez et al., 2001); *Vaccinia* virus (McMahon-Pratt et al., 1993; Ramos et al., 2008) although concerns on their safety and efficacy have been raised. A comprehensive review of the recombinant candidates for leishmanial vaccination during the last decades has been published (Nagill & Kaur, 2011). Most of them have only been tested in animal models and only a reduced number went through clinical trials. One of them, Leish-111f, a recombinant protein with three subunits (Coler et al., 2002), induced a significant protection in murine models of CL and VL when formulated with MPL-SE but failed in the prevention of CanL under natural conditions (Kumar & Engwerda, 2014). In spite of this drawback Leish-111f/MPL-SE is the first defined candidate to be tested in healthy human volunteers in the USA (Fäber & Moll, 2013) and clinical studies have been performed in Brazil, Peru, Colombia and in VL patients in India (Vélez et al., 2009; Nascimento et al., 2010; Llanos-Cuentas et al., 2010; Chakravarty et al., 2011). Another recombinant vaccine, Leish-Tec®, has been registered in Brazil for CanL. The vaccine contains the recombinant A2 antigen from *Leishmania* amastigotes and saponins as adjuvant and a recent study has shown similar efficacy profiles as Leishmune® (Fernandes et al., 2014).

Third generation vaccines (i.e. DNA vaccines) could overcome some of the constraints of native or recombinant protein-based vaccine candidates. Their reduced cost, easy manufacturing and conservation, and stability make this approach attractive. Furthermore they have shown to exhibit a high immunogenic capacity to booster both cellular and antibody immune responses, and multiple genes encoding more than one antigen from one *Leishmania* spp, antigens from different *Leishmania* spp, and even cytokines or adjuvants can be incorporated. Bacterial plasmids containing unmethylated CpG (cytosine-phosphodiester-guanine), as vaccine vectors, provide an additional advantage since CpG motifs have shown to trigger an innate immune response characterized by the production of a predominantly Th1-type cytokines, with increase

of IFN-γ and higher levels of CD8+ T-cell (Klinman, 2004). Nagill & Kaur (2011) and more recently Kumar & Engwerda (2014) have summarized the state of the art of DNA vaccination to control CL and VL.

Scientific community efforts to produce a safe vaccine against leishmaniasis, stable, efficacious, specific, easily manufactured and capable of eliciting long lasting immunological memory continue. However, up to now despite the investments made and the suggestive results using some of the approaches effective immunoprophylaxis of human leishmaniasis has not been yet achieved. By its part the registered vaccines for CanL do not protect against the infection but rather limit the severity of the disease. Thus chemotherapy still is the main available tool to control the extension of this infection in humans and dogs.

1.7.4. Chemotherapeutic control of leishmaniasis

1.7.4.1. Current scenario of the chemotherapy of leishmaniasis

In spite of the high number of drugs, over 25, approved for use in *Leishmania* infections chemotherapy is far from ideal. This large number constitutes an evidence of the shortcomings of the present-day antileishmanial chemotherapy. In the last decades there was a feeling of optimism within the scientific community as new drugs and new formulations of old drugs were undergoing clinical trials (Croft et al., 2006). Actually, since 2002 miltefosine and paromomycin have been registered for the treatment of visceral leishmaniasis. Combination therapy is now increasingly being explored in clinical trials (http://www.dndi.org/diseases-projects/clinical-trial-protocols.html) as a new approach, endorsed by the WHO (2010), to overcome default treatment options.

Nevertheless, the chemotherapeutic arsenal available is still scarce and the efficacy of some compounds is limited and others exhibit high toxicity and severe side effects. Moreover, socio-economic conditions allowing treatment administration -most drugs need monitoring, hospitalization and parenteral administration- are lacking in remote regions. Additionally, variability in the sensitivity of different *Leishmania* species and isolates has been described. More concerning is the high rate of clinical unresponsiveness due to the development of resistance to the most commonly used drug for the past 60 years (i.e. pentavalent antimonials) (Croft & Coombs, 2003; Croft et al., 2006). Furthermore, first line drugs against these infections

have a high price (Sundar & Chatterjee, 2006) making them simply unaffordable under the economic conditions prevailing in many areas of the world where leishmaniasis is endemic.

This scenario has favoured the research and development (R&D) efforts towards the discovery of new treatments for leishmaniasis. However, up to now, the attempts for rational therapy –parasite target-oriented compounds- have yielded only limited success and the deep knowledge on the physiology and molecular biology of *Leishmania* has not yet fostered the development of new drugs. Actually the compounds used in both human medicine and veterinary clinics or those under clinical study are more a consequence of incidental findings than of focused research (Guerin et al., 2002). In 2004 WHO/TDR identified liposomal amphotericin, miltefosine and paromomycin as the most promising anti-*Leishmania* compounds (www.who.int/tdr/diseases/leish). However, none of these compounds is new. Amphotericin B (AmB), in addition to its antifungal properties, has been used as an effective treatment against leishmaniasis for decades; miltefosine (hexadecylphosphocholine) was originally developed as an antineoplastic agent; the aminoglycoside antibiotic paromomycin (aminosidine) has been used as antimicrobial since the 1960s of the past century. Presently, these three drugs and the pentavalent antimonials still constitute the chemotherapeutic agents of reference for the treatment of leishmaniasis.

Pentavalent antimonials -sodium stibogluconate and meglumine antimoniate- are the standard treatment for leishmaniasis in most parts of the world although resistance and treatment failure in the Bihar state (India) and the neighbouring Nepal have been a matter of concern (Croft et al., 2006). These compounds are still effective in other countries alone or in combination although variable drug sensitivity of *Leishmania* has been reported. Other drawbacks of antimonial therapy include length of treatment and toxicity in HIV coinfected patients (Sundar & Chakravarty, 2013).

In the past years, the major chemotherapeutic breakthrough has been the approval of miltefosine as the first oral drug for the treatment of leishmaniasis (Sundar et al., 2002). Although it has showed promising results in clinical trials, there are important concerns regarding its long half-life (>150 h) and the possible emergence of resistance (Bryceson, 2001) in addition to its teratogenicity. This precludes its use during the reproductive life, unless a contraception method is applied (Sindermann & Engel, 2006). Under laboratory conditions, the relatively easy selection of resistant clonal lines of *L. donovani* has been reported (Seifert et al., 2003). Dorlo et al. (2012b) pointed out the high cost of the complete 28 day treatment in monotherapy and also demonstrated that children were relatively underexposed to miltefosine compared to adults using the standard chemotherapeutic schedule. Oral administration and the

absence of control treatment programs may increase the proportion of patients which do not complete the treatment or do a suboptimal compliance of it leading to a high risk of parasite resistance emergence (Sundar & Chakravarty, 2013). Miltefosine relapse probability has been estimated to be of ca. 21% and it has been associated to low levels of drug exposure (Dorlo et al., 2014). Furthermore, only 10 years after its registration, there are increasing evidences of treatment failure, possible resistance emergence and reinfection under field conditions (Rijal et al., 2013; Ostyn et al., 2014).

In 2006 paromomycin was the latest drug registered for VL. Paromomycin is a low cost compound, classified as "orphan drug" (FDA, EMEA) sponsored by the Institute for One World Health (www.iowh.com), after a long time of being used as antimicrobial. Short time course parenteral administration, affordable cost, efficacy and low toxicity make it a potential first line drug against leishmaniasis. Clinical trials carried out in India have established the efficacy of intramuscular route for paromomycin administration. However, there have been regional differences in clinical drug efficacy trials (Sundar & Chakravarty, 2013) and induction of resistant *L. donovani* strains *in vitro* (Maarouf et al., 1998) suggests the possibility of resistance emergence if used in monotherapy.

Amphotericin B deoxycholate is highly efficacious against *Leishmania* infections (particularly *L. donovani* and *L. infantum*) presenting excellent cure rates (i.e. 97%). It is the treatment of choice in India for refractory VL (Mishra et al., 1992) and recommended for PKDL (WHO, 2010). No resistant isolates have been reported even after prolonged therapy in humans coinfected with VIH and *L. infantum* (Durand et al., 1998), although some resistant lines of *L. donovani* and *L. mexicana* have been obtained *in vitro* under pharmacological pressure (Espuelas et al., 2000; Al-Mohammed et al., 2005). However, high toxicity, adverse effects during administration, requiring hospitalization and monitoring, and high cost are the main shortcomings of its therapeutic use. To reduce toxicity several lipid formulations have been developed and three are commercially available [i.e. liposomal amphotericin B (AmBisome®), amphotericin B lipid complex (Abelcet®) and amphotericin B cholesterol dispersion (Amphotec™ formerly known as Amphocil™).

Liposomal amphotericin B (AmBisome®), approved in both USA and Europe is considered the best treatment option for VL caused by *L.donovani* and *L.infantum* (WHO, 2010) in terms of security and efficacy rates (Bern et al., 2006) and is recommended in HIV coinfected patients (Croft & Yardley, 2002). Actually it has been considered that preparations of lower toxicity of AmB would make this compound an almost ideal treatment of visceral leishmaniasis in humans (Alvar et al., 2006). The high price of the treatment with AmBisome® or other lipid-based

formulations is unaffordable in low-income areas. Recently there has been an agreement for price reduction of the liposomal formulation in developing endemic countries (Olliaro & Sundar, 2009). Despite this, high cost of liposomal AmB still restricts its general use in endemic areas (Table 2).

Table 2. Drugs currently used for the treatment of VL (Griensven et al., 2010).

	Manufacturer* (trade name of drug)	Regimen	Clinical efficacy	Resistance	Toxicity	Cost of drug course (US$)	Disadvantages
Pentavalent antimonials (sodium stibogluconate, meglumine antimoniate)	Sodium stibogluconate: Albert David (SSG), GlaxoSmithKline (Pentostam). Meglumine antimoniate: Sanofi Aventis (Glucantime)	20 mg antimony per kg bodyweight daily for 20–30 days (depending on geographic area), intravenous or intramuscular	35–95% (depending on geographic area)	Treatment failure up to 60% (Bihar, India)	Moderately toxic: cardiac effects, pancreatitis, nephrotoxicity, hepatotoxicity	53 (generic) to 198 (branded SSG)	Quality control; length of treatment; painful injection; toxicity; resistance in India
Amphotericin B	Bristol Meyers Squibb (Fungizone); generic companies	1 mg/kg every other day for up to 30 days (15 mg/kg total dose), intravenous	>97% for all regions	Not documented	Moderately toxic: nephrotoxicity (inpatient care required)	About 21 (generic)	Need for slow intravenous infusion; dose-limiting nephrotoxicity; heat instability
Liposomal amphotericin B	Gilead (AmBisome)	5–20 mg/kg total dose in 4–10 doses over 10–20 days, intravenous	Asia: >97%; India, single dose: 91%; Africa: not fully established	Not documented	Nephrotoxicity (limited)	280 (preferential) for 20 mg/kg dose; about 3000 (non-preferential)	Price; need for slow intravenous infusion; heat stability (needs to be stored below 25°C)
Miltefosine	Paladin, Montreal, Canada (Impavido)	2–2·5 mg/kg daily over 28 days (India only), oral	Asia: 94% (India); Africa: 60% (single field study), 93% in patients not infected with HIV and excluding those lost to follow up	Only in laboratory isolates	Gastrointestinal effects (20–55% of patients, usually mild), nephrotoxicity, hepatotoxicity, possibly teratogenic	About 74 (preferential), about 150 (non-preferential)	Price; possibly teratogenic; potential for resistance (half-life); poor patient compliance
Paromomycin sulphate	Institute for OneWorld Health;† Gland Pharma, Hyderabad, India	15 mg/kg daily for 21 days (India only), intramuscular	Asia: 94% (India); Africa: under evaluation	Only in laboratory isolates	Nephrotoxicity, ototoxicity, hepatotoxicity (all relatively rare)	About 15	Efficacy varies between and within regions; potential for resistance?

*Marketing authorisation holder. †Paromomycin was granted orphan drug status by the US Food and Drug Administration and the European Medicines Agency in 2005. Adapted with permission from the Drugs for Neglected Diseases initiative from data presented during Fourth World Congress on Leishmaniasis (Feb 3–7, 2009).

*Miltefosine treatment failure in the field has been recently documented in India and Nepal (Rijal et al., 2013; Ostyn et al., 2014).

1.7.4.2. Chemotherapy of canine leishmaniasis (CanL)

Chemotherapy of infected animals, particularly in the Mediterranean, includes the use of the same compounds employed in the treatment of the human infection (Oliva et al., 2004; Noli & Auxilia, 2005). Chemical structures of the main antileishmanial compounds are illustrated in Figure 14. A large body of information has been obtained by veterinary practitioners, especially

with pentavalent antimonials and combinations, along their clinical practice. However, this experience has hardly been published and the treatment conditions were mostly not controlled, thus failing to meet statistical standards and rendering the data difficult to interpret.

The drugs more frequently employed are pentavalent antimonials, particularly meglumine antimoniate, administered as monotherapy or in combination and miltefosine- amphotericin B and paromomycin have also been used among other compounds- (Vexenat et al., 1998a; Vexenat et al., 1998b; Noli & Auxilia, 2005; Oliva et al., 2010; Podaliri Vulpiani et al., 2011). The variability of treatment schedules (number of doses, total dose administered, administration route, duration of treatment), sometimes contradictory results (i.e. with AmBisome and others) or the use of different systems of post-treatment monitoring have demanded standardized treatment protocols. International efforts –particularly from Spain, Italy and Brazil- for standardization of diagnosis, management, control and prognosis of CanL have recently increased (Baneth, 2013).

In high endemicity regions it is estimated that 30-70% of infected dogs if untreated will develop the disease within the following 2 to 3 years (Roura et al., 2013). None of the drugs available for CanL treatment achieves parasite clearance; neither prevents relapses nor transmission to sandflies. Although clinicopathological improvement or clinical cure can be achieved relapses are frequent as a consequence of parasites' survival within the host (Noli, 1999; Baneth & Shaw, 2002). Prognosis of the animals subjected to antileishmanial treatment is highly unpredictable and depends on the initial clinicopathological and physiological status of the infected dogs, particularly, if present, the degree of renal dysfunction (Roura et al., 2013). Accurate classification of the clinical stage of the disease and the presence of intercurrent infections are crucial when establishing a therapeutic protocol or anticipating the final outcome of the infection (Oliva et al., 2010; Solano-Gallego et al., 2011). Actually it has been stated that affected dogs treated with meglumine antimoniate followed by a subsequent treatment at the time of recurrences have a 75% probability of living four additional years (Noli, 1999).

All therapeutic regimens established for CanL must be accompanied by supportive therapy, appropriate diet and a monitoring system during chemotherapy (clinical and haematological parameters, biochemical profile, serum protein levels, liver and, particularly, renal functionality markers and anti-*Leishmania* antibody levels) (Podaliri Vulpiani et al., 2011). The patient should be periodically re-evaluated to detect disease progression or regression (Roura et al., 2013). Succinctly, there is not only a need for effective treatment schedules with the existing chemotherapeutic agents and for adequate surveillance but also for the discovery of new drugs.

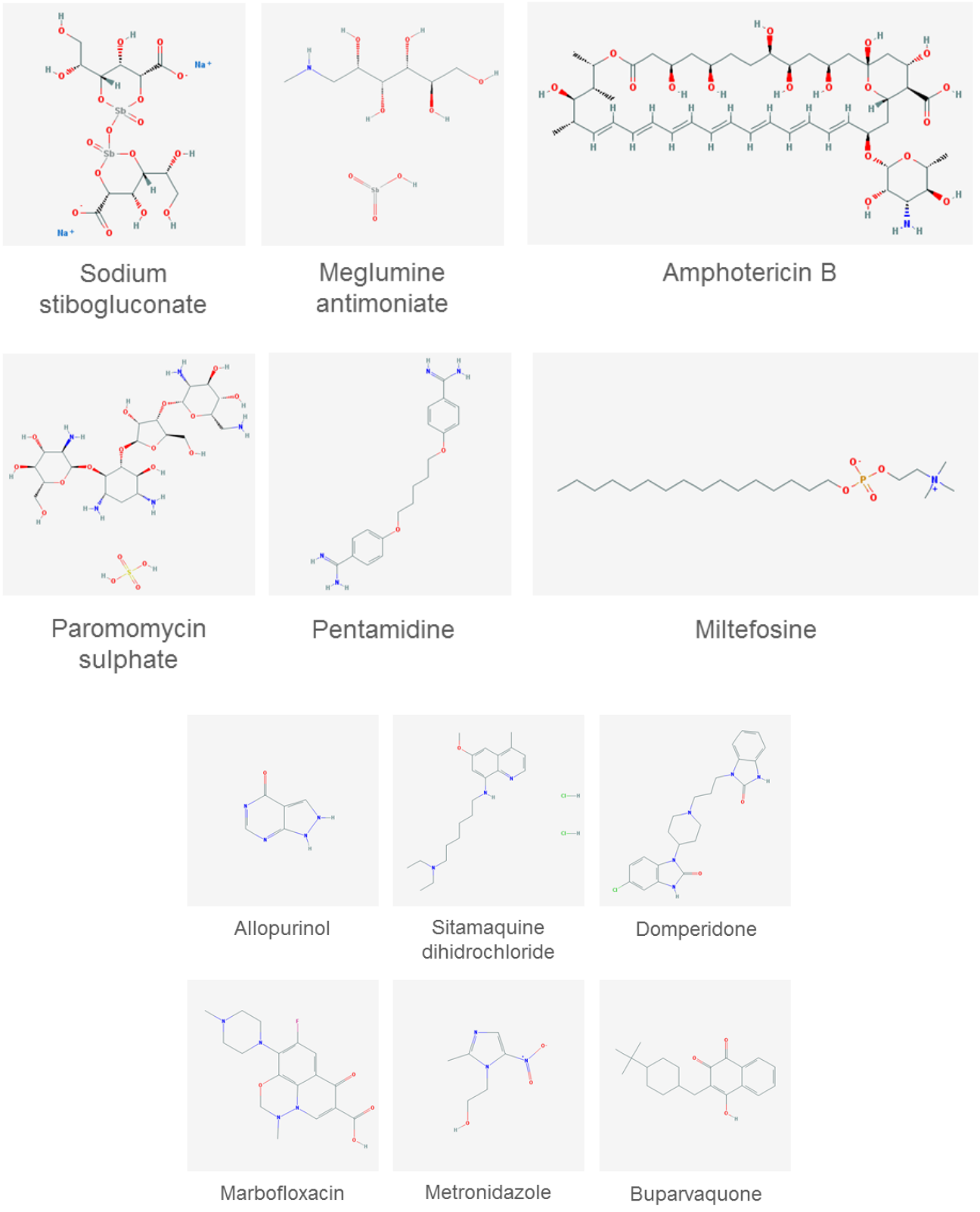

Figure 14. Chemical structures of some relevant compounds with antileishmanial activity. Main compounds employed in the chemotherapy of VL are pentavalent antimonials (i.e. sodium stibogluconate and meglumine antimoniate), miltefosine, amphotericin B and paromomycin. Chemical structures were obtained from Pubchem.

Pentavalent antimonials

Pentavalent antimonials (SbV) have been available since the 1920s and for the last 60 years have been the first line drug for the treatment of leishmaniasis. The development of this less toxic antimonial form is attributed to Brahmachari, Schmidt and Kikuth among others and led to the synthesis in 1937 of antimony gluconate (i.e. Solustibosan®) and in 1945, sodium stibogluconate (i.e. Pentostam®). Soon after the launch of Pentostam, Aventis developed another carbohydrate complex form of SbV, meglumine antimoniate (Glucantime®). Nowadays, there are two marketed formulations, N-methylglucamine (meglumine) antimoniate, containing 85 mg of SbV/mL (Glucantime®, Aventis, France), and sodium stibogluconate (Pentostam® GlaxoSmithKline, UK) with 100 mg of SbV/mL. There are also some cheaper generic antimonial formulations undergoing clinical trials (Croft & Yardley, 2002).

Despite the continued use of antimonials against *Leishmania* for over 50 years, their mechanism of action remains poorly understood and several potential targets have been proposed. To date, still is not clear whether the final active form of pentavalent antimonials is SbIII or whether both oxidation states (SbV and SbIII) present biological activity (Frezard et al., 2013). Two main models have been proposed to address pentavalent antimonials mode of action: (i) the prodrug model, in which SbV will act as a prodrug and will be further reduced in host cells to the more active and toxic trivalent antimonium (SbIII) (Figure 15); and (ii) direct antileishmanial activity of SbV (i.e. inhibition of type I topoisomerase) (Frezard et al., 2013). Interestingly, dramatic differences have been described in the IC_{50}s for SbV against extracellular stages (>64 μg/mL) and intracellular amastigotes (8.8-10.5 μg/mL) of *L. donovani* (Vermeersch et al., 2009).

Some studies reported that a variable amount of SbIII -the most active and toxic form of the antimonial- could be present in the marketed preparations, and this could partially explain the toxicity of the drug (Croft & Yardley, 2002). SbV has been suggested to act as a prodrug, being converted to the more active antileishmanial and toxic SbIII form in the host. Nevertheless, this link between toxicity for the host and antileishmanial activity of SbIII forms has been challenged, since only a marginal effect on *Leishmania* of the residual SbIII in pentavalent antimonial drugs administered could be observed *in vivo* (Dzamitika et al., 2006).

Leishmanicidal activity of antimonials has been related to the selective inhibition of enzymes required for energy metabolism (glycolysis and Krebs cycle) and fatty acids oxidation (Berman et al., 1985). However, SbIII killed promastigotes of *L.mexicana in vitro*, were not affected by SbV and neither the antimonial inhibited hexokinase, phosphofructokinase, pyruvate kinase, malate dehydrogenase and phosphoenolpyruvate carboxykinase (Mottram & Coombs, 1985).

Comparable results were obtained in *L.tropica* promastigotes without any noticeable effect of Glucantime® on the activity of hexokinase and phosphofructokinase (Foulquie et al., 1999). Another possible mechanism of action of antimonials (SbV) is the inhibition of type I DNA topoisomerase (Chakraborty & Majumder, 1988), but no relationship has been found by Walker and Saravia (2004) between leishmanicidal activity of SbIII and inhibition of topoisomerase I in *L. donovani*. In addition, it has been shown that SbIII interferes with trypanothione metabolism by two different mechanisms, namely rapid efflux of intracellular trypanothione and glutathione, and inhibition of trypanothione reductase (Wyllie et al., 2004). Most experimental work has been carried out *in vitro* or *ex vivo* and in some cases with promastigotes. Consequently, in the infected host other mechanisms could be involved.

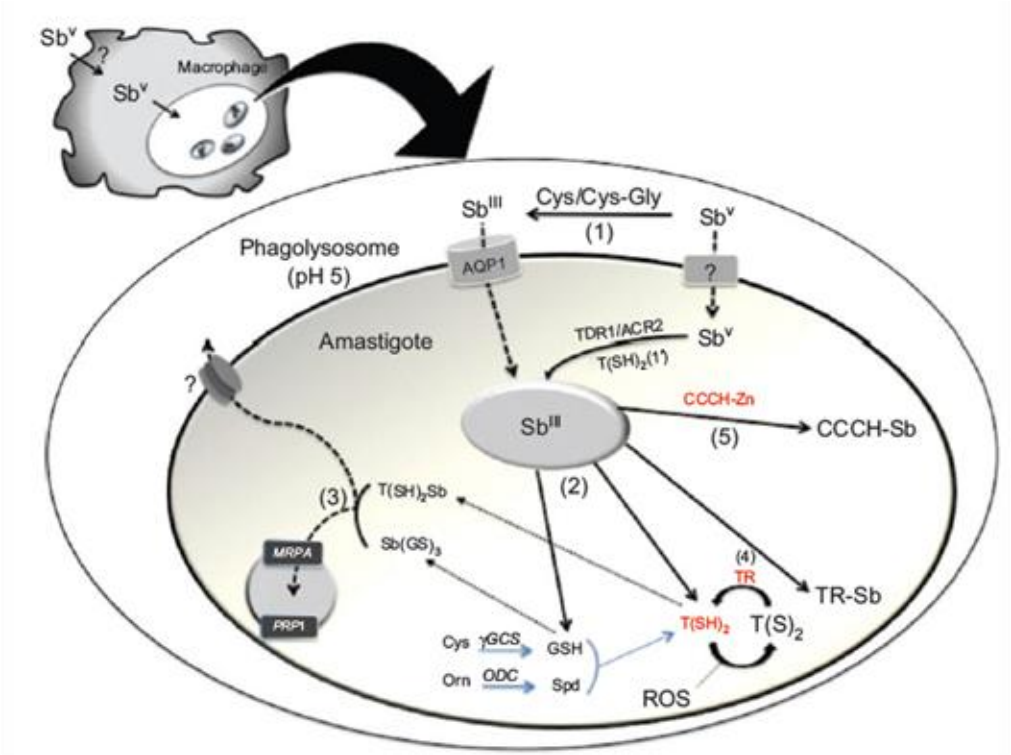

Figure 15. Prodrug model suggested by Frezard et al. (2013) for the mechanism of action of pentavalent antimonials. Reduction of SbV to SbIII may be mediated (1) by the thiols Cys or Cys-Gly as reducing agents within the phagolysosomes, (1') by $T(SH)_2$ as the predominant thiol within parasite or by the thiol-dependent reductase 1 (TDR1) or antimoniate reductase 2 (ACR2); (2) formation of complexes between SbIII and thiols (GSH, $T(SH)_2$); (3) sequestration by ABC transporter multidrug resistance protein A (MRPA) or possibly pentamidine resistance protein 1 (PRP1) into intracellular organelles or active extrusion by a non-identified plasma membrane transporter; (4) binding of SbIII to the active site and inhibition of trypanothione reductase, increasing intracellular ROS production; (5) ejection of Zn(II) from zinc-finger protein by competition with SbIII, resulting in the interference with post-transcriptional regulation of gene expression; (?) means a mechanism not yet elucidated. GSH, glutathione; YGCS, enzyme y-glutamylcysteine synthase; $T(SH)_2$,trypanothione; Spd, spermidine; ODC, ornithine decarboxylase; TR, trypanothione reductase; AQP1, aquaglyceroporin; TDR1, thiol dependent reductase 1; ACR2, antimoniate reductase 2; MRPA, multidrug resistance protein A; Orn, ornithine; ROS, reactive oxygen species.

As mentioned earlier in this review resistance emergence against antimonials is of particular concern in the state of Bihar, India. Mechanisms underlying development of resistance are not completely understood although antimony-resistant lines of *Leishmania* have shown to have increased levels of trypanothione and this likely increases conjugation to the metalloid. This conjugate can either be sequestered inside an organelle through a MRPA (multidrug resistance protein A) transporter or effluxed outside the cell possibly via another ATP-binding cassette (ABC) transporter (Ouellette et al., 2004).

Both Pentostam® and Glucantime® are usually employed in the control of leishmaniasis although N-methylglucamine (meglumine) antimoniate, administered by parenteral or intramuscular route, is the SbV formulation most frequently used for treating leishmaniasis in humans and dogs, particularly in the Mediterranean basin (Oliva et al., 2010). On occasion, they have been recommended to prevent the development of the disease in asymptomatic infected dogs. This pre-symptomatic treatment could, in addition, induce an important reduction of the available parasites for sandflies (Gradoni et al., 1987; Mancianti et al., 1988) thus diminishing the risk of transmission to other dogs or humans.

The use of antimonials is restricted by the side effects, mainly nephrotoxicity (Veiga et al., 1990; Baneth & Shaw 2002), reported. However, toxicity of antimonials is a controversial issue, since the apparent toxicity disappears when the doses are applied more separately (Valladares et al., 1998). Actually, antimonials have a short-half life (21, 42 and 122 min after intravenous, intramuscular and subcutaneous administration, respectively) and are quickly eliminated by urine and do not accumulate; after 6-9 h the 80-95% of meglumine antimoniate is eliminated through the kidneys (Tassi et al., 1994). Possibly the renal toxicity after administration of the antimonial reported in some studies is due to previous kidney damage caused by intraglomerular deposition of circulating immunocomplexes inducing glomerulonephritis (Mancianti et al., 1989; Poli et al., 1991). Oliva et al. (2010) concluded that no scientific evidence is presently available that supports a genuine nephrotoxicity of antimonials in dogs. Other side effects observed after administration include painful local swellings at the site of the injection, gastrointestinal disturbances, locomotory problems, joint stiffness, anorexia and fatigue (Noli, 1999; Baneth & Shaw, 2002; Denerolle & Bourdoiseau, 1999). Apathy has been observed during the first days post-treatment (Ferrer & Roura, 2012).

Meglumine antimoniate induces a rapid improvement of the clinical condition of the infected dogs, temporary recovery of cell-mediated immune response (Bourdoiseau et al., 1997) and a differential IgG_1/IgG_2 pattern up to 5-12 months post-treatment (Fernández-Pérez et al., 2003). This is observed in 75-90% of the animals treated. However, parasitological cure of the dogs is

not achieved and relapses are the rule in ca. 75% of the cases in the following 6 to 8 months post-treatment (Slappendel & Teske, 1997; Gramiccia et al., 1992; Ikeda-García et al., 2007). As observed in human leishmaniasis, the presence of resistant strains of *L. infantum* to meglumine antimoniate in dogs has sporadically been reported (Gramiccia et al., 1992; Carrió & Portús, 2002); this resistance development could be related to inappropriate under-dosage of the drug.

As mentioned, treatment schedules in canine leishmaniasis are variable and this holds also true for meglumine antimoniate dosing. Doses from 40-75 mg/Kg, twice a day for 4 weeks or 75-100 mg/Kg/day administered by subcutaneous injection are currently employed (Tassi et al., 1994; Denerolle & Bourdoiseau, 1999; Noli, 1999; Manna et al., 2008; Solano-Gallego et al., 2011). Separation of daily doses is recommended on the basis of the pharmacokinetic properties to sustain tissue levels. Treatments are supposed to be repeated after relapses and a prolonged treatment (2-3 additional weeks) could be used if no improvement of the animal is observed. Meglumine antimoniate is used as monotherapy, although practitioners favour its combination with allopurinol and to a lesser extent with miltefosine (see below). The most frequent treatment schedule used by veterinary clinicians is meglumine antimoniate (100 mg/Kg/day for 4 weeks) plus allopurinol (10 mg/Kg/per os, twice a day, six to twelve months). Dogs treated with the combination show a prolonged period of clinical remission (Denerolle & Bourdoiseau, 1999). Comparable results have been obtained in naturally infected dogs with *L.infantum* treated with the combination of allopurinol (15 mg/Kg *per os* twice a day until clinical improvement, followed by allopurinol plus sodium stibogluconate, 30 mg/Kg/day, subcutaneously for 1 month, followed by allopurinol at the same dose up to eight months) (Pasa et al., 2005). Adverse effects of the combination are those of the drugs administered in monotherapy.

Some drug delivery systems (DDS), particularly liposomes, have been employed. These formulations have shown promising results as they retain the leishmanicidal ability of this compound and reduce its potential toxicity (Valladares et al., 2001). Despite this apparent success, Schettini et al. (2005) observed that treatment with liposomal forms could not provoke the complete elimination of parasites either. Other formulations of sodium stibogluconate in non-ionic surfactant vesicles have been tested on rodent models (mouse and hamster) and dogs. In all cases the encapsulated forms increased efficacy with lower toxicity when compared to the free form (Nieto et al., 2003).

Allopurinol

Allopurinol is a structural analogue of hypoxanthine that was first used against leishmaniasis in the 1980's. This drug (e.g. Zyloric®) is traditionally used in humans for the treatment of gout as it reduces serum and urinary uric acid concentrations. Its mechanism of action is based on the inhibition of enzymes of purine metabolism such as xanthine oxidase.

In vitro efficacy of allopurinol and allopurinol ribonucleoside against *Leishmania* amastigotes was low (ED_{50} 54-96 μM and 86-213 μM, respectively) and studies in CL murine models revealed also a moderate to low efficacy of the compound *in vivo* (Neal et al., 1985). In *Leishmania*, but not in mammals, this compound is also metabolized to various nucleosides and nucleotides which exert toxicity via incorporation into RNA (Nelson et al., 1979a; 1979b).

Allopurinol does usually not generate side effects, and thus is recommended in individuals with chronic nephritis due to leishmaniasis (Plevraki et al., 2006) or in locations where first choice drugs are not available. Nevertheless, it has been shown that this leishmaniostatic compound alone has poor efficacy in terms of parasite elimination (Cavaliero et al., 1999; Koutinas et al., 2001; Oliva et al., 2010) and does not prevent infection of healthy individuals (Saridomichelakis et al., 2005). In spite of this limitation it has been used in canine leishmaniasis both in monotherapy or combined with antimonials.

Doses are variable ranging from 5-30 mg/Kg/every twelve hours *per os*. The treatment should be maintained for at least six months (Noli, 1999; Oliva et al., 2010) and is recommended to be preferably continued for 12 months (Roura et al., 2013). The most frequently used dose is 10 mg/Kg/twice a day (Cavaliero et al., 1999; Solano-Gallego et al., 2011). Side effects are not common but hepatic and renal function must be monitored during prolonged treatment since xanthinuria and xanthine urolythiasis can occur particularly in dogs with hepatic disease (Saridomichelakis et al., 2005). A moderate clinical improvement of the animals treated with allopurinol has been described (Cavaliero et al., 1999; Vercammen et al., 2002) and 20 mg/Kg/day, one week every month, of this compound were effective to maintain clinical remission in naturally infected dogs. Additionally, allopurinol has been reported to ameliorate renal function, preventing deterioration of glomerular filtration rate in infected dogs with proteinuria, provided that there is no renal insufficiency (Plevraki et al., 2006).

Since parasitological cure is not achieved (Koutinas et al., 2001), relapses appear once the administration of allopurinol is interrupted (Ginel et al., 1998; Cavaliero et al., 1999). The comparatively poor efficacy of allopurinol monotherapy demands combination with antimonials or miltefosine, except for cases with severe renal injury (Solano-Gallego et al., 2011).

Miltefosine and Alkylphosphocholines

Miltefosine (hexadecylphosphocholine) was originally developed as an antineoplastic by its ability to induce apoptosis selectively in tumour cells. The use of the oral formulation for the treatment of solid tumours was discontinued due to dose-limiting gastrointestinal side effects in cancer patients (Dorlo et al., 2012a).

First *in vitro* and *in vivo* activity of miltefosine against *Leishmania* was described by Croft et al. (1987). Interspecific variability to miltefosine has been demonstrated in *Leishmania* parasites; *L.donovani* intracellular amastigotes (EC_{50} 3.3-4.6 μM = 1.3-1.9 μg/mL) were more susceptible than *L.major* (EC_{50} 31.6-37.2 μM = 31.6-37.2 μg/mL) and other cutaneous species of *Leishmania* (Escobar et al., 2002). Furthermore, remarkable intraspecific variability of miltefosine efficacy was observed in different *L.donovani* isolates from Nepal (EC_{50} 0.04-8.7 μg/mL) and Peru (EC_{50} 8.4 and >30 μg/mL) (Yardley et al., 2005). *L infantum* canine and human isolates also displayed variable susceptibility to miltefosine (IC_{50} for intracellular amastigotes from 4.12 to 69.5 μM) (Maia et al., 2013). Some other derivatives of alkyllysophospholipids such as edelfosine or ilmofosine have also shown antileishmanial activity (in promastigotes and amastigotes) *in vitro* (Escobar et al., 2002; Azzouz et al., 2005). Efficacy of oral miltefosine against VL was first demonstrated *in vivo* in a murine model of *L.donovani* (Kuhlencord et al., 1992) and Le Fichoux et al. (1998) described that miltefosine was also able to reduce by 89% the parasite burden in spleen and liver in BALB/c mice infected with *L. infantum*.

Miltefosine mechanism of action is not completely known and several potential targets have been proposed, probably indicating a multitarget mode of action (Figure 16) (Dorlo et al., 2012). Activity of hexadecylphosphocholine has been mainly linked to a disturbance of lipid-dependent cell signalling pathways and an apoptotic-like cell death. Miltefosine altered the ether lipid metabolism and the synthesis of some virulence-linked surface molecules of *Leishmania* [i.e. glycosylphosphatidylinositol (GPI) anchor biosynthesis] (Lux et al., 1996). Moreover, miltefosine-resitant strains have shown decreased ergosterol levels and modification of fatty-acids (i.e. length and level of unsaturation) suggesting that fatty-acid and sterol metabolism could be also affected by miltefosine treatment (Rakotomanga et al., 2005). Miltefosine treated promastigotes (Paris et al., 2004; Marinho et al., 2011) and amastigotes (Verma & Dey, 2004) showed phenotypic characteristics of an apoptotic-like cell death similar to that described for metazoans. Mitochondrial dysfunction in miltefosine-treated promastigotes has also been examined (Santa-Rita et al., 2004). As reviewed by Dorlo et al. (2012a), miltefosine, besides its antileishmanial activity, has demonstrated immunomodulatory properties by inducing the

release of some cytokines (e.g. IL-12, IFN-γ, TNF-α), NO production in macrophages, and enhancement of IFN-γ receptors, therefore, increasing IFN-γ response in infected Mφ promoting an IL-12 mediated Th1 response. However, other authors did not observe an up-regulatory effect in MHC-II or co-stimulatory molecules or an induction of IL-12, IL-10 and TNF-α in *L.major* infected dendritic cells (Griewank et al., 2010). Selection of miltefosine-resistant *Leishmania* promastigotes (15-fold less susceptible to miltefosine) (Pérez-Victoria et al., 2003b) and amastigotes (Hendrickx et al., 2014) under laboratory conditions has been relatively easy. Increased drug efflux and defect in drug internalization have been proposed as possible mechanisms of resistance (Pérez-Victoria et al., 2003a, 2003b).

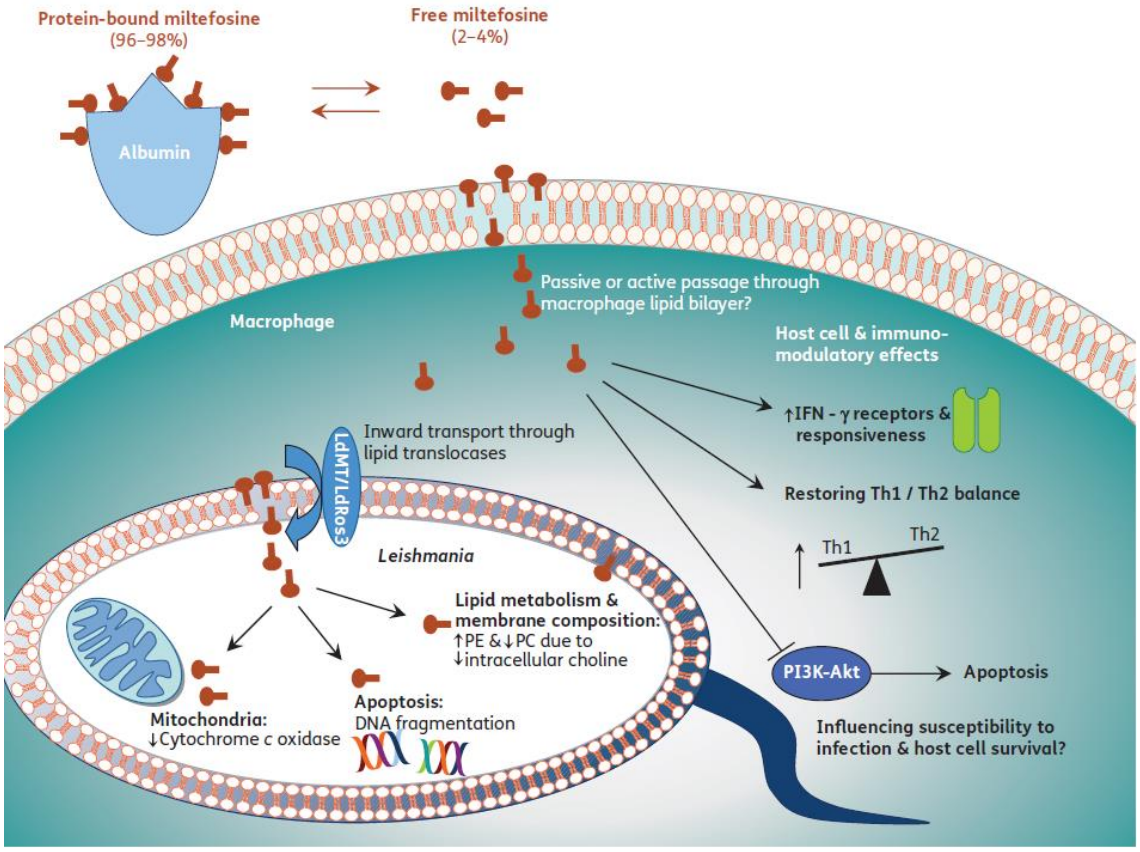

Figure 16. The various proposed mechanisms of action of miltefosine against *Leishmania* inside the macrophage. PC, phosphatidylserine. Image from Dorlo et al., 2012a.

Promising results (i.e. 94 % cure rate) in phase II and phase III clinical trials in India lead to the approval of miltefosine in 2002 for the treatment of VL in humans in India (Sundar et al., 2006). Impavido® is the commercially available oral miltefosine for humans and there is also a

marketed veterinary formulation to treat CanL, Milteforan®, registered for its use in European countries but not approved by the FDA (Oliva et al., 2010). Gastrointestinal adverse effects (i.e. vomiting and diarrhoea) have been described as the main acute toxic effects of miltefosine in humans or dogs (Olliaro et al, 2003).Treatment of naturally infected dogs with *L. infantum* using 2 mg/Kg/day miltefosine for 28 consecutive days yielded clinical improvement of the animals, recovery of pre-treatment haematologic abnormalities (50%) and seronegativity (IFAT test) in ca. 50% of pre-trial seropositive dogs (Woerly et al., 2009). Comparable results were obtained by Mateo et al. (2009) using the same treatment schedule. Another study carried out in Brazil evaluated miltefosine treatment using three different therapeutic regimens (100 mg/animal, administered daily over a 28 day period; 200 mg/animal, administered daily over a 28 day period; and 100 mg/animal, administered daily for 45 days) in naturally infected dogs (n=14) during the following 24 months post-treatment. Although clinical improvement and recovery was evident, parasitological cure was never achieved; and 6 months after treatment all animals presented a significant increase in parasite load. The authors discourage clinicians from using miltefosine in the treatment of CanL in endemic areas of Brazil (Andrade et al., 2011).

Combination of miltefosine with allopurinol (2 mg/Kg/day, oral administration miltefosine plus 10 mg allopurinol/Kg/day *per os*, PO) for 30 days induced a rapid improvement of the clinical condition of severely affected dogs (one week after starting the treatment) and recovery of normal renal function after two weeks (Manna et al., 2005). Impressive clinical recovery with progressive reduction of *L. infantum* burden in lymph node aspirates and increase of IFN-γ was observed during an extended follow-up period after various miltefosine-based treatment regimens; however parasitological clearance was not achieved, as evidenced by PCR performed 24 months after treatment (Manna et al., 2005). In 2009, Manna et al. evaluated in 28 infected dogs the efficacy of the combination of miltefosine (2 mg/Kg/day, PO) with allopurinol (10 mg/Kg/day, PO) for 30 days, followed by allopurinol alone at the same dosage for 12 months. Eight dogs received a second miltefosine cycle and 4/8 relapsed, and although clinical amelioration was observed, even two miltefosine cycles failed to eliminate the infection.

The main limitation of miltefosine is the induction of fetal malformations which restricts its use to non-fertile individuals or those under contraceptive treatment (Sinderman & Engel, 2006). In addition, the potential of this compound to generate resistant *Leishmania* under field conditions is high and treatment failure and relapse rates are increasing (Rijal et al., 2013; Sundar & Chakravarty, 2013; Ostyn et al., 2014). The ability of miltefosine-exposed *Leishmania* to overexpress a transporter protein expelling the drug from the parasite, the long half-life (>150 h) of the compound and the prolonged treatment required, often associated with subtherapeutic drug levels, favour rapid emergence of resistances (Pérez-Victoria et al., 2006).

Treatment failure has been recently associated to exposure of parasites to low levels of the drug (Dorlo et al., 2014). Other risk factors as reinfection, emergence of resistant *Leishmania* isolates or reduced treatment quality should be also assessed as possible relapse sources. Decreased efficacy over the years has been highlighted by several authors (Ostyn et al., 2014) with final cure rates of 96.7% (Jha et al., 1999), 82% (Bhattacharya et al., 2007) and 72% (Rahman et al., 2011). Moreover, oral administration of miltefosine, a logistic advantage to treat human leishmaniasis in remote regions even with poor sanitary standards, implies lacking control of resistance development (Sundar & Chakravarty, 2013). Similar considerations can be made and should be evaluated in a veterinary scenario.

Another alkylphosphocholine analogue, oleylphosphocholine (OlPC, Dafra Pharma Research & Development, Belgium) has demonstrated to be equipotent to miltefosine *in vitro* and orally effective and tolerable in a hamster model of VL (Fortin et al., 2012), in a murine model of CL (Fortin et al., 2014), delivered intralesionally in tattooed liposomes (Shio et al., 2014) and in dogs with CanL (Hernández et al., 2014). No information is available about the teratogenicity, pharmacokinetics -in particular regarding the half-life of the compound- and resistance selection under laboratorial conditions of this miltefosine analogue.

Amphotericin B

Amphotericin B (AmB) is a broad spectrum polyene macrolide antibiotic obtained from the fermentation of the fungus *Streptomyces nodosus*. Its leishmanicidal capacity was discovered in the 60s of the past century (Croft et al., 2006). The compound was mainly used as antifungal, especially for the treatment of systemic mycoses. Its mechanism of action has been related to the ability to bind to sterols, preferentially ergosterol, in cell membranes of *Leishmania* and fungi. The binding of AmB to sterols results in perturbation of the cell membrane structure and formation of pores composed of AmB-sterol aggregates causing membrane depolarization and leakage of ions as K^+ leading to cell death (Brajtburg et al., 1990; Ramos et al., 1996). Actually, this primary mechanism of action could explain why clinically significant resistance to AmB is so rare (Gray et al., 2012). However, some studies point toward a more complex mode of action since pore formation does not necessarily lead to cell death (Baginski & Czub, 2009). AmB can produce cellular auto-oxidation and generation of free radicals (Lamy-Freund et al., 1985). Thus, cell death could be a result of oxidative stress and ionic membrane permeability (Brajtburg et al., 1990b). AmB-resistant *Leishmania* lines have been obtained *in vitro* and resistance has been correlated to an increased cell membrane fluidity and substitution of ergosterol by one of its precursors (mainly, cholesta-5,7,24-trien-3β-ol) which presents lower

affinity for AmB (Mbongo et al., 1998). Moreover, an increased expression of MDR1 (multidrug resistant protein 1 transporter) -an ABC transporter- was evident in resistant parasites, suggesting a higher AmB efflux. Antioxidant defence in resistant parasites was also modified as shown by the up-regulation of the thiol metabolic pathway (Purkait et al., 2012)

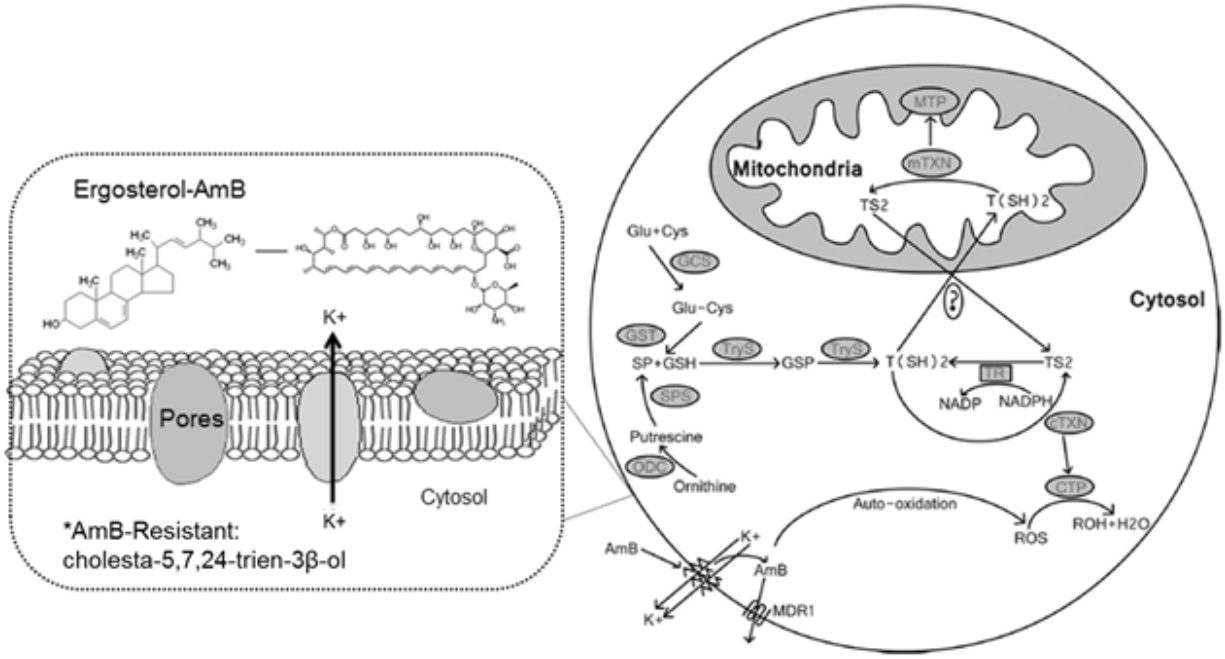

Figure 17. Amphotericin B mechanism of action in *Leishmania* and cellular events conferring amphotericin B resistance. AmB binds to ergosterol-containing membranes in *Leishmania* parasites, causing membrane depolarization and ionic leakage leading to cell death. Oxidative stress contributes to cell injury and death of *Leishmania*. In resistant strains ergosterol is replaced by cholesta-5,7,24-trien-3β-ol (with low AmB affinity) in the cell membrane. However, it is possible that certain amount of AmB molecules can enter the cell, but some may also be pumped out by MDR1. Remaining intracellular AmB auto-oxidizes generating ROS production. ROS production may be counteracted by the up-regulated tryparedoxin cascade of the thiol metabolic pathway. GSH, glutathione; Cys, cysteine; $T(SH)_2$, trypanothione, SP, spermidine; ODC, ornithine decarboxylase; TR, trypanothione reductase; TryS, trypanothione synthetase; SPS, spermidine synthetase; GCS, glutamylcysteine synthetase; GST, Glutathione S-transferase; cTXN, tryparedoxin; CTP, cytidine triphosphate; ROS, reactive oxygen species; MDR1, multidrug resistant protein 1. Modified from Purkait et al., 2012.

AmB presents excellent *in vitro* activities against promastigotes and intracellular amastigotes of *L.donovani* (IC_{50} 0.1-0.4 μM) (Vermeersch et al., 2009) and *L.infantum* (IC_{50} 0.04-0.71 μM) (Maia et al., 2013). AmB is marketed in a colloidal suspension of AmB deoxycholate to be administered parenterally. A large number of studies have shown the leishmanicidal efficacy of this compound, although its high toxicity, adverse effects during infusion and cost hamper its general use. Toxicity for mammals has been attributed to the similarity between ergosterol from the *Leishmania* membranes and cholesterol, the major sterol

in mammalian cells, causing nonspecific binding and thereby altering potassium permeability. AmB affinity for sterols, and therefore toxicity, depends on its state of aggregation: the monomeric form of AmB does not interact with ergosterol, whereas its affinity increases with aggregation (Gruda & Dussaul, 1988). Moreover, the sterol composition of the membrane also influences the selectivity of AmB for ergosterol (Barwicz & Tancrède, 1997), which could contribute to differential sensitivity of *Leishmania* species to AmB.

Amphotericin has been employed in human medicine as a second choice drug against leishmaniasis but its use is increasing due to the emerging resistance to antimonials (Croft & Yardley, 2002; Croft et al., 2006). To reduce its adverse effect in the mammalian host many drug delivery systems (DDS) have been developed (Sundar & Chakravarty, 2013). The leishmanicidal drug reaches the actual intracellular location where *Leishmania* amastigotes multiply. In turn, the AmB trapped in large-size liposomes can no longer interact with mammalian sterols and accordingly the toxicity for the host is substantially lowered. The most successful has been AmBisome®, a formulation of AmB in sonicated liposomes. Another liposomal preparation (Fungisome), developed in India in 2003, indeed yielded most promising results in kala-azar patients (100% cure after one month and 90% sustained cure 6 months after treatment) with doses of 10 mg/Kg (twice 5mg/Kg) (Mondal et al., 2010). Non-liposomal DDS of AmB in clinical use are Amphocil®, a colloidal dispersion of AmB in cholesterol, and Abelcet®, a lipid complex which, in fact, was the first lipid formulation of AmB that was approved by the FDA for the treatment of fungal infections (Berman et al., 1992; Berman et al., 1998; Guerin et al., 2002).

New affordable formulations (e.g. AmB lipid emulsion, ABLE) have shown encouraging results in phase II studies. In September 2014 Sundar et al. published a phase III clinical trial in India (326/500 patients completed the study) which evaluated the efficacy and safety of single dose parenteral infusion of ABLE (15 mg/Kg) *versus* liposomal AmB. Results showed that ABLE was well tolerated [i.e. infusion-related problems were comparable in both groups, severe side effects were scarce (ABLE: 0.3%; L-AmB: 1.6%) and hepato-nephrotoxicity was not observed in either group] and had favourable efficacy [i.e. clinical improvement was comparable (ABLE: 98.9%; L-AmB: 98.4%) and cure was achieved in 85.9% in the ABLE treated group compared to 98.4% with L-AmB]. Single-dose administration is a clear asset in terms of treatment compliance, access and cost.

Over the last years alternative DDS of AmB of lower cost and higher stability (i.e. niosomes, nanodisks, polymers conjugates, micro/nano-polymeric particles) have been tested against experimental leishmaniasis. Nanospheres of poly(ε-caprolactone) with AmB have been

employed *in vitro* against *L.donovani* amastigotes (Espuelas et al., 2002). These nanospheres, coated with poloxamer 188, have also been used to treat fungal infections (Espuelas et al., 2003). AmB conjugated with arabinogalactan (Golenser et al., 1999) and or associated to nanodisks (Nelson et al., 2006) displayed low toxicity and high leishmanicidal activity in mice infected with *L. major*. Using a polysaccharide matrix with anionic lipids, AmB retained the leishmanicidal activity against *L.donovani* infections and was substantially less toxic in a mouse model (Loiseau et al., 2002). Similarly, lecithin microemulsions (Moreno et al., 2001) or nanospheres of egg albumin (Santhi et al., 1999) had lower toxicity than the free antibiotic. Also superaggregated AmB, obtained by heat treatment, has a reduced toxicity (Petit et al., 1999; Bau et al., 2003). Human albumin microspheres containing AmB have been tested *in vitro* and *in vivo* models (hamster) of *L.infantum* infection. This low cost DDS showed a reduced toxicity (10-fold) when compared to AmB deoxycholate and similar leishmanicidal properties (Sánchez-Brunete et al., 2004; Ordóñez-Gutiérrez et al., 2007).

Dogs naturally infected with *L.infantum* have been treated with different dosage regimens of AmB (1 to 2-5 mg/Kg, twice per week, slow intravenous infusion in normal saline followed by 10 mL/Kg mannitol 20%). All animals receiving a total dose over 10 mg/Kg were apparently cured after treatment, and 14 out of 17 were PCR-negative (Lamothe, 2001). With the same emulsion all dogs were clinically cured and five months post treatment the 38% of the dogs were positive by PCR (Cortadellas, 2003). The main side effect of AmB associated to the treatment of canine leishmaniasis is nephrotoxicity caused by renal vasoconstriction and possibly by its direct action on renal epithelial cells (Baneth & Shaw, 2002). It may also cause fever, vomiting, anorexia and periphlebitis (Noli & Auxilia, 2005). AmBisome® (3-5 doses of 3-3.3 mg/Kg, intravenous injection) administered to naturally infected treated dogs elicited a rapid clinical improvement although parasite clearance was only achieved in one dog (Oliva et al., 1995). Despite its excellent cure rates in treated dogs it is generally not used due to the complexity of preparation and the need of monitoring during administration (Oliva et al., 2010).

It has often been stated that the use of AmB for the treatment of canine leishmaniasis is not recommended, since parasite clearance is not achieved and resistance development appears possible (Oliva et al., 2004, 2010; Alvar et al., 2006). Nevertheless, its mechanism of action does not likely favours resistance emergence (Gray et al., 2012) and no resistant strains of *L. infantum*, despite the long history of AmB use in human medicine, have ever been identified in humans (Durand et al., 1998; Maia et al., 2013) or dogs (Maia et al., 2013). This restriction is indeed surprising since AmB was, and still is, the preferred treatment for systemic fungal infections in humans and domestic animals including dogs (Vorathavorn et al., 2013) and

frequent exposure of *L. infantum* to this antibiotic must be rated as more than likely over the fifty years since its introduction.

Paromomycin (aminosidine)

Paromomycin (PM) is an aminoglycoside antibiotic produced by *Streptomyces* spp. This low cost compound shows both antibacterial and antiprotozoal activity (Baneth & Shaw, 2002). It is the last antileishmanial drug approved for the treatment of VL in India (Sundar et al., 2007) and parenteral and intramuscular formulations are available for the treatment of VL and topical formulations have been used for CL (Sundar & Chakravarty, 2013). Parenteral formulations seem an attractive alternative to antimony treatment in endemic areas. There is a consensus that monotherapy with 15 mg/Kg/day for 20 days is well tolerated and efficacious (i.e. cure rates 80-90%) (Jha et al., 1998). Nonetheless, toxicity, relapses and *in vitro* selection of resistant strains are the main drawbacks for its use in monotherapy (Seifert, 2011; Hendrickx et al., 2014).

Antileishmanial susceptibility to paromomycin has been proven variable among different *Leishmania* spp. This drug resulted more active against intracellular amastigotes of *L.major* and *L.tropica* (i.e. ED_{50}s in the range of 1-5 μM) than to *L. mexicana* (ED_{50}, 39 μM), *L.braziliensis* (ED_{50}, 12 μM) or *L.donovani* (ED_{50}, 6-18 μM) (Croft et al., 2006). Mechanism of action of aminosidine in *L.donovani* promastigotes has been related to its effect on RNA synthesis and on membrane permeability affecting its fluidity and the membranous lipid metabolism (Maarouf et al., 1997a). Mitochondrial dysfunction has been also observed in paromomycin exposed promastigotes (Maarouf et al., 1997b; Jhingran et al., 2009). Stable and infective resistant *Leishmania* strains have been easily developed under laboratory conditions (Maarouf et al., 1998). Resistant parasites have shown *in vitro* decreased drug uptake (Jhingran et al., 2009) and more recently Bhandari et al. (2014) confirmed an increase in membrane fluidity and paromomycin efflux, accompanied by a decrease in intracellular drug accumulation, and a superior tolerance to nitrosative stress at the promastigote and amastigote stages. In addition, an increased survival capacity was observed in paromomycin resistant strains in this study.

Topical paromomycin has shown antileishmanial activity in experimental murine models of CL (El-On & Hamburger, 1987) and resulted effective in the treatment of *L.major* infected patients (i.e. 74.2% achieved parasitological cure after 10 days of topical treatment) (El-On et al., 1992). A parenteral formulation of this drug reduced the parasite burden in a model infection of *L. infantum* in mice, although the efficacy was lower than that obtained with antimonials (Gangneux et al., 1997). Different trials have shown the clinical efficacy of aminosidine for the

treatment of CanL (Poli et al., 1997; Oliva et al., 1998; Vexenat et al., 1998a). However, aminosidine is not likely suited for monotherapy given the relapses observed 50-100 days after treatment with a dose of 20 mg/Kg/day and the casualties associated with higher doses (80 mg/Kg/day) (Vexenat et al., 1998a). Nephrotoxicity and ototoxicity represent the main adverse effects (Oliva et al., 2010). In 2005, Noli & Auxilia proposed a dosage of 5 mg/Kg twice daily for 3-4 weeks although they anticipated that relapses should be expected within a few months. Lately, two studies carried out in healthy and *L. infantum* naturally infected dogs evaluated the efficacy, safety and pharmacokinetics of aminosidine (15 mg/Kg/day, once daily, subcutaneous route, 21 days). Improvement of the clinicopathological status of the animals was observed with this regimen although infection persisted in tissues (Athanasiou et al., 2013). Single-dose aminosidine was well tolerated, without signs of nephrotoxicity, and resulted in effective serum concentrations of the drug.

A combined therapy of aminosidine and antimonials appears more realistic, since interference between these antileishmanial agents has not been observed and renal function was not altered after treatment of healthy Beagle dogs (Belloli et al., 1999). Oliva et al. (2010) suggested a combination of aminosidine (5 mg/Kg, subcutaneous, daily for 3 weeks) plus 60 mg meglumine antimoniate (intramuscular, twice a day for 4 weeks). In murine models of infection, the combination of antimonials and aminosidine proved to be more efficacious than the individual drugs, but still the parasites persisted in liver and spleen and toxicity was not abrogated (Gangneux et al., 1997). Various combinations of paromomycin with other drugs are now being explored for the treatment of leishmaniasis *in vitro* (Morais-Teixeira et al., 2014; PM + miltefosine, Das et al., 2014), in animal models [PM + stearylamine (SA)-bearing phosphatidylcholine (PC) liposomes, Banerjee et al., 2011] and in clinical trials (PM + gentamicin, Sosa et al., 2013; PM + sodium stibogluconate, Musa et al., 2012).

Pentamidine

Pentamidine isethionate is an aromatic diamidine used to treat pneumonia caused by *Pneumocystis carinii*, an opportunistic fungal infection frequent in inmunocompromissed patients and also described in dogs (Sukura et al., 1996). The drug also has antiparasitic properties against *Leishmania*, *Trypanosoma* and *Plasmodium* (Pearson & Hewlett, 1985). Currently, pentamidine is still used for the treatment of first stage human African trypanosomiasis (HAT) (Babokhov et al, 2013), for some forms of CL, and is considered a second-line drug for the treatment of VL (Seifert, 2011). In the 1980s, this drug was used for the treatment of refractory VL in India; it has been abandoned due to safety concerns as it has been

associated with the induction of insulin-dependent diabetes mellitus and low efficacy (Jha et al., 1991). A formulation for parenteral use (Pentacarinat®) is available. A closely related compound, pentamidine dimetasulfonate (Lomidine®), is also marketed (Baneth & Shaw, 2002; Guerin et al., 2002). The mechanism of action of pentamidine is believed comprise induction of changes in DNA conformation as a consequence of its double binding to DNA and ubiquitin (Nguewa et al., 2005), inhibition of polyamine metabolism and interference with purine metabolism (Calonge et al., 1996; Johnson et al., 1998).

In CanL, an apparently complete clinical and immunologic recovery has been observed after pentamidine treatment (Rhalem et al., 1999) but relapses occur. Treatment of dogs with pentamidine was carried out during the 80s but was discontinued as a result of adverse side effects including irritation at the injection site, vomiting, diarrhoea, hypersalivation, tachycardia, hypotension and anaphylactic shock (Baneth & Shaw, 2002; Oliva et al., 2010). As with other antileishmanial compounds, various DDS have been investigated (nanospheres of polylactic acid and polymethacrylate containing pentamidine), and the results obtained in mouse models of *L. infantum* infections showed a significant increase of efficacy when compared to the free compound (Durand et al., 1997a, 1997b).

Domperidone

Domperidone is a dopamine D2 receptor antagonist used as an antiemetic and prokinetic compound. Marketed as Motilium®, oral domperidone has been used since the 1980s in humans for the treatment of gastrointestinal disorders (Brogden et al., 1982) and immunomodulatory properties have been also ascribed to this molecule. Domperidone transiently increases prolactinemia (Rovensky et al., 1995, 1996). Prolactin is known to induce the increase of the CD4+ Th1 cellular subpopulation releasing IL-2, IL-12, IFN-γ, TNF-α, and thus, resulting in activation of Mφ and natural killer (NK) cells (Sabaté et al., 2014). Cell-mediated immune response has been classically related to protection against *Leishmania*.

Gómez-Ochoa et al. (2009) reported the development of a Th1-cell mediated immune response in naturally *L.infantum* infected dogs (n=98) after oral treatment with domperidone (1mg/Kg, twice daily for 30 days) and its efficacy in controlling and diminishing clinical signs. Domperidone, combined with other antileishmanials (e.g. meglumine antimoniate or furazolidone), has been used to treat CanL by *L.infantum* (Lanaro, 2012) and by *L.(V.)braziliensis* (Passos et al., 2014). Although infection was not cleared, relapse rate was decreased and improvement of the clinical signs was accelerated. Prophylactic use of domperidone (0.5 mg/Kg/day 30 consecutive months, every 4 months) has been explored in

healthy dogs (n= 90) during a 21-month follow-up period; results showed a 7-fold reduction of the risk of developing an active infection (Sabaté et al., 2014).

An oral veterinary formulation is available in Spain since 2012, Leishguard® (Esteve), for the prevention and control of disease progression in early stages of CanL. Adverse effects described are mild and include galactorrhea, gastrointestinal disturbances (diarrhoea, anorexia, and abdominal pain) and apathy. Nonetheless, its use in humans has been recently restricted by the EMA (European Medicines Agency) (EMA/129231/2014) by its association with heart failure (Hondeghem, 2013) this probably leading to a re-evaluation of its use in veterinary therapeutics.

Sitamaquine (WR-6026) and 8-aminoquinolines

The 8-aminoquinolines were the first group of compounds specifically synthesized as antiplasmodials. United States military initiated a large-scale research programme, in the 1940s, with the aim of synthesizing less toxic and more efficacious antimalarials. This programme finally rendered three new compounds: pentaquine, isopentaquine and, the most effective, primaquine (Grewal, 1981). Since their discovery, this series of compounds has aroused interest in the search for new antileishmanials (e.g. 6-methoxy-8-alkylpiperazinoalkylaminoquinoline derivatives: Beveridge et al., 1958; lepidines: Kinnamon et al., 1978; NPC1161B: Nanayakkara et al., 2008; tafenoquine: Yardley et al., 2010).

Sitamaquine (WR-6026), an 8-aminoquinoline analogue, was synthesized at the Walter Reed Army Institute (WRAIR) and since 2002 is in clinical development by GlaxoSmithKline (GSK, UK) for the oral treatment of VL (Yeates, 2002). Mechanism of action of the molecule remains unknown. Sitamaquine accumulates inside acidocalcisomes of *Leishmania*, yet the antileishmanial effect is not related with its accumulation (López-Martín et al., 2008). Loss of mitochondrial membrane potential (Vercesi et al., 1992) and oxidative stress (Carvalho et al., 2011) have also been observed in treated cells. Coimbra et al. (2010) demonstrated that sitamaquine has high affinity towards anionic phospholipids and interacts with lipid monolayers in *L.donovani* promastigotes. These authors proposed that sitamaquine diffuses across the cell membrane according to an energy-independent electrical gradient and a sterol-dependent process. The drug can be effluxed in an energy-dependent manner, suggestive of the existence of a still uncharacterized transporter.

In vitro experiments proved that sitamaquine had activity (ED_{50}, 2.9-19.0 µM) against a variety of *Leishmania* spp. (Garnier et al., 2006). It has been proposed that variable

susceptibility can be related to the anionic phospholipid and sterol composition of different *Leishmania* spp plasma membranes (Coimbra et al., 2010). Recently, in an *in vitro* drug combination study carried out on intracellular amastigotes of *L. infantum*, sitamaquine's IC_{50} resulted of 2.92 ± 0.20 µM (Mesquita et al., 2013). Kinnamon et al. (1978) tested sitamaquine in a hamsters infected with *L.donovani* and results showed that this compound was more active than sodium stibogluconate. Conversely, no activity was observed after topical administration in a murine model of CL (BALB/c, *L.major*) (Garnier et al., 2006).

This compound has completed phase II clinical trials in Kenya (cure rate: 83%: Wasunna et al., 2005) and India (cure rate: 87%: Jha et al., 2005). Sitamaquine properties as a drug candidate against *Leishmania* have been reviewed elsewhere (Loiseau et al., 2011). Main side effects described in these trials were abdominal pain, headache, vomiting, cyanosis and, more concerning, methemoglobinemia (not in Kenya) and renal toxicity with doses > 2.5 mg/Kg. In a clinical trial in HIV infected patients methemoglobinemia was dose-limiting (3/6 patients had methemoglobine levels >20%) (Petty et al., 1999). Nonetheless, another phase II clinical trial carried out in Brazil with *L. infantum* infected patients showed discouraging results as low cure rates and lack of increasing efficacy with escalating doses were observed (Dietze et al., 2001). Moreover, even though its half-life is short (i.e. 29.1 h in humans) (Yeates, 2002), there is a risk of resistance emergence in the field as stable resistant strains of *L.donovani* can be easily obtained under laboratory conditions (Bories et al., 2008) and these cells show a change in drug accumulation and lipid metabolism (Imbert et al., 2014).

Data regarding pharmacokinetics and bioavailability in Beagle dogs defined oral sitamaquine as a drug of relatively high systemic clearance, large volume of distribution (100% orally available), short half-life and low systemic availability (4%), probably due to pre-systematic liver metabolism (Taylor et al., 1991). Toxicity studies in dogs after oral administration of sitamaquine revealed a mean of 30% methemoglobine in the treated animals (3 mg/Kg/day for 4 weeks) (Levine et al., 1997) and of 20.7% after a 4 consecutive day treatment with 0.0116 mmol/Kg sitamaquine (Anders et al., 1988). Whether or not sitamaquine is a good candidate for the treatment of CanL needs further research including metabolites involved and toxicity.

Marbofloxacin and fluoroquinolones

Fluoroquinolones, which target DNA topoisomerase type II and gyrase, are a group of broad spectrum antibiotics extensively used for the treatment of human respiratory and urinary infections (e.g. ciprofloxacin). DNA topoisomerases have been proposed as interesting

molecular targets in the search for new antikinetoplastid agents. These enzymes play key roles in cellular functions and there is evidence that human and parasite topoisomerases are sufficiently distinct, therefore making them a potential chemotherapeutic target (Das et al., 2004). Fluoroquinolones have demonstrated leishmanicidal (Croft & Hogg, 1988) and trypanocidal activity by promoting the formation of protein DNA covalent complexes (Nenortas et al., 1999).

Marbofloxacin is a synthetic third generation fluoroquinolone antibiotic approved for veterinary use that has shown *in vitro* antileishmanial activity (Oliva et al., 2010). A trial to assess its efficacy was carried out in Greece in naturally infected dogs with *L.infantum* (n=24) and results suggested amelioration of the clinical status in the treated animals (Rougiers et al., 2008). In 2012, Rougiers et al., reported the results obtained in a field trial with marbofloxacin (n=72 dogs) in different endemic areas (i.e. Spain, Italy and France) with a follow-up period of 12 months. Regimen used in the study was a dose of 2 mg/Kg/day Marbocyl®, orally administered for 28 consecutive days. Clinical improvement was evident after 3 months in 61% of the treated animals and 10 animals were clinically cured after 3 months. However, none of the animals achieved parasite clearance and relapse rates were high (20/38 dogs 5.5 months after treatment completion).

Metronidazole and azoles

Azoles are inhibitors of the ergosterol synthetic pathway. Ergosterol is the main sterol existing in Fungi, *Leishmania* spp. and *Trypanosoma* spp. (Croft et al., 2006). This similarity explains the interest antifungal azoles have raised as potential antileishmanial agents. A number of azoles (e.g. ketoconazole, imidazole, itraconazole, metronidazole, fluconazole, posaconazole) have been investigated for activity against *Leishmania* spp. although stage and species sensitivity variation have been observed *in vitro*, *in vivo* and in clinical trials (Croft & Yardley, 2002; Paniz Mondolfi et al., 2011; Sundar & Chakravarty, 2013). This disparity of results observed between promastigotes and amastigotes has been related to the ability of the latter to take up cholesterol from the host cell under drug pressure (Roberts et al., 2003).

In CanL their efficacy has been lower than that of SbV antimonials (Baneth & Shaw, 2002). Metronidazole has also been tested in combination with spiramycin (Pennisi et al., 2005) or enrofloxacin (Bianciardi et al., 2004) has been evaluated in the treatment of CanL. None of the combined therapies cleared the infection. Moreover, clinical efficacy was moderate and better results were obtained in the antimonial treated groups.

Recently, fexinidazole, a compound patented by Merial (patent application nº 20140213624), has been proposed as a potential oral drug candidate for the treatment of VL (Wyllie et al., 2012). In fact, currently DND*i* is recruiting participants in Sudan for a phase II/III trial to study its efficacy against VL caused by *L.donovani* (clinicalTrial.gov identifier: NCT01980199). Results obtained with this nitroimidazole against *L.donovani* in murine models have shown a decrease in parasite burden of 98.4%. The biological activity and pharmacokinetics in dogs suggest the interest of this drug as a good candidate for further testing against CanL.

Buparvaquone and derivatives (naphtoquinones)

Buparvaquone (BPQ) is a hydroxynaphtoquinone currently marketed as Butalex® for the treatment of theileriosis in cattle. *Theileria* is an intracellular Apicomplexa that parasitizes T and B lymphocytes. *In vitro* antileishmanial activity of BPQ and other naphtoquinones was described in the early 1990s. In spite of encouraging *in vitro* activity, limited success was observed in a murine model of *L donovani* infection (62% reduction of parasite burden in liver) (Croft et al., 1992). Due to BPQ's physicochemical properties (i.e. low aqueous solubility and high lipophilicity) its phosphate prodrugs (i.e. buparvaquone-3-phosphate, 3-phosphonooxymethyl-buparvaquone) were studied in an attempt to increase bioavailability. Strong *in vitro* inhibitory activities were observed against *Leishmania* spp. (Mäntylä et al., 2004). *In vivo* studies, on cutaneous and visceral mouse models of leishmaniasis, revealed the efficacy of different topical formulations of BPQ for CL and that buparvaquone-3-phosphate was the most active antileishmanial molecule against *L.donovani* murine infection (Garnier et al., 2007).

A liposomal BPQ formulation containing phosphatidylserine (BPQ-PS-LP) has recently been proved efficacious *in vitro* against promastigotes and amastigotes of *L.chagasi* and its therapeutic efficacy has also been explored in a hamster model. This formulation (0.33 mg/Kg/day for eight days) reduced spleen (89.4%) and liver (67.2%) parasite burden and, although *in vitro* efficacy against intracellular *Leishmania* spp. amastigotes of free BPQ was high (IC_{50}=1-4 µM), BPQ (20 mg/Kg/day, 8 days), failed to cure hamsters (Reimão et al., 2012). Information regarding efficacy of BPQ to treat CanL is scarce. Only one study is available and BPQ (5 mg/Kg/day for 12 days) was ineffective (Vexenat et al., 1998b).

1.7.5. Current strategies to improve the chemotherapy of VL

On previous grounds the need for novel and efficacious antileishmanial treatments seems evident. In 2005, Pink et al. thoroughly reviewed the current scenario regarding the challenges in drug discovery against parasitic neglected diseases. They concluded that the overall cost for developing a new antiparasitic or anti-infectious agent is not higher or technically more demanding than that of other drugs targeting different pathologies. Certainly, the attrition rate in the development of new therapeutic agents is very high (Herper, 2013). However, in the case of antiparasitic agents this situation is not so evident. In fact, it has been determined that success rate in some cases (i.e. antimalarials) is higher than that of other medications. In spite of the high morbidity and mortality rates of these diseases investment has been limited (Figure 18). In the time-period corresponding to 1975-1999 more than 1300 new drugs or presentations were launched to the market and only 13 of them were indicated for the treatment of neglected diseases caused by helminths and protozoa. Malaria, trypanosomiasis, leishmaniasis and tuberculosis together are responsible for 5% of the world's disease burden and, in 2000, only 0.1% of the global health budget was dedicated for the discovery of drugs against these diseases.

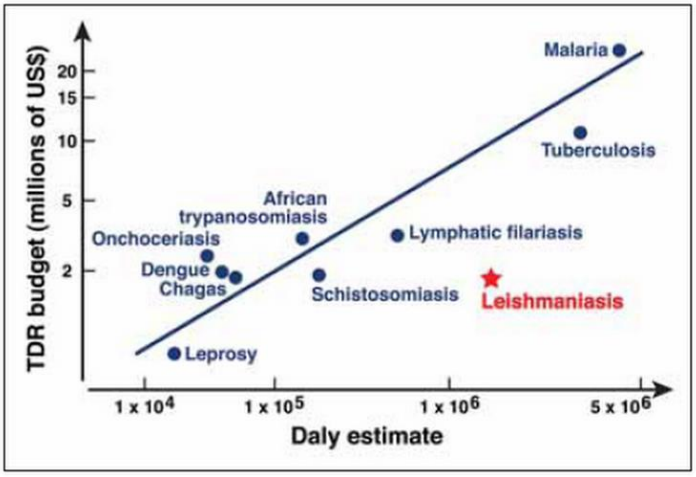

Figure 18. TDR investment in neglected diseases in relation with disability-adjusted life years (DALY) estimates. The DALY estimates determine the level of WHO/TDR funding for research; as a disease has higher prevalence and incidence, the higher the budget for its research and development. Image from Handman et al., 2008.

Given the comparatively low research and development (R&D) effort on drug discovery it is not surprising that the majority of compounds employed in the treatment of leishmaniasis were originally developed for other indications. This opportunistic "piggy-back" strategy (“new indications for old drugs”), although less expensive, is a useless tool for the identification of

new drug targets (e.g. better use of the genome sequence of *Leishmania*, distinct metabolic pathways and regulation systems) and for the introduction of entirely different chemical compounds. In the face of resistance emergence and treatment failure, this trend seems to be slowly changing. Some pharmaceutical companies and foundations (e.g. Bill & Melinda Gates Foundation) have growing interest in the development of compounds for non-profitable diseases (Handman et al., 2008). Moreover, the establishment of some public-private partnerships (e.g. Drugs for Neglected Diseases Initiative, DND*i*; Institute for One World Health, IOWH) has increased the research efforts for rationale antiparasitic chemotherapy development in the last years (Pink et al., 2005). Besides, novel academia-industry partnerships as a mean to increase innovation in pharmaceutical R&D are becoming more frequent and highly successful if adequately funded (e.g. Cancer Research Technology, UK). It is suggested that academia together with the biotech industry can form a new "front end" to the early discovery phases of drug development; further preclinical, clinical development phases and commercialization can be then taken by pharma companies (Tralau-Stewart et al., 2009). An analysis on the actual reasons underlying the scarce efficiency of the drug discovery by pharmaceutical companies is beyond the purpose of this review. Probably the causes are multiple and besides those described previously and the anticipated low returns of antiparasitic drugs, other factors related to their R&D structure and internal expenses' distribution would be involved (Cuatrecasas, 2006).

In the particular case of leishmaniasis current therapy is insufficient to control disease and infection in endemic areas. Present day chemotherapy has serious shortcomings including toxicity and side effects induced by some drugs; high cost of the most effective presentations, length of treatments and resistance phenomena described in many *Leishmania* isolates. In the absence of efficacious vaccines, novel therapeutic options are compulsory (Sundar & Chakravarty, 2013).

The search for novel, safe, affordable and effective antileishmanial chemotherapies has followed different strategies. Approaches can be classified as short-to-medium term (i.e. new indications for existing drugs, combination therapy, improvements in known drugs and compound classes) or long-term (e.g. discovery of new synthetic or natural agents, immunochemotherapy) (Pink et al., 2005; Croft et al. 2006; Seifert, 2011; Mendes-Roatt et al., 2014). In addition, incorporation of drug delivery systems (DDS) as a complementary strategy to develop new formulations of old and novel compounds and combinations represents another approach for therapy improvement (Pham et al., 2013). We will focus, in our review, on the three most suggestive strategies, namely combination therapy, development of DDS and the discovery of new drugs.

1.7.5.1. Combination therapy

In the coming years, even supposing new drugs are being developed, there are fair evidences to support that compounds now available for leishmaniasis are going to still constitute the main chemotherapeutic arsenal against the disease with its limitations (see Table 2). Thus, already miltefosine efficacy has diminished in the field and resistance is a growing concern as in the case of antimonials among other drugs (Sundar & Chakravarty, 2013). Combination therapy has recently attracted interest in the treatment of neglected diseases (e.g. malaria, tuberculosis, HIV, leishmaniasis) as a mean of prolonging life-span of existing drugs (Bryceson, 2001).

It is assumed that the combination of chemically different entities can reduce needed dosage or length of treatment, thus lowering associated toxicity, attaining higher compliance and, therefore, decreasing direct and indirect costs ensuing less burden on the health systems (Griensven et al., 2010). Moreover, combined therapy may be suitable to diminish resistance emergence and increase treatment efficacy (Croft et al., 2006) improving therapy options in complicated situations such as HIV coinfection (WHO, 2010) or in solid organ transplants (Antinori et al., 2008). From a pharmacologic perspective, distinct mechanisms of action are recommended. A similar pharmacokinetic profile would be propitious as development of double-resistant parasites is improbable (Griensven et al., 2010). Another advantage would be short half-life and a rapid elimination phase, as selection of resistant strains is more probable to occur when parasites are exposed to subtherapeutic concentrations of the drugs (Stepniewska & White, 2008).

There are clear cut differences in the exploration of combination therapy in human and veterinary medicine. The information on the combined use of effective antileishmanial drugs on humans or even in preclinical studies is very scarce although there is a growing number in the last years (e.g. Seifert & Croft, 2006; Serrano-Martín et al., 2009; Seifert, 2011). On the contrary, in the veterinary arena this strategy has been traditionally used for the treatment of CanL as a relatively successful and safe chemotherapeutic approach (e.g. Oliva et al., 2010; Solano-Gallego et al., 2011). However, drugs combined, dosage and schedules were generally based on empirical observation and not on the knowledge of the drug-drug-host interaction. A more thorough development would need a deep understanding on the actual behaviour of the drugs combined (e.g. mechanism of action, pharmacokinetics).

Generally speaking drugs in combination can exert effects greater than, equal to, or less than the sum of their individual potencies. Relationships between molecules are therefore classified as synergistic, additive or antagonistic. The classic approach to study the nature of the drug-drug interaction is the "chequerboard" approximation, in which two serial dilutions of the test

compounds are used to calculate the fractional inhibitory concentration (FIC) index. This parameter will quantify if the effect of combination is synergistic (i.e. FIC > 1) or antagonistic (i.e. FIC< 1). However, this straightforward and popular method is prone to reproducibility errors thus leading to contradictory conclusions (Odds, 2003). To overcome the limitations of the chequerboard approaches some models of interaction have been developed (Tallarida, 2006). Among them, a modified three-dimensional analytical method to characterize drug-drug interactions, developed by Prichard et al. (1993), and the combination index (CI) method developed by Chou & Talalay (1984). This method is based on the multiple drug effect equation derived from the median-effect principle and the mass action law and allows the quantification of synergism among other parameters (Chou, 2010). The fine understanding of the interaction between drugs administered simultaneously to an infected host would allow the establishment of more accurate dosage and schedules.

Table 3. Clinical trials on combination therapy for VL (Griensven et al., 2010).

	Study design	ClinicalTrials.gov registration	Country	Study period	Patients enrolled	Drug combinations studied	Definitive cure (95% CI) at 9 months in intention-to-treat analysis
Completed trials							
Sundar et al[31]	Phase 2, randomised, non-comparative, group-sequential trial	NCT00370825	India	2008–08	181 adults: 45 each in groups A, C, D and E; 46 in group B	Group A: SD L-AmB 5 mg/kg alone; group B: SD L-AmB 5 mg/kg followed by miltefosine 100 mg for 14 days; group C: SD L-AmB 5 mg/kg followed by miltefosine 100 mg for 10 days; group D: SD L-AmB 3·75 mg/kg followed by miltefosine 100 mg for 14 days; group E*: SD L-AmB 5 mg/kg followed by miltefosine 100 mg for 7 days	Group A: 91% (78–97%); group B: 98% (87–100%); group C: 96% (84–99%); group D: 96% (84–99%); group E: 98% (87–100%)
Planned or ongoing trials							
DNDI (VLCOMBO-07 trial)[33]	Phase 3, randomised, open-label, non-inferiority trial	NCT00696969	India, Bangladesh, and Nepal	2008–09 (India), 2009–10 (Bangladesh, Nepal)	624 adults and children†	Group 1: AmB 1 mg/kg every other day for 30 days; group 2: SD L-AmB 5 mg/kg followed by miltefosine 2·5 mg/kg for 7 days; group 3: SD L-AmB 5 mg/kg followed by paromomycin 15 mg/kg for 10 days; group 4: miltefosine 2·5 mg/kg plus paromomycin 15 mg/kg for 10 days	..
Banaras Hindu University[32]	Phase 2, non-randomised, open-label, historical control, safety/efficacy trial	NCT00371995	India	Ongoing	150 adults and children†	SD L-AmB 5 mg/kg followed by miltefosine 2·5 mg/kg for 14 days	..
TDR‡	Phase 2	..	Bangladesh	Planned	150†	SD L-AmB 5 mg/kg followed by miltefosine 2·5 mg/kg for 14 days	..
DNDi (LEAP0104A/B trial)[34]	Phase 3, randomised, open-label, active control, safety/efficacy trial	NCT00255567	Kenya, Ethiopia, Sudan, and Uganda	2004–09 (LEAP0104A), 2007–10 (LEAP0104B)	1100 adults and children†	Active comparator group 1: SSG 20 mg/kg for 30 days; group 2: paromomycin 15 mg/kg (LEAP0104A) or 20 mg/kg (LEAP0104B) for 21 days;§ group 3: SSG 20 mg/kg plus paromomycin 15 mg/kg for 17 days	..
DNDi (VLCOMBO trial)¶	Phase 2, exploratory, randomised, open-label trial	..	Sudan and Kenya	Planned for 2009–10	189 adults and children; 63 in each group	L-AmB followed by miltefosine; L-AmB followed by SSG; miltefosine 2·5 mg/kg for 30 days.	..

*Non-randomised group assigned when it had become apparent that all other regimens were effective. †Estimated. ‡B Arana, WHO Special Programme for Research and Training in Tropical Diseases, personal communication, Nov 3, 2009. §Due to poor outcomes on paromomycin 15 mg/kg in monotherapy in Sudan in LEAP0104A, the dose for paromomycin monotherapy was increased to 20 mg/kg in LEAP0104B. ¶Our unpublished data. AmB=amphotericin B. DNDi=Drugs for Neglected Diseases initiative. L-AmB=Liposomal amphotericin B. LEAP=leishmaniasis in east Africa platform. SD=single-dose. SSG=sodium stibogluconate. TDR=WHO Special Programme for Research and Training in Tropical Diseases. VLCOMBO=visceral leishmaniasis combination treatment.

Data available from recent human drug combinatory clinical trials are encouraging. Several phase II trials have identified combined therapeutic regimens that have resulted efficacious, well-tolerated, safe and of shorter duration than those of the equivalent monotherapy regimens. Table 3 resumes the completed and on-going studies (in 2010) on combination therapy for VL in East Africa and India. Irrational use of antimonials has probably led to the appearance of resistance and unresponsiveness particularly in Bihar, India (Croft et al., 2006). Caution should be taken as *in vitro* resistance to drug combinations has been already reported in *L.donovani* promastigotes (García-Hernández et al., 2012). Therefore, in the absence of alternative compounds, close and effective monitoring and regulatory policies are essential to avoid misuse and guarantee proper implementation of the regimens.

1.7.5.2. Drug Delivery Systems (DDS)

Drug delivery systems (DDS) were designed to provide an alternative mean of administering single or combined compounds -old and new drugs- enhancing drug efficacy while minimizing side effects. DDS are therefore used to improve drug solubility; modify ADMET (absorption, distribution, metabolism, excretion and toxicity) profile (e.g. improved absorption, increased residency time); enhance target localization in cells (e.g. macrophages) or tissues; prevent degradation in biological fluids (e.g. gastric degradation) allowing the exploration of new administration routes; and reduce toxicity-related adverse effects. Different DDS systems developed for the treatment of VL have been recently reviewed elsewhere (Pham et al., 2013). Liposomes and nanoparticles are suitable carriers for VL chemotherapeutic agents as these particles are phagocytised by *Leishmania* host cells in target organs (liver and spleen). Figure 19 illustrates a schematic representation of different DDS.

The DDS more extensively used in the treatment of VL is Liposomal-amphotericin B (i.e. L-AmB, AmBisome®) (Sundar & Chakravarty, 2013). As described in previously in this review (see AmB section) other lipid based DDS have been used for AmB release. Liposomal-encapsulated antimonials have also been developed (Alving et al., 1978; Schettini et al., 2005). Liposomes were developed by Bangham in 1985 and commercialized for the first time in 1986 as the liposomal-anti-aging product Capture (Dior) (Müller et al., 2000). They are vesicles formed from one or more phospholipid bilayers with an internal aqueous core. Liposomal-based formulations have been reported to prolong *in vivo* the circulation time of the drug, therefore, modifying its toxicity and distribution. Liposomes are readily internalized by cells of mononuclear phagocytic system (MPS) and this is a clear advantage in the treatment of intracellular parasites as *Leishmania*. Moreover, liposomal surface can be modified to improve

macrophage uptake (e.g. mannosilated, fucosylated or phosphatidylserine-coated liposomes) (Reimão et al., 2012; Pham et al., 2013). Among limitations it has been indicated the high price and toxicity of liposomes, linked to leakage of free drug during administration (Berman, 1997), or the possibility of development of accumulation-related side-effects due to prolonged concentration of drugs in the liver (Alving, 1983).

Niosomes as carriers are a good alternative to liposomes. Niosomes are vesicles, similar to the latter, composed of non-ionic surfactants and cholesterol, which are cheaper, biodegradable, relatively nontoxic, and chemically more stable, therefore, offering a better drug retention. They may also release the drugs in a controlled manner (Kazi et al., 2010). Biodegradable polymeric nanoparticles (NPs) were developed in the 1970s (Pham et al., 2013). They are more stable than liposomes and also offer controlled release properties, can target different organs/tissues and are also used in DNA gene therapy.

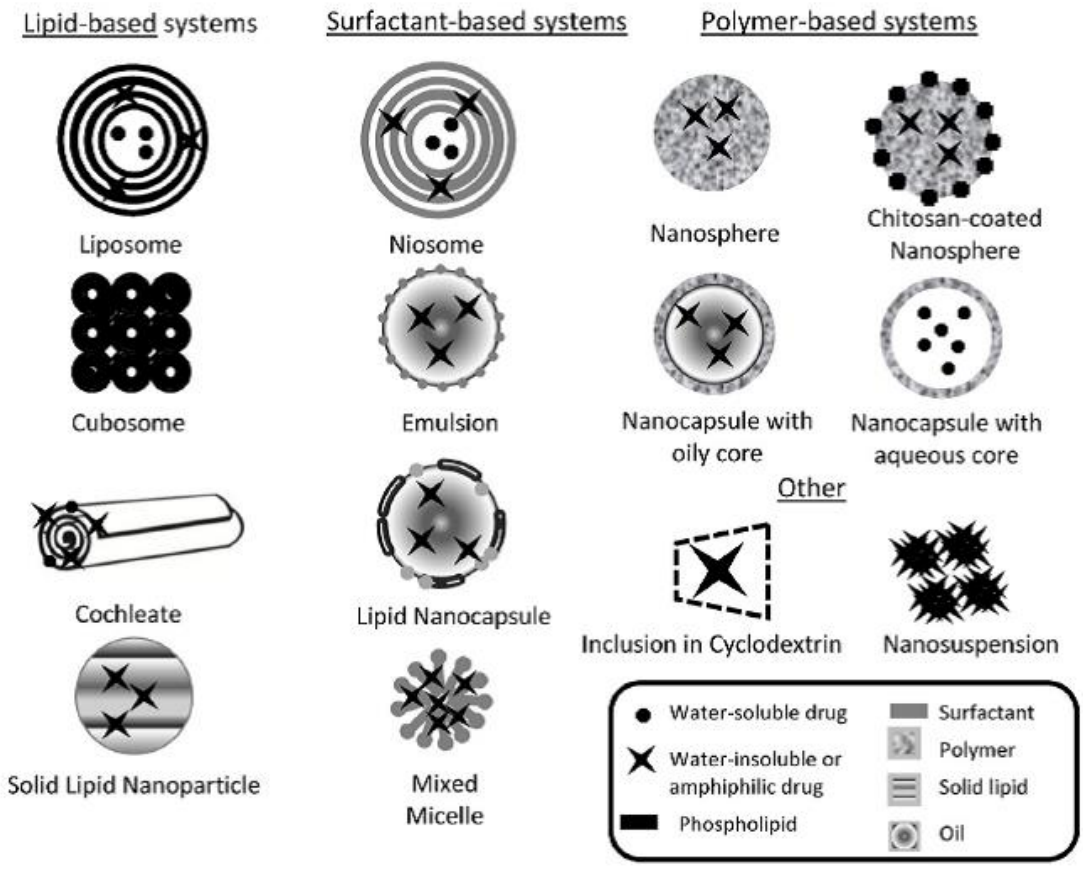

Figure 19. Schematic illustration of different drug delivery systems (DDS) (not to scale). Image from Pham et al., 2013.

Moreover, NPs are rapidly internalized by macrophages. NPs (100-1000 nm) can be classified into nanospheres or nanocapsules. Different hydrophobic polymers have been used for NPs synthesis [e.g. poly (lactic acid), PLA; poly (glycolic acid), PGA; poly (lactide-co-glycolide), PLGA; poly (cyanocrylate); polyalkylcyanocrylates, PACA] (Soppimath et al., 2001). Second-generation NPs -included NPs based on chitosan, gelatin, sodium alginate, hydrophilic biodegradable polymers- try to slow down macrophage uptake and act as carriers to other tissues (e.g. poly (ethylene glycol), PGE; polys(ε-caprolactone)s (Pham et al., 2013). Müller et al. (2000) highlighted that the number of marketed NPs containing products is scarce mainly due to cytotoxic effects of polymers and the lack of suitable massive scale production. Solid lipid NPs, introduced in the 1990s, are a secure alternative to classic NPs and large scale production is feasible making them attractive carriers (Müller et al., 2000). This DDS as other lipid-based carriers are rapidly cleared from plasma to liver and spleen yet their use has not been deeply explored in the treatment of parasitic diseases (Pham et al., 2013). Encouraging results were obtained with AmB (Lemke et al., 2005). Critical reviews on the use of DDS against *Leishmania* have been published by Romero & Morilla (2008) and Pham et al. (2013), the latter particularly focused on the development of oral drugs.

1.7.5.3. New Drugs

No ideal drug to control leishmaniasis, human or canine, is presently available. Many of the drugs currently used against leishmaniasis are expensive and toxic, and the treatments of choice are losing their effectiveness due to the emergence of resistance (Handman et al., 2008). As previously stated the geographical distribution of leishmaniasis has not been reduced and even re-emergence phenomena related to human migrations (e.g. wars, famine) and bioclimatic changes have been described. Therefore the need for new antileishmanial drugs still is a challenge for the industrial sector and the academia.

By both scientific and economic reasons drug discovery processes quickly integrated the advances from biomedical sciences, particularly molecular biology, and engineering. It was assumed that the use of the powerful techniques developed would reduce costs and the time invested to obtain safer drugs, affordable and in a shorter discovery time-line (Koehn & Carter, 2005) besides providing a rational approach to drug discovery. After the realization that the vast majority of drug targets are proteins, molecular biology exerted a profound influence on drug

discovery, through parasite gene cloning and expression, identification of differential genes of hosts and parasites, identification of new targets and the search for small "druggable" molecules (Handman et al., 2008). Moreover, these available tools would fit the discrete and iterative process prevailing in drug discovery and could be easily adapted, through robotics, to the molecular target identification and validation by High Throughput Screening (HTS) (Figure 20). It has been assumed that this process together with the criteria for suitable anti-parasite hits, leads and drug candidates (Table 4) would offer adequate tools for development of new drugs (Pink et al., 2005).

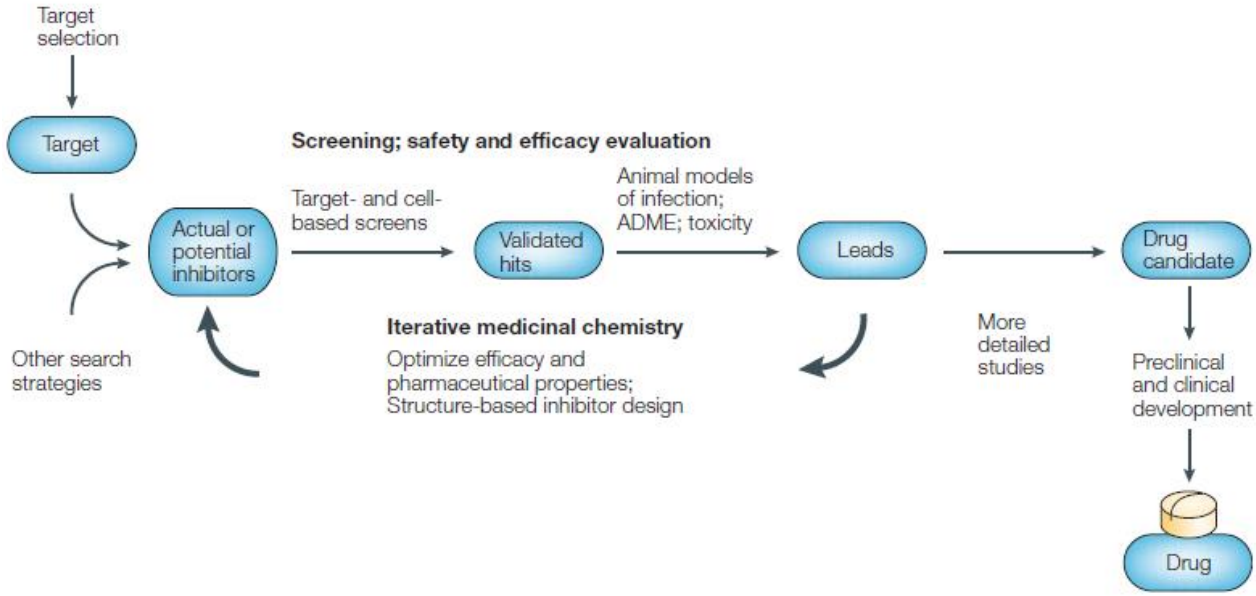

Figure 20. Drug discovery process and main stages. Identification and validation of molecular targets. If the target is unknown, compounds are tested for activity against the whole parasite. Molecules (actual or potential inhibitors) are screened in target or cell-based assays. Hits are defined as compounds that have shown activity against the whole parasite and that can be considered for further *in vivo* testing in animal models. Analyses of the pharmacokinetic and pharmacodynamic (PK/PD) properties of the molecule are also initiated at this stage. Compounds that have shown *in vivo* activity without overt toxicity and that have been considered "druggable" are defined as leads. Leads often need optimization of efficacy and ADME properties and re-evaluation of toxicity (thick arrows: lead optimization). Once a compound reaches the stage for testing in humans is defined as a drug candidate. From now on the drug candidate enters in preclinical and clinical studies following the typical drug development pathway. From Pink et al., 2005.

HTS allows analysis of massive compound libraries in a short period of time. Its combination with the availability of newly synthesized compounds by combinatorial chemistry put on the bench millions of molecules to be screened. The high attrition rate of this non-directed screening could be reduced by selecting focused compound collections (e.g. compounds with defined activity against related parasites or biochemical activity against enzymes or receptors known as molecular targets of the parasite) or computational filtering strategies [e.g. favourable ADME (absorption, distribution, metabolism, and excretion) predictions, biochemical profile of the molecule] (Handman et al., 2008).

Structure-based drug discovery has impressively progressed in the last decade. Availability of 3D structure, in theory, should facilitate *in silico* virtual screens and compound docking, predicting structure activity relationships (SARs) and, therefore, enabling the search or synthesis of the adequate molecule or analogues. While many suitable "drug-like" (i.e. sharing certain characteristics that act as drugs like shape, size, solubility) molecules are produced, synthetic chemical compounds sometimes generate compounds likely to be toxic, unstable, highly reactive or mutagenic (Pink et al., 2005).

Despite the intellectually appealing nature of these approaches the success rate has been modest. The extraordinary advances in our knowledge of the basic biology of Kinetoplastida, particularly in *Leishmania* (e.g. genomic, proteomic, metabolomics), use of bioinformatics and *in silico* design have not yet resulted in the development of new antileishmanial drugs. Steverding (2010), in a critical review on the historical development of chemotherapy against infections by *Trypanosoma brucei*, concluded that the rational design of drugs against this disease had only produced a single compound, eflornithine (α-difluoromethyl ornithine, DFMO), in fact developed as antiproliferative for human cancer.

It is expected that refinement of selection criteria of screened libraries, the increasing knowledge of drug-target interaction and the growing industry-academia collaborative efforts would render this synthetic chemically-based approach a useful system to identify new potentially effective drugs. Nevertheless, up to now the control of leishmaniasis still relies on “old” drugs and presently no new drugs are foreseen commercially available at the moment.

Table 4. Criteria for anti-parasite hits, leads and drug candidates (Pink et al., 2005).

HIT
Start of a screening campaign • Active *in vitro* against protozoa $IC_{50} \leq 1\mu g/mL$* (nM- low μM range)* • Selective (minimum of ten times more active against parasite than against mammalian cells)
LEAD
Start of a screening campaign • Active *in vivo* against parasites ≤ 100 mg/Kg* • Not overtly toxic in animals at the efficacious dose • Active *in vitro* against relevant parasite types (e.g. drug-resistant parasites) • Chemically tractable (analogues can be obtained) Candidate for ***lead optimization***: more stringent criteria of selection • Active *in vitro* with activity similar to that of existing drugs • Active *in vivo* against parasites in the relevant animal model (e.g. chronic or late-stage disease) when delivered by a relevant route (oral is preferred) in an acceptable formulation at a reasonable dose (<< 100 mg/Kg)* • Good selectivity against several mammalian cell lines
DRUG DEVELOPMENT CANDIDATE
After succeeding lead optimization process compound that seems also to fulfil other essential criteria • Activity *in vivo* comparable or exceeding that of standard drugs in the most relevant animal models • Activity against desired range of parasites (e.g. different spp, drug-resistant strains) • Pass early toxicity/mutagenicity (e.g. Ames Test) criteria • Acceptable metabolic profile *in vitro* and *in vivo* • Acceptable pharmacokinetic profile • Amenable to cost-effective scale-up • Preferably: well understood mode of action
CLINICAL DEVELOPMENT CANDIDATE
• Drug development candidate for which additional criteria have been met in studies of detailed pharmacology, pharmacokinetics/absorption, distribution, metabolism and excretion, mutagenicity and toxicity, formulation, scale-up for production, cost of goods

*Values may vary depending on parasite, assay and compound.

1.7.5.3.1. New drugs from natural origin

Historically, natural products from different origins (e.g. plants, marine organisms), have been an importance source of new therapeutic agents and are the basis of traditional medicine in the past and also, presently, in large areas where human leishmaniasis is endemic. The investigation of natural products for novel therapeutics peaked in the pharmaceutical industry between 1970-1980 this leading to the fact that between 1981 and 2002 the 49% of the new chemical entities (NCEs) introduced in the market were natural products, semi-synthetic natural products analogues or synthetic compounds based on natural products (Koehn & Carter, 2005). Despite this success during the past decades the research carried out by the industry on these molecules has dropped (Newman, 2008; Li & Vederas, 2009). Several reasons could account for this lack of interest, including the complex molecular interactions in natural sources, difficult integration of these mixtures in HTS and concerns about the proprietary rights on the identified molecules. Figure 21 illustrates the long process for natural product drug discovery and the generic scheme for bioassay-guided fractionation required when working with crude extracts to obtain a pure compound.

Nonetheless, the important contribution of drugs from natural sources is still true in the post HTS and post-genomic era (Harvey, 2008) since between 2005 and 2007 thirteen natural product-derived drugs were approved by the Food and Drug Administration (FDA) and five of them were the first members of new classes (Li & Vederas, 2009). Moreover, in the period 1981-2006, only one NCE obtained by *de novo* combinatorial chemistry, the kinase inhibitor sorafenib for renal carcinoma, got the approval of FDA (Newman, 2008). This low productivity output of the so-called "HTS-Combichem" strategy has put under scrutiny the R&D orientation of pharmaceutical companies. Rather than stressing the differences (e.g. time length of drug development, labour investment, efficacy and efficiency) between both approaches, synthetic chemistry *versus* natural source molecules, a more eclectic strategy should be followed by combining their strengths and minimizing the weaknesses.

The major bottleneck in natural product drug discovery still is the fractionation and purification of active molecules from a complex matrix. Today, difficulties inherent to the analysis of complex mixtures of natural origin have been lessened by the availability of powerful techniques (Figure 21b) (e.g. Mass spectrometry and Nuclear Magnetic Resonance, Ion Cyclotron Resonance Mass Spectrometry, Frontal Affinity Chromatography) and natural-product databases (Koehn & Carter, 2005; Newman, 2008; Li & Verderas, 2009). Another difficulty inherent to this drug discovery approach is that often active principles represent ca. 1% of the total extract; thus, yielding enough quantity of pure compound is a challenge,

particularly when the source are marine organisms (Koehn & Carter, 2005). Complementation of HTS, *in silico* design and bioguiding will enhance the efficiency of drug discovery using natural products and molecules displaying activity against *Leishmania* (Tiuman et al., 2011; Schmidt et al., 2012a, 2012b; Singh et al., 2014).

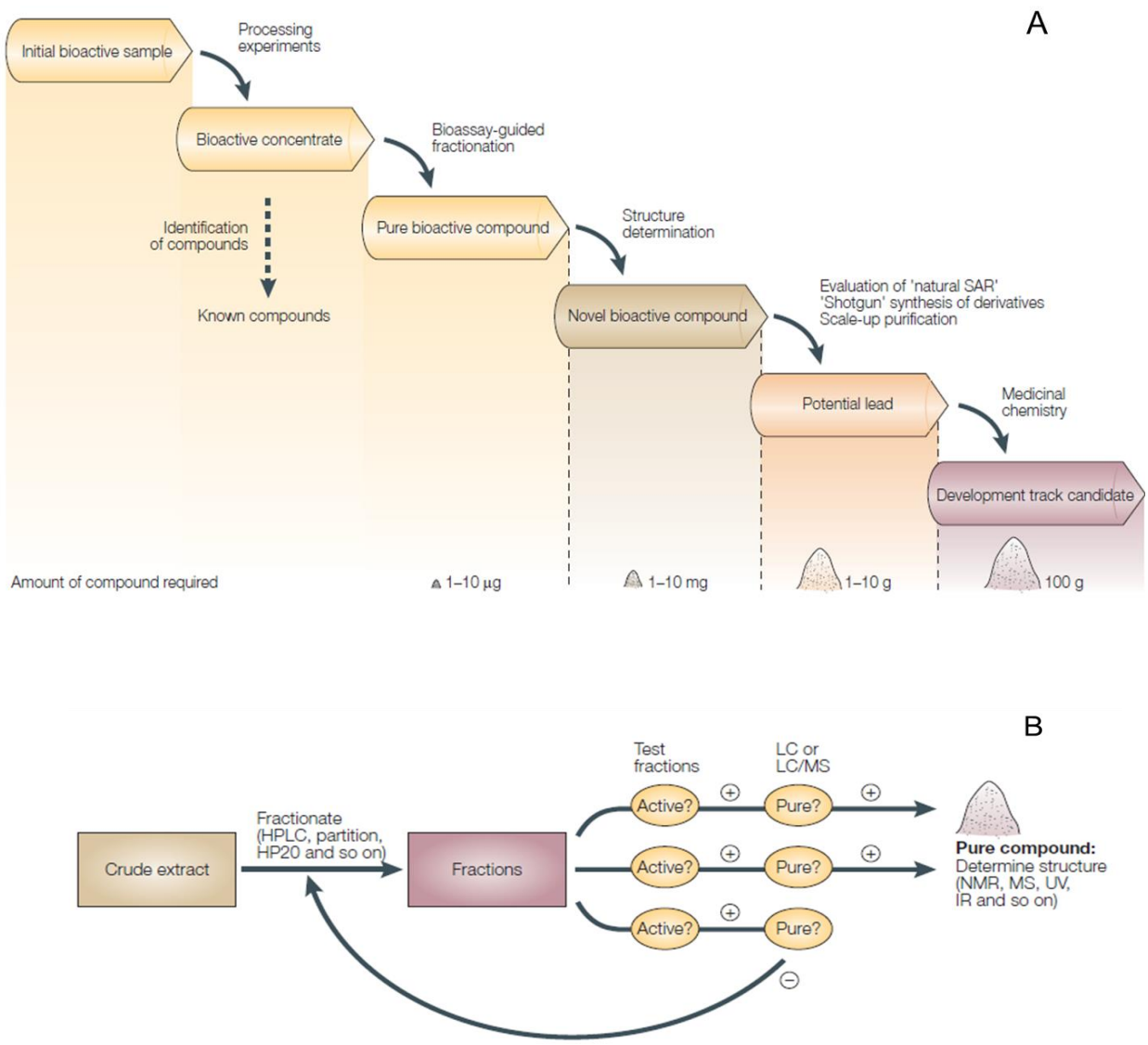

Figure 21. Chemical process of natural product drug discovery and bioguided fractionation scheme for purifying compounds from crude extracts. (**A and B**) A crude extract from the natural source is obtained, concentrated, fractioned and purified ideally yielding a single biologically active pure compound. This process (**B**) requires several cycles of fractionation to finally obtain a pure compound. When the pure active compound satisfies selectivity and potency requirements, (**A**) SAR (structure-activity relationship) studies are carried out and the purification process is scaled up. Once the feasibility of modulating biological response through synthetic modification is established, the hit is declared a lead and proceeds to the lead optimization process by traditional medicinal chemistry. *HPLC, high-performance liquid chromatography; HP20, a solid phase adsorber; NMR, nuclear magnetic resonance; LC/MS, liquid chromatography/mass spectrometry; UV, ultraviolet; IR, infrared spectroscopy. From Koehn & Carter, 2005.

1.7.5.3.2. Allicin

Garlic (*Allium sativum*), among other plants of the Family Alliaceae, is a rich source of organosulfur compounds (1-3%). Historically it has been used as a traditional remedy against parasites, fungi, bacteria and viruses (Ankri & Mirelman, 1999; Corzo-Martínez et al., 2007). Nevertheless, even though organosulfur compounds had been proposed as the active components of crushed garlic cloves (Augusti & Mathew, 1974), it was not until 1944 that Cavallito et al. isolated and identified the constituent responsible for the antibacterial activity of garlic: allicin.

The chemistry of garlic is complex and it appears to have been originally developed as a potential defence against microorganisms. In intact cloves cysteine-sulfoxides, mainly alliin, is enclosed in a separated compartment from alliinase. When a garlic clove is crushed, chopped or suffers any other external aggression (e.g. fungal or bacterial invasion), enzyme and substrate get into contact. Alliinase catalyses the conversion of alliin into the biologically active allicin, that is subsequently transformed into other volatile thiosulphinates. Thiosulphinates are responsible for the characteristic odour of garlic which is absent in intact cloves (Ankri & Mirelman, 1999). Allicin is extremely reactive and has a short half-life rapidly decomposing into other active organosulphur compounds as DAS (diallyl sulfides), DADS (diallyl disulfides), DATS (diallyl tri- and tetrasulfides), ajoene and vinyldithiines (Figure 22).

The absorption and metabolism of allicin and related compounds are poorly understood (Lawson & Wang, 2005). Moreover, although allicin has been proven active *in vivo*, it is unknown if allicin or another derived metabolite is actually reaching the target organs. Allicin has never been detected in blood, urine, or stool suggesting its rapid metabolism (Lawson, 1998). Allyl methyl sulphide detection in the breath has been proposed as a potential indicator of its bioavailability (Lawson & Wang, 2005). Radiolabelled studies performed in rats after oral administration of the closely related molecule DADS revealed that they were absorbed and transformed into allyl mercaptan, allyl methyl sulphide, allyl methyl sulphoxide and allyl methyl sulphone as these metabolites could be detected in stomach, plasma, urine and liver (Germain et al., 2002).

Allicin (diallyl thiosulfinate = 2-Propene-1-sulfinothioic acid S-2-propenyl ester) has shown antibacterial activity against different bacteria including *Helicobacter pylori* (O'Gara et al. 2000; Cañizares et al. 2004) and methicillin-resistant *Staphylococcus aureus* (MRSA) (Cutler & Wilson, 2004) besides a full range of medicinal and antimicrobial effects (Lawson 1998; Ankri and Mirelman 1999). Some reports on the antifungal (*Candida*, *Aspergillus*) (Shadkchan et al., 2004; Khodavandi et al. 2011) properties *in vitro* and *in vivo* of this molecule have been

published. The antiproliferative effect of allicin has been also shown in *Entamoeba* (Ankri et al., 1997), *Plasmodium* (Coppi et al., 2006) and *Trypanosoma* (Waag et al. 2010). McClure et al. (1996) observed that allicin, besides other garlic extracts, was effective against the *in vitro* growth of *L. mexicana* and *L.chagasi* promastigotes but no further testing was carried out. In spite of the recognised therapeutic value of garlic and garlic extracts, research was hampered by the instability of allicin and the rapid transformation of the molecule to related compounds (e.g. ajoene, vinyldithiins) this precluding more refined experimentation. The recent availability of a stable allicin preparation would allow the exploration of the antileishmanial effect of this low molecular weight compound.

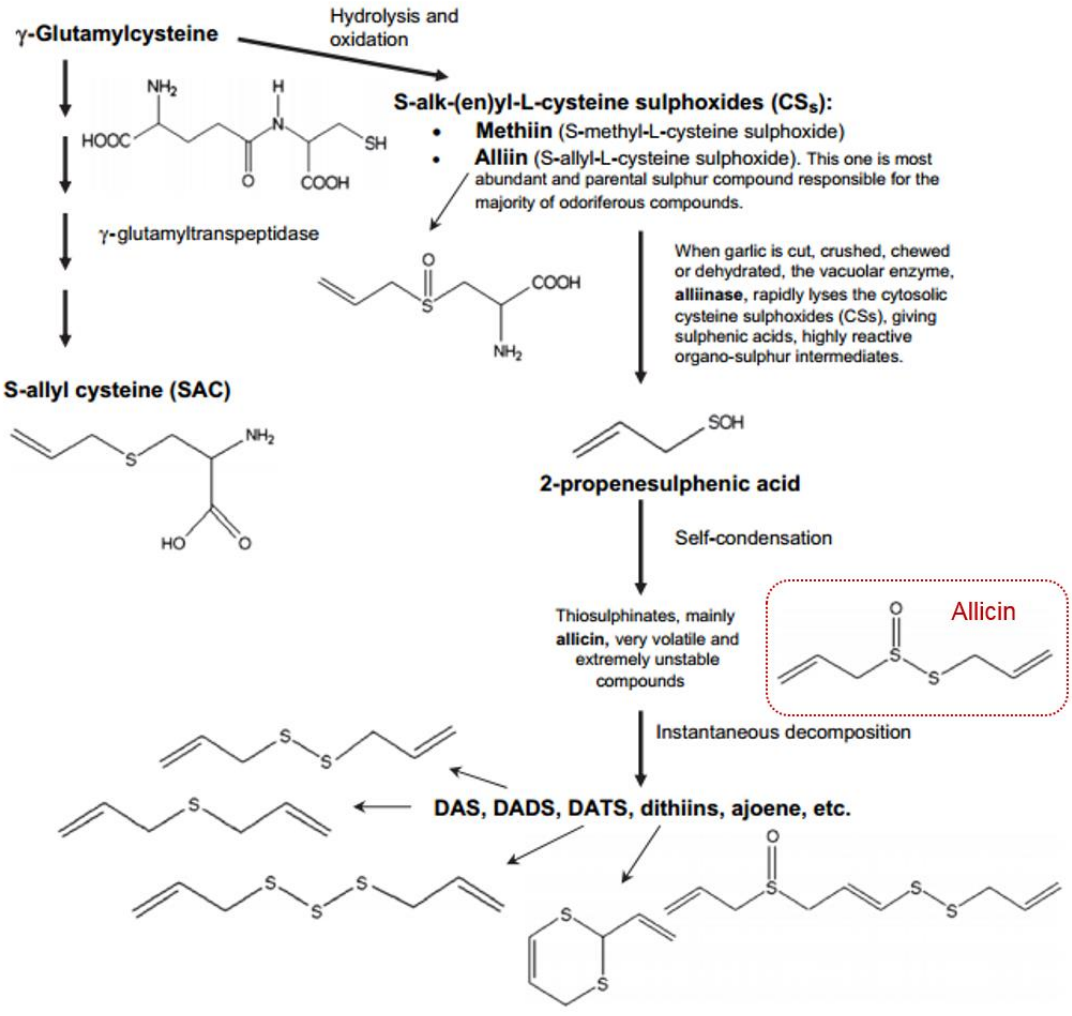

Figure 22. Proposed metabolism of allicin. From Corzo-Martínez et al., 2007.

1.8. Objectives

On the grounds of the above reviewed state of the art in the chemotherapy of leishmaniasis our research project had the following objectives:

i. Setting a reliable and affordable phenotypic platform for discrete screening of putative antileishmanial drugs *in vitro* and *ex vivo* by improving the quantification method in promastigotes and the intramacrophage infection rates of *Leishmania infantum* amastigotes.

ii. Evaluation of the potential antileishmanial effect of allicin on promastigotes and amastigotes of *Leishmania*.

iii. Exploration of the potential synergy of micromolar allicin and Amphotericin B (AmB), *in vitro* and *ex vivo*, on the inhibition of *Leishmania* proliferation.

iv. Efficacy of allicin and its combination with AmB in the control of experimental visceral leishmaniasis in hamster by *L.infantum*.

v. Determination of the mechanism of action of allicin involved in the inhibition of the multiplication of *Leishmania*.

1.9. References

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CHAPTER 2

Improvement of 96-well microplate assay for estimation of cell growth and inhibition of *Leishmania* with Alamar Blue

2.1.a. Resumen

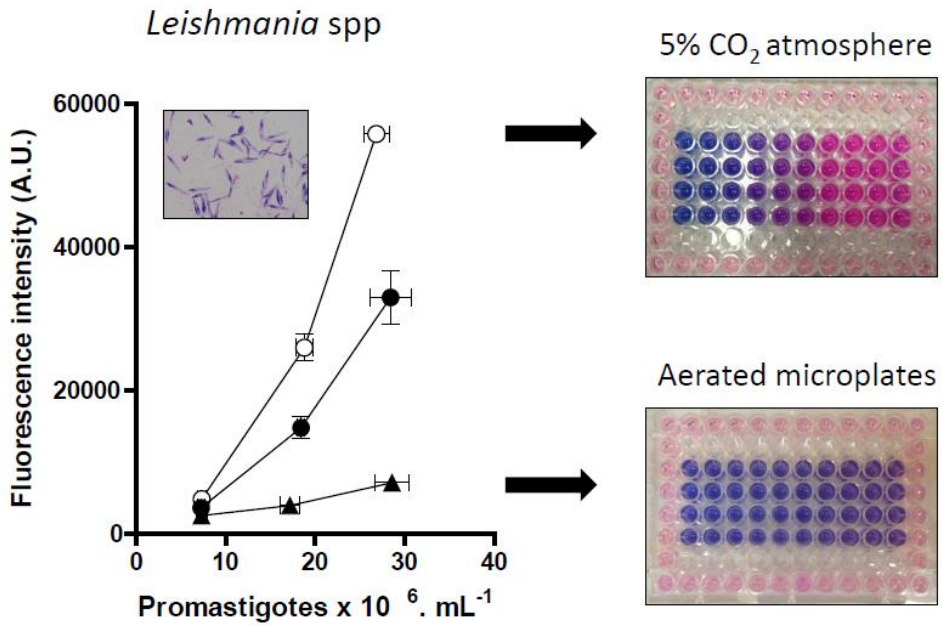
Mejora de método Alamar Blue 96 pocillos para la determinación de la multiplicación e inhibición de Leishmania

En este capítulo se examina el valor del indicador redox Alamar Blue, método basado en la reducción de resazurina, en la determinación de la proliferación celular de promastigotes de *Leishmania* en placas de 96 pocillos. Además, el ensayo fue comprobado con anfotericina B (AmB) y alicina. El método fue ensayado en promastigotes de *L.infantum* y *L.donovani* empleando distintas condiciones de cultivo (fase aérea variable, presencia de rojo fenol, distintas densidad celular inicial, diferentes tiempo de incubación, tamponado con Hepes). Los resultados demostraron que la fase gaseosa en la que se cultivaban los promastigotes era un factor crítico. Los mejores resultados se obtuvieron con concentraciones iniciales del inóculo de 2,5 x 10^5 promastigotes/pocillo, un periodo de incubación de hasta 72 h y presencia de una atmósfera de 5% CO_2 o reducción de la fase gaseosa disponible (bolsas plásticas selladas, placas con film adhesivo). La fluorometría resultó más sensible que la espectrofotometría y permitió detectar densidades celulares menores en los cultivos. El tamponado del medio de cultivo con 20 mM Hepes mejoró de forma notable los resultados. Los recuentos de promastigotes de ambas especies de *Leishmania* se correlacionaron con los valores de intensidad de fluorescencia. Los valores de la concentración inhibitoria (CI_{50}) de AmB y alicina determinados mediante recuentos celulares en cámara de Neubauer y fluorometría fueron comparables. Este indicador resulta sencillo, puesto que implica un solo paso, su precio es económico y permite su uso en la determinación de la sensibilidad diferencial a fármacos de distintos aislados o especies de *Leishmania* en un formato de 96 pocillos.

2.1.b. Abstract

The value of resazurin-based Alamar Blue redox indicator to determine multiplication of *Leishmania* promastigotes in 96-well microtiter plates was examined. In addition, assay was validated with amphotericin B (AmB) and allicin. The method was tested on *L.donovani* and *L.infantum* promastigotes under different culture conditions (variable air-phase, presence of phenol red, initial cell density, incubation time, use of Hepes buffer). Results showed that the gas-phase of promastigote cultures was critical. The method yielded consistent results with initial plating cell densities of 2.5 x 10^5 promastigotes/well, up to 72 h incubation and 5% CO_2 atmosphere or reduced air availability (sealed plastic bags, film-sealed microplates). Detection of low numbers of promastigotes and earlier results could be obtained using fluorimetry instead of spectrophotometry. The addition of 20 mM Hepes improved the results. Fluorescence intensity correlated to promastigotes number in both *Leishmania* spp. Inhibitory concentration (IC_{50}) values for AmB and allicin using cell counting and fluorimetry were comparable. Under these conditions this one-step, low-cost redox indicator can be used in drug sensitivity assays and studies of differential proliferation rates of *Leishmania* isolates or strains in a 96-well format.

Graphical Abstract



2.2. Introduction

Visceral leishmaniasis is a parasitic disease caused by *Leishmania donovani* and *L. infantum* (= *L.chagasi*) (Kinetoplastida). The infection affects both humans and dogs in large areas of the world (i.e. India, Mediterranean Basin, and South America) and it is fatal unless treated. Current first-line chemotherapy of leishmaniasis relies on a rather limited arsenal of drugs, most of which have serious side-effects including nephro- and hepatotoxicity and teratogenicity. Therefore, the identification of new molecules or formulations is an urgent need and has been recognised by WHO as one of the research areas where a sustained effort has to be made (Alvar et al., 2006).

It is assumed that *in vivo* models have superior predictive value than *in vitro* models, and that screening using intracellular amastigotes are more convenient than axenic amastigotes and promastigotes (Sereno et al., 2007; Vermeersch et al., 2009; De Muylder et al., 2011; Gupta and Shakya, 201). In spite of the limitations the promastigote stage is currently used and screening with this parasitic stage has been exploited as a first-step to identify "hit" and "lead" anti-leishmanial compounds in undirected massive screening [High Throughput Screening (HTS)] of chemical libraries (Sharlow et al., 2009; Siqueira-Neto et al., 2010; Walker et al., 2011).

There are several *in vitro* systems available to determine promastigotes proliferation of *Leishmania* spp (i.e. reporter gene assays, enzymatic determinations, H^3 -thymidine incorporation, colorimetric methods). Among colorimetric methods, resazurin-based Alamar Blue entails several advantages. First, it is simple to use as it requires only a one-step procedure. Other benefits reported are its low cost, environmentally friendly composition and transferability to field sites if necessary (Räz et al., 1997). Unlike other assays, this redox indicator is relatively non-toxic to cells and can be used with long incubation periods (up to 72 h) (Fumarola et al., 2004). This indicator has been extensively used in the related genus *Trypanosoma* (Räz et al., 1997; Rolón et al., 2006; Sykes and Avery, 2009) and in some *Leishmania* spp (Mikus and Steverding, 2000; de Oliveira-Silva et al., 2008; Shimony and Jaffe, 2008; Kulshrestha et al., 2013). Recently, this method has been adapted using HTS with *Leishmania* and two different platforms and a 384-well format (Sharlow et al., 2009; Siqueira-Neto et al., 2010).

Our laboratory has been engaged on the study of the anti-leishmanial antiproliferative effect of different molecules. While Alamar Blue could be easily employed to determine the cytotoxicity for the murine cell line J774 (Wert et al., 2011), results obtained with *Leishmania* promastigotes were inconsistent, since resazurin reduction did not correlate with cell counts.

Given the lack of experimental details given in the available literature dealing with *Leishmania*, our aim was to examine the value of Alamar Blue to determine the multiplication and growth inhibition of *L. donovani* and *L. infantum* promastigotes under different culture conditions (variable air-phase, cell density and incubation time). Results showed that optimal conditions of Alamar Blue assay with promastigotes in 96-well microtiter plates included a 5% CO_2 atmosphere, the presence on 20 mM Hepes in the culture medium and an initial concentration of promastigotes of ca. 2.5 x 10^5 /mL. With these conditions, reduced resazurin (resorufin) measured by fluorimetry provided an accurate estimation of promastigotes multiplication and could be used for drug screening and IC_{50} estimation**.**

2.3. Material and methods

Parasites

An autochthonous isolate of *L. infantum* (UCM 9), obtained from affected dogs in the area of Madrid (Spain) by the Clinical Service of the Department of Animal Health, Faculty of Veterinary Medicine (Universidad Complutense), and Khartoum 1246 isolate from *L. donovani*, provided by Dr. Toraño (Department of Immunology, Instituto de Salud Carlos III, Madrid) were routinely maintained as promastigotes in RPMI 1640 medium (Lonza) at 26 °C supplemented with heat inactivated (30 min, 56 °C) foetal bovine serum (FBS) (Sera Laboratories International) and 100 U/ml penicillin + 100 μg/ml streptomycin (BioWhittaker) in 25 ml culture flasks.

Chemicals

Alamar Blue was purchased from AbD Serotec. Allicin (2-Propene-1-sulfinothioic acid S-2-propenyl ester) was obtained as liquid Allisure® from Allicin International Ltd (Rye, East Sussex, UK) at a concentration of 5000 ppm and kept at a temperature of -80˚C until used. Amphotericin B (AmB) was obtained as fungizone (Sigma).

Promastigote assays

Depending on the experiment promastigotes were cultured in flat-bottomed 96-well cell culture microtiter plates with lid (Costar, Corning), in microtiter plates wrapped with Parafilm®, or in plates sealed with Thermal adhesive film for PCR plates (Simport). For comparative purposes promastigote cultures were also done in 1.5 mL eppendorf® tubes. Cultures were carried out at 26 ºC in aerated culture chamber or incubated in a 95% air/5% CO_2 humidified atmosphere. Culture media (RPMI 1640) with and without additional 20 mM Hepes were employed depending on the experiment.

Alamar Blue assay

Concentration of resorufin, the product of reduction of resazurin, in the *Leishmania* cultures was determined following the manufacturer's recommendations by reading the absorbance (A) at 570 and 600 nm, and fluorescence (550 nm excitation wavelength, 590 nm emission wavelength) in a FLUOstar Omega (BMG Labtech) fluorimeter. Fluorescence intensity was expressed as arbitrary units (A.U.). Briefly, mid-log phase promastigotes were added to the wells of microtiter plates or eppendorf tubes up to a volume of 200 µL/well. After 24 h, 20 µL Alamar Blue (10% v/v) was added and the cultures were kept for 24, 48 or 72 additional hours. Absorbance and fluorescence intensity were determined every 24 h. Promastigote counts were carried out in Neubauer improved chambers and cell viability was assessed by trypan blue exclusion staining. Untreated cultures, wells without cells and the maximal concentration of the drugs, and wells with culture medium and Alamar Blue (10% v/v) were included as controls. All experiments were performed at least in triplicate.

Statistical analysis

Results were expressed as means ± standard deviation. Data were compared by analysis of variance (one- and two-ways ANOVA) and GLM analysis using GraphPad Prism5. Differences were considered significant when $p<0.05$. Figures were also prepared with GraphPad Prism5.

2.4. Results

Determination of optimal cell density

Different concentrations (10^4, 2.5 x10^4, 5 x10^4, 7.5 x 10^4, 10^5, 2.5 x10^5, 5 x10^5, 7.5 x 10^5, 10^6) of mid-log phase promastigotes of *L.donovani* and *L.infantum* were added in a final volume of 200 µL/well in 96-microtiter plates. Cultures were carried out at 26 ºC in plates with lid under a 5% CO_2 atmosphere or in film-sealed plates. After 24 h incubation, Alamar Blue was added and the plates were kept for 24, 48 or 72 h. For each time determination a plate was used. Resazurin was effectively reduced to resorufin in the medium, evidenced by the higher levels of absorbance and, particularly, fluorescence related both to the initial promastigotes density and the time of culture. Absorbance determinations, especially with low initial promastigote concentration (<10^5 promastigotes/well) were more variable (not shown). This variability was not observed when cultures were kept in the CO_2 atmosphere. Results were more consistent when resorufin concentration was determined by fluorimetry. Fluorescence was significantly higher (ca. 2 times) when cultures were exposed to 5% CO_2 (Figure 1A) as compared to those performed in film-sealed plates (Figure 1B). Highest levels of fluorescence were obtained with initial inoculums of 2.5 to 5 x 10^5 promastigotes/well from both *Leishmania* species, after 72 h and exposition to CO_2.

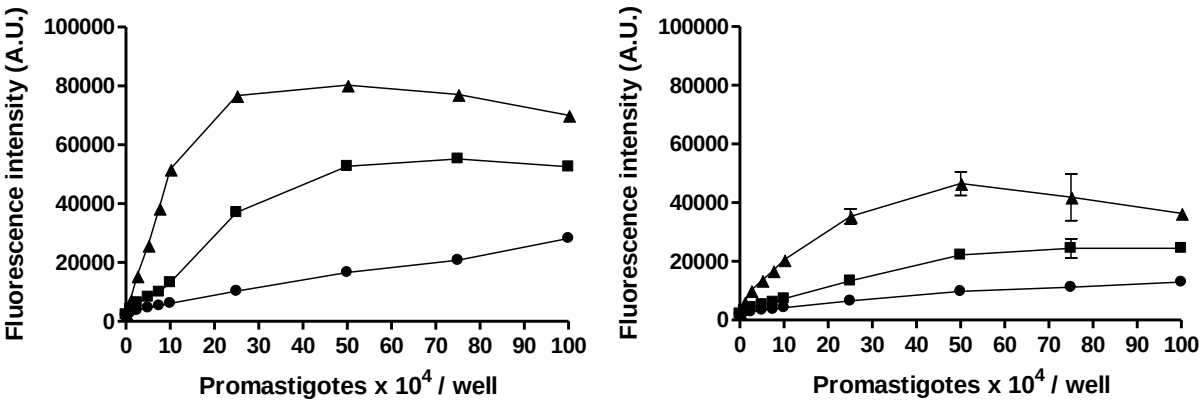


Figure 1: Effect of incubation time with Alamar Blue on the fluorescence curves with promastigotes of *L.donovani*. Increasing initial plating concentrations of promastigotes were incubated with Alamar Blue (10% v/v) and the fluorescence intensity determined (arbitrary units, A.U.) (Excitation wavelength: 560 nm, emission wavelength: 590 nm). **Figure 1A**: in the presence of 5% CO_2. **Figure 1B**: film-sealed microplates. Results are means ± standard deviation. ●: 24 h; ■: 48 h and ▲ : 72 h. Results are means ± standard deviations of 3 determinations.

Correlation between Alamar Blue reduction and promastigotes multiplication

Preliminary results obtained in our laboratory allowed the use of the redox indicator to determine the proliferation of *Leishmania* promastigotes in culture tubes. However, as shown above, for a given initial number of promastigotes and time of incubation, significant differences ($p<0.05$) were found in the concentration of resorufin estimated by absorbance and, particularly, fluorimetry depending on the exposition to CO_2 or to a limited air phase in the sealed microtiter plates. To rule out the possibility of resazurin reduction being an inaccurate estimation of *Leishmania* multiplication, promastigotes (2.5 x 10^5 /well) were cultured in 96-well plates under a CO_2 atmosphere or under air phase. For comparative purposes parallel cultures were done in eppendorf tubes. In all cases cell multiplication was estimated by fluorimetry and cell counting of viable *Leishmania* in Neubauer chamber. A set of cultures was employed for each time determination (24, 48, 72 and 96 h).

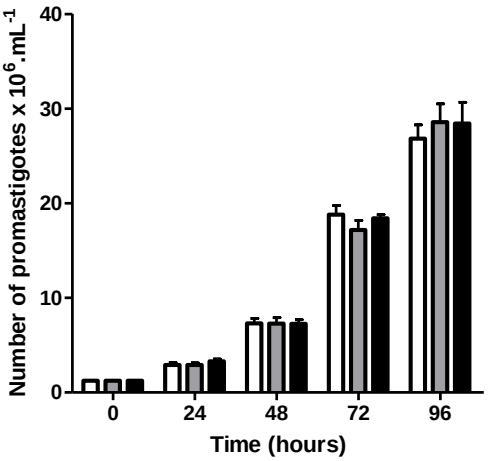


Figure 2A: Proliferation of *Leishmania* promastigotes under different culture conditions [White bars: CO_2 atmosphere; grey bars: aerated cultures; solid bars: eppendorf tubes]. Promastigotes were seeded (2.5 x 10^5 promastigotes/well) on day 0. Alamar Blue was added 24 h later. Results are means ± standard deviations of 3 determinations.

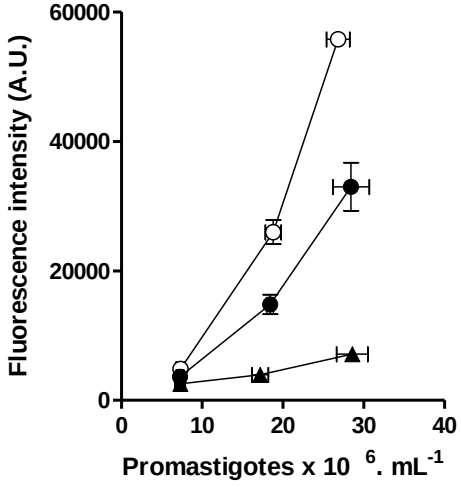


Figure 2B: Relationship between fluorescence intensity (A.U.) and promastigote counts at different times and culture conditions, ○: 5% CO_2 atmopshere; ●: aerated microplates; ▲ : eppendorf tubes. Results are means ± standard deviations of 3 determinations.

Figure 2 shows that no significant differences (p>0.05) were found between cultures in the experiment irrespective of the exposition to 5% CO_2 or to an unlimited air phase. After 96 h, all cultures reached values ca. 2.7 x 10^6 promastigotes/mL in both *Leishmania* spp (Figure 2A). However, resazurin concentration determined by fluorimetry was strongly dependent on the culture conditions (Figure 2B). Highest levels of fluorescence were seen in cultures exposed to CO_2 and the lowest values were present using standard culture conditions with air atmosphere. Cultures in eppendorf tubes, with a limited amount of air, displayed intermediate values. These results were consistent with the absence of colour change in the standard microtiter plates in spite of the active multiplication. These results suggested the importance of the air phase of the cultures in the usefulness of Alamar Blue method to determine *Leishmania* proliferation and also the need of standardization of the assay.

Effect of atmosphere and buffer on resazurin reduction by promastigotes of Leishmania

Promastigotes of *L.infantum* and *L.donovani* were cultured in 96-well standard microtiter plates exposed to 5% CO_2, in standard microplates, in microplates wrapped with Parafilm, in microplates in sealed plastic bags or film-sealed microplates for PCR. Moreover parallel cultures were done in eppendorf tubes to monitor cell viability (trypan Blue exclusion dye) and multiplication (counting in Neubauer chamber). Cultures with standard RPMI medium and medium with 20 mM Hepes were employed. Absorbance and fluorescence were determined 24, 48 and 72 h after Alamar Blue addition.

As expected time-dependent fluorescence increases were observed in all culture conditions and both *Leishmania* species earlier than absorbance variations (not shown) in the determinations carried out. Figure 3 shows the results obtained with *L. infantum* and similar values were obtained for *L.donovani*.

It was found that the presence of 5% CO_2 or limited air availability was critical to get the highest fluorescence values. Microplates with lid exposed to air had the lowest levels, comparable to those found in Parafilm wrapped plates. By its part, 96-well plates cultured in a CO_2 incubator displayed the highest resorufin concentrations in all time determinations carried out. In cultures done in eppendorf tubes, film-sealed microplates and plastic-sealed microplates intermediate values of absorbance and intensity of fluorescence were obtained. Moreover, the presence of additional 20 mM Hepes improved the resazurin reduction in all culture conditions,

particularly when film-sealed microtiter plates and eppendorf tubes were employed (p<0.01-p<0.001).

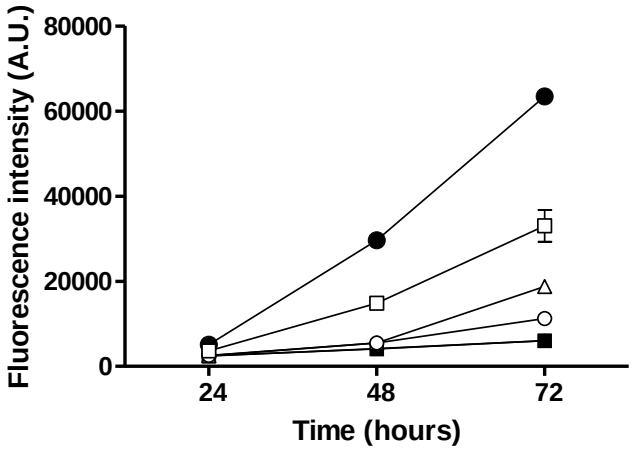


Figure 3: Fluorescence intensity (A.U.) of promastigote cultures of *L.donovani* with additional buffer (20 mM Hepes) at different times of incubation. All cultures except □ were carried out in 96-well microplates. ●: 5% CO_2 atmosphere ;■:aerated microplates; ▲: Parafilm wrapped plates; ○: plastic bag-sealed microplates; ∆: Film-sealed microplates; □: eppendorf tubes. Results are the means ± standard deviations of 3 experiments.

Value of resazurin reduction measured by fluorimetry for drug screening in Leishmania

Value of resazurin transformation by fluorimetry for drug screening in 96-well microtiter plates was tested with two compounds with antileishmanial activity, namely allicin and AmB. In all cases 2.5 x 10^5 promastigotes/well for both species were used. In the case of allicin, 10, 30, 60 and 120 µM concentrations were used and microplates in CO_2 or aerated incubator, and with and without additional 20 mM Hepes in the medium, were used. Parallel counts were done in Neubauer haemocytometer with cultures in eppendorf tubes exposed to the same allicin concentrations.

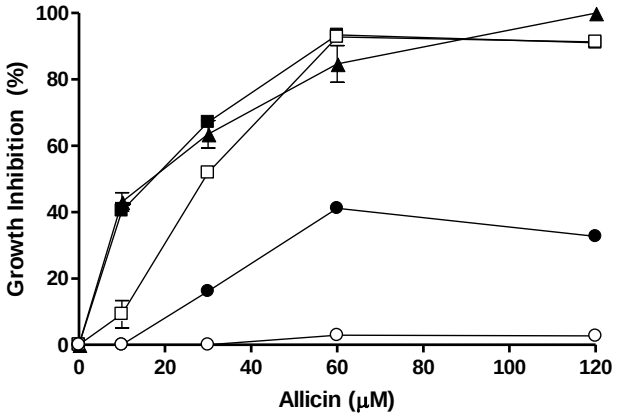


Figure 4A: Growth inhibition of *L.donovani* promastigotes in the presence of allicin (24 h) estimated by cell counts and fluorescence intensity (A.U.). ▲ : cell counts; ■ : 5% CO_2 atmosphere and 20 mM Hepes; □ : 5% CO_2 atmosphere; ●: aerated microplates and 20 mM Hepes; ○: aerated microplates. Results are means ± standard deviations of 3 experiments

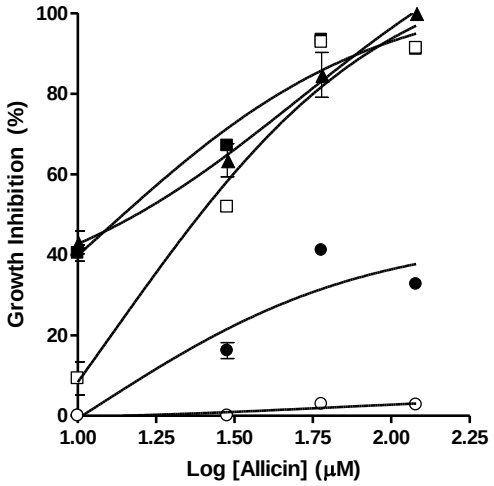


Figure 4B: Best-fit representation of the effect of allicin on growth inhibition of *L.donovani* promastigotes, determined by fluorescence and cell countings. ▲: cell counts; ■ : 5% CO_2 atmosphere and 20 mM Hepes; □ : 5% CO_2 atmosphere; ●: aerated microplates and 20 mM Hepes; ○: aerated microplates. Results are means ± standard deviations of 3 experiments.

Cultures were treated for 24 h with allicin and absorbance and fluorescence were monitored at 24, 48 and 72 h after exposure to the drug. A range of AmB (0, 0.001, 0.002, 0.003, 0.004, 0.005, 0.006, 0.007, 0.008, 0.009, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.5, 1 and 10 µM) was tested with the optimal assay conditions found (5% CO_2 , 20 mM Hepes). Fluorescence and absorbance were measured 72 h after exposure to the antibiotic.

Figure 4A shows the relationship between the allicin concentration and the growth inhibition of *L.donovani* promastigotes determined by cell counting and fluorescence of the cultures. Addition of 20 mM Hepes improved the detection of fluorescence in aerated microplates whereas no correlation between growth inhibition and resorufin levels in the cultures was observed without Hepes in this case. Our results clearly showed that Alamar Blue reduction closely correlated to microscopic cell counts and viability, in the allicin concentration range examined, provided that a 5% CO_2 atmosphere and 20 mM Hepes were present (Figure 4B). Comparable results were obtained for *L.infantum*. Inhibition of multiplication of *Leishmania* promastigotes in the presence of AmB confirmed the value of the resorufin level with fluorimetry using the assay conditions described in the screening and IC_{50} determination of this antileishmanial drug. Actually, for the particular *L.infantum* isolate employed, an approximate IC_{50} of 0.07µM was obtained by cell counting whereas using the best-fit values with fluorescence determinations the value obtained was 0.0618 µM (Figure 5). Figure 6 shows a representative result of the effect of CO_2 and 20 mM Hepes on the reduction of resazurin.

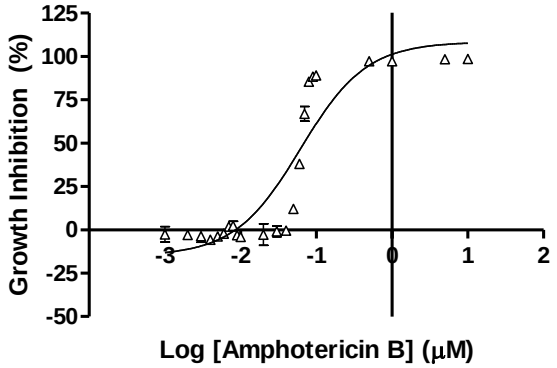


Figure 5: Determination of IC_{50} of amphotericin B for *L.donovani* promastigotes using Alamar Blue reagent. Experimental conditions included an initial plating concentration of 2.5 x 10^5 promastigotes/well, 5% CO_2 atmosphere and 20 mM Hepes. Results are the means ± standard deviations of 3 experiments.

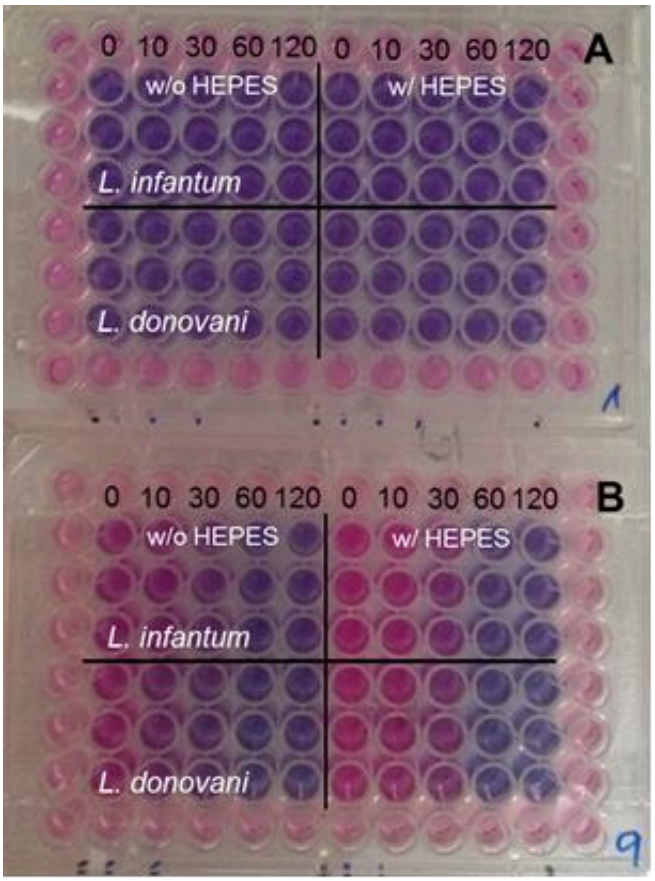


Figure 6: Representative image of *L.infantum* and *L.donovani* promastigote cultures exposed to different concentrations of allicin (0, 10, 30, 60 and 120 μM), with and without 20 mM Hepes, in the presence of 5% CO_2 atmosphere (**B**) or in air exposed 96-well microplates (**A**). Marginal wells were not used to avoid edge effect.

2.5. Discussion

Present results, with the optimal conditions described, show that Alamar Blue, and presumably other resazurin-based methods, is an accurate assay to determine the multiplication of *Leishmania* promastigotes and growth inhibition elicited by antileishmanial drugs. Assay conditions examined in a checkerboard manner showed that several factors were relevant to obtain consistent results, namely an initial plating density of promastigotes ca. 2.5 x 10^5, besides the presence of a 5% CO_2 atmosphere and additional buffering of the medium with 20 mM Hepes. Our results on the optimal cell density were comparable to the findings by Mikus and Steverding (2000) with *L.major* and Shimony and Jaffe (2008) with *L.donovani* and support the lower reduction rate of *Leishmania* compared to *T. cruzi* epimastigotes (Rolón et al., 2006) and bloodstream trypanosomes (Räz et al., 1997). A linear fluorescence response was observed with lower plating densities of promastigotes and therefore the method could probably be used in 72 h experiments in the range of 5 x 10^4 to 10^5 promastigotes/well. The plateau observed with higher cell densities and incubation times are probably related to the conversion of resorufin to colorless and non-fluorescent hydroresorufin (O'Brien et al., 2000).

Alamar Blue method has been successfully employed to monitor the growth of mammalian cells (Ansar Ahmed et al., 1994; Nakayama et al., 1997; O'Brien et al., 2000; Sykes and Avery, 2009). Most of the assays have been performed in a 95 % air / 5 % CO_2 atmosphere, including those carried out in African trypanosomes (Räz et al., 1997; Sykes and Avery, 2009), or in plastic sealed bags (Martin et al., 2003). More than probably the performance of a redox indicator added to a culture medium would be affected by the gas-phase although this factor apparently has not been considered in the previous contributions on *Leishmania* (de Oliveira-Silva et al., 2008; Shimony and Jaffe, 2008; Vermeersch et al., 2009; Kulshrestha et al., 2013) and *T. cruzi* epimastigotes (Rolón et al., 2006) using 96-well microtiter plates or, more recently with the HTS with 384-well format, using *L.major* (Siqueira-Neto et al., 2010). Our results showed that, using the 96-well plate format incubation in a 5 % CO_2 gas phase yielded accurate results of cell growth and inhibition in *Leishmania* (r^2 = 0.9713). The carbon dioxide dissolved in the medium allowed the efficient reduction of resazurin to the fluorescent resorufin. Actually, O'Brien et al. (2000) highlighted the need of CO_2 in the medium when reduction is occurring in order to capture electrons. In the absence of 5% CO_2 additional buffering with Hepes could circumvent some of the limitations provided that a limited volume of gas phase was present (capped eppendorf tubes, film sealed plates, sealed plastic bags). Earlier and more sensitive estimation of resorufin was obtained by fluorimetry and thus should be preferred to spectrophotometry. The significantly higher sensitivity of fluorimetry allowed the earlier

identification of anti-leishmanial activity of allicin. Promastigote multiplication and inhibition could be easily detected by fluorescence after 48 h whereas observation of significant absorbance variations needed 72 h as observed by Mikus and Steverding (2000).

Visceral leishmaniasis, both human and animal, caused by *L.donovani* and *L.infantum* (=*L.chagasi*) continues being a challenge for medical doctors and veterinary clinicians in many regions of the world. Available drugs have important shortcomings (nephrotoxicity, hepatotoxicity, gastrointestinal disturbances) and, moreover, in some areas *Leishmania* strains resistant to first line drugs have been described (Croft et al., 2006). Identification of new potentially effective drugs or preparations requires robust and reliable screening methods. *In vivo* tests of efficacy must be reduced at a minimum by both ethical and economic reasons and therefore *in vitro* screening of potentially useful compounds is the first step to identify "hits and leads". Obviously, the best *in vitro* model to test antileishmanial activity of compounds is done on the intracellular amastigote within macrophages (Sereno et al., 2007). However, intracellular amastigotes screening is only available to some laboratories, species such as *L. donovani*, and specially *L.infantum*, have a slow rate of division (Gupta and Shakya, 2011) and some *Leishmania* isolates from clinical cases do not infect the macrophage cell lines employed. By its part, promastigotes are easy to culture and they share with amastigotes many metabolic pathways and thus some of the most commonly used antileishmanial agents, such as AmB or miltefosine, are effective against both parasite stages. Therefore, promastigote screening is useful (Sharlow et al., 2009) before testing in intracellular amastigotes, and *in vivo*, and can be employed to monitor susceptibility of clinical isolates to some drugs (i.e. Miltefosine, Kulshrestha et al., 2013).

Among the available methods (^{3}H-thymidine incorporation, microscopic counting, quantitative PCR, enzymatic and colorimetric methods) to determine proliferation of *Leishmania* promastigotes, resazurin-based assays such as Alamar Blue can be carried out in a single step, are unaffected by the red phenol from the culture medium and have the advantage of low cost and negligible toxicity. Validation with *L.donovani* and *L.infantum* promastigotes showed that this method could be advantageously used in trials of cell proliferation and drug screening, including the determination of approximate IC_{50} values against promastigotes. Conditions described for *Leishmania* included the presence of 5% CO_2, or reduced air availability, to allow the efficient reduction of resazurin to resorufin, in a promastigote-density related manner. The method was improved by fluorimetry although spectrophotometry could also be employed. Given the accuracy (fluorescence vs. promastigotes density), the low number of promastigotes needed and results obtained in the determination of IC_{50} for AmB and allicin as

compared to the results with microscopic counting (i.e. Corral-Caridad et al., 2012), this 96-well microplate could be adapted for a 384-well format**.**

2.6. **Acknowledgements**

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CHAPTER 3

Effect of allicin on promastigotes and intracellular amastigotes of *Leishmania donovani* and *L.infantum*

3.1.a. Resumen

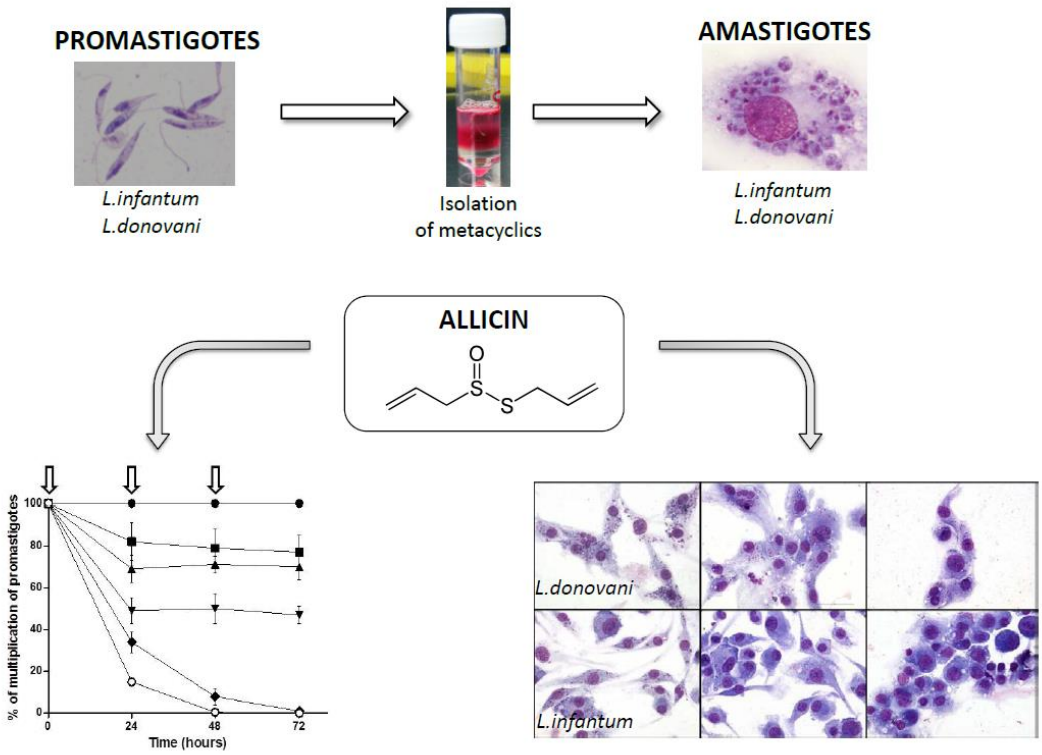
Efecto de alicina sobre promastigotes y amastigotes intracelulares de Leishmania donovani y L.infantum

La actividad anti-*Leishmania* de alicina (dialil tiosulfinato) ha sido ensayada *in vitro* frente a promastigotes de *Leishmania donovani* y *L.infantum*. Asimismo, se han realizado infecciones intracelulares de *Leishmania in vitro* en macrófagos murinos de la línea celular J774 y *ex vivo* en macrófagos peritoneales de BALB/c empleando un método modificado para el aislamiento de promastigotes metacíclicos. El compuesto ha demostrado un efecto inhibitorio dosis y tiempo dependiente sobre la multiplicación de promastigotes de *L.donovani* y *L.infantum.* Por primera vez ha sido descrito el efecto inhibitorio de alicina sobre amastigotes intracelulares de *Leishmania.* El valor de la concentración inhibitoria (CI_{50}) del compuesto obtenido por nosotros se encontró en la horquilla micromolar (10-30 µM) para ambas *Leishmania* spp. El efecto anti-*Leishmania* de alicina no fue debido a la presencia de ajoeno como producto de su degradación como se confirmó mediante espectrometría de masas. La actividad de la molécula frente a promastigotes y amastigotes de *Leishmania* se incrementó cuando se administró en dosis repetidas cada 24 h. Dos administraciones consecutivas de 5 µM alicina lograron inhibir *ca.* 50% la proliferación de amastigotes intracelulares de *Leishmania*. La escasa toxicidad que ha demostrado este compuesto frente a células de mamífero apunta al interés de esta molécula en terapia combinada para el tratamiento de infecciones por *Leishmania*.

3.1.b. Abstract

Anti-leishmanial activity of allicin (=diallyl thiosulphinate) has been tested *in vitro* against promastigotes and intracellular amastigotes of *Leishmania donovani* and *L. infantum*. Macrophage infections have been carried out *in vitro* in the murine cell line J774 and *ex vivo* with peritoneal macrophages from BALB/c mice with a modified method to isolate metacyclic promastigotes. The compound has shown a significant *in vitro* effect on the multiplication of promastigotes of *L.donovani* and *L.infantum* in a time- and dose-dependent manner. It has been shown for the first time the inhibition of multiplication of intracellular amastigotes of *Leishmania* by allicin. Inhibitory concentrations of the compound were in the micromolar range (10-30 µM) for both *Leishmania* species. Antileishmanial effect of allicin apparently was not related to products of degradation of the molecule as assessed by mass spectrometry analysis. Inhibitory activity of allicin against promastigotes and intracellular amastigotes increased when the compound was added to the cultures every 24 h. Two administrations of 5 µM allicin inhibited by ca. 50% the proliferation of *Leishmania* amastigotes. Low toxicity for mammalian cells of this compound suggests the interest of exploring the value of allicin in combined therapy against leishmanial infections.

Graphical abstract



3.2. Introduction

Human *Leishmania* infections are present in all inhabited continents, with around 12 million people infected, 2 million new cases every year and 350 million people living in areas at risk (WHO, 2000). Leishmaniasis is a spectral infection with a range of clinical presentations, from self-healing dermal infections to visceral fatal processes. Visceral leishmaniasis caused by *L.donovani* and *L.infantum* = *L.chagasi* is a severe disease whose distribution has expanded frequently associated to HIV infection (Desjeux, 2004). In addition to human cases, canine leishmaniasis by *L.infantum* (Mediterranean Basin) and *L.chagasi* (South America) constitute a first order pathology in veterinary clinics besides its zoonotical importance as reservoirs. The main control system of leishmaniasis in both humans and dogs is chemotherapy. Liposomal amphotericin (AmB), paromomycin and miltefosine were considered the most promising drug for chemotherapy of leishmaniasis (www.who.int/tdr/diseases/leish). However, these drugs have some important shortcomings, including high toxicity and teratogenicity of some of them, absence of parasitological cure in most cases and unaffordable prices for some of the compounds (Sundar and Chatterjee, 2006). Moreover, in some areas resistant *Leishmania* strains to commonly used drugs have emerged (Croft and Coombs, 2003; Croft et al., 2006). Therefore, new drugs or combinations are needed (Alvar et al., 2006; Chappuis et al., 2007; Mishra et al., 2007).

This scenario favours the exploration of alternative drugs. Among them, allicin (diallyl thiosulfinate = 2-Propene-1-sulfinothioic acid S-2-propenyl ester), a natural product present in plants of the Family Alliaceae, including garlic, has shown antibacterial activity against *Helicobacter pylori* (O'Gara et al., 2000; Cañizares et al., 2004) and methicillin-resistant *Staphylococcus aureus* (MRSA) (Cutler and Wilson, 2004) besides a full range of medicinal and antimicrobial effects (Lawson, 1998; Ankri and Mirelman, 1999). Reports on the antifungal (*Candida*) (i.e. Khodavandi et al., 2011) and antiprotozoal (Mirelman et al., 1987: *Entamoeba*; Coppi et al., 2006: *Plasmodium*; Waag et al,. 2010: *Plasmodium* and *Trypanosoma*) activity of this molecule have been published. Ankri and Mirelman (1999) referred to unpublished results on the inhibition of *Leishmania major* growth by 30 μM allicin. Moreover, McClure et al. (1996) observed that allicin, besides other garlic extracts, was effective against the *in vitro* growth of *L. mexicana* and *L. chagasi* promastigotes but no further testing was carried out. On these grounds we have tested the potential antiproliferative effect of allicin on promastigotes and amastigotes of the two major causative agents of visceral leishmaniasis, *L. donovani* and *L. infantum*. Our results showed that micromolar concentrations of allicin are able to inhibit the proliferation of both stages and species.

3.3. Material and methods

Parasites

An autochthonous isolate of *L. infantum* (UCM 9), obtained from affected dogs in the area of Madrid (Spain) by the Clinical Service of the Department of Animal Health, Faculty of Veterinary Medicine (UCM), and "Khartoum 1246" isolate from *L. donovani*, provided by the Department of Immunology, Instituto de Salud Carlos III, Madrid, were routinely maintained as promastigotes in RPMI 1640 medium (Lonza) at 26 °C supplemented with heat inactivated (30 min at 56 °C) foetal bovine serum (FBS) (Sera Laboratories International), 100 U/mL penicillin + 100 µg/mL streptomycin (BioWhittaker) and 1% L-glutamine in 25 ml culture flasks.

Promastigote assay

Promastigotes were cultured in 10 ml sterile polystyrene tubes. Allicin was obtained as liquid Allisure® from Allicin International Ltd (Rye, East Sussex, UK). Dilutions of the compound were added up to 2 mL final volume. After 24, 48 and 72 h at 26 °C, live promastigotes were counted in an improved Neubauer chamber. Results from treated cultures were expressed as % of the multiplication obtained in untreated control cultures. Cultures were performed at least in triplicate and cultures treated with 0.1 µM amphotericin B (AmB) were included as control.

Cell cytotoxicity assay: MTT assay

MTT assay was employed to determine the toxicity of allicin for both J774 cells and mouse peritoneal macrophages. Peritoneal macrophages were isolated from female BALB/c mice. Cell suspension with RPMI-1640 medium (supplemented with 10% heat-inactivated FCS, 1% penicillin/streptomycin, 1% L-Glutamine) were seeded in 96 well flat-bottomed cell culture plates (Corning). Plates were incubated overnight in a humidified atmosphere 5% CO2/air atmosphere at 37 °C to ensure cell adherence. After 24 h, cells (4 x 10^6 peritoneal macrophages/mL; 5 x 10^3/mL J774 cells) were treated with increasing concentrations of allicin (1-120µM). Non-treated cells were included as a negative control. At the end of the drug exposure period (24 h), the medium from all the wells was removed and the cells were fed with 200 µL of fresh medium, and plates were incubated for another 24 h. The viability of cells was determined by the MTT reduction assay as described before (Plumb, 2004). Briefly, 200 µL of

fresh medium and 50µL of the MTT (Sigma) solution (5mg/mL in PBS) were added to all wells; plates were incubated for 4 h protected from light in a humidified atmosphere at 37ºC. The remaining formazan crystals were dissolved by adding 200 µL of DMSO (Sigma) and 25 µL/well of Sorensen's glycine buffer were added to stop the reaction. Absorbance was read at 570 nm. The approximate IC_{50} concentration was determined as the allicin concentration required to reduce the absorbance to half that of the untreated control wells (considered as 100% viable). Three independent experiments, with eight replicates, were carried out for assessing the cytotoxicity of allicin with this method.

Amastigote assay

In the amastigote assay, infection was carried out using a modification of the methods developed by us (Méndez et al. 1996, Wert et al. 2011) and the procedure described by Yao et al. (2008) to isolate metacyclic promastigotes. Depending on the experiment, infections with *Leishmania* were carried out *in vitro*, using the murine macrophage cell line J774, and *ex vivo*, with peritoneal macrophages from BALB/c mice. Stationary phase *Leishmania* promastigotes (day 7) were centrifuged without brake (365 x*g*, 10 min) through a Ficoll gradient (0, 10% in Medium 199, and 30% in sterile PBS). Parasites recovered from the interphase between 0% and 10% Ficoll were washed with fresh medium and opsonised with 15% normal mouse serum in a solution 1:1(v/v) RPMI medium and HBSS, 0.15 mM $CaCl_2$ and 1 mM $MgCl_2$ for 30 min at 37 ºC. Murine J774 cells (2.5 $x10^4$ cells/well) were cultured in 8-well Lab-Tek chambers (Nunc), for 16 h with mitomycin C (Calbiochem) at 1 µg/mL was to avoid cell proliferation. The washed parasites were added to the macrophage cultures (parasite: macrophage ratio 10:1) and maintained at 33 ºC in 5% CO_2 overnight without mitomycin. Non-internalized promastigotes were eliminated by thorough washing three times with fresh medium. Compounds were added at appropriate concentrations and maintained for 24 or 48 h depending on the experiment carried out.

In the *ex vivo* experiments, peritoneal macrophages were obtained from female BALB/c mice and cultured as described above except for the absence of mitomycin treatment. Macrophage concentration was adjusted to 2.5 x 10^5/well up to a final volume of 200 µL/well. Metacyclic promastigotes were isolated and opsonised as previously described and parasite: macrophage ratio was 5:1. After a contact period overnight, Labtek plates were washed with RPMI 1640 and the compounds were added at different concentrations and times, and kept at 37 ºC for 24, 48 or 72 h depending on the experiment. Slides were fixed and stained (May-

Grünwald-Giemsa, Merck Darmstadt) and the number of amastigotes/100 cells and % of infected cells was determined. Cultures were performed at least in triplicate.

Solid phase microextraction (SPME) and Gas Chromatography/Mass Spectrometry (GC/MS)

The SPME technique was carried out using an 85 μm Light Blue Carboxen/PDMS fibre coated with polydimethylsiloxane. Samples (100 μL) from the experimental cultures were prepared in extraction vial of 20 mL. RPMI, PBS and internal standards (1μL) were included as control samples. Extraction was performed at 40 ºC during 60 min under agitation (250 rpm) using an automatic injector Multifibre Change CTC Analysis model Mps2xl. Thermal desorption of analytes from the fibre was carried out in the chromatograph injector port at 220ºC in splitless mode. GC was performed with an Agilent Technologies model 5975C gas chromatograph, at a constant flow of 1 mL/min for 5 min using a VA624 column (60 m x 0.25 mm; 1.4 μm). Oven temperature programme employed included an initial isothermal time at 35 ºC for 5 min, followed by a 5ºC/min ramp up to 240 ºC setting during 30 min in isothermal mode. MS was performed with Agilent Technologies model 7890A mass analyser without solvent delay in scan mode and in the 35-650 m/z mass range. Analysis was performed at the MS Unit- UCM.

Transmission electron microscopy (TEM)

The specimens were fixed for 3 h in a solution of glutaraldehyde 2.5% + paraformaldehyde 4% in PBS at 4ºC followed by 2% osmium tetroxide + 3% potassium ferrocyanide for 1 h, room temperature, darkness. Samples were dehydrated with increasing concentrations of acetone in water and soaked in resin SPURR (Fedelco Madrid). The resin was polymerized at 70°C for 72 h. Thin slices, 0.5 μm thick, were dyed with Richardson's methylene blue and observed by light microscopy. Ultra-thin slices, 70 nm thick, were treated with 2% uranyl acetate, as well as Reynold's lead citrate solution and examined with a Zeiss EM 902, transmission electron microscope (Carl Zeiss) at the SME-UCM.

Statistical analysis

Statistical significance of differences between mean values obtained in the different groups was performed by one-way and two-way ANOVA, repeated measures analysis and Bonferroni test. Differences were considered significant when $p<0.05$.

3.4. Results

Allicin inhibits the multiplication of Leishmania promastigotes

Micromolar concentrations of allicin effectively inhibited the growth of promastigotes of *L.infantum* and *L.donovani* in a dose- and time-dependent manner ($p<0.0001$) (Figure 1A and 1b). Only a moderate anti-leishmanial activity (ca. 30-40% reduction as compared to control) was observed with the lowest concentration (30 µM) whereas 60 µM and 120 µM provoked a reduction over 80% of the initial population of promastigotes after 24 h ($p<0.05$). Addition of 120 µM allicin completely eliminated the promastigotes after 72 h. There was a certain recovery of *Leishmania* cultures treated with 30 µM allicin from day 1 onwards. Microscopical observation showed active cell division with this concentration (not shown) and the motility of promastigotes was higher after 72 h than in similarly treated cells during 48 h.

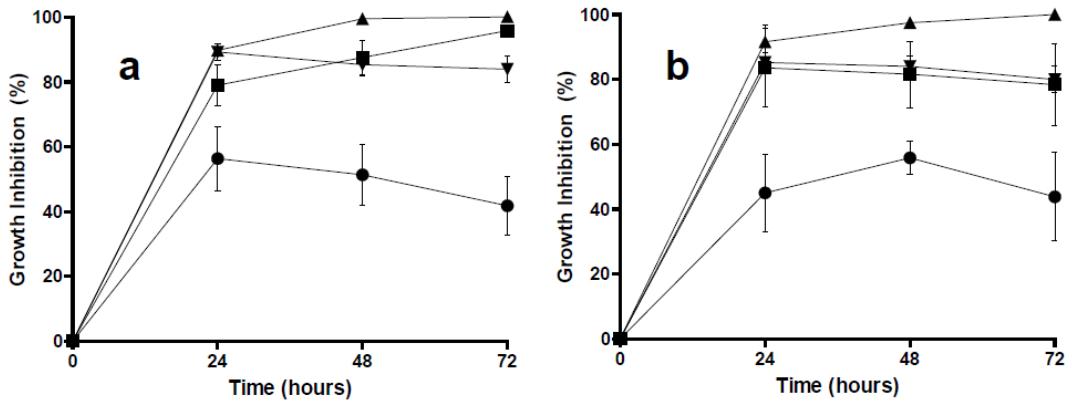


Figure 1. Effect of allicin on the proliferation of *Leishmania infantum* (**Figure 1A**) and *L.donovani* promastigotes (**Figure 1B**). Compounds were added at time 0. ●: 30 µM; ■: 60 µM; ▲: 120 µM; ▼: amphotericin (0.1 µM). Results are the means of three experiments ± standard deviation.

As expected, 0.1 µM amphotericin provoked a reduction between 80 and 90% of the promastigote number after 24 h. Treatment with allicin (60 and 120 µM) provoked important ultrastructural alterations of *Leishmania* promastigotes (Figure 2). Cellular swelling with reduction of electrodense material in the cell cytoplasm and generalized vacuolisation were observed. In addition, there was deformation and loss of integrity of membranes from both mitochondrion and kinetoplast, and chromatin condensation in the nucleus and kDNA.

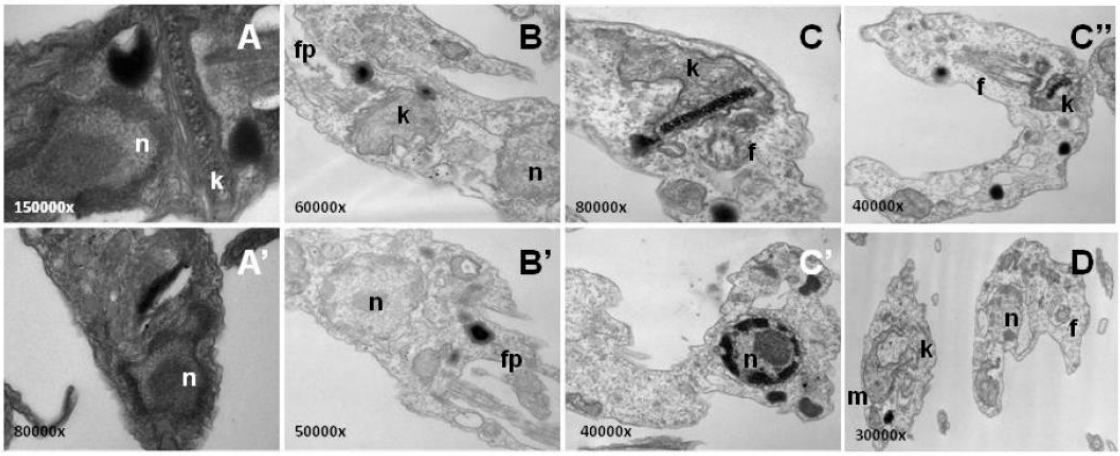


Figure 2. Transmission electron micrographs of promastigotes of *Leishmania infantum* treated with 60 µM (**B** and **B'**), 90 µM (**D**) and 120 µM (**C, C', C''**) as compared to untreated control cultures (**A** and **A'**). Treated promastigotes showed generalized vacuolization, reduction of electrodense material in the cytoplasm, cellular swelling (**C''**), loss of integrity of mitochondrial and kinetoplast membranes (**B** and **C**), and chromatin condensation in the nucleus (**C'**) and kDNA (**C**). n: nucleus, k: kinetoplast, fp: flagellar pocket, f: flagellum, m: mitochondrion. Magnifications are given on each micrograph.

Toxicity of allicin for mammalian cells

Toxicity of a range of allicin concentrations on the murine macrophage cell line J774 and mouse peritoneal macrophages was examined (Figure 3) with MTT method. Apparently, all concentrations of allicin affected cell viability (p<0.0001) and 10 µM almost completely arrested cell proliferation of J774 after 24 h (Figure 3A). However, results obtained with MTT did not correlate with the microscopical examination of May-Grünwald-Giemsa stained cultures. Cultures treated up to 60 µM allicin did not show significant microscopic alterations as can be observed in Figure 4 (C and C'). The apparent discrepancy could be related to the lack of discrimination between cytotoxic and cytostatic effects from these colorimetric methods. On these grounds toxicity was tested in non-dividing primary cells, mouse peritoneal macrophages (Figure 3B). Results showed that allicin did not induce any significant (p<0.0001) toxicity up to

a concentration of 60 µM and the cell viability was reduced only by 10% after exposition of macrophages to this concentration.

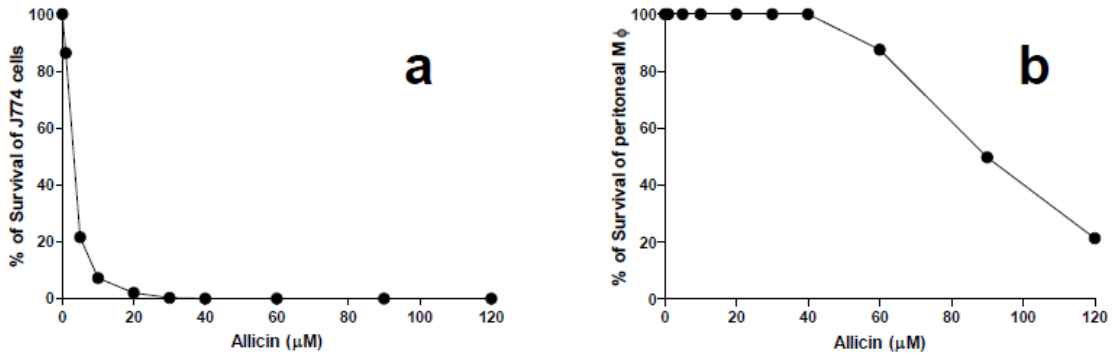


Figure 3: Effect of different concentrations of allicin on the viability of J774 cells (**Figure 3A**) and BALB/c mice peritoneal macrophages (**Figure 3B**). Cytotoxicity was determined by MTT assay. Results are means of eight determinations.

Allicin also inhibits the multiplication of intracellular amastigotes of Leishmania

The apparent low toxicity prompted us to examine the anti-leishmanial activity of allicin on intracellular amastigotes of *Leishmania*. Given our initial experimental design, the effect of allicin was tested on previously infected J774 cells. The modification of the infection procedure developed was highly successful since ca. 70% of the J774 cells were infected with *Leishmania* amastigotes. Results obtained showed that the compound was effective against *Leishmania* amastigotes multiplication after 24 h incubation. This anti-leishmanial effect was observed in both *L.donovani* and *L.infantum* in spite of the higher amastigote burden in *L.donovani* infections (Figure 4). Apparently the compound behaved as leishmaniostatic since no notable reduction in the infection rate was appreciated in any of the species tested whereas a significant (p<0.05) reduction in the amastigote burden was induced. Antileishmanial effect of allicin was dose-dependent (Figure 5) and dose-response curves yielded an approximate IC_{50} value of 10 µM for both species.

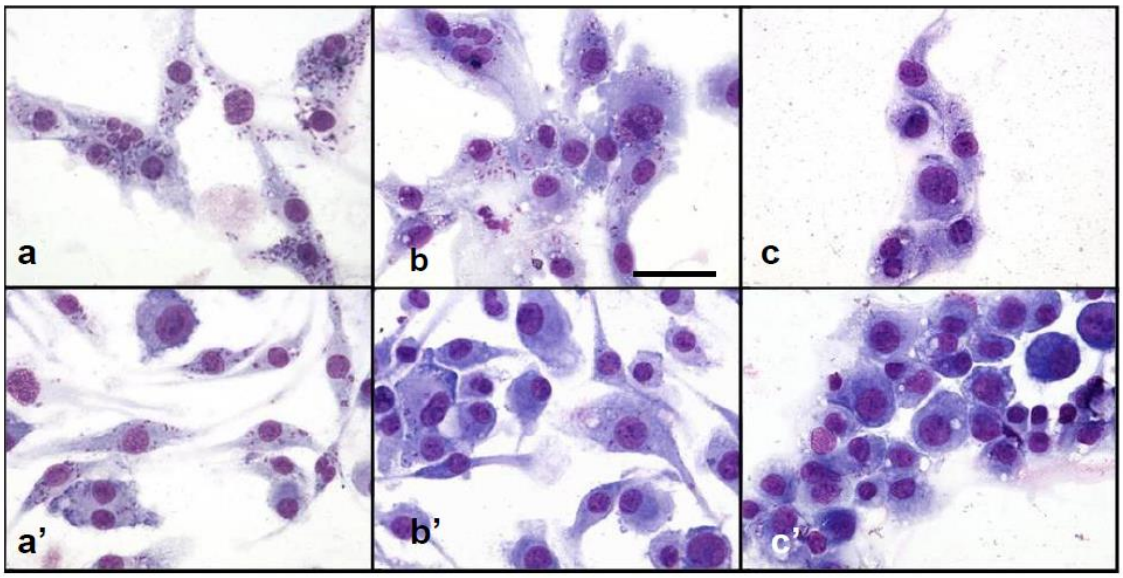


Figure 4. Effect of allicin on the infection of J774 macrophages with *Leishmania donovani* (upper row) and *L.infantum* (lower row): **a** and **a'**: infected and untreated control cultures; **b** and **b'**: cultures treated with 30 µM allicin; **c** and **c'**: cultures treated with 60 µM allicin. Micrographs are representative images stained with May-Grünwald-Giemsa of the triplicate cultures. Bar: 50 µm.

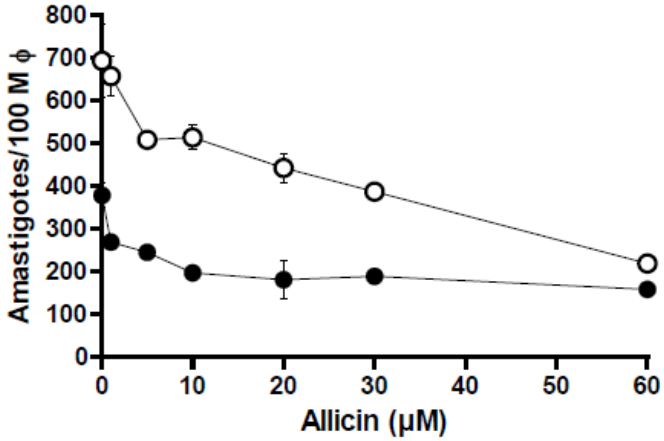


Figure 5. Effect of the single administration of different concentrations of allicin on the intracellular amastigote burden of J774 cells infected with *Leishmania donovani* (empty circles) and *L. infantum* (filled circles). Results are the mean ± standard deviation of three experiments.

Thermal degradation of allicin

The apparent recovery of promastigotes cultures of *L. donovani* and *L. infantum* treated with 30 µM allicin (see Figure 1) suggested the possibility of degradation of the compound in the experimental conditions established. Mass spectrometry analysis of allicin in RPMI 1640 medium, exposed for 24 h at the culture temperatures for promastigotes and amastigotes, 26 ºC and 37 ºC respectively, showed that the molecule was degraded at both temperatures (Figure 6). Our technique did allow us to detect 19 peaks in fresh untreated allicin preparation (Figure 6a) whereas only 12 peaks were found after treatment at 37 ºC for 24 h (not shown). Thermal degradation products included diallyl sulphides (DAS), diallyl disulphides (DADS) and a vinyldithiin although no evidence of allicin or ajoene was obtained. Significantly simpler chromatograms were observed in cultures containing allicin (120 µM) treated at 26 ºC. Only DADS (Figure 6b) and 2-Butoxyethyl acetate and Benzaldehyde, 3-5 dimethyl were found; the last two compounds corresponded to the culture medium, RPMI 1640 (Figure 1c).

Consistently with the degradation of allicin, anti-leishmanial activity of the preparation was greatly improved when this product was freshly added every 24 h. Figure 7 shows the results obtained with *L.donovani* and comparable results were obtained with *L.infantum* (not shown). All concentrations induced a reduction in the number of promastigotes, after 24 h exposition to the drug, in a concentration related manner ($p<0.05$) and no recovery of the cultures could be appreciated along the experiment. A different pattern of inhibition was observed onwards (48 h and 72 h) depending on the concentration of allicin. Cultures with the lower concentrations tested (1 µM, 5 µM and 10 µM) did not multiply for the duration of the experiment. By its part, the antileishmanial effect of 30 µM and 60 µM, added every 24 h, provoked a strong reduction of the promastigote number and, eventually, promastigote clearance with the highest concentration tested after 48 h. These results suggested a leishmaniostatic effect of allicin at low concentrations whereas concentrations over 30 µM behaved as leishmanicidal for promastigotes under our conditions.

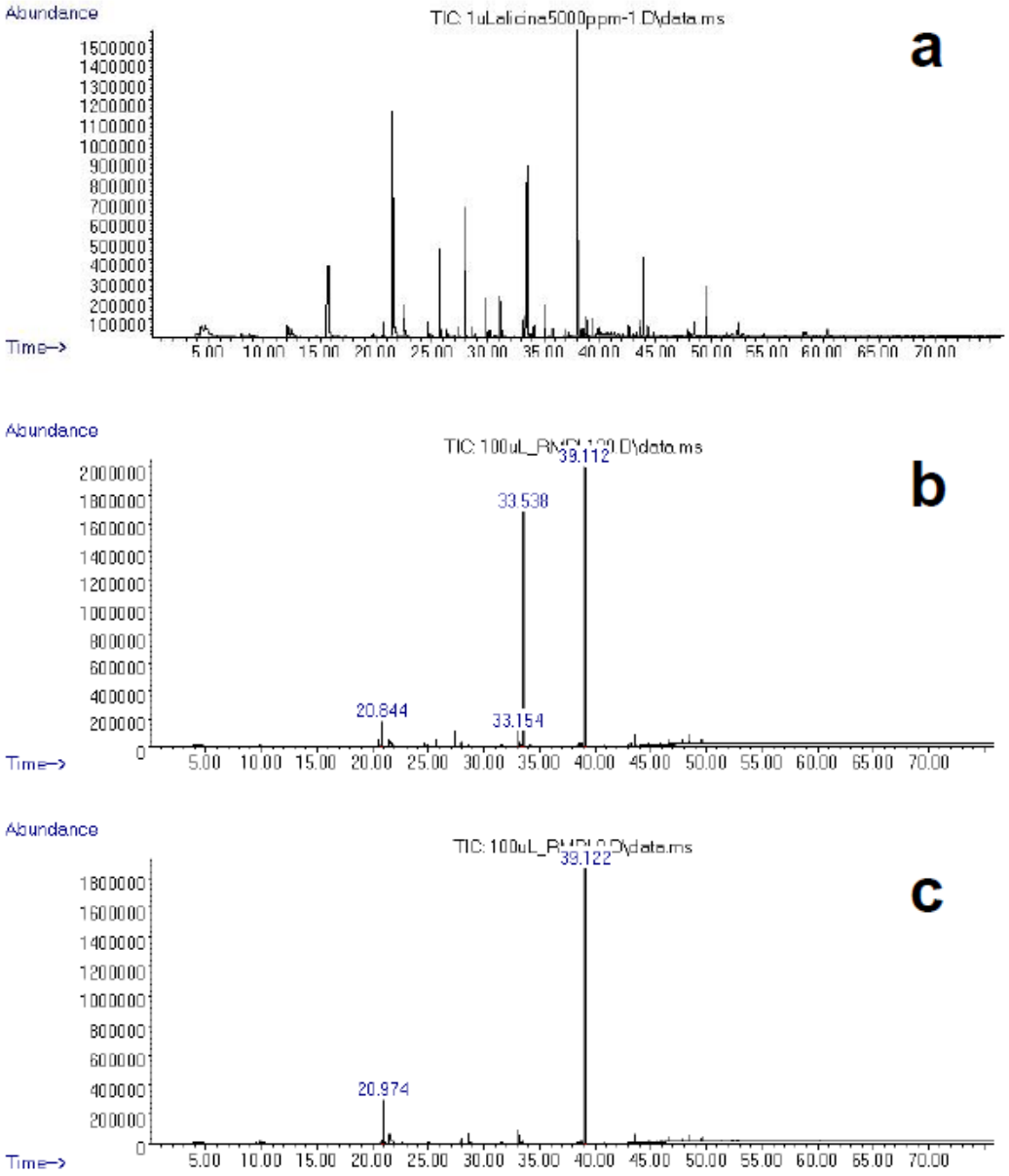


Figure 6. Mass spectrometry representative chromatograms of 120 µM allicin + RPMI 1640 medium treated for 24 h at 26ºC (**b**). For comparative purposes fresh untreated allicin (5000 ppm) (**a**) and RPMI 1640 medium treated for 24 h at 26 ºC (**c**) chromatograms are included.

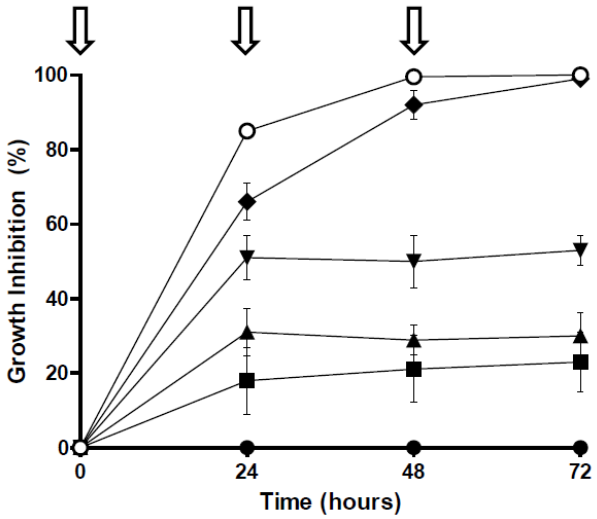


Figure 7. Effect of different concentrations of allicin added every 24 h on the multiplication of *Leishmania donovani* promastigotes. Arrows: days of treatment with allicin. ●: untreated control cultures; ■: 1 µM; ▲ : 5 µM; ▼ : 10 µM; ♦: 30 µM; ○: 60 µM. Results are means ± standard deviation of three experiments.

The effect of the addition of allicin every 24 h on the actual stage causing the leishmanial infection in mammals, amastigotes, was evaluated in an *ex vivo* assay with mouse peritoneal macrophages infected with *L.donovani* (Table 1).The modification of the infection procedure was very efficient since over 90% of the macrophages were infected with amastigotes after 24 h. Infection rate was only slightly affected by both treatment with allicin, and time. The effect of the compound on the number of macrophages infected was moderate and actually with the highest concentration used (30 µM), after 72 h infection rate was 60 ± 6.5% as compared to 86 ± 2.6 % in the untreated cultures (p<0.05). More relevant was the effect of allicin on the intracellular amastigote burden. Untreated control cultures displayed active multiplication along the experiment, reaching 965.7 ± 59.9 amastigotes/100 macrophages from the initial burden of 489 ± 24.2 after 24 h. Antiproliferative effect of allicin was concentration and time dependent (p<0.0001, F=12.64). Both amastigote burden (number of amastigotes/ 100 macrophages) and relative parasite growth inhibition were significantly reduced with all concentrations of allicin (p<0.05 to p<0.01). After 24 h, 10 µM allicin reduced by ca. 40% the number of amastigotes as compared to the untreated control cultures and over 67% after 72 h (p<0.05), with three administrations of allicin. More importantly, 5 µM allicin significantly (p=0.0014) reduced by ca. 50% the number of intracellular *Leishmania* amastigotes after 48 h.

Table 1. Effect of allicin replaced every 24 h on the multiplication of intracellular amastigotes of *Leishmania donovani* in peritoneal macrophages. Values are means ± standard deviation.

Allicin (μM)	Amastigotes/ 100 Mφ	% of infection	% Parasite growth inhibition
24h			
0(a)	489.0 (24.2)^{bcde}	91.7 (3.5)	0.00
1(b)	418.7 (28.5)^{ed}	91.0 (5.5)	14.38(5.84)^{cde}
5(c)	346.7 (32.5)^{ae}	81.7 (2.5)*	29.11(6.65)^{bde}
10(d)	292.0 (16.7)^{ab}	74.0 (4.0)*	40.29(3.41)^{bce}
30(e)	177.3 (6.8)^{abc}	70.0 (1.0)*	63.74(1.39)^{bcd}
48h			
0 (a)	781.0 (64.9)^{bcde}	79.0 (7.5)	0.00
1(b)	632.0 (58.5)^{acde}	77.0 (6.0)	19.08(7.49)^{cde}
5(c)	402.0 (21.3)^{abe}	70.0 (4.5)	48.53(2.73)^{be}
10(d)	373.3 (61.2)^{abe}	79.3 (3.7)	52.20(7.84)^{be}
30(e)	228.3 (22.0)^{abcd}	70.7 (2.5)	70.76(2.81)^{bcd}
72h			
0(a)	965.7 (59.9)^{bcde}	86.0 (2.6)	0.00
1(b)	670.7 (56.7)^{acde}	82.7 (4.0)	30.55(5.87)^{cde}
5(c)	436.3 (39.5)^{abde}	72.0 (5.5)*	54.82(4.09)^{bde}
10(d)	317.7 (7.5)^{abc}	65.0 (1.0)*	67.10(0.77)^{bc}
30(e)	267.7 (29.6)^{abc}	60.0 (6.5)*	72.28(3.07)^{bc}

Asterisks (*) represent values significantly different (p<0.05) to the untreated control cultures. Different superscripts within a column for each time (24h, 48h, 72h) represent differences (p<0.05 to p<0.01) between cultures treated with allicin.

3.5. Discussion

A significant inhibitory effect of allicin on the multiplication of both *L. donovani* and *L.infantum* has been found by us. Inhibition was time- and dose-dependent against promastigotes and intracellular amastigotes *in vitro* (J774 cells) and *ex vivo* (mouse peritoneal macrophages). Very few reports have been published on the effect of allicin against *Leishmania* promastigotes. The approximate IC_{50} value obtained (10- 30 µM, 24 h exposition) by us for promastigotes from both species is in the line of the value indicated by Ankri and Mirelman (1999) (30 $\mu g.mL^{-1}$) for promastigotes of *L. major* and slightly higher than that reported by McClure et al. (1996) for *L.chagasi* and *L.mexicana*. Allicin, in the micromolar range, effectively inhibited the multiplication of amastigotes within J774 murine line cells and peritoneal macrophages. As far as we know this is the first report on the inhibitory effect of allicin against intracellular amastigotes.

Mechanism of action of allicin has been related to the rapid reaction with thiol groups (Rabinkov et al., 1998) but the actual intracellular targets responsible of the cytostatic / cytocidal effects are largely unknown. It has been reported that allicin induces apoptosis (Oomen et al., 2004) and our TEM results showed condensation of nuclear chromatin and kDNA and loss of integrity of mitochondrial and kinetoplast membrane. Ledezma et al. (2002) also observed nuclear alterations in *L.amazonensis* promastigotes treated with the related compound ajoene. Antioxidant systems of glutathione and trypanothione/ trypanothione reductase participate in the protection of *Leishmania* against the toxic effect of nitrogen-derived reactive species from macrophages (Romão et al. 2006). Interference of allicin with these thiol-redox proteins could lead to amastigote destruction and this possibility should be explored. Besides this interaction other cell targets (i.e. other thiol-dependent enzymatic systems, cysteine proteinases, microtubule disruption) (Ankri and Mirelman, 1999, Prager-Khoutorsky et al., 2007, Miron et al., 2010, Waag et al., 2010) should be involved since comparable IC_{50} values for allicin, administered as a single dose, were found by us in intracellular amastigotes in J774 cells and extracellular promastigotes.

The modified method employed in the macrophage infection with *L.infantum* metacyclic promastigotes (Ficoll gradient + opsonisation with 15% mouse serum) allowed to obtain higher yields than those achieved with previously used methods (Méndez et al., 1996, Wert et al., 2011). Comparative advantage of the methodology was particularly evident in the *ex vivo* experiments with mouse peritoneal macrophages and provides us with a valuable tool for *in vitro* drug screening against intracellular amastigotes of this species.

Under our conditions, toxicity of allicin in the concentration range used was low and no significant effect on mouse macrophages was observed up to 40 µM as estimated by MTT method. Moreover, the absence of substantial toxicity of the compound was confirmed by microscopical examination without any noticeable cell alteration up to 60 µM in both J774 cells and BALB/c peritoneal macrophages. A lack of correlation between the results of toxicity obtained with the colorimetric method in J774 cells and the findings in peritoneal macrophages was found. This apparent inconsistency could be related to the limitations of colorimetric methods to distinguish between cytostatic and cytotoxic effects of a drug (Plumb, 2004). Our results showed that allicin is a potent cytostatic agent but of scarce toxicity for mammalian cells as confirmed by the results obtained in non-dividing peritoneal macrophages. In addition it stresses the need of performing direct microscopic observation of the cells besides indirect methods when testing potentially useful drugs against intracellular parasites.

Administration of allicin every 24 h greatly increased the anti-leishmanial activity of the compound. This finding suggested the degradation of the diallyl thiosulfinate at 26º and 37 ºC and is consistent with the reported thermo-sensitivity of the molecule (McClure et al., 1996; Cañizares et al., 2004). The degradation of the allicin preparation employed by us after 24 h at 26º C and 37 ºC was confirmed by mass spectrometry. Allicin is a highly reactive thiosulfinate which is rapidly transformed to other types of organosulfur compounds, particularly diallyl disulfides (DADS), diallyl sulphides (DAS), ajoene and vinyldithiins (Amagase, 2006). Our results showed the presence of sulphides and vinyldithiins but not ajoene (E or Z) and allicin were found in any of the analysis. Simpler chromatograms of culture samples are probably related to the lower concentrations of allicin (<120 µM) as compared to the standard sample. The absence of allicin and ajoene could be due to their short half-life at the culture temperatures (26 ºC and 37 ºC) (i.e. ajoene, less than 1 min) and the analytical method employed. It is possible that less stringent analysis (HPLC) (Lanzotti, 2006) would allow the detection of these compounds. It is noteworthy to indicate that most studies on the degradation of allicin have been carried out with free allicin whereas our experiments were performed with a stabilized form of this molecule. Whether or not the actual antileishmanial agent in our studies is allicin or some other degradation product requires further experimentation. It has been indicated that thermal sensitivity of allicin at physiological temperature as well as its rapid transformation to other types of organosulphur compounds (Amagase, 2006) could rule out its therapeutic use. However, it has been shown that decline of allicin is accompanied by its transformation into other compounds (i.e. ajoene) (Fujisawa et al., 2008) with strong anti-leishmanial activity (Ledezma et al., 2002). Moreover, the metabolism of allicin, and therefore its bioavailability, is unknown for the most part. Recently, oral and peritoneal administration of methanolic extracts

of garlic significantly reduced the *L.donovani* burden in spleens of hamster infected with *L.donovani* (Wabwoba et al., 2010).

Chemotherapy of leishmaniasis is far from ideal (Alvar et al., 2006; Chappuis et al., 2007) and new drugs and combinations are required. Value of IC_{50} found by us *in vitro* and *ex vivo* (5-10 µM) against intracellular amastigotes possibly excludes allicin as monotherapy against leishmanial infections. However, low cost and scarce toxicity of this compound would allow its use in combined therapy with other well established anti-leishmanial drugs.

In conclusion, allicin (=diallyl thiosulphinate) has shown a significant *in vitro* effect on the multiplication of promastigotes of *L.donovani* and *L.infantum*. Allicin exhibited a scarce toxicity for J774 cells and mouse macrophages. Moreover, it has been shown for the first time the inhibition of multiplication of intracellular amastigotes of *Leishmania*. Consistent infections of macrophages (J774 cells, mouse peritoneal) with *L.infantum* have been obtained with a modified method to isolate metacyclic promastigotes. Inhibitory concentrations of allicin were in the micromolar range for both *Leishmania* species and stages. More stable galenic preparations are probably needed. However, low toxicity for mammalian cells of this compound suggests the interest of testing this molecule *in vivo* in combined therapy against leishmanial infections.

3.6. Acknowledgements

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CHAPTER 4

In vitro synergistic effect of Amphotericin B and Allicin on *Leishmania donovani* and *L.infantum*

4.1.a. Resumen

Efecto sinérgico de anfotericina B y alicina sobre L.infantum y L.donovani

La monoterapia actual para el tratamiento de la leishmaniosis presenta importantes efectos adversos y se han identificado resistencias frente a los compuestos de primera línea. La anfotericina B (AmB) ha mostrado una excelente eficacia frente a *Leishmania* y hasta la fecha no se han detectado resistencias frente al antibiótico. No obstante, su toxicidad ha limitado su uso generalizado. Los resultados obtenidos han demostrado que la combinación de alicina y AmB ha resultado de moderadamente sinérgica a sinérgica con concentraciones bajas (0,07 µM AmB + 35,45 µM alicina indujo una inhibición del crecimiento del 95%). Ninguno de los tratamientos ensayados demostró citotoxicidad sobre macrófagos murinos en la horquilla de concentraciones ensayada. Alicina, AmB o la combinación de ambos no modificaron los porcentajes de infección de los macrófagos infectados por *Leishmania* spp. Alicina mejoró la actividad de AmB sobre amastigotes intracelulares de *L.donovani* y *L.infantum*. La combinación de 0,05 µM AmB +10 µM alicina indujo una reducción de *ca.* 45% en el número de amastigotes intracelulares, permitiendo reducir a la mitad la dosis necesaria del antibiótico para alcanzar la concentración inhibitoria 50 (IC_{50}) de AmB en monoterapia. Estos resultados apuntan al interés de explorar esta combinación *in vivo* y reducir, por tanto, la toxicidad asociada a la AmB empleada en monoterapia.

4.1.b. Abstract

Current monotherapy against visceral leishmaniasis has serious side effects and resistant *Leishmania* strains have been identified. Amphotericin B (AmB) has shown an extraordinary anti-leishmanial efficacy without emergence of resistance; however toxicity has limited its general use. Results obtained showed, using a fixed ratio analysis, that the combination of diallyl thiosulfinate (allicin) and AmB ranged from moderately synergic to synergic at low concentrations (0.07 µM AmB + 35.45 µM allicin induced a 95% growth inhibition). None of the treatments, alone or in combination, had noticeable adverse effect on macrophages (Mφ) in the concentration range examined (Allicin: 0.5, 1, 5 and 10 µM; AmB: 0.05, 0.075, 0.1 µM). Allicin, AmB or the combination did not affect the infection rate (% of infected Mφ) of *Leishmania*. Allicin enhanced the activity of AmB on intracellular amastigotes of *L.donovani* and *L.infantum* (*ca.* 45% reduction on amastigote burden with 0.05 µM AmB +10 µM allicin) this representing near a twofold reduction in the IC_{50} value of the antibiotic added alone. Results point towards the interest of testing this combination *in vivo* thus reducing the toxicity associated to the monotherapy with AmB.

4.2. Introduction

Human *Leishmania* infections are present in all inhabited continents, with around 12 million people infected, 2 million new cases every year, and estimates of around 350 million people living in areas at risk (http://who.int/health-topics/leishmaniasis.htm). Leishmaniasis is a spectral infection with a range of clinical presentations, from self-healing dermal infections to deadly processes. Cutaneous leishmaniasis is by large the most common disease, but visceral leishmaniasis [caused by *Leishmania donovani* and *L. infantum* (=*L. chagasi*)] is also present in all continents, and the infections produce severe disease. The distribution of these infections has greatly expanded, and coinfection of *Leishmania* and HIV is very frequent (Desjeux, 2004). In addition to human cases, canine leishmaniasis caused by *L. infantum* (Mediterranean Basin) and *L. chagasi* (South America) constitutes a first-order pathology in veterinary clinics besides the zoonotical importance of dogs as reservoirs. The main control system of leishmaniasis in both humans and dogs is chemotherapy. However, antileishmanial drugs have some important shortcomings, including high toxicity and teratogenicity in some cases, absence of parasitological cure in most cases, and unaffordable prices for some of the compounds and presentations. Moreover, in some areas, *Leishmania* strains resistant to commonly used drugs, particularly antimonials, have emerged (Croft et al., 2006). Liposomal amphotericin (AmB), paromomycin, and miltefosine were considered the most promising drugs for chemotherapy of leishmaniasis (http://www.who.int/tdr/diseases/leish). However, for the large part, these drugs were introduced over 40 years ago, and new active compounds or combinations against *Leishmania* are needed (Alvar et al., 2006; Chappuis et al., 2007).

The pharmaceutical industry has experienced a contraction during recent years, resulting in very few companies being present in the market. It is anticipated that investment and intercompany competition and consequently the launch of new antiparasitic agents will be reduced (Woods & Knauer, 2010). One of the alternative chemotherapeutic approaches is the use of combinations of effective existing drugs whose toxicity precluded them from being widely used with chemically unrelated compounds of reduced toxicity. The antibiotic AmB, besides being the standard treatment for systemic fungal infections, has shown an extraordinary antileishmanial efficacy. Its main mechanism of action, binding to ergosterol-containing membranes of *Leishmania* (Seifert, 2011), probably explains the lack of significant emergence of resistance to the compound. However, toxicity has limited its general use, and different low toxicity preparations have been developed (i.e., liposomes), but their high price limits their standard use (Sundar & Chatterjee, 2006). Some low-cost vehicles for the antibiotic have been tested *in vitro* and *in vivo* [i.e., albumin microspheres (Sánchez-Brunete et al., 2005) and polylactic-co-glycolic acid (PLGA) (Ordóñez-Gutiérrez et al., 2007; Van de Ven et al., 2012),

although without further development. For its part, allicin (diallyl thiosulfinate = 2-propene-1 sulfinothioic acid S-2-propenyl ester), a natural product present in plants of the family Alliaceae, with antibacterial (Cutler & Wilson, 2004), antifungal (*Candida*) (Khodavandi et al., 2011), and antiprotozoal (*Plasmodium* and *Leishmania* promastigotes) (McClure et al., 1996; Waag et al., 2010) activities, has been found to exhibit a notable antileishmanial effect on the intracellular stages of *L. donovani* and *L. infantum* without substantial cytotoxicity for mammalian cells (Corral-Caridad et al., 2012).

On these grounds, our approach was the exploration of the potential synergistic or additive antileishmanial effect of the combination of AmB and allicin (low micromolar concentrations of AmB plus micromolar allicin), thus avoiding the toxic concentrations needed with AmB in monotherapy. Results obtained *in vitro* against both promastigotes and intracellular amastigotes of *L. donovani* and *L. infantum* showed that allicin significantly enhanced the leishmanicidal activity of AmB and therefore reduced the required amount of AmB to eliminate intracellular infection of Mφ by *Leishmania*.

4.3 Material and methods

Parasites

L. infantum (MCAN/ES/2001/UCM-9) is an autochthonous isolate obtained at the Clinical Service of the Department of Animal Health, Faculty of Veterinary Medicine (UCM), from a naturally infected dog (Madrid, Spain). *L. donovani* isolate Khartoum 1246 (MHOM/SD/43/124) was provided by A. Toraño (Department of Immunology, Instituto de Salud Carlos III, Madrid, Spain). Both species were routinely maintained as promastigotes in 25-ml culture flasks at 26°C in RPMI 1640 medium (Lonza Group) supplemented with 10% heat-inactivated (30 min at 56°C) fetal bovine serum (FBS; Sera Laboratories International) and 100 U/ml of penicillin plus 100 μg/ml of streptomycin (BioWhittaker).

Drugs

Fungizone (deoxycholate-dispersed amphotericin B) was a gift from Bristol-Myers Squibb. Stabilized allicin was obtained as liquid Allisure (5,000 ppm) from Allicin International Ltd. (Rye, East Sussex, UK). For the *in vitro* experiments, 10 mM stock, solutions of amphotericin B deoxycholate (AmB) in dimethyl sulfoxide (DMSO; Sigma) were prepared. Further dilutions were freshly made in RPMI 1640 medium. The final DMSO concentration never exceeded 0.5%

in the culture medium and had no effect on cell growth. Dilutions of allicin were directly performed in culture medium.

Promastigote assay

Cell counts

Preliminary tests to determine the 50% inhibitory concentrations (IC_{50}s) of the individual drugs were performed. For the combination assay, the IC_{50}s of the single drugs, previously determined by cell counts, were combined. *Leishmania* log-phase promastigotes were cultured in 10-ml sterile polystyrene tubes (initial concentration, 10^6 parasites/ml; 2 ml/tube) and treated with 0.07 μM AmB or 30 μM allicin or the combination of both (0.07 μM AmB plus 30 μM allicin). Cultures were counted 24, 48, and 72 h post-treatment in a Neubauer chamber, and the viability was determined with trypan blue exclusion dye [0.4% in phosphate-buffered saline (PBS)]. Experiments were performed in triplicate.

Alamar Blue assay for promastigote proliferation and synergism of the drug combination

Mid-log-phase promastigotes were seeded in 96-well culture plates (Corning) at an initial plating density of 2.5 x 10^5 cells/well (200 μl/well). Cultures were exposed for 24 h to various concentrations of the drugs and their combinations. Promastigote proliferation and inhibition were determined with Alamar Blue reagent (AbDSerotec Ltd.) (10%, v/v) by following the manufacturer's recommendations by reading fluorescence intensity (550-nm excitation wavelength and 590-nm emission wavelength) in a FLUOstar Omega (BMG Labtech) fluorimeter after 72 h of incubation with the dye. Untreated cultures, wells without cells and the maximal concentration from each compound and their combinations, and wells with culture medium and Alamar Blue (10%, v/v) were included as controls. Results for growth inhibition were expressed as percentage of growth in untreated control cultures. Cultures were performed at least in triplicate.

Alamar Blue assays with increasing concentrations of the single drugs were performed first, and appropriate drug combination ratios were tested according to the results obtained. Drugs were combined at constant ratios [0.01:5, 0.01:10, and 0.01:20 (μM AmB/ μM allicin)] by following a modified fixed-ratio method (Seifert & Croft, 2006), and their dose-effect relationships were assessed by the Chou-Talalay combination index (CI) method using CalcuSyn software (Chou & Hayball, 1996; Chou, 2008). This method is based on the multiple-drug effect equation derived from the median-effect principle and the mass action law. Results

provide a quantitative determination for synergism (CI < 1), additivity (CI = 1), and antagonism (CI > 1). The software allows the calculation of the dose reduction index (DRI), a parameter which indicates the fold dose reduction allowed in a drug combination to reach a given degree of inhibition compared to the drug as a single agent. It provides other dose-effect-related parameters such as the potency (D_m) of the drugs alone or in combination, this representing the concentration which inhibits cell growth by 50%, and the shape of the dose-effect curve (*m*), where *m* of 1, > 1, and < 1 indicate hyperbolic, sigmoidal, and flat sigmoidal curves, respectively. The linear correlation coefficient (*r*) of the median-effect plot represents the conformity of the data to the mass action law.

Cytotoxicity assay for mammalian cells

Toxicity for mammalian cells was determined *ex vivo* in murine (female BALB/c mice; Harlan) peritoneal Mφ isolated by peritoneal lavage, resuspended in RPMI 1640 medium supplemented with 10% FBS, and seeded in 96-well culture plates (4 x 10^6 cells/ml; 200 µl/well). Plates were incubated overnight to allow cell adherence at 37°C in a 5% CO2–95% air mixture. Different concentrations of allicin (10, 30, and 40 µM) plus AmB (0.1 and 0.5 µM) were added to the cultures. At the end of the drug exposure period (24 h), the medium was removed; wells were replenished with fresh medium and incubated for another 24 h. The viability of cells was determined by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay as described previously (Plumb, 2004). Briefly, 50 µl of MTT (Sigma) solution (5 mg/ml in sterile PBS) was added to the wells, and the plates were kept for 4 h at 37°C in a humidified atmosphere protected from light. Formazan crystals were dissolved with 200 µl of DMSO and 25µl/well of Sorensen's glycine buffer (0.1 M glycine and 0.1 M NaCl, adjusted to pH 10.5 with 1 M NaOH) and added to stop the reaction. Absorbance was read at 570 nm in a microplate reader. Untreated cells and wells without cells and the maximal concentration from each compound were included. Three independent experiments were carried out.

Amastigote assay and determination of the synergism of the drug combination

Infection of Mφ was carried out with the modification developed by us (Méndez et al., 1996; Corral-Caridad et al., 2012). In brief, 2.5 x 10^4 J774 cells/well (200 µl/well) were cultured in 8-well Lab-Tek chambers (Nunc) for 16 h to ensure adherence. Stationary-phase *Leishmania* promastigotes (day 7 to 10) were centrifuged (370 x*g*, 10 min, without brake) in a 5810R centrifuge (Eppendorf) through a Ficoll gradient (0, 10% in medium 199, and 30% in sterile

PBS). Metacyclic parasites recovered were washed and opsonized with 15% normal mouse serum (Jackson ImmunoResearch) in a solution (1/1, v/v) of RPMI medium and Hanks' balanced salt solution (HBSS; Sigma), 0.15 mM $CaCl_2$, and 1 mM $MgCl_2$ for 30 min at 37°C and 5% CO_2. Infection was carried out overnight at 33°C at a parasite/Mφ ratio of 10:1 in 5% CO_2. Non-internalized promastigotes were removed by thorough washing, and fresh media containing the appropriate compounds dilutions were added to the wells. Cultures were treated with AmB (0.05, 0.075, and 0.1 μM) and allicin (0.05, 1, 5, and 10 μM) administered alone and in combination for 48 h. Unless otherwise stated, mitomycin C (Calbiochem) at 1 μg/ml was added to avoid cell line proliferation. Untreated cultures, cultures treated with mitomycin C, and cultures treated with 0.5% DMSO were included as negative controls. Slides were fixed and stained (May-Grünwald Giemsa; Merck Darmstadt), and the number of amastigotes/100 cells and % of infected cells were determined. Drug activity was expressed as percentage of growth inhibition compared to that in untreated cultures. Three independent experiments were performed at least in triplicate.

Statistical analysis

Graphs were generated and statistical analysis was performed using GraphPad Prism 5. Statistical significance of differences between mean values obtained in the different groups was determined by repeated-measures generalized linear model analysis, one-way analysis of variance (ANOVA), and Bonferroni test. Differences were considered significant at a *p* value of < 0.05.

4.4. Results

Figure 1 shows the results obtained by cell counting in the preliminary test on the effect of the combination of AmB and allicin. Similar results were obtained with both species, *L. donovani* (Figure 1A) and *L. infantum* (Figure 1B). Both compounds, AmB (0.07 μM) and allicin (30 μM), reduced by more than 50% the *Leishmania* growth after 24 h of exposure, although a certain recovery of cell multiplication was observed on the 48 h and 72 h samplings. Combination of AmB (0.07 μM) and allicin (30 μM), however, provoked the cell death of the majority of the leishmanial population, and actually, after 72 h, *L. infantum* counts reached a mere 3 x 10^3/ml, this representing a reduction of more than 99% compared to the untreated cultures. These results were suggestive of a positive interaction (additive or synergic) between the compounds.

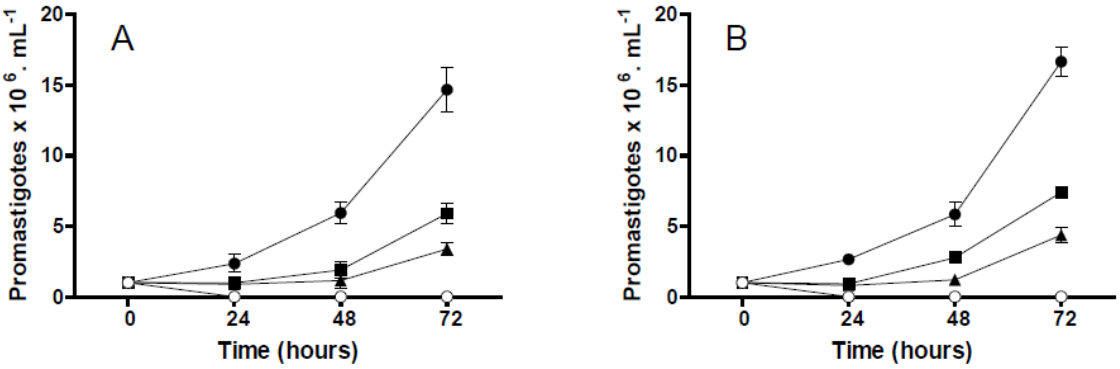

Figure 1. Effect of 30 µM allicin (■), 0.07 µM AmB (▲) and the combination (30 µM allicin + 0.07 µM AmB) (○) on the multiplication of *L.donovani* (**Figure 1A**) and *L.infantum* (**Figure 1B**) promastigotes after 24, 48 and 72 hours. (●): Untreated control cultures. Data are means ± standard deviation of an experiment in triplicate.

To determine the effect of the combination, drugs were tested at constant micromolar ratios (0.01:5, 0.01:10, and 0.01:20) (AmB/allicin); growth inhibition was estimated with the Alamar Blue fluorimetric method. The two *Leishmania* species behaved similarly; Table 1 shows the results obtained for *L. infantum* promastigotes. In all cases, data obtained agreed with mass law action (r) and inhibition followed a sigmoid curve ($m > 1$). D_m values, corresponding to IC_{50}s, obtained for the individual drugs tested were in the range of the previously estimated concentrations (22.1 µM for allicin and 0.09 µM for AmB). Growth inhibition results showed the significantly higher effect of the combination with allicin even with 0.01 µM AmB. Thus, whereas AmB administered alone did not induce at this concentration any inhibition of promastigotes' multiplication, the combination with 20 µM allicin provoked a 37.5% growth reduction. This effect of allicin was more evident with 0.02 µM AmB, since when the antibiotic was combined with 20 µM allicin, 50% inhibition was reached (IC_{50}), and with 40 µM allicin, 89% (IC_{90}) of the multiplication was stopped. Similarly, an AmB concentration of 0.08 µM, close to the IC_{50} of this compound, with 40 µM allicin reduced almost completely the multiplication of the promastigotes. It is noteworthy that microscopic examination of the promastigotes subjected to the combinations showed that they were not viable and that no recovery of the cultures was found (data not shown).

Table 1. Effect of AmB and allicin and their combination on promastigotes of *Leishmania infantum* determined by Alamar Blue assay.

Combination Ratio D_1-D_2	*AmB* (μM)	*Allicin* (μM)	Growth Inhibition (%)	Parameters		
				m	*D*m (μM)	*r*
(0.01:5)	0.0025	1.25	3.5 ± 0.59			
	0.005	2.5	3.4 ± 0.98			
	0.01	5	3.5 ± 0.45			
	0.02	10	3.6 ± 2.84			
	0.04	20	15.0 ± 1.32			
	0.08	40	99.0 ± 0.03			
	0.16	80	99.0 ± 0.02	2.655	11.71	0.90
(0.01:10)	0.0025	2.5	3.1 ± 1.54			
	0.005	5	3.3 ± 1.49			
	0.01	10	4.0 ± 2.02			
	0.02	20	52.0 ± 2.22			
	0.04	40	97.0 ± 1.65			
	0.08	80	99.0 ± 0.11			
	0.16	160	99.0 ± 0.12	1.998	21.96	0.91
(0.01:20)	0.0025	5	1.42 ± 1.05			
	0.005	10	2.09 ± 1.13			
	0.01	20	37.5 ± 1.52			
	0.02	40	89.0 ± 2.31			
	0.04	80	99.0 ± 0.07			
	0.08	160	99.0 ± 0.04			
	0.16	320	99.0 ± 0.02	2.478	26.30	0.94

Drugs were combined at constant ratios (0.01:5, 0.01:10, and 0.01:20) and their dose-effect parameters were assessed by the Chou-Talalay method (Chou & Hayball, 1996) using CalcuSyn software. Dm (median-effect dose) signifies the potency and it is the concentration which inhibits cell growth by 50%; m is the shape of the dose-effect curve, where m values of 1, > 1, and < 1 indicate hyperbolic, sigmoidal, and flat sigmoidal curves, respectively; r represents the linear correlation coefficient of the median-effect plot (conformity of the data to the mass action law principle). The m, *D*m (μM), and r values for AmB (drug 1 [D_1]) were 1.451, 0.09, and 0.86, respectively, and those for allicin (D_2) were 2.066, 22.13, and 0.90, respectively. Results are expressed as the means ± standard deviations of three independent experiments.

The combined effect of the two compounds in terms of synergism-antagonism was complex, as shown at Table 2. Generally speaking, all combinations allowed reduction of the concentrations required for AmB (DRI), and synergism (CI < 1) was evident only with the fixed ratio (0.01 μM AmB plus 5 μM allicin) (Figure 2). Combination allowed a 7- to 10-fold reduction of the AmB dose required to provoke a 90 to 95% reduction in the promastigotes' multiplication.

Table 2. Dose-effect relationships of the combination of AmB and allicin on *Leishmania infantum* promastigotes.

Drug combination ratio D_1+D_2	% Growth Inhibition (ED_n)	CI values	DRI value		Dose required (µM)	
			AmB	*Allicin*	*AmB*	*Allicin*
(0.01:5)	50	**0.79 (moderate synergism)**	3.89	1.89	0.023	11.69
	75	**0.65 (synergism)**	5.48	2.13	0.035	17.69
	90	**0.54 (synergism)**	7.73	2.39	0.053	26.75
	95	**0.49 (synergism)**	11.52	2.74	0.071	35.45
(0.01:10)	50	1.23 (moderate antagonism)	4.14	1.01	0.022	21.94
	75	1.16 (slight antagonism)	5.31	1.03	0.037	36.49
	90	1.09 (nearly additive)	6.81	1.05	0.061	60.71
	95	1.05 (nearly additive)	8.06	1.07	0.085	85.82
(0.01:20)	50	1.33 (moderate antagonism)	6.92	0.84	0.015	31.22
	75	1.19 (slight antagonism)	9.47	0.91	0.023	47.48
	90	1.07 (nearly additive)	12.96	1.01	0.036	72.23
	95	0.99 (nearly additive)	16.05	1.06	0.048	96.07

Drugs were combined at constant ratios (0.01:5, 0.01:10, and 0.01:20), and their dose-effect relationships were assessed by the Chou-Talalay method (Chou & Hayball, 1996) using CalcuSyn software. CI was calculated by the combination index equation. CI of < 1, = 1, and > 1 indicate synergism, additive effect, and antagonism, respectively, at different effective doses (ED_{50}, ED_{75}, ED_{90}, and ED_{95}). Synergistic effects (CI < 1) are in bold. DRI indicates the fold dose reduction allowed in a drug combination to reach a given degree of inhibition compared to the drug as a single agent. Computer-simulated dose-required values of each drug in combination to reach a given effect level are included.

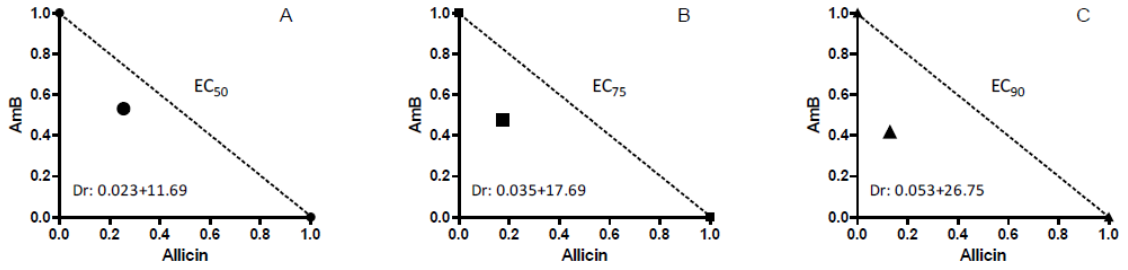

Figure 2. Representative normalized isobolograms of the interaction of AmB with allicin at a fixed ratio (0.01 µM AmB to 5 µM allicin). Lines intersect at the x and y axes at the normalized concentrations corresponding to the EC_{50} (●) (**A**), EC_{75} (■) (**B**), and EC_{90} (▲) (**C**) when the compounds were administered alone. The same symbols are used for the concentrations found with the AmB-plus-allicin combination to elicit the same effect as the drugs added alone (EC_{50}, EC_{75}, and EC_{90}). Dr, dose required (µM AmB + µM allicin); EC, effective concentration.

Given the results obtained with the extracellular stage of *Leishmania*, interaction was tested on intracellular amastigotes. Toxicity of the combination of AmB plus allicin was assayed on peritoneal Mφ from BALB/c mice. None of the concentrations of allicin employed (10 through 40 μM) in the combination induced any significant toxicity as assessed by MTT assay (Figure 3). However, 0.5 μM AmB, irrespective of the allicin concentration used, significantly ($p < 0.001$) inhibited the viability of Mφ, more than 60%. As expected, no toxicity for mammalian cells was found with 0.1 μM AmB. This value is in the range of reported IC_{50}s for *Leishmania* and supported the exploration of the combined treatment with allicin on the multiplication of intracellular amastigotes of *Leishmania* given the apparent absence of toxicity of the dialylsulfide up to 40 μM.

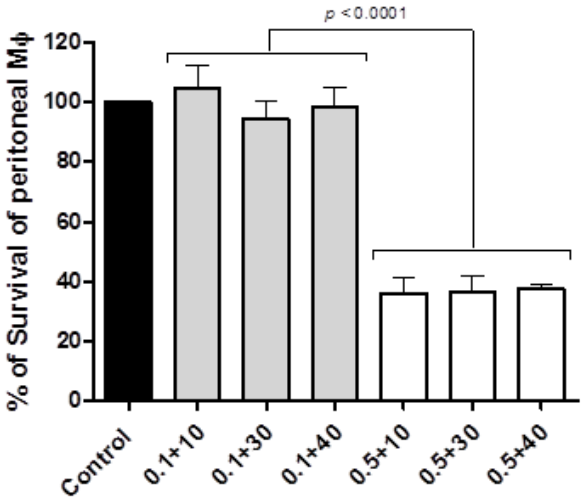

Figure 3. Effect of the combination of AmB and allicin on the *ex vivo* survival of mouse peritoneal macrophages (Mφ). Numbers on the *x* axis represent the concentration (μM) of AmB plus concentration (μM) of allicin. Solid bar, untreated control Mφ; gray bars, Mφ cultures treated with 0.1 μM AmB; white bars, Mφ cultures treated with 0.5 μM AmB. Results are the means ± standard deviations of three independent experiments.

Only concentrations below 0.1 μM AmB and 10 μM allicin were tested (Table 3). For comparative purposes, the effect of both compounds administered alone was also included. Since antileishmanial effect was determined by cell counting, drugs were combined at a non-constant ratio in a checkerboard manner. As expected, AmB alone showed an approximate IC_{50} of 0.1 μM, and the maximal concentration of allicin (10 μM) reduced by ca. 30% the multiplication of amastigotes. Tested concentrations of the drugs, added alone or in combination, did not significantly affect the viability and infection rate of Mφ under our experimental conditions; infection rates ranged from 47.3% ± 3.8% to 67.0% ± 3.5%, and the results obtained with the two *Leishmania* species (*L. infantum* and *L. donovani*) were similar (data not shown). An interaction between the compounds was found from moderately synergistic with the lowest concentrations to antagonistic with the highest concentration used (0.1 μM AmB plus 10 μM allicin) (Figure 4A). In spite of this deduced interaction (CI), the most significant finding of the experiment was that 10 μM allicin could induce ca. 50% reduction of amastigotes' multiplication when administered with 0.05 μM AmB. It should be

pointed out that for the particular *L. infantum* isolate employed, this AmB concentration administered alone had almost no effect (7.3% ± 5.5% growth inhibition) on the multiplication of intracellular amastigotes. This represents a 2-fold reduction in the dose required for the antibiotic.

Table 3 Effects of AmB, allicin, and their combination on the multiplication of intracellular amastigotes of *Leishmania infantum* in J774 cells.

Drug combination non-constant ratio (μM)		% of Infection	% Growth Inhibition	CI value	DRI value	
AmB	*Allicin*				*AmB*	*Allicin*
0	0	64.7 ± 2.5	0			
0.05		67.0 ± 3.5	7.3 ± 5.5			
0.075		64.7 ± 4.5	25.8 ± 5.1			
0.1		58.3 ± 2.1	50.1 ± 5.5			
	0.05	60.3 ± 2.1	1.3 ± 4.2			
	1	61.7 ± 5.7	15.7 ± 4.6			
	5	56.7 ± 3.1	24.9 ± 1.8			
	10	63.0 ± 4.4	32.8 ± 3.9			
0.05	0.05	62.7 ± 3.1	26.1 ± 3.1 **	**0.75 (moderate synergism)**	1.51	11.28
	1	60.0 ± 4.0	28.5 ± 4.4 **	**0.80 (moderate synergism)**	1.55	6.04
	5	56.7 ± 1.5	34.2 ± 6.3 ***	1.30 (moderate antagonism)	1.67	1.42
	10	56.3 ± 1.5	45.6 ± 8.5 ***	1.58 (antagonism)	1.91	0.94
0.075	0.05	56.0 ± 9.5	47.4 ± 4.5 ***	**0.82 (moderate synergism)**	1.29	19.68
	1	54.0 ± 8.2	55.8 ± 10.9 ***	**0.79 (moderate synergism)**	1.42	11.96
	5	59.0 ± 4.4	54.8 ± 2.0 ***	1.13 (nearly additive)	1.41	2.34
	10	59.0 ± 2.6	54.0 ± 3.6 ***	1.58 (antagonism)	1.39	1.15
0.1	0.05	47.3 ± 3.8	61.4 ± 3.8 *	**0.90 (slight synergism)**	1.13	27.58
	1	53.3 ± 3.8	61.5 ± 3.3 *	**0.95 (nearly additive)**	1.13	13.86
	5	53.0 ± 3.6	64.5 ± 5.0 **	1.18 (slight antagonism)	1.17	3.00
	10	47.7 ± 1.5	64.9 ± 0.9 **	1.50 (antagonism)	1.18	1.51

Drugs were combined at a non-constant ratio and their dose-effect relationships assessed by the Chou-Talalay method (Chou & Hayball, 1996) using CalcuSyn software. CI was calculated by the combination index equation. CI of < 1, = 1, and > 1 indicate synergism, additive effect, and antagonism, respectively, at the different drug combination doses. Synergistic effects (CI < 1) are in bold. DRI indicates the fold dose reduction allowed in a drug combination at the different data points compared with each drug alone. Statistical differences between the effects of AmB alone and the combination of AmB plus allicin are indicated as follows: *, $p < 0.05$; **, $p < 0.001$; and ***, $p < 0.0001$. Values are means ± standard deviations.

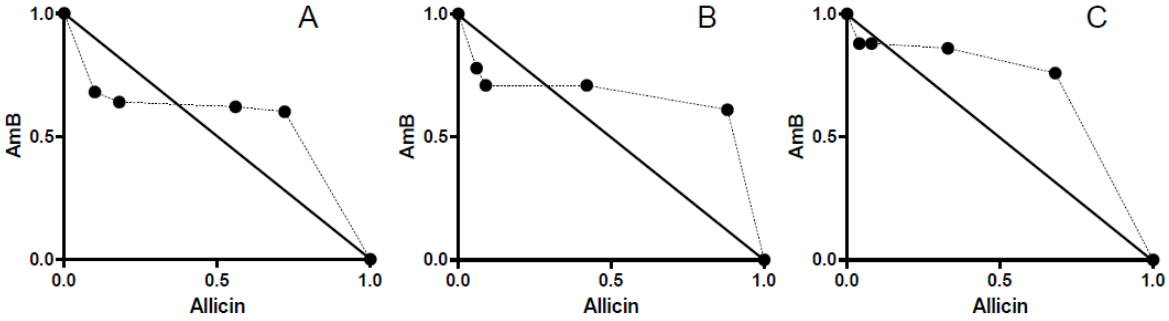

Figure 4. Representative isobolograms of the *in vitro* interaction between AmB and allicin on intracellular amastigotes of *L. donovani*. Results correspond to the non-constant ratio experiments using 0.05 µM (**A**), 0.075 µM (**B**), and 0.1 µM (**C**) AmB and 0.05, 1, 5, and 10 µM allicin. Black circles indicate the IC_{50} of the combination at the concentrations on the *x* and *y* axes.

4.5. Discussion

The aim of this work was the evaluation of the *in vitro* antileishmanial effect of the combination of AmB and allicin with the final purpose of reducing the required doses of the antibiotic and therefore the toxicity associated with its use. Results showed, using a fixed-ratio analysis with the extracellular stage (Chou & Hayball, 1996; Chou, 2008) and a checkerboard analysis with amastigotes, that the combination of the two compounds was from moderately synergic to synergic at low concentrations against both promastigotes (0.07 µM AmB plus 35.45 µM allicin induced 95% growth inhibition) and amastigotes (ca. 45% reduction with 0.05 µM AmB plus 10 µM allicin); this represents nearly a 2-fold reduction in the IC_{50} of the antibiotic added alone (Corral-Caridad et al., 2012; Vermeersch et al., 2009). The differences observed between promastigotes, with a 7- to 11-fold reduction of AmB, and amastigotes, with a ca. 2-fold reduction, support an interest in using intracellular amastigotes in drug screening (Ordóñez-Gutiérrez et al., 2007; Sereno et al., 2007; Vermeersch et al., 2009).

It is clear that *in vitro* and *ex vivo* tests measure only the antiparasitic effect and that no direct prediction of the effect *in vivo* can be drawn from the results (Seifert et al., 2011). Analysis showed that some drug combinations were classified as antagonist in spite of reaching a highly inhibitory effect in amastigotes and particularly promastigotes. This finding, previously observed in anti-*Plasmodium* screening (Wiesner et al., 2002; Fivelman et al., 2004), suggests that even drugs associated with an indifferent effect *in vitro* could be useful therapeutic partners *in vivo*.

In addition, differential interaction between AmB and allicin found in promastigotes and amastigotes of *Leishmania* could give us some clues on the mechanism of action of the drugs used. The main mechanism of action of AmB against *Leishmania* is related to the preferential binding to ergosterol (Seifert, 2011), which impairs the permeability of membranes, resulting in loss of small cations and causing cell death (Berman et al., 1998; Larabi et al., 2003). This mechanism is also responsible for the fungicidal activity of the antibiotic.

The mechanistic basis of the antiproliferative, and in particular the antileishmanial, activity of allicin (Corral-Caridad et al., 2012; Plumb, 2004) is not clearly understood, although several cellular targets have been incriminated (Rabinkov et al., 1998). Apparently allicin produces an enhancement of fungicidal activity of AmB by inhibiting ergosterol trafficking from the plasma membrane to the vacuole membrane in *Candida* (a member of the Fungi) (Ogita et al., 2009). *Leishmania* also has considerable amounts of ergosterol on the plasma membrane. Resistance to AmB in clinical isolates of *L. donovani* has been associated, besides the presence of cholesta-5,7, 24-trien-3β-ol instead of ergosterol, with the upregulation of the thiol metabolic pathway (Purkait et al., 2012). No thiol contents have been yet determined by us in allicin-treated *Leishmania*, but interference with thiols has been considered the main mechanism of action for allicin (Rabinkov et al., 1998; Miron et al., 2010). The synergy observed could be related to the impairment of the thiol metabolic pathway provoked by allicin, thus enhancing the effect of AmB. Whether or not this enhancement of AmB effect in the presence of allicin is responsible for the synergism found by us in amastigotes and, particularly, promastigotes of *Leishmania* needs further experimentation, which is under way.

Chemotherapy of leishmaniasis, both human and animal, is not yet solved in spite of the substantial efforts made on basic aspects of *Leishmania* biology. An alternative approach to monotherapy is the combination of drugs using well-established chemotherapeutic agents (i.e., miltefosine plus amphotericin B or antimonials plus amphotericin B) (Olliaro, 2010); in addition, these drug combinations have been common in veterinary clinical practice.

Our results showed the apparently synergistic effect of allicin and AmB in the micromolar range. Data obtained with drug combinations *in vitro* have many limitations in spite of the dose-effect relationship analysis carried out (i.e., unforeseen interactions and pharmacokinetics) (Chou. 2008). However, the 2-fold reduction in the required dose of AmB against intracellular amastigotes and the possibility of using higher non-toxic doses of allicin (> 10 μM) suggest exploration of this combination *in vivo*.

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CHAPTER 5

Efficacy of low doses of amphotericin B plus allicin against experimental visceral leishmaniasis

5.1.a. Resumen

Eficacia de dosis bajas de Alicina y Anfotericina B en el tratamiento de la leishmaniosis visceral experimental

Objetivos: Valorar la eficacia de la combinación de alicina y desoxicolato de anfotericina (AmB) en la quimioterapia de las infecciones por *Leishmania infantum* con el objetivo final de reducir la dosis de AmB en el tratamiento de la leishmaniosis visceral.

Métodos: Los cricetos fueron infectados por vía intraperitoneal (ip) con *L.infantum* (10^7 promastigotes en fase estacionaria). A partir del día 45 postinfección los animales fueron tratados (ip) con AmB (1 o 5 mg/kg/día), alicina (5 mg/kg/día) o con una combinación de AmB (1 mg/kg/día) + alicina (5 mg/kg/día) durante 5 días. Se determinaron las alteraciones clínicas y fisiológicas, y se valoró la respuesta de anticuerpos (IgG, IgG_1, IgG_2). La carga parasitaria de estimó mediante dilución límite y los niveles de AmB en plasma, riñón, bazo e hígado se valoraron mediante HPLC.

Resultados: No se apreciaron signos clínicos ni alteraciones hepatorrenales. AmB, a la dosis de 1 mg/kg/día, no eliminó la infección mientras que en dos de los animales tratados con 5 mg/kg/día de alicina no se pudo aislar *L.infantum*. La combinación (5 mg/kg/día alicina + 1 mg/kg/día AmB) redujo la carga parasitaria en >95%. La actividad de la combinación frente a la infección por *L.infantum* fue comparable ($p<0,05$) al tratamiento estándar con AmB (5 mg/kg/día).

Conclusiones: Alicina (5 mg/kg/día durante 5 días) redujo de forma significativa la carga de *Leishmania* en el bazo e hígado de los cricetos infectados. La administración conjunta de alicina (5 mg/kg/día durante 5 días) y AmB (1 mg/kg/día durante 5 días) mostró un efecto aditivo parcial en la reducción de carga parasitaria en los dos órganos.

5.1.b. Abstract

Objectives: To evaluate the efficacy of the combination of allicin and amphotericin deoxycholate (AmB) in the chemotherapy of *Leishmania infantum* infection with the final aim of reducing the dose of AmB in the chemotherapy of visceral leishmaniasis.

Methods: Hamsters were intraperitoneally (ip) infected with *L.infantum* (10^7 stationary phase promastigotes). On day 45 post-infection animals were treated ip with AmB (1 or 5 mg/kg/day), allicin (5 mg/kg/day) or a combination of AmB (1 mg/kg/day)+allicin (5 mg/kg/day) for 5 days. Animals were clinically and biopathologically monitored and the antibody response (IgG, IgG_1, IgG_2) was determined. Parasite burdens were estimated by limiting dilution and AmB biodistribution was determined by HPLC in plasma, kidney, spleen and liver.

Results: No clinical signs or liver and kidney alterations were observed. AmB (1 mg/kg/day) did not clear the *Leishmania* infection and no parasites were detected in two animals treated with 5 mg/kg/day allicin. Combination therapy (5 mg/kg allicin+1 mg/kg AmB) reduced the *L.infantum* burden by > 95%. Antileishmanial activity of the combination was comparable ($p<0.05$) to the standard AmB treatment (5 mg/kg).

Conclusions: Allicin alone (5 mg/kg/day for 5 days) significantly reduced the *Leishmania* burden in spleen and liver of infected hamsters. Co-administration of allicin (5 mg/kg/day for 5 days) and AmB (1 mg/kg/day for 5 days) showed a partial additive effect on the reduction of leishmanial burden in both target organs.

5.2. Introduction

Visceral leishmaniasis, a disease that is fatal unless treated, is caused by *Leishmania donovani* and *Leishmania infantum* (=*L.chagasi*) and is responsible for ~40000 human deaths per year (http://www.who.int/leishmaniasis/). As such, this infection is second only to malaria among human parasitic diseases. Moreover, *L.infantum* infection is a first-order pathology in veterinary medicine since in endemic regions infection reaches a prevalence sometimes exceeding 25% of the total dog population, besides its role as a reservoir for the human population. Visceral leishmaniasis is among the frequent co-infections found in immunodepressed patients (e.g. HIV+ or solid organ transplant recipients). No available vaccine for humans has been developed and the present immunoprophylaxis of dog infections has important drawbacks (e.g. annual revaccination, adverse effects). Chemotherapy, both of humans and dogs, is imperfect and relies on the use of drugs discovered >50 years ago. In addition, important resistances have been described against some of the first-line drugs (i.e. antimonials) employed in endemic areas (Croft et al., 2006). The long half-life of some of the alternative treatments (e.g. miltefosine) and easy selection of resistant lines of *L.donovani* to others (e.g. paromomycin) (Maarouf et al., 1998) suggests that emergence of resistance has to be expected when used in monotherapy.

Amphotericin B (AmB) has been extensively used against systemic fungal infections and as a second-line treatment against leishmaniasis. It is a very effective antileishmanial drug and the main mechanism of action that has been described, binding to ergosterol-containing *Leishmania* membranes, does not favour the appearance of resistance (Seifert, 2011; Gray et al., 2013). The main inconvenience of this drug has been the toxicity associated with its use as the deoxycholate (Laniado-Laborín and Cabrales-Vargas, 2009; Hamill, 2013), but this limitation has been partially circumvented by the use of low-toxicity vehicles (e.g. lipid carriers) (Alvar et al., 2006). However, the high price of liposomal preparations makes the treatment unaffordable in most endemic areas for human use and simply irrelevant for veterinary purposes. Among the possible strategies to manage the problems with the currently available antileishmanial compounds -inefficacy, price, toxicity and induction of resistance- combination therapy and the use of new molecules have been indicated as research areas worth of exploration (Chappuis et al., 2007).

Allicin (2-propene-1-sulfinothioic acid S-2-penyl ester; dialylsulphur) has shown activity against some protozoa, including *Leishmania* (McClure et al., 1996; Waag et al., 2010). The main mechanism of action of allicin is related to its high permeability through cell membranes and interaction with thiols (Miron et al., 2000). Stabilized allicin has shown a significant

antiproliferative effect, in the micromolar range, against amastigotes (the stage present in the infected hosts) of *L.donovani* and *L.infantum* without evidence of toxicity for mammalian cells (Corral-Caridad et al., 2012). Apparently allicin enhances the antifungal (*Candida*) activity of AmB by inhibiting ergosterol trafficking (Borhijan et al., 2009). Recently, a synergistic effect of low concentrations of allicin and AmB has been reported against these species using an intracellular *ex vivo* model (mouse macrophages and amastigotes). We have tested the effect of the combination of AmB at a 5-fold reduced dose with allicin on experimental infections of hamsters with *L.infantum*. The final aim of the research is the reduction of the required dose of AmB and therefore the toxicity associated with the antibiotic if used in monotherapy of leishmaniasis.

5.3. Material and methods

Leishmania culture media and drugs

L.infantum (MCAN/ES/97/10.445) zymodeme MON-1 was obtained from a naturally infected dog and has been routinely maintained by subpassage in hamsters and as promastigotes in RPMI 1640 medium (Lonza) in 25 mL culture flasks at 26°C supplemented with 10% heat-inactivated (30 min, 56°C) fetal bovine serum (FBS; TDI), 1% L-glutamine (Lonza) and 100 U/mL penicillin + 100 µg/mL streptomycin (Lonza). Fungizone® (deoxycholate-dispersed amphotericin B) was from Bristol-Myers Squibb and stabilized allicin was obtained as liquid Allisure® (15000 ppm) from Allicin International Ltd.

Experimental infection of hamsters and follow-up

Female hamsters were purchased from Janvier SAS. At the time of their arrival their weight ranged from 80 to 90 g. After a quarantine period of 30 days, animals were randomly allocated to six matched groups of six hamsters each except group 1 (five animals). Group 1 was the uninfected control group; group 2 was infected but untreated; group 3 was treated with 5 mg/kg/day AmB for 5 days; group 4 was treated with 1 mg/kg/day AmB for 5 days; group 5 was treated with 5 mg/kg/day allicin for 5 days; and group 6 was treated with 1 mg/kg/day AmB plus 5 mg/kg/day allicin for 5 days.

Dilutions of the compounds were made with 5% dextrose solution to a final volume of 500 µL. All treatments were administered by the intraperitoneal (ip) route. Animals, except those in group 1, were infected with 10^7 stationary phase promastigotes in 500 µL by ip injection on day

0 of the experiment. From day 45 post-infection (p.i.) onwards, the hamsters were treated with the appropriate drug or drug combination. The uninfected control group received the same volume (200 μL) of dextrose 5% solution. On day 30 p.i. one of the animals in the infected, untreated group (group 2) was euthanized to assess the infection. Ten days after the last treatment animals were anaesthetized with isoflurane, bled by intracardiac puncture and sacrificed by anaesthetic overdose.

Experimental animals were dissected and the liver, kidneys and spleen were removed, weighed and used to estimate the parasite burden (liver and spleen) and to perform the pharmacological determinations. During the entire experiment the animals were housed at the facilities of the Instituto de Salud Carlos III, Majadahonda, Madrid (ISCIII) with a 12 h light/dark cycle, fed with pellets and given water ad libitum. Animals were observed daily for undesired reactions to the infection and treatments and body weight was determined weekly. Blood samples (serum and plasma) were obtained at the end of the experiment to determine the levels of renal and liver function markers [urea, creatinine, glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and alkaline phosphatase (ALP)] by standard clinical laboratory techniques (DLV Laboratorio Veterinario, Madrid). Experimental design and procedures were approved by the ethics committee of the ISCIII and followed the recommendations of EC Directive 2010/63/EU.

ELISA

The peripheral specific antileishmanial antibody response (IgG, IgG_1 and IgG_2) was determined by ELISA using serum samples from all experimental animals. Microtitre 96-well plates (Nunc) were coated with 25 μg/mL (50 μL/well) of soluble *L.infantum* extract or fixed promastigotes (10^8 promastigotes/mL; 50 μL/well) overnight at 4°C. For the latter, mid-log-phase promastigotes were fixed with 0.025% formaldehyde solution (Panreac) for 2 h at room temperature (RT) and washed three times with PBS. To ensure cell adhesion, prior to blocking, promastigote-coated plates were centrifuged (500 g, 10 min, RT).

Plates were blocked with 2% BSA (100 μL/well) at 37°C for 1 h. Animal sera were used at dilution 1/50 (50 μL/well), as established by prior ELISA chequerboard titrations (not shown), and incubated for 2 h at 37°C. After washing, secondary antibodies (50 μL/well) were added and the plates were incubated for 1 h at RT. Antibodies were diluted in PBS-Tween at 1/2000 dilution for total IgG [Goat Anti-Hamster IgG(H+L)-HRP, Southern Biotech] and at 1/1000 dilution for biotinylated mouse anti-Armenian hamster IgG_1 (Abcam) and mouse anti-Armenian hamster IgG_2 (Abcam), both of which react with heavy chains of Syrian hamster IgG isotypes.

After washing, IgG_1 and IgG_2 plates were further incubated with 50 µL/well HRP-conjugated streptavidin (Southern Biotech) at a dilution of 1/2000 and incubated for 30 min at RT. Finally, a 1 mg/mL solution of O-phenylenediamine (Sigma) and H_2O_2 (1/1000) were added (100 µL/well). The reaction was stopped with 50µL of 3N H_2SO_4 and absorbance was read at 492nm.

Efficacy of treatments

Besides clinical observation of the experimental hamsters, at the end of the experiment the antileishmanial efficacy of drugs and combinations was estimated by determining the parasite burden by limiting dilution assay as previously described (Castro et al., 2011). Briefly, livers and spleens were removed, weighed and homogenized in Schneider's medium (Sigma) supplemented with 20% heat-inactivated FBS, 2% sterile human urine, 20 mM HEPES (Lonza), 1% L-glutamine (Lonza) and 100 U/mL penicillin+100 µg/mL streptomycin (Lonza). Organ homogenates were filtered through a cell strainer to ensure a single-cell suspension. Further dilutions of the homogenate were done in the same medium to a final concentration of 10 mg/mL; 200 µL/well of this cell suspension was added to the first well of a 96-well culture plate (Corning) and serial 4-fold dilutions were made across the plate. Plates were incubated for 2 weeks at 26°C and the presence of parasites was assessed by microscopy. The parasite burden (number of parasites/g of organ) was calculated as described by Buffet et al. (1995).

Amphotericin B biodistribution in treated hamsters

Blood samples were obtained at the end of the experiment from group 3 (hamsters treated with 5 mg/kg AmB), group 4 (treated with 1 mg/kg AmB) and group 6 (treated with 1 mg/kg AmB + 5 mg/kg allicin). Plasma was obtained and samples were stored at –20°C until analyses were performed.

Plasma samples (100 µL) were spiked with meloxicam (Fagrón SL) as internal standard at a final concentration of 10 µg/mL. Two extractions were carried out with methanol (300 µL each) and a third extraction with acetonitrile (300 µL). After every extraction, the mixture was vortexed and centrifuged at 9000 rpm, 10 min, 4°C. The supernatants were collected (300 µL×3) and evaporated off in a concentrator (Savant, SpeedVac®) at 35°C. Samples were reconstituted with 200 µL of a 1:1 mixture of methanol:mobile phase (acetonitrile:acetic acid:water) (52:4.3:43.7, v/v/v). The reconstituted samples were centrifuged (9000 rpm, 5 min) and the supernatants were analysed by the validated HPLC method described below.

HPLC was equipped with a Jasco PU-1580 pump, a Jasco AS-2050 Plus autosampler and a Jasco UV-1575 UV–visible detector. Integration of the peaks was performed with the program Borwin 1.5 for PC (JMBS Developments). AmB was isocratically eluted using a Thermo Hypersil BDS C18 reverse-phase column (200×4.6 mm, 5 μm) and a mobile phase as above with a flow rate of 1 mL/min. Absorbance was monitored at 406 nm and the injection volume was set at 100 μL. Under these conditions, the relative retention times of AmB and the internal standard were 7.9 and 5.1 min, respectively. Plasma AmB concentrations were calculated from linear regression calibration curves of the AmB/internal standard peak height ratio. The linear range in plasma was 0.01 to 10 μg/mL (y=0.2191x−0.0026; r^2=0.9986).

Liver samples (0.5 g) were spiked with meloxicam (10 μL at 200 μg/mL) as internal standard. Samples were homogenized with PBS (pH 7.4, 0.25 mL). Then, AmB was extracted with methanol (1 mL). The mixture was vortexed and then centrifuged (5000 rpm, 20 min). The supernatants were filtered through a 0.45 μm filter (Millipore® Millex PVDF) and then analyzed by the HPLC method previously described. Liver AmB concentrations were calculated from linear regression calibration curves of the AmB/internal standard peak height ratio. Similarly, kidney samples (0.15 g) and spleen samples (0.075 g) were analysed. Spleen samples were pooled (three animals/group) to obtain the required amount of tissue.

Statistical analysis

Graphs and statistical analysis were performed using GraphPad Prism 5. The statistical significance of differences between mean values obtained in the different groups was determined by one-way analysis of variance followed by Tukey's test. In all cases a p value of $p<0.05$ was considered significant.

5.4. Results

Clinical follow-up

No clinical signs were noted during the experiment as a consequence of either infection with *L.infantum* alone (group 2) or infection and treatments administered. Similarly, no important alterations were found in any of the biochemical parameters determined in the experimental hamsters (Figure 1) since renal (urea, creatinine) and liver function markers (ALP and GOT/GPT ratio) were comparable in all experimental groups irrespective of infection and the treatments administered.

The only exception was the blood urea nitrogen level in infected, untreated hamsters (group 2), which was significantly higher than levels in uninfected control animals and animals receiving AmB (5 mg/kg), allicin (5 mg/kg) or the allicin+AmB combination. However, creatinine values were in all cases <0.2 mg/dL. ALP levels were in the range of 214-251 U/L and the GOT/GPT index was <1.0 in all groups. These results suggest that allicin, AmB or the combination (allicin+mB) with the doses and treatment schedule followed in our experiment did not induce any significant toxicity in the infected animals.

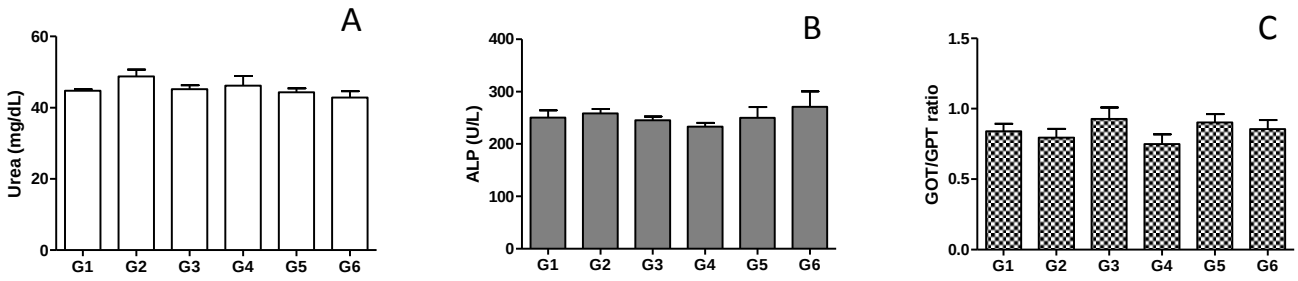

Figure 1. Values of renal and kidney function markers at the end of the experiment. G1, uninfected control; G2, infected with *Leishmania infantum*; G3, infected and treated with 5 mg/kg/day AmB; G4, infected and treated with 1 mg/kg/day AmB; G5, infected and treated with 5 mg/kg/day allicin; G6, infected and treated with 1 mg/kg/day AmB + 5 mg/kg/day allicin. ALP, alkaline phosphatase; GPT, serum glutamate pyruvate transaminase; GOT, serum glutamic oxaloacetic transaminase. Data are means + SEM.

Antibody response

Comparable results were obtained with the two antigens, although higher OD values were obtained with the cell-based assay. *L.infantum* infection elicited a significant rise in anti-*Leishmania* IgG against the soluble extract of the parasite (Figure 2) and fixed promastigotes (Figure 3) as assessed by the results observed in infected + untreated hamsters (group 2).

Using fixed promastigotes of *Leishmania*, animals treated with the lowest AmB dose (group 4) displayed values not significantly different from those found in infected, untreated hamsters (group 2) (Figure 3A). However, animals treated with allicin alone (group 5) or together with a low dose of AmB (group 6) showed significantly lower antibody values ($p<0.05$). Values in animals treated with the combination were not different from those in animals treated with standard dosage of AmB (group 3; 5 mg/kg/day, 5 days) ($p>0.05$) or the uninfected control group (group 1).

The pattern found for the specific IgG_2 response was more complex (Figures 2B and 3B). Excluding the uninfected control hamsters, the lowest antibody values were found in animals receiving 5 mg/kg/day AmB (group 3) and the highest IgG_2 levels in hamsters treated with allicin alone (group 5). Moreover, group 3 hamsters were significantly different ($p<0.05$) from the group treated with allicin alone (group 5) but not from the infected control group (group 2) or those treated with the lowest AmB dose (group 4) or the combination of allicin+AmB (group 6). The IgG_1 response was very low in all groups and no significant differences between them were found (Figures 2C and 3C).

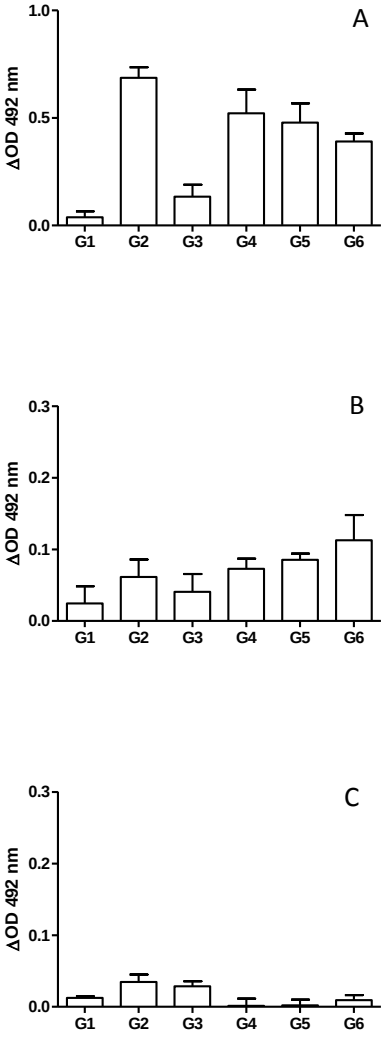

Figure 2. Serum specific antibody responses against soluble *Leishmania infantum* antigen (SLA) estimated by ELISA on day 60 of the experiment. (**A**) Total IgG (H+L). (**B**) IgG_2. (**C**) IgG_1. G1–G6, experimental groups as in Figure 1. ΔOD, increase in OD.
Values are means ± SEM.

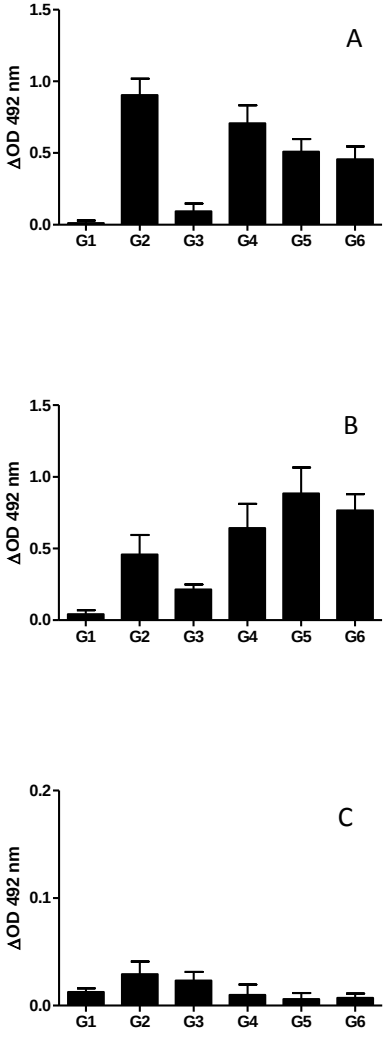

Figure 3. Serum specific antibody response to fixed promastigotes of *Leishmania infantum* estimated by ELISA on day 60 of the experiment. (**A**) Total IgG (H+L). (**B**) IgG_2. (**C**) IgG_1. G1–G6, experimental groups as in Figure 1. ΔOD, increase in OD.
Values are means ± SEM.

Liver and spleen Leishmania burden

Figure 4 shows the results obtained in the determination of *L.infantum* burden in liver and spleen. The infection procedure (ip administration) was efficient and all infected control hamsters (group 2) had detectable infections. Spleen burdens of *Leishmania* (~ 6.0 $\log_{10}$ units) were, in general, significantly higher than those found in the liver (~ 3.0 $\log_{10}$ units). The negative control group (group 1) did not show any parasites.

The curative effect of AmB was dependent on the dose administered. With the highest dose (5 mg/kg AmB; group 3) no parasites were detected in two animals and the average reduction of *L.infantum* burden was 94.5% in the spleen and 90.6% in the liver. However, none of the hamsters treated with 1 mg/kg AmB (group 4) was free of infection, although the parasite burden was reduced by ~ 70% in the spleen and a similar reduction was found in the liver.

Allicin, administered alone (group 5), cleared *Leishmania* infection in two of the hamsters and both liver and spleen parasite burdens were significantly lower than those found in the infected, untreated group ($p<0.05$). Results with the combination of 1 mg/kg AmB and 5 mg/kg allicin) (group 6) showed a partially additive effect. The reduction in *Leishmania* burden was significantly ($p<0.05$) higher than that obtained with 1 mg/kg AmB and slightly better than that obtained with the higher dose of allicin (5 mg/kg). In three out of six hamsters in group 6 no *Leishmania* were recovered from the liver and in two out of six no parasites were detected in the spleen. Results in terms of organ clearance were similar to those found with the standard AmB treatment (5 mg/kg AmB).

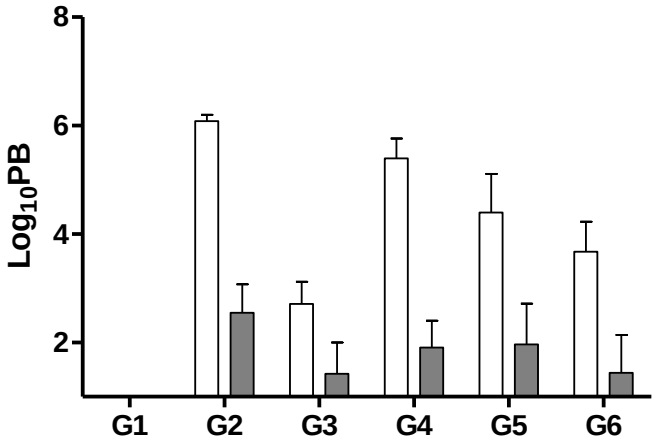

Figure 4. *Leishmania infantum* burden (parasite burden, PB) determined by limiting dilution. G1–G6, experimental groups as in Figure 1. White bars, spleen burden; grey bars, liver burden. Values are means ± SEM.

Amphotericin B pharmacokinetics

Very low AmB plasma concentrations were found in all experimental animals (Figure 5). However, AmB was detected in all organs analysed (kidney, liver and spleen). Animals in group 3 (5 mg/kg AmB) exhibited significantly higher AmB levels in liver, kidney and spleen than animals in groups 4 (1 mg/kg AmB) and 6 (1 mg/kg AmB+5 mg/kg allicin) but no differences were observed in plasma among groups. AmB levels in group 6 hamsters were higher than those in group 4, although the difference was not statistically significant.

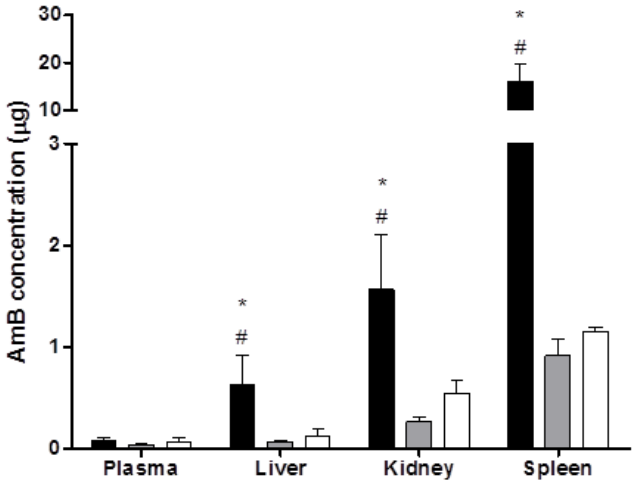

Figure 5. Amphotericin B (AmB) concentrations in plasma, liver, kidney and spleen of hamsters infected and treated with 5 mg/kg/day AmB (black bars) (group 3), 1 mg/kg/day AmB (grey bars) (group 4) and 1 mg/kg/day AmB + 5 mg/kg/day allicin (white bars) (group 6). Values are means ± standard deviation. Statistically significant differences (p<0.0001) were found between groups 3 and 4 (*) and between groups 3 and 6 (#).

5.5. Discussion

Successful chemotherapy of leishmaniasis remains a challenge in spite of the research that has been done on the fundamental biology of the aetiological agents. In practice only a handful drugs are currently used and clinical isolates of *L. donovani* have shown notable resistance to the pentavalent antimonials (Chakravarty and Sundar, 2010). Moreover, given the relatively easy selection of resistant *Leishmania* lines to paromomycin (Maarouf et al., 1998) and miltefosine (Seifert, 2011), together with the oral administration of the latter and risks associated with self-medication, clinical resistance after monotherapy with these drugs will probably appear (Maltezou, 2010).

AmB is probably the best existing drug against leishmaniasis, with cure rates exceeding 95% (Chappuis et al., 2007; Paila et al., 2010) and negligible levels of resistance after being employed as the reference drug against systemic fungal infections for >50 years (Gray et al., 2013). The main mechanism of action of AmB is still controversial (Ramos et al., 1996; Pucadyil et al., 2004; Chattopaddhyay and Jarufulla, 2011), although it is generally considered that binding of AmB to ergosterol in the *Leishmania* membrane is the first or the unique event (Gray et al., 2013) causing cell destruction. The few reports of clinical resistance in *Leishmania* are related to a modification of ergosterol to the closely related lipid cholesta-5,7,24-trien-3β-ol (Purkait et al., 2012).

To cope with the shortage of available drugs against leishmaniasis, combination therapy with a variety of drugs and schedules (Chunge et al., 1985; Murray and Hariprashad, 1996) has some potential advantages, such as delaying or preventing the development of resistance and shortening of treatment regimens (Seifert and Croft, 2006). Since the main shortcoming of AmB is toxicity (Laniado-Laborín and Cabrales-Vargas, 2009; Hamill, 2013), our approach used a combination of allicin with the antibiotic AmB with the aim of reducing the amount of AmB required and therefore the potential toxicity of the antibiotic.

The experimental model employed (*L.infantum* and hamster) produced consistent infections with significantly higher parasite burdens in the spleen than in the liver, thus confirming its value (Binzahim et al., 1993; Requena et al., 2000; Melby et al., 2001; Dea-Ayuela et al., 2007; Moreira et al., 2012) and its suitability for *in vivo* testing of antileishmanial agents. Moreover, the limiting dilution assay allowed the detection of the parasite 2 months after infection in both the spleen and the comparatively less parasitized liver.

No clinical signs or biopathological alterations were observed in any of the infected or infected+treated animals, indicating the lack of toxicity of AmB and allicin at the doses administered. The levels of ALP found in our experiment were higher than the reference range of 50–186 U/L. This could be related to the age of the hamsters, since immature animals have higher ALP levels than adults due to release of the enzyme from growing bone (Washington and van Hoosier, 2012). The alteration in blood urea nitrogen level in group 2 hamsters provided evidence of renal compromise in infected animals. Moreover, higher values of urea, observed in all groups compared with the reference range (12–26 mg/dL), were probably due to diet (i.e. high protein content) and sex (i.e. female) (Washington and van Hoosier, 2012).

In our experiment, 5 mg/kg allicin administered by the ip route, to avoid the potential degradation induced by the conditions in the stomach, elicited a clear antileishmanial effect in hamsters since two out of six animals were cleared of *L.infantum* in both spleen and liver. As far

as we know this is the first evidence of the antileishmanial activity of allicin *in vivo* without apparent toxicity in infected, treated animals. The combination of allicin+AmB significantly improved the efficacy of low-dose AmB (group 4), and was slightly more efficacious than allicin at 5 mg/kg. The low-dose combination of AmB+allicin (group 6) cleared the leishmanial infection in three out of six hamsters. Moreover, the reduction in the *Leishmania* spleen burden was almost as great as that obtained with standard AmB chemotherapy in this model (5 mg/kg, 5 days, ip) (96.57% versus 94.5%) (Manandhar et al., 2008). These results suggest a partial additive antileishmanial effect of the combination *in vivo*. Results obtained with either drug administered alone to hamsters support the predictive value of the *in vitro* and *ex vivo* model (*Leishmania*-infected macrophages) for drug screening of antileishmanial molecules (Sereno et al., 2007).

No information on the mechanism of action of the combination against *Leishmania* is available. However, in fungi (e.g. *Candida albicans*) the enhanced fungicidal activity of this combination (Kim et al., 2012) has been related to the inhibition of ergosterol trafficking by allicin in the presence of non-lethal concentrations of AmB (Borhijan et al., 2009; Ogita et al., 2009). AmB levels in treated hamsters suggested, but did not demonstrate conclusively, that allicin improves the accumulation of the antibiotic in the target organs, and this should be explored.

Resolution of *Leishmania* infection after vaccination or successful therapy seems to be associated with a Th2-to-Th1 switch with a dominant IgG_2 response (Melby et al., 2001; Murray et al., 2003; Rama Íñiguez et al., 2006; Samant et al., 2009; Gupta et al., 2012). Our results showed a degree of correlation between total IgG (H+L) and *Leishmania* burden (Requena et al., 2000). However, hamsters treated with 5 mg/kg AmB (group 3) had lower levels of specific IgG_2 than animals treated with 1 mg/kg AmB. This suggests that the relationship of the IgG_1 and IgG_2 pattern to the outcome of leishmanial infections in hamsters is not clear. In fact, hamsters treated with AmB showed antibody levels comparable to those of infected, untreated hamsters (Asthana et al., 2013). In addition, slightly different patterns were obtained with fixed promastigotes and soluble extracts of *Leishmania*. These variations are probably related to the different sets of antigens recognized by the sera of infected hamsters. Allicin apparently enhances proinflammatory responses (IFN-γ, TNF and IL-12p70) in mice (Feng et al., 2012), which could explain the relatively high IgG_2 response in animals treated with allicin or allicin+AmB. Whether or not the role of these cytokines is similar in mice and hamsters or, alternatively, allicin has immunostimulating properties should be clarified. More research is needed (refining of the dosage and time schedule), but our results point to the interest of this

combination as a means of reducing the dose of AmB needed in the chemotherapy of leishmaniasis.

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CHAPTER 6

Antileishmanial activity of allicin: a mechanistic approach

6.1.a. Resumen

Mecanismo de acción de alicina frente a Leishmania

La actividad antimicrobiana y antiparasitaria de alicina ha sido ampliamente estudiada aunque los mecanismos de acción implicados en su actividad frente a *Leishmania* no han sido explorados. En esta contribución presentamos las alteraciones ultraestructurales y bioquímicas provocadas por esta molécula sobre los promastigotes de *Leishmania infantum*. Los estudios de microscopía electrónica de transmisión mostraron que alicina indujo importantes modificaciones que incluyeron el aumento de tamaño de la mitocondria, invaginaciones de la membrana mitocondrial interna y pérdida de integridad. Se observaron asimismo dilatación de la membrana nuclear y vacuolización citoplasmática generalizada. La exposición de los promastigotes a alicina causó la producción de niveles elevados de especies reactivas de oxígeno (ROS), tanto en citoplasma como en mitocondria, no controladas por tioles intracelulares; colapso del potencial de membrana mitocondrial, disminución de la producción de ATP y elevación de calcio citosólico. La incubación de los promastigotes con SYTOX Green mostró que la disminución de ATP no estaba asociada al incremento de la permeabilidad de la membrana celular. La tinción con Anexina V y yoduro de propidio (PI) demostró que alicina no indujo la externalización de fosfatidilserina en la membrana. Además, el análisis TUNEL permitió observar que alicina no provocó fragmentación de ADN. Estos resultados indicaban que alicina indujo necrosis celular en *Leishmania*. El análisis del ciclo celular, mediante tinción con PI, mostró que alicina detenía el ciclo celular en la fase G_2/M. Se concluye que la diana primaria de alicina es la mitocondria e induce estrés oxidativo, no controlado por la defensa antioxidante, que provoca una disfunción mitocondrial, incremento de los niveles citoplasmáticos de calcio y una catástrofe bioenergética que conduce a la necrosis celular y detención del ciclo celular en fase premitótica.

6.1.b. Abstract

Antimicrobial activity of allicin has been extensively studied although the precise mechanism of action underlying its antileishmanial effect has been virtually unexplored. Here we report the ultrastructural and biochemical alterations caused by diallyl thiosulphinate in *Leishmania infantum* promastigotes. Transmission electron microscopy showed that allicin induced relevant morphological changes, including mitochondrial swelling, invaginations in the inner mitochondrial membrane and loss of internal integrity. Other important alterations observed involved swelling of the nuclear membrane and an intense vacuolization of the cytoplasm. Exposure of the parasites to allicin led to high production of intracellular and mitochondrial reactive oxygen species (ROS) which was not controlled by thiol homeostasis, collapse of the mitochondrial membrane potential, reduced production of ATP and elevation of cytosolic calcium. The incubation of the promastigotes with SYTOX Green revealed that decrease of ATP was not associated with plasma membrane permeabilization. Annexin V and propidium iodide (PI) staining indicated that allicin did not induce phosphatidylserine exposure on the plasma membrane. Moreover, TUNEL analysis demonstrated that allicin did not provoke DNA fragmentation. These results point towards a necrotic type of cell death in *Leishmania* after exposure to diallyl thiosulphinate. In addition, analysis of the cell cycle with PI staining showed that allicin induced cell cycle arrest in the G_2/M phase. We conclude that allicin primarily targets mitochondria and induces oxidative stress, uncontrolled by the antioxidant defense of the cell, which leads to mitochondrial dysfunction, increase of cytosolic calcium levels and a bioenergetic catastrophe leading to cell necrosis and cell cycle arrest in the premitotic phase.

6.2. Introduction

Leishmaniases are vectorial parasitic diseases of mammals, including humans, caused by species of the genus *Leishmania* (Protista). Infections are present in all inhabited continents and their prevalence has been estimated around 12 million people infected, with an annual incidence of 2 million and 350 million people living in areas at risk (WHO, 2010). Leishmaniases are, for the most part, transmitted by some species of sandflies (Insecta, Psychodidae) and therefore their epidemiology is highly dependent on the environmental conditions regulating the presence, abundance and dynamics of vector populations. Leishmaniasis presents a range of clinical forms, from cutaneous self-healing infections to fatal unless treated visceral processes, mainly related to the *Leishmania* species involved and the immune status of hosts (WHO, 2010). Presently, it is considered the second most lethal parasitic disease, after malaria, visceralizing species being responsible of 20,000 to 40,000 human deaths per year (Alvar et al., 2012). In the last years a rise in human prevalence has been found and it has been related to the changing epidemiological patterns (e.g. non-vectorial transmission, lower investments in public health, climate change, and human migrations). Recent estimates based on databases and statistical modelling rise the current human population to 1.71 billion and 1.69 billion individuals living in areas suitable for cutaneous leishmaniasis and visceral leishmaniasis respectively (Piggot et al., 2014).

Control of leishmaniasis relies mainly on chemotherapy using antimonials, amphotericin B, miltefosine and paromomycin (Sundar & Chatterjee, 2006). However, these drugs have several shortcomings including high price, long treatments and side effects such as toxicity and teratogenicity (Sundar & Chakravarty, 2013) and this research area is hardly attractive for industry (low returns, long development required, high costs). In addition, in some of the most affected areas of the world (i.e. northeastern India), resistance to the treatment of choice (antimonials) is widespread (Croft et al., 2006); therefore there is an urgent need of new molecules, both synthetic and natural (Croft & Olliaro, 2011).

Allicin (diallyl thiosulfinate) and related compounds have been shown to inhibit the multiplication of several neoplastic cell lines and the viability and growth of different tumors (Knowles & Milner, 2001; Arunkumar et al., 2006; Chu et al., 2012). In addition it has shown antibacterial (O'Gara et al., 2000; Cañizares et al., 2004; Cutler & Wilson, 2004), antifungal (Khodavandi et al., 2011a; Lemar et al., 2005) and antiprotozoal activity (Mirelman et al., 1987; McClure et al. 1996; Coppi et al., 2006; Waag et al., 2010). More recently, antiproliferative activity of allicin has been shown against intracellular amastigotes of *Leishmania* (Corral et al., 2012) and *in vivo* experimental infections with *L.infantum* (Corral et al., 2014). Allicin easily

diffuses across cell membranes and it has been described to react with thiol groups (Rabinkov et al., 1998) and some other intracellular targets have also been incriminated (e.g. cysteine proteases, microtubules disruption) (Ankri and Mirelman, 1999; Prager-Khoutorsky et al., 2007; Miron et al., 2010; Waag et al., 2010) but the actual mechanism of action of allicin and the type of death induced are for the most part unknown. It has been reported that allicin induces p53-mediated autophagy of Hep G2 human liver cancer cells (Chu et al., 2012) and apoptosis through caspase activation (Oomen et al., 2004) and via Nrf2 (Zhang et al., 2010). Nevertheless, the closely related compound diallyl disulfide causes cell cycle arrest in the G_2/M checkpoint in HCT-15 (Knowles & Milner, 2001) and PC-3 (Arunkumar et al., 2006) cell lines.

Information in unicellular eukaryotes is scarce although allicin seems to inhibit the expression of silent information regulator 2 (SIR2) gene (ortholog to mammalian SIRT1) (Khodavandi et al., 2011b) thus inhibiting the hyphae formation in *Candida* (Low et al., 2008). In addition, one of the metabolites of allicin, allyl alcohol, induces oxidative stress in this fungus. On these grounds our aim was to explore the mechanistic basis of its antileishmanial activity. Preliminary transmission electron microscopy (TEM) studies of *Leishmania* promastigotes exposed to sublethal concentrations of allicin showed that the most altered organelle was the unique mitochondrion from this kinetoplastid (Corral et al., 2012). Present results indicate that allicin induces in *Leishmania* promastigotes ROS generation with mitochondrial dysfunction with a collapse of the mitochondrial membrane potential ($\Delta\Psi m$) and rapid elevation of cytosolic Ca^{2+} levels. These events lead to a bioenergetic catastrophe with fall of mitochondrial ATP production and cell necrosis with no evidence of apoptotic-like markers.

6.3. Material and Methods

Drugs

Allicin (2-Propene-1-sulfinothioic acid S-2-propenyl ester) was purchased as liquid Allisure® from Allicin International Ltd. (Rye, East Sussex, UK) at a concentration of 5000 ppm and stored at -80˚C until used.

Parasite culture and maintenance

The canine isolate of *L.infantum* (MCAN/ES/2001/UCM9) was employed in all the experimental procedures. Promastigotes were routinely cultured in 25 mL culture flasks at 27ºC

in RPMI 1640 modified medium (Lonza) supplemented with 10% heat-inactivated (30 min at 56°C) fetal bovine serum (TDI laboratories) and 100 U/mL of penicillin plus 100 µg/mL of streptomycin (BioWhittaker).

Ultrastructural study by Transmission Electron Microscopy (TEM)

Leishmania untreated and allicin treated cultures (27ºC, 24 h allicin exposure) were centrifuged (3000xg, 10 min, 4ºC) and the resulting cell pellet was fixed for 90 min at 4ºC in 2% glutaraldehyde (Sigma) in 0.1 M sodium cacodylate buffer (pH 7.4). Cells were washed in the same buffer containing 4.5% sucrose. Parasites were post-fixed for 1 h in the dark at 4ºC in a solution containing 1% osmium tetroxide in 0.1 M cacodylate buffer and washed in the same buffer. Samples were dehydrated with increasing concentrations of ethanol in Milli-Q water (10 min incubations in 50% and 70% EtOH at 4ºC and 90% and 100% EtOH at room temperature (RT). To enhance contrast, en-bloc staining was carried out as an intermediate step with 1% uranyl acetate solution in 70% EtOH for 45 min at 4ºC in the dark. Samples were embedded in Epon 12 resin and polymerized (24 h at 45ºC and 48 h at 60ºC). After polymerization, Epon blocks were sectioned with an ultramicrotome (Leica EM UC6). Ultrathin sections, 50-60 nm thick, were mounted on 300-mesh grids, stained with 1 % uranyl acetate aqueous solution and lead citrate and observed using a TEM/STEM Philips Tecnai 12 electron microscope at the Instituto de Salud Carlos III facilities (Majadahonda, Madrid).

Measurement of Reactive Oxygen Species (ROS) generation

Intracellular ROS levels were measured using the cell permeable probe H2DCFDA (2',7'-dichlorodihydrofluorescein diacetate, Molecular Probes). This non-fluorescent compound passively diffuses into cells where is retained upon intracellular hydrolization by esterases. Oxidation of the compound by ROS results in the formation of the fluorescent product dichlorofluorescein (DCF). This probe can be oxidized in the presence of different ROS species such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$) and peroxynitrites among others.

Experiments were carried out in triplicate following a modified protocol described by Fonseca-Silva et al. (2011). Briefly, 2 x 10^6/mL mid-log phase promastigotes were incubated at 27ºC for 3h in the absence or presence of increasing concentrations of allicin (15-120 µM). Parasites were washed in phosphate saline buffer (PBS) (Lonza) and resuspended in 1 mL of PBS (2 x 10^6/mL) and incubated with 20 µM H2DCFDA for 20 min at 37ºC, 5% CO_2. Aliquots of 200 µL/well were transferred to a 96-well solid flat bottomed black microtiter plate (Costar,

Corning) and fluorescence intensity was measured in a FLUOstar OPTIMA microplate reader (BMG Labtech) using excitation/emission wavelength of 500 nm/490 nm. Antimycin A (5 µM) (Sigma) was used as a positive control of ROS generation.

Measurement of mitochondrial ROS generation

Superoxide anion (O_2^-) production was assayed fluorimetrically using the mitochondrial targeted probe MitoSox Red (Molecular probes) as described previously by Carvalho et al. (2010) with some modifications. This cell-permeable dye is positively charged and reacts in the presence of O_2^- rendering a red-fluorescent product when oxidized.

Cells (10^7 promastigotes/mL) were loaded with 1 µM MitoSox Red for 30 min at 27 ºC in HBSS (Ca/Mg) (Hank's Balanced Salt Solution with calcium and magnesium, Gibco). Parasites were washed in HBSS and then treated with allicin (15-120 µM) for 3h at 27ºC. After treatment cells were washed and resuspended in HBSS (10^7 promastigotes/mL). Aliquots of 200 µL/well were transferred to a 96-well solid black microtiter plate (Costar, Corning) and fluorescence intensity was recorded in a FLUOstar OPTIMA microplate reader (BMG Labtech) with an excitation wavelength of 510 nm and emission of 580 nm. Untreated cultures and cultures treated with 5 µM antimycin A (Sigma) were included as controls. Three experiments were carried out in triplicate.

Measurement of free cytosolic calcium

Cytosolic Ca^{2+} in promastigotes was monitored using Fluo 4AM dye (Molecular Probes) using a modified protocol previously described by Serrano-Martín et al. (2009). Exponentially growing cultures were washed twice with the following loading buffer: 137 mM NaCl, 4 mM KCl, 1.5 mM KH2PO4, 8.5 mM Na2HPO4, 11 mM glucose, 2 mM $CaCl_2$, 0.8 mM $MgSO_4$ and 20 mM HEPES-NaOH, pH 7.4 (Sigma).

A total of 10^7 cells/mL were preloaded with 5 µM Fluo 4AM for 60 min at 27ºC in the same loading buffer. Pluronic F-127 (0.02%, Molecular Probes) was added to facilitate the dispersion of the nonpolar AM ester as recommended by the manufacturer. Parasites were washed twice with loading buffer to allow complete intracellular de-esterification of the AM esters and cells were further incubated for 15 min at 27ºC. Fluorescence was excited using a 485 nm filter and read through a 520 nm long-pass emission filter in a microplate reader (FLUOstar OPTIMA, BMG Labtech). To measure intracellular Ca^{2+} responses, baseline fluorescence was monitored

before the addition of the different stimuli. Cells were treated with increasing concentrations of allicin (15-120 µM) and fluorescence intensity was recorded for 1 h every 5 min. Fluorescence intensity measurements were normalized according to Takahashi et al. (1999). Maximal fluorescence values were obtained by permealizing cells with 0.5% Triton X-100 under the saturating Ca^{2+} environment. Minimal fluorescence was measured after chelation of Ca^{2+} with 8 mM EGTA. Two independent experiments were carried out in triplicate.

Measurement of mitochondrial transmembrane potential (ΔΨm)

Changes in the ΔΨm were analyzed by flow cytometry using the cationic lipophilic dye 5,5_,6,6_-tetrachloro-1,1_,3,3_-tetraethylbenzimidazole carbocyanide iodide (JC-1) according to manufacturer's instructions (Molecular Probes). As a positive charged molecule, this dye selectively accumulates in mitochondria in inverse proportion to ΔΨm according to the Nernst equation (Perry et al., 2011). Healthy cells, with functional mitochondrial electron transport, are negatively charged and will accumulate more dye. When the ΔΨm collapses JC-1 dye will be accumulated in its monomeric form within the cytosol of the cells. The JC-1 monomer, which predominates in cells with depolarized ΔΨm, emits green fluorescence (530 nm or in FL-1 in FACS analysis), whereas the J-aggregates, which are formed in mitochondria with high potentials ≥140 mV, emit red fluorescence (590 nm or in FL-2). The ratio between red/green fluorescence intensities (FL-2/FL-1; 590 nm/530 nm) allows assessment of ΔΨm fluctuations (Keil et al., 2011).

After 3 h of treatment with allicin (15-120 µM, 27ºC) promastigotes were washed in PBS and resuspended (2 x 10^6/mL) in 1 mL of PBS containing JC-1 dye at a final concentration of 6 µM. Parasites were incubated in the dark for 20 min at RT. After incubation, parasites were washed twice in PBS to eliminate the non-internalized dye. Non-treated cells and cells treated with 100 µM of the mitochondrial uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP, Sigma) were included as controls. Measurements were performed using a FACScan flow cytometer and analyzed with CellQuest software (Becton Dickinson).

Measurement of cellular ATP levels

Quantification of ATP levels was carried out using the CellTiter-Glo luminescent assay (Promega) as previously described by Manzano et al. (2011). This assay generates a luminescent signal which is directly proportional to the amount of ATP present. Briefly, mid-

log phase promastigotes (2 x 10^6/mL) were incubated at 27ºC in RPMI supplemented medium in the presence of 15, 30, 60, 90 and 120 µM allicin for 3 h. Untreated cultures and cultures treated with 20 mM sodium azide (Sigma) which inhibits mitochondrial oxidative ATP generation were included as controls. After the drug exposure period, a 30 µL aliquot of the parasite suspension was transferred to a 96-well solid white flat bottomed microtiter plates (Costar, Corning) and an equal volume of CellTiter-Glo was added to each well. Plates were incubated in the dark for 10 min at room temperature (RT) and luminescence was measured using a FLUOstar Omega microplate reader (BMG Labtech). Three independent experiments were carried out in triplicate.

Determination of plasma membrane integrity

Cell membrane permeabilization was determined using the SYTOX Green nucleic acid stain (Molecular Probes) as described previously (Lynn et al., 2011) with modifications. Intact cells are impermeable to SYTOX Green dye which penetrates compromised membranes and binds to nucleic acids. Mid-log phase promastigotes were washed twice in HBSS and parasites (2 x 10^6 promastigotes/mL) were incubated (15 min, 27ºC) in the dark with 2 µM SYTOX Green. Cells were washed in HBSS and incubated in the presence of increasing concentrations of allicin (0, 15, 30, 60, 90 and 120 µM) for 3 h at 27ºC. An aliquot of the parasite suspension (200 µL/well) was transferred to a 96-well solid black microtiter plate (Costar, Corning) and fluorescence intensity was measured in a FLUOstar OPTIMA microplate reader (BMG Labtech) with excitation and emission wavelengths of 520 nm and 500 nm, respectively. Control for maximum fluorescence (100% membrane permeabilization) was obtained by the addition of 0.5% Triton X-100. Three independent experiments were carried out in triplicate.

Determination of Trypanothione reductase (TryR) activity in parasite lysates

Assessment of TryR activity was carried out using the colorimetric method described by van den Bogaart et al. (2014). TryR is a NADPH-dependent oxidoreductase which reduces trypanothione ($T[S]_2$) to dihydrotrypanothione ($T[SH]_2$). Within the cells, trypanothione is re-oxidized when inactivating free radicals and other reactive oxygen species thus playing a critical role in the parasite antioxidant defense. Measurement of TryR activity was monitored by coupling the regeneration of $T[S]_2$ to the reduction of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB, Sigma). One molecule of DTNB re-oxidizes $T[SH]_2$ forming two molecules of yellow

thionitrobenzoate ion (TNB^{2-}) which can be quantified spectrophotometrically (Hamilton et al., 2003). Figure 1 shows a scheme of the reaction:

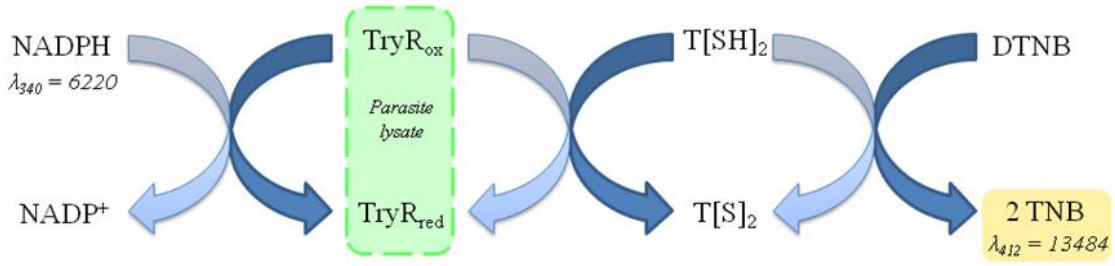


Figure 1. TryR activity assay principle: regeneration of $T[S]_2$ by DTNB-coupled reaction (Modified from van den Bogaart et al., 2014).

Leishmania logarithmic promastigotes (2 x 10^6/mL; 50 mL) were grown in 75 cm^2 culture flasks and exposed to different allicin concentrations (30, 60 and 120 µM) for 3 h at 27ºC. Untreated cultures were included as negative controls. The tricyclic neuroleptic drug clomipramine HCl (Sigma) has previously been reported to selectively inhibit TryR (Ondarza et al., 2000). Positive controls consisting in cultures treated with 10 µM clomipramine HCl (3h, 27ºC) were also included in the experiment. After the drug exposure period, cells were washed twice in PBS and cultures were concentrated (2 x 10^7/mL; 1mL/eppendorf). Samples were centrifuged (10000 rpm, 5 min, RT) and supernatants were discarded. Pellets were incubated for 15 min at RT with 1 mL of lysis buffer consisting of 1 mM EDTA, 40 mM HEPES, 50 mM Tris-HCl (pH 7.5), and 2% v/v Triton X-100 (Sigma). Prior to lysis, buffer was supplemented with 1 mM protease inhibitor phenylmethanesulfonyl fluoride (PMSF, Sigma). Stock solutions and further dilutions of NADPH (Sigma), $T[S]_2$ (Bachem) and DTNB (Sigma) were stored and prepared as described by van den Bogaart et al. (2014).

To determine TryR activity, an aliquot (375 µL) of the parasite lysate was transferred into a new microcentrifuge tube, followed by the sequential addition of NADPH (125 µL/sample), $T[S]_2$ (375 µL/sample) and DTNB (125 µL/sample) yielding final concentrations of 200, 75 and 100 µM, respectively. A blank was included for each sample consisting of the same reaction mixture without the substrate. $T[S]_2$ was replaced by an equal volume of 0.05M Tris buffer (pH 7.5). Samples were incubated at 27ºC protected from light and absorbance (λ= 412 nm) was determined for 90 min every 15 min in a UV Mini 1240 spectrophotometer (Shimadzu). Blank values were subtracted from all the samples. TNB ion formation is equivalent to TryR activity.

Determination of non-protein thiol levels: GSH, T[SH]$_2$ and Cys

Reagents and materials

L-cysteine (Cys) (97%), glutathione (GSH) (99%), 2,3-dimercapto-1-propanol (DMP) (98%), methanesulfonic acid (MSA) (99.5%), tris (2-carboxyethyl)phosphine hydrochloride (TCEP-HCl) (98%), diethylenetriaminepentaacetic acid (DTPA) (99%) were purchased from Sigma. Trypanothione (T[SH]$_2$) (>99%) was obtained from Bachem AG, monobromobimane (mBBr) was from Toronto Research Chemicals.

Acetonitrile (MeCN) and methanol (MeOH) (HPLC-grade) were provided by Scharlab and trifluoroacetic acid (TFA) (HPLC-grade, 99.5%) was from Apollo Scientific. Analytical grade reagents: 3-[4-(2-hydroxyethyl)-1-piperazinyl) propane sulfonic acid (HEPPS) (99%) was from AppliChem and sodium hydroxide (NaOH) was from Thermo-Fisher Scientific. Water was purified with a Milli-Q system from Millipore. All solutions used in the HPLC were passed through a 0.45 μm nylon filter before use.

Experimental procedure

Exponentially growing promastigotes (2 x 10^6/mL; 50-100 mL) were harvested in triplicate and treated for 24 h with increasing allicin concentrations at 27ºC. Cell suspensions were washed twice in PBS and frozen (2 x 10^7 cells; 50 μL PBS) at -80ºC until the time of analyses.

Standards and stock solutions were prepared as described below. Briefly, GSH, Cys, and T[SH]2 stock solutions were prepared in Milli-Q water at a concentration of 8mM, 8 mM and 15 mM, respectively. DMP stock solution was prepared in Milli-Q water at a concentration of 8 mM. A stock solution of mBBr was prepared in acetonitrile at a concentration of 50 mM and 20 mM TCEP solution was made in 200 mM HEPPS buffer (pH 8.2). All solutions were aliquoted and stored at -20 ºC in the dark until the time of analysis. Appropriate aliquots of Cys, GSH and T(SH)$_2$ stocks were mixed and diluted with extraction solution (6.3 mM DTPA with 0.1% TFA) to construct a calibration curve of each thiol with concentration ranging between 0.05 and 6.2 nmol/mL. The internal standard, DMP, was prepared at a final concentration of 0.75 nmol/mL.

The extraction of thiols was performed in acid media using as extraction solvent 50 μL of 6.3 mM DTPA (0.1% TFA) that was added to microcentrifuge tubes containing the pellets resuspended in PBS. After vortex mixing, *Leishmania* extracts were immediately frozen in liquid N_2 and thawed three times to fully release cellular content. The supernatant was collected by centrifugation (13,000 rpm, 10 min) and subsequently derivatized.

The derivatization procedure followed was based on the method described by Minocha et al. (2008). Firstly, 10 µL of 20 mM TCEP and 246 µL of 200 mM HEPPS buffer (6.3 mM DTPA, pH 8.2) were mixed to obtain a sulfur reducing solution that was subsequently added to 100 µL aliquots of *Leishmania* sample extracts or standards; 4 µL of 75 µmol/L DMP was also added as an internal standard. Then, the mixtures were incubated at 45 ºC in a water bath for 10 min to guarantee the reduced state of thiols before mBBr derivatization. For derivatization of thiols, 10 µL of 50 mM of mBBr was added to the vials containing samples or standards and incubated in the dark for 30 min at 45 ºC in a water bath. Finally, 100 µL of 1 M MSA were added to stop reaction and derivatized samples were analyzed by HPLC-DAD/FLD [High-performance liquid chromatography (HPLC) equipped with both diode-array detector (DAD) and a fluorescence detector (FLD)].

Chromatographic separation of the thiols was performed on a SynergiTM Hydro-RP (250 mm × 4.6 mm, 4 µm) HPLC column from Phenomenex (Torrance, CA, USA). A gradient program was used with the mobile phase, combining solvent A (Milli-Q water with 0.1% TFA) and solvent B (MeCN with 0.1% TFA) as follows: 10% B (5 min), 20.6% B (6.7 min), 31.1% B (13.6 min) and 100% B (5 min). The column was equilibrated with 10% of solvent B for a total of 8 min before next injection. Analyses were performed at a flow rate of 1.0 mL/min and the column temperature was kept at 40 ºC. The injection volume was 100 µL and all the compounds elute within 30 min. The DAD detector was set at both 290 and 380 nm and the excitation and emission wavelengths of the FLD detector were set at 380 nm and 470 nm, respectively. Quantification was performed using internal calibration and peak area measurements. Multiple analyses were performed using blanks and standards to determine detection limits, response linearity and reproducibility of the protocol.

Annexin V and Propidium Iodide staining

Externalization of phosphatidylserine (PS) upon the outer leaflet of the plasma membrane has been considered a common marker of apoptosis in eukaryotic cells. This calcium regulated process takes place as an early event in the apoptotic dying cascade. PS translocation in the cell surface serves as a specific signal for phagocytic clearance without triggering an inflammatory response (Balasubramanian et al., 2007). Annexin V, a Ca^{2+}-dependent phosphatidil-binding protein with high affinity for PS, was used for PS labeling. Evaluation of plasma membrane integrity with propidium iodide (PI) staining allowed the discrimination between necrosis and apoptosis.

PS exposure in *Leishmania* promastigotes was analyzed using the Annexin V-FLUOS Staining kit (Roche) according to manufacturer's instructions. Briefly, cells (2 x 10^6/mL) were incubated at 27ºC in the presence of allicin (30-120 µM) for 3, 12, 24 and 48 h. Cells were washed twice in cold PBS and the resulting pellet resuspended in 100 µL of HEPES buffer containing Annexin-V and PI (2 µL of each). Samples were incubated at RT in the dark for 15 min and analyzed by flow cytometry (FACScan, Becton Dickinson) with CellQUEST software. Cultures incubated at 45ºC overnight were used as positive death controls.

In situ DNA fragmentation detection and cell cycle analysis

A biochemical hallmark of late apoptosis stages is internucleosomal DNA fragmentation. Chromatin cleavage by endonucleases originates nucleosomal DNA fragments that can be labeled in situ by the terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick-end labelling (TUNEL) method. TUNEL was performed using an APO-BrdU™ TUNEL Assay Kit (Invitrogen) following manufacturer's recommendations. Bromodeoxyuridine (BrdU) is a synthetic thymidine analog. This assay is based on the ability of TdT enzyme to incorporate labeled-dUTP (BrdU) to 3'-hydroxyl ends of DNA breaks (Gavrieli et al., 1992). BrDU incorporation can be labeled and quantified by flow cytometry measuring fluorescence intensity emitted by a conjugated anti-BrdU antibody.

Briefly, promastigotes (2 x 10^6/mL) were treated with allicin for 48 and 72 h and washed twice with cold PBS. Parasites were fixed in 1% paraformaldehyde (4ºC, 15 min), washed and incubated at -20ºC overnight in 70% ethanol. After washing, cells were incubated (60 min, 37ºC, darkness) in DNA-labeling solution to allow TdT-mediated DNA-BrdU binding. Cells were washed twice in rinsing buffer and incubated (30 min, RT, darkness) with Alexa Fluor 488 conjugated anti-BrdU antibody. Prior to flow cytometric analyses (FACScan, CellQUEST software). PI/RNase A staining solution was added to examine cellular DNA content and study cell cycle progression.

Statistical analysis

Statistical analysis and graphs were performed with GraphPad Prism 5 software. Statistical significance of differences was determined by one-way analysis of variance (ANOVA) and Bonferroni post-test. Differences were considered significant at a p value of < 0.05.

6.4. Results

Transmission Electron Microscopy (TEM) studies of allicin treated Leishmania promastigotes

Normal morphology and structural organization of *Leishmania* promastigotes is shown in untreated controls (Figure 2A, 2A' and 2A''). Promastigotes incubated in the presence of different concentrations of allicin for 24 h showed important morphological and subcellular alterations (Figure 2B-2J).

Electron micrographs of treated parasites revealed significant ultrastructural changes in the mitochondrion and kinetoplast (Figure 2B, 2C, 2E, 2F and 2I). Allicin treated promastigotes presented intense mitochondrial swelling and loss of electron dense material in the mitochondrial matrix, particularly evident in Figures 2B and 2I. In some images, mitochondrial cristae could be recognized (Figure 2B and 2F). Indeed, a complete loss of internal integrity of this structure was observed with high allicin concentrations (Figures 2B and 2I). Moreover, invaginations of the inner mitochondrial membrane could be seen in some micrographs (Figures 2B and 2I).

A second relevant morphological change took place in the nucleus. Swelling of the nuclear membrane was evident in all drug-treated cells (Figures 2B, 2D, 2E, 2G, 2H and 2J). Peripheral chromatin condensation in the nucleus was found in some micrographs (Figures 2B, 2G and 2H). Extensive alterations in the structure and organization of the cytosol were found after treatment with allicin. Cytoplasm vacuolization (Figures 2B, 2C, 2D, 2F, 2H, 2I and 2J), dramatic changes in cytosolic density (loss of electron dense material) (e.g. Figures 2G, 2H and 2J) and an increase in the number of cytoplasmic vesicles, probably autophagosomal structures, were observed in allicin exposed parasites (Figures 2B, 2C, 2D, 2F, 2I and 2J). Glycosomes, megasomes and acidocalcisomes could be observed in untreated controls (Figure 2A''). Presence of glycosomes or other electrodense vesicles was remarkably reduced in treated parasites. However, acidocalcisomes persisted in treated promastigotes (Figures 2H, 2I and 2J).

No significant alteration could be observed in the flagellum or flagellar pocket (not shown). Ultrastructural internal changes and cell swelling were evident after allicin exposure; nevertheless, cellular membrane integrity seemed to be conserved in treated cells (Figures 2G and 2H).

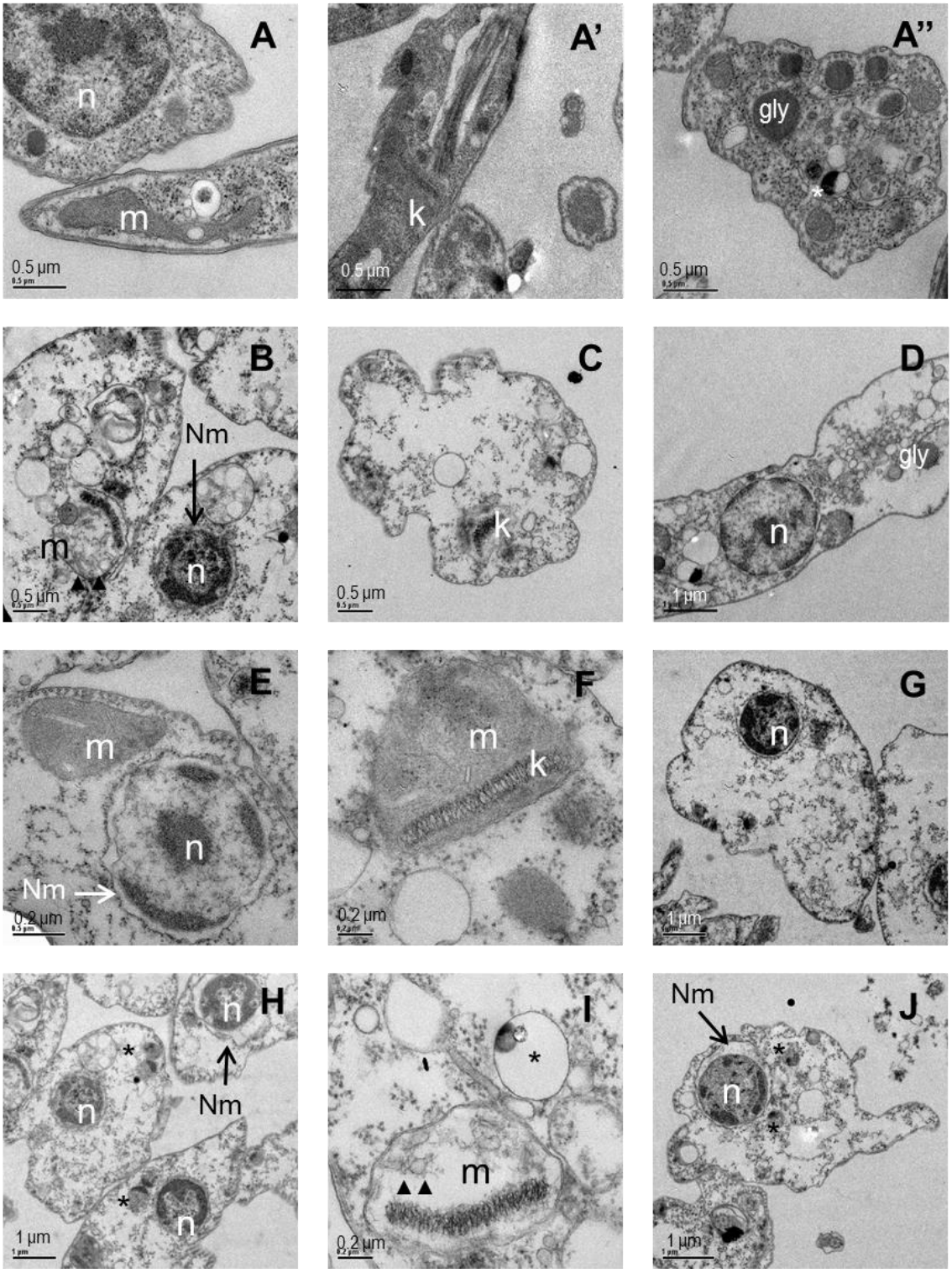


Figure 2. Electron micrographs of untreated (**2A, 2A'** and **2A''**) and treated (24 h exposure to allicin) *Leishmania* promastigotes. Allicin treated parasites (24 h drug exposition): 60 µM (**2D, 2E, 2F** and **2H**), 90 µM (**2B, 2C** and **2I**), 120 µM (**2G** and **2J**). m: mitochondrion; k: kinetoplast; n: nucleus; Nm: nuclear membrane; gly: glycosomes; *: acidocalcisomes; ▲▲ : inner mitochondrial membrane invaginations.

ROS production of promastigotes in the presence of allicin

Experimental approach only included the exposition to different concentrations of allicin in the micromolar range. The molecule triggered the elevation of intracellular fluorescence, after 3h treatment with allicin, as assessed by the synthesis of dichlorofluorescein (DCF) this evidencing the presence of high levels of hydroxyl radicals, hydrogen peroxide and peroxynitrites (Figure 3A). Intracellular ROS generation was concentration dependent and reached over 5-fold production in the presence of 90 µM allicin. It is noteworthy to indicate that the exposition of promastigotes to the estimated EC_{50} value (30 µM) elicited a ROS production similar to the value reached by the positive control (5 µM antimycin A).

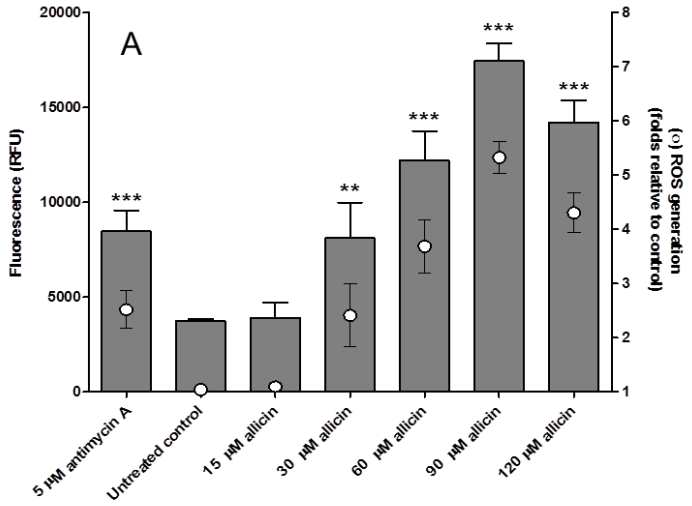


Figure 3. Allicin induces ROS generation in *Leishmania* promastigotes in a concentration-dependent manner. **Figure 3A**, intracellular generation of ROS estimated by H2DCFDA in promastigotes treated for 3h with 15-120 µM allicin, untreated control cultures and positive control exposed to 5 µM antimycin A.

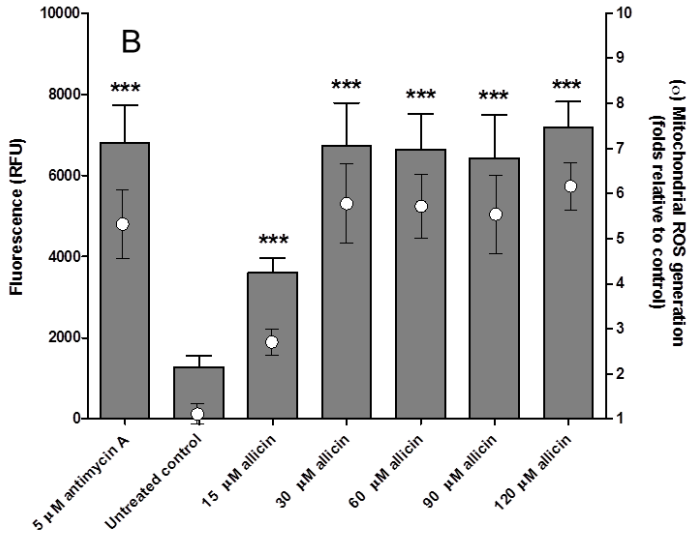


Figure 3B, effect of allicin on mitochondrial ROS generation by MitoSox Red in promastigotes treated for 3 h. Figure shows the results in Relative Fluorescence Units (RFU) and folds relative to untreated control cultures. Results shown correspond to means ± standard deviation (S.D.) of three experiments in triplicate and asteriks represent significant differences related to untreated cultures (***: p<0.0001).

Availability of a mitochondrial targeted probe (MitoSox Red) allowed the estimation of superoxide anion production. Similarly to the ROS production determined by the H2DCFDA probe, allicin effectively induced a strong elevation of superoxide production in mitochondria of treated cells (Figure 3B). This induction was very notable even with moderate allicin concentrations and a 6-fold increase was found with a concentration of 30 µM. It should be indicated that this increase, in the region of the levels reached by the positively treated promastigotes (5 µM antimycin), was maintained with higher allicin concentrations (60-120 µM).

Effect of allicin on the levels of cytosolic Ca^{2+} in Leishmania promastigotes

It has been shown that oxidative stress causes mitochondrial depolarization in *Leishmania* (e.g. *L.donovani*) by increasing cytosolic Ca^{2+} levels. Moreover, the role of this cation and the fine regulation of its cellular concentration are critical. On these grounds, the cytosolic levels of calcium in *L.infantum* promastigotes treated with increasing concentrations of allicin (15-120 µM) were determined at different times (0-60 min). For comparative purposes results obtained were normalized using as baseline the fluorescence before adding stimuli.

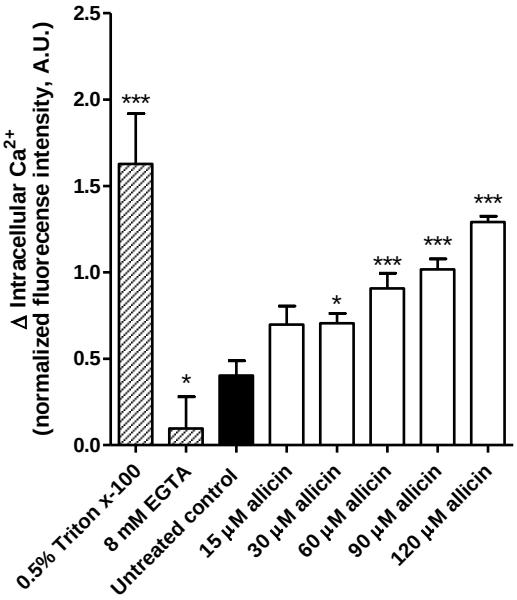


Figure 4. Intracellular Ca^{2+} increases in response to allicin stress. *Leishmania* promastigotes were treated with allicin (15-120 µM) for 20 min. Calcium levels were determined by Fluo 4AM preloading of cells (60 min) in loading buffer with 0.02% Pluronic F-127. Maximal fluorescence was obtained with cells treated with 0.5% Triton X-100 and minimal after chelation with 8 mM EGTA. Data presented corresponds to the average increase of normalized fluorescence intensity of two independent experiments in triplicate (mean ± S.D.). Asterisks represent significant differences compared to untreated control promastigotes (*: p<0.05; ***: p<0.001).

Addition of allicin induced a rapid increase of cytosolic Ca^{2+} in a dose-dependent manner for a given post treatment time. Figure 4 shows the results obtained after 20 min. Chelation with 8 mM EGTA inhibited this increase whereas the permeabilization of the cell membrane of *Leishmania* with 0.5% Triton X-100, in a saturated Ca^{2+} environment, showed the maximal

cytosolic concentration. The increase of calcium was very rapid and after 20 min cells treated with the EC_{50} concentration (30 µM allicin) reached ca. 43% of the maximal value obtained with the positive control.

Mitochondrial membrane potential of allicin-treated promastigotes

Evaluation of mitochondrial transmembrane potential (ΔΨm) of *L.infantum* promastigotes treated with allicin was carried out using the cationic dye JC-1 and its selective accumulation in mitochondria inversely related to ΔΨm. A net negative charge of mitochondrial membrane is a characteristic of healthy cells thus allowing the concentration of the cationic dye. JC-1 aggregates emit red fluorescence at higher potential whereas with membrane potentials below 140 mV remains as a monomer within the cytoplasm emitting green fluorescence.

The potential can be altered by some intracellular events (e.g. ROS production) in *Leishmania* and our results showed an increase in mitochondrial ROS production in the presence of allicin, even at low micromolar concentrations. To asses changes in the ΔΨm in the presence of allicin it was considered relevant to determine the ratio between red fluorescence (590 nm or FL-2) and green fluorescence (530 nm or FL-1). Fluorescence intensity as determined by FACS analysis (Figure 5A) showed that the FL-2/FL-1 ratio (red/green fluorescence ratio) was significantly affected, in our experimental conditions, in a dose-related manner since only 15-30 µM allicin induced a 23-30% reduction of the ratio and this value was below 90% with allicin concentrations >90 µM.

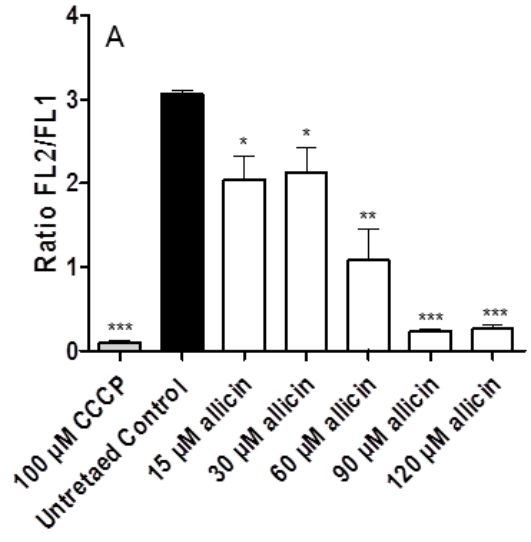


Figure 5. Allicin provokes the loss of ΔΨm in *L.infantum* promastigotes. Cells were exposed for 3 h to 15-120 µM allicin and fluctuations of ΔΨm were determined by flow cytometry of cells preloaded with JC-1. Fluorescence intensity was determined by FACS analysis (green, FL-1 and red, FL-2). Untreated and positive (100 µM CCCP treated cells, 3 h) cultures were included. **Figure 5A**: Effect of allicin on FL-2/FL-1 ratio. Fluorescence intensity was determined by FACS analysis. (FL-2: J-aggregates and in FL-1: JC-1 monomers). Untreated control cells and promastigotes exposed to the mitochondrial membrane uncoupler CCCP were included as controls. Data represents the mean ± S.D of three determinations. Asterisks represent significant differences to the untreated control cultures (*: $p<0.05$; **: $p<0.005$; ***: $p<0.001$).

Actually, the reduction with 60 µM was higher than that found with the CCCP treated control. Furthermore, the depolarization of the mitochondria evidenced by the ΔΨm fall was supported by the shift of the *Leishmania* population towards the right in the FL-1 channel (green fluorescence) as observed by FACS analysis (Figure 5B).

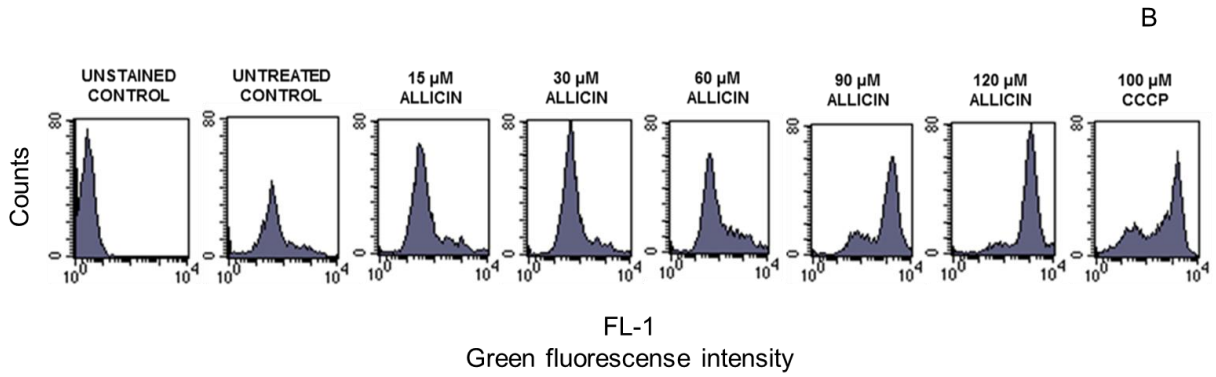


Figure 5B. Representative FACS analysis of monomeric JC-1 (green fluorescence intensity, FL-1) levels in promastigotes of *L.infantum* treated with allicin, untreated or subjected to the mitochondrial uncoupler CCCP (100 µM).

Cellular ATP levels of Leishmania promastigotes treated with allicin

Allicin induced a dose-dependent notable fall of ATP levels in *Leishmania*, since exposure to 30 µM allicin (3 h) reduced ATP levels over 40% (2.59 ± 0.16 Relative Luminescence Units, RLU, in 30 µM allicin-treated promastigotes versus 4.55 ± 0.27 RLU in untreated control cultures). The decrease of ATP levels caused by 60 µM allicin was significantly higher (p<0.001) than the fall provoked by 20 mM sodium cyanide (Figure 6A).

To ascertain that this fall of ATP was not due to membrane leakage, cellular membrane permeability of treated promastigotes was evaluated with SYTOX Green. Exposition to allicin (≥ 90 µM) induced altered permeability of plasma membrane of promastigotes of *Leishmania* (p<0.001); 120 µM allicin yielded ca. 50% increased permeability from that obtained with the positive control (0.5% Triton X-100) at least as assessed by SYTOX Green internalization (Figure 6B). Interestingly, low concentrations of diallyl thiosulfinate, and in particular in the range of EC_{50} values for this parasite stage (ca. 30 µM), did not result in any significant alteration of the cell membrane. These results point towards the fact that the diminished intracellular ATP generation observed in treated cells (<60 µM; i.e. drug concentrations that did not induce a notable damage of the plasma membrane) was probably due to a direct action of allicin on mitochondria.

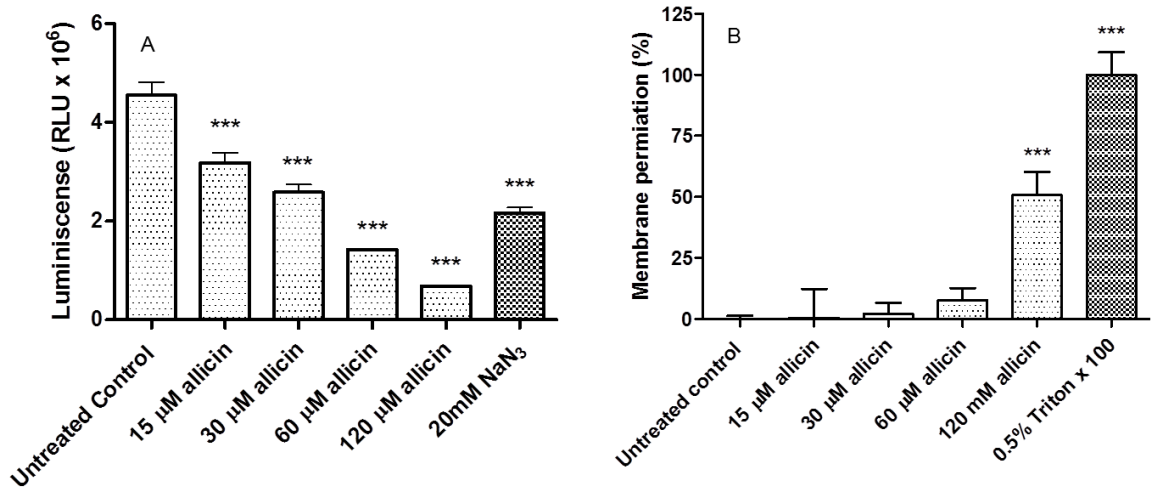


Figure 6A. Allicin induces the fall of ATP production by *Leishmania*. Effect of allicin (15-120 µM, 3 h) on ATP levels of *L.infantum* promastigotes determined by the CellTiter-Glo luminescent assay. Untreated control cells and cells treated with 20 mM sodium azide as inhibitor of mitochondrial oxidative generation of ATP were included. Data presented corresponds to one representative experiment of the three independent experiments in triplicate carried out. **Figure 6B.** Effect of allicin on membrane permeabilization. Promastigotes of *Leishmania* were incubated with allicin (15-120 µM) for 3 h and the permeability to SYTOX Green nucleic acid stain determined. Untreated and positive samples exposed to 0.5% Triton X-100 were included. Data represents means ± S.D. of three independent experiments carried out in triplicate. Asterisks represent significant differences to the untreated control cultures (***: $p<0.001$).

Effect of allicin on trypanothione reductase (TryR) activity and non-protein thiol levels in Leishmania

Trypanothione reductase (TryR) plays a fundamental role in the antioxidant defense of the cells through the reduction of trypanothione, the unique thiol present in Kinetoplastida including *Leishmania*. Given the strong induction on ROS production by allicin treated promastigotes (see above), it was considered relevant to know whether the enzymatic activity was affected by the diallylsulphide and also its possible impact on the intracellular levels of non-protein thiols. Exposure of *Leishmania* promastigotes to allicin for 3 h provoked a dose-dependent reduction of TryR activity in parasite lysates of treated cells. Residual activity in the presence of the lowest allicin concentration (30 µM) only reached a 66.67% of that found in untreated control cultures; 120 µM allicin reduced the TryR activity by a 80%, inhibition higher than that induced by the specific inhibitor clomipramine. Remaining TryR present in the leishmanial lysates displayed standard enzymatic kinetics (Figure 7).

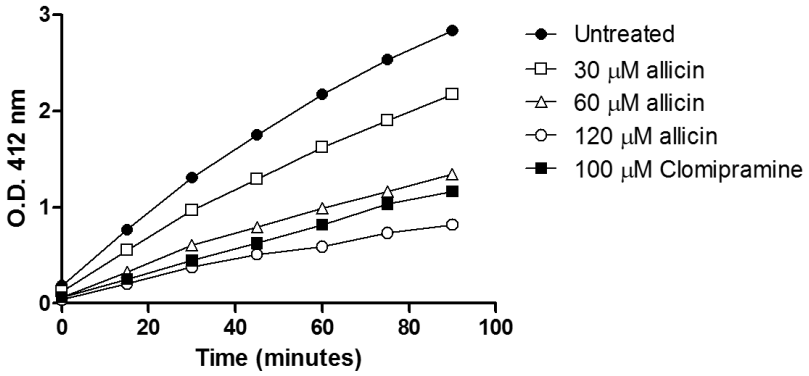


Figure 7. Inhibition of TryR activity of *Leishmania* by allicin. Promastigotes treated for 3 h with varying concentrations of allicin (30, 60 and 120 μM) were lysed and the enzyme activity was determined by the sequential addition of 200μM NADPH, 75 μM trypanothione and 100 μM DTNB. Thionitrobenzoate ion (TNB^{2-}) produced was proportional to TryR activity and was quantified spectrophotometrically (412 nm) at different time points. Untreated control cultures and cultures treated with the TryR inhibitor clomipramine (100 μM) were included. Data shown correspond to the mean values of a representative experiment, from the two executed, carried out in duplicate.

No clear figure emerged, in the three experiments carried out, when the levels of GSH, $T[SH]_2$ and Cys from *Leishmania* promastigotes exposed to allicin were analyzed, variability was high among the experiments. However, the tendency suggests that non-protein thiol levels increased with allicin treatment (≤ 60 μM) (Table 1). Higher allicin concentrations induced a reduction of the analyzed thiols. $T[SH]_2$ levels remained ca. 1.0 and 2.0 nmol/10^8 cells irrespective of the allicin concentration; only with 100 and 120 μM allicin, when TryR activity was reduced over 80% (see above), the levels of the thiol fell. By its part, the levels of GSH showed a pattern of increased levels with low allicin concentrations (< 30-50 μM), up to 2.5-3.0 nmol/10^8 cells, followed by reduced levels with higher concentrations of the drug. Levels of cysteine were more variable between experiments although in all determinations there was a steady increase with higher drug concentrations.

Table 1. Levels of non-protein thiols (cysteine, trypanothione and glutathione) in promastigotes of *L.infantum* promastigotes treated with allicin (10-120 μM).

Allicin (μM)	**nmol/10^8 cells**					
	Cys		**GSH**		**$T[SH]_2$**	
	mean	S.D.	mean	S.D.	mean	S.D.
0	0.723	0.194	0.723	0.126	1.162	0.130
10	0.914	0.214	0.914	0.146	1.591	0.192
20	1.179	0.684	1.179	0.211	1.998	0.231
30	1.448	0.801	1.448	0.194	2.235	0.135
40	1.371	0.962	1.371	0.154	2.162	0.173
50	1.561	0.711	1.561	0.184	2.365	0.112
60	1.869	0.909	1.869	0.173	2.217	0.206
80	1.675	0.849	1.675	0.166	3.076	0.255
100	1.656	1.079	1.656	0.138	0.655	0.069
120	1.771	0.762	1.771	0.100	0.216	0.012

Non-protein cellular thiols of *Leishmania* were separated, identified and quantified using HPLC-DAD-FLD. This approach was modified to improve reproducibility and sensitivity. Several isocratic and gradient elution conditions were evaluated using different acetonitrile/water mixtures containing TFA. Experimental parameters such as peak weight, resolution (Rs) and retention times (rt) have been compared to select the adequate separation procedure.

The optimized separation was performed in gradient elution mode that yields Rs higher than 2.0 and rt of 6.70, 11.94, 15.90 and 26.43 min for Cys, GSH, $T[SH]_2$ and DMP, respectively. The RTs were reproducible between runs. Figure 8 shows a representative chromatogram of a standard mixture of the thiols. Thiols were quantified using an internal standard (DMP) calibration model using an eight-point calibration curve, which covered three-log concentration range (0.05 – 6.2 nmol/mL) for the three monothiols Cys, GSH and $T(SH)_2$. The r^2 value for standard curves was >0.999 for all thiol compounds tested.

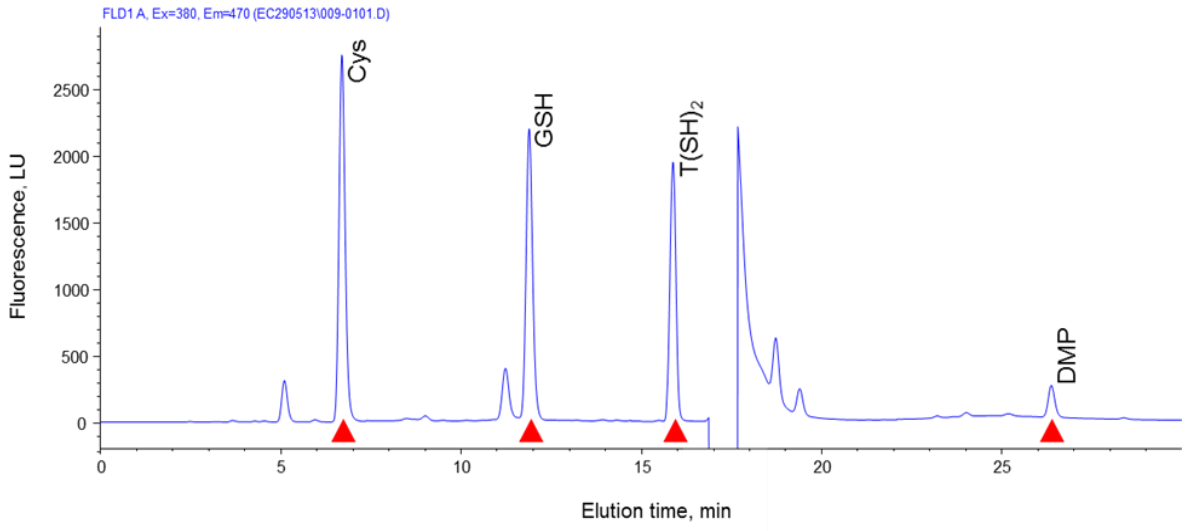


Figure 8. HPLC elution profile of MBBr derivatized thiols. Standard solution of Cys, GSH and $T[SH]_2$ prepared at 3 nmol/mL; 0.075 nmol/L of DMP was added as an internal standard.

Externalization of phosphatidylserine in allicin-treated Leishmania promastigotes

Results obtained in the TEM study showed some alterations in the nuclear structure of the treated cells, these including the chromatin condensation in the periphery of the nucleus. Therefore it was considered relevant to evaluate the possibility of allicin inducing apoptosis in *Leishmania*. One of the most frequently used early markers of apoptosis is the externalization of phosphatidylserine (PS) by apoptotic cells. Figure 9 shows the results obtained, by FACS, after 48 h treatment with 30, 60 and 120 µM allicin. Results obtained in the first three determinations (3, 12 and 24 h) did not yield significant results.

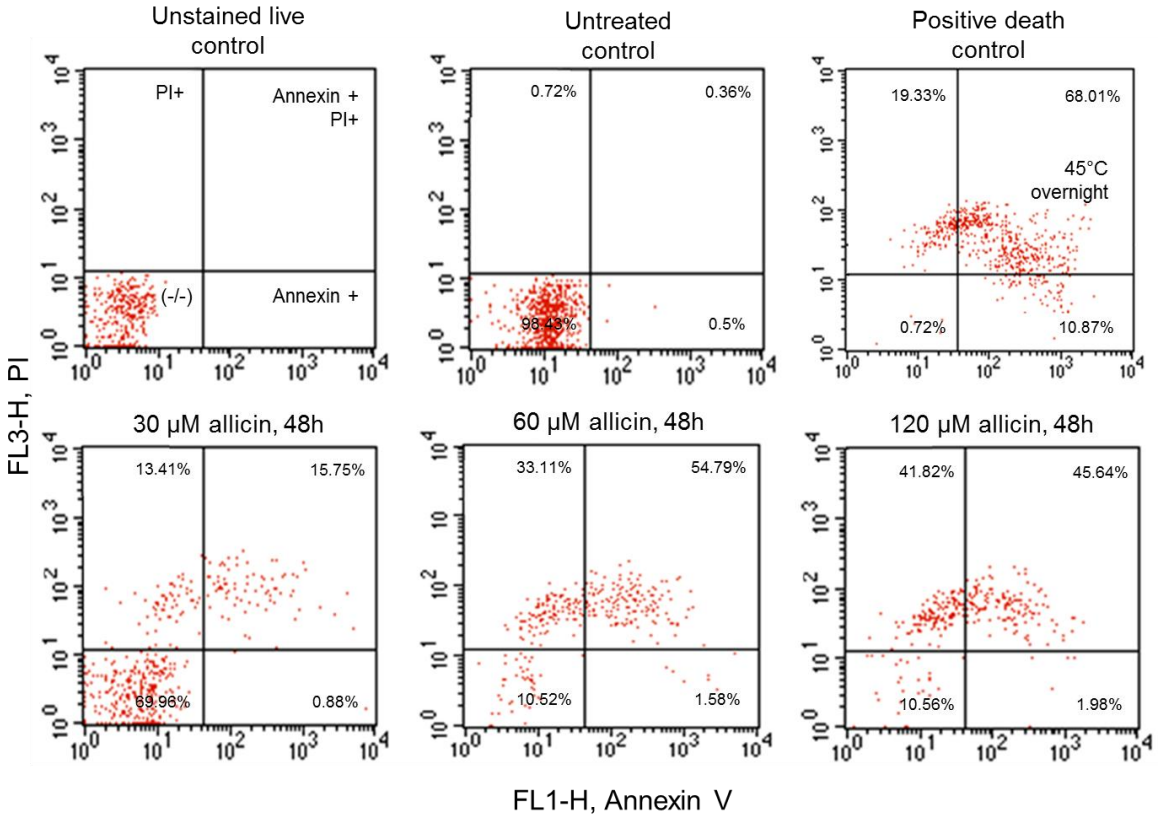


Figure 9. PS externalization in *Leishmania* promastigotes is not detected with Annexin V and PI (propidium iodide) after treatment of promastigotes with allicin. *L.infantum* promastigotes were exposed to allicin (30, 60 and 120 µM) for 3, 12, 24 and 48 h and subsequently stained with Annexin V-FITC + PI and analyzed by flow cytometry. Data correspond to the samples exposed for 48 h. Lower row shows the allicin-treated cultures and the upper row includes unstained cells, untreated control cells and cells exposed overnight at 45 ºC. Percentages are shown in each quadrant. (-/-): healthy cells; Annexin+: apoptotic cells; Annexin+/PI+: late apoptotic or early necrotic cells; PI+: necrotic cells.

Using the EC_{50} concentration of the compound (30 µM) a shift of the cell population from the bottom left quadrant (Annexin V or PS-/PI-), characteristic of untreated healthy control cells, towards the upper left (PI+) and upper right quadrants (PS+/PI+) was observed. This shift was increased with higher concentrations of allicin and only a residual population remained double negative even with 120 µM. It is noteworthy to indicate that no annexin single positive (PS+) population was found with any of the concentrations and times of treatment. These results point towards the absence of apoptotic cell death in *Leishmania* as a consequence of allicin treatment. In our case, cell distribution with the drug showed a necrotic cell death pattern similar to that found in *Leishmania* subjected to thermal treatment (45ºC, overnight).

Effect of allicin on in situ DNA fragmentation of Leishmania

Intranucleosomal DNA fragmentation is characteristic of late apoptosis and this fragmentation remains stable. Using the dUTP nick-end labelling (TUNEL) method, FACS analysis of cultures of *Leishmania* promastigotes treated for 48 and 72 h with allicin (30, 60 and 120 µM) showed that no DNA fragmentation was evident in our treated cells (Figure 10A and 10B). There was a shift in the FL1-H axis but this shift was present in all cultures irrespective of the treatment and the concentration of allicin. A small and possibly non-significant apoptotic population was observed after 48 h in the cultures with the highest allicin concentration (>60 µM). In the 72h sample a significant fraction of the population displayed an apparent phenotypic fragmentation pattern. However cellular distribution was similar in both treated and untreated control cultures. These results obtained with the marker of late apoptosis reinforce the lack of evidence of apoptotic-like death in *Leishmania* after treatment allicin.

Allicin induces G_2/M phase cell cycle arrest of Leishmania infantum promastigotes

In order to determine the effect of allicin on the progression of the cell cycle of *L.infantum* promastigotes, cells were treated with different concentrations of the antileishmanial (30, 60 and 120 µM) at different exposure times (48 and 72 h) and analyzed by flow cytometry using PI staining. Allicin induced notable changes in the distribution of cell cycle phases of synchronized cultures. These alterations were time and dose dependent as shown in Figure 10C. FACS analysis of untreated control cultures showed 1.3% of cells in Sub-G_1 phase in 48 h cultures and 0.17% in 72h cultures. Allicin treatment did not induce the appearance of the Sub-G_1 peak, characteristic of apoptotic cells, as the highest concentration produced a minimal change in the

G_0/G_1 phase % (i.e. 48 h: 1.24% and 72 h: 1.20%). Untreated controls presented a large population in the G_0/G_1 phase (i.e. 48 h: 72.42% and 72 h: 62.48%) whereas the allicin treated cells showed a dose dependent increase in the G_2/M phase cell population and a reduction of the cells in G_0/G_1 phase. Allicin 120 µM (3h) induced a marked shift in the G_2/M gate (i.e. 48 h: 23.83% and 72 h: 48.90%). Figure 10D illustrates the cell cycle phases and their analysis.

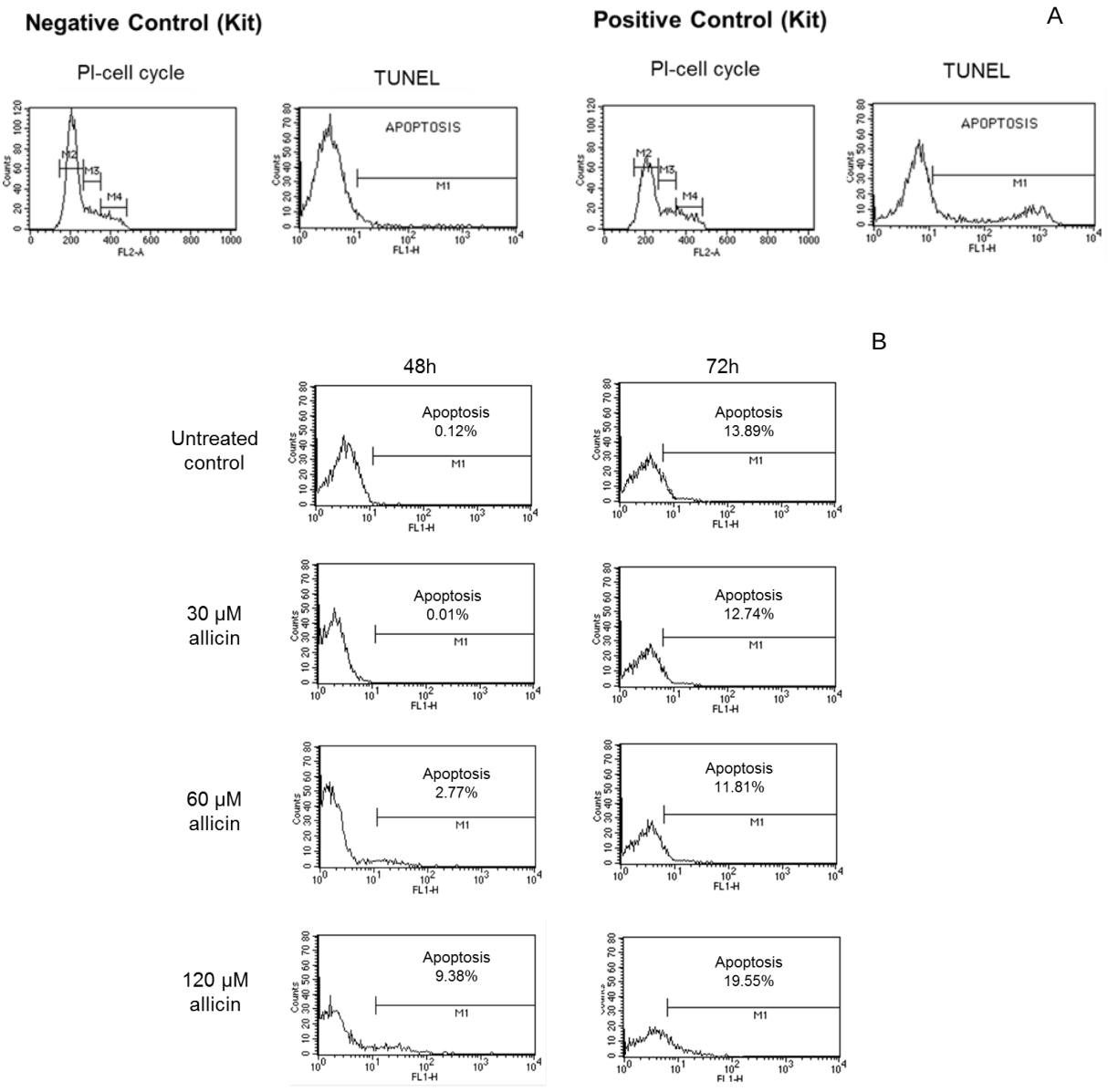
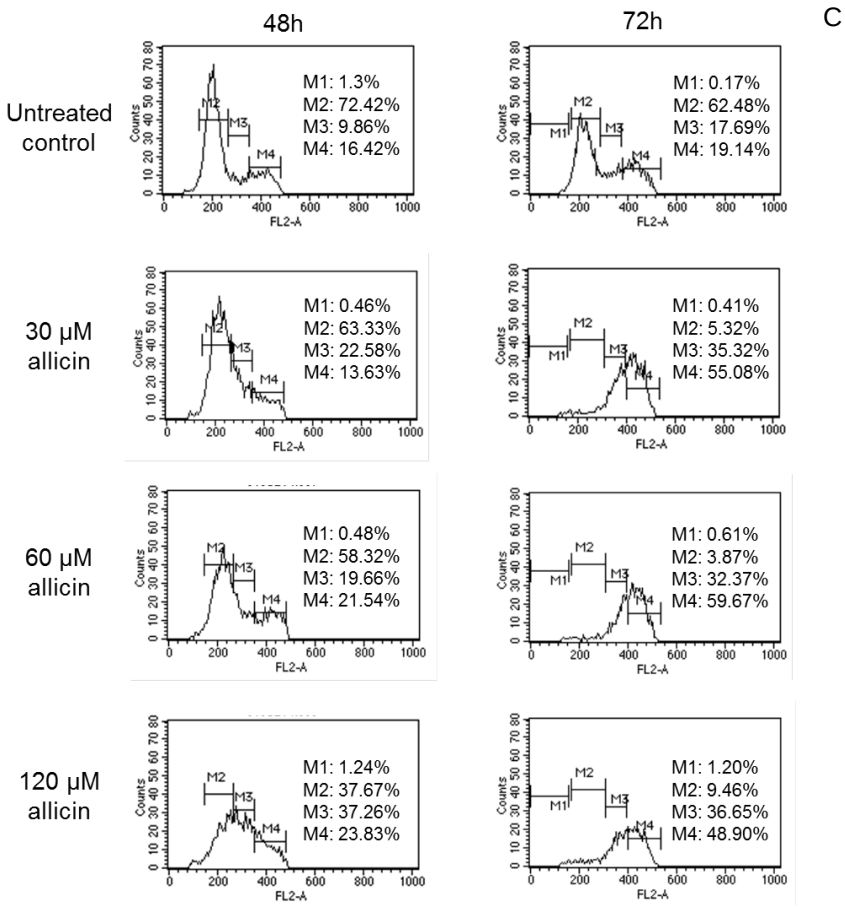


Figure 10. Allicin does not provoke oligonucleosomal DNA fragmentation but it induces cell cycle arrest at G_2/M phase in *Leishmania*. **10A**, Apoptotic control cells of APO-BrdU™ TUNEL Assay Kit. **10B,** analysis of DNA fragmentation in *Leishmania*. Promastigotes of *L.infantum* treated with allicin (30, 60 and 120 µM) for 48 and 72h and the incorporation of bromodeoxyridine (BrUD) (TUNEL) analyzed by flow cytometry with Alexa Fluor 488 conjugated anti-BrDU antibody. Untreated cell cultures, positive and negative controls were included.



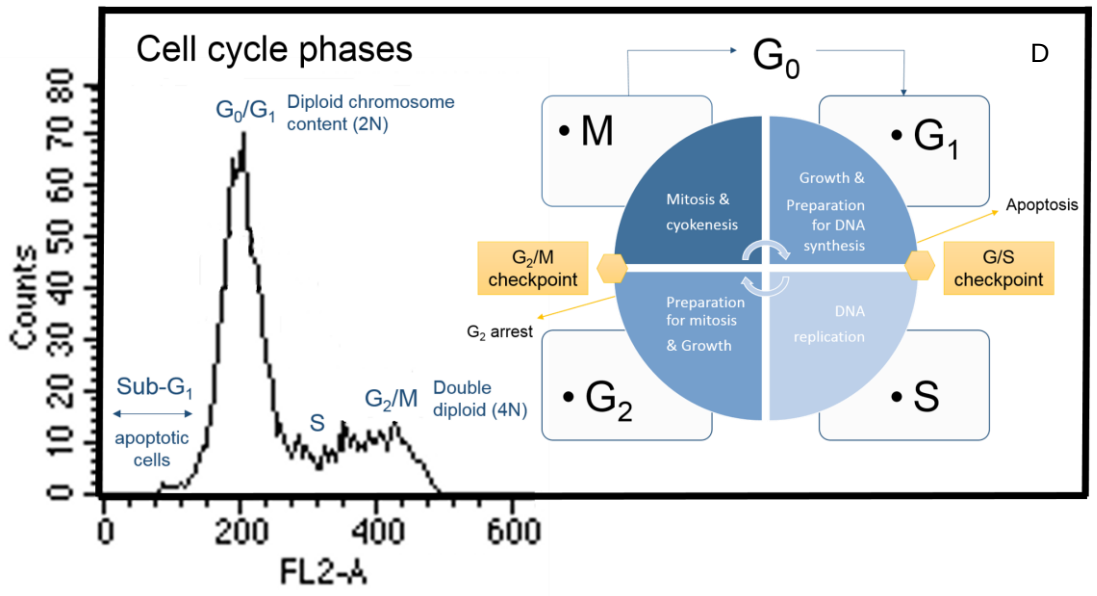


Figure 10C, Cultures of *L.infantum* promastigotes treated with allicin (30, 60, 120 µM) (48 and 72h) from the TUNEL assay were incubated with PI/RNAse staining solution and DNA contents and cell cycle progression determined. Untreated promastigotes were included as controls. **10D,** Cell cycle analysis: phases and interpretation.

6.5. Discussion

Our experiments showed that allicin induced morphological alterations in vital cellular organelles. Significant ultrastructural TEM changes were observed compared to normal morphology and organization of *Leishmania* (e.g. Pimienta & de Souza, 1985; de Souza, 2008). This is relevant since microscopy is a golden standard to determine intracellular targets of toxic agents and the type of cell death.

Micromolar concentrations of the molecule did not provoke detectable lesions in the plasma membrane of promastigotes treated for 24 h (even with the higher concentration employed 120 μM). The absence of morphological alterations in the cell membrane was in agreement with the scarce permeability to SYTOX Green found, with values below 5%, after 3 h treatment with 30 μM allicin and only significant increases in membrane permeability with concentrations ≥ 90 μM allicin, concentrations well above the cytostatic concentration found (30 μM) (Corral et al., 2012). There was intense cytoplasmic vacuolization, with loss of electron dense material and neoformation of cytosolic vesicles, comparable to that described by Ledezma et al. (2002) in *L.amazonensis* promastigotes treated with the related compound ajoene. Similar vesicular formations were described as autophagosomes in *Trypanosoma cruzi* epimastigotes treated with cysteine protease inhibitors (Engel et al., 1998), in *Leishmania* spp promastigotes exposed to monoterpenic aldehydes (Machado et al., 2012) and in *L.amazonensis* promastigotes and amastigotes treated with 22,26-Azasterol (Rodrigues et al., 2002). The latter study (i.e. Rodrigues et al., 2002) linked the presence of autophagosomes to an intensive remodeling process of intracellular organelles irreversibly damaged by the drug.

Another significant alteration observed in the TEM analysis was the swelling of the nuclear membrane. The same type of nuclear alteration was described in *Leishmania* by Machado et al., (2012) after treatment with citral (a compound present in essential oil of *Cymbopogon citratus*), by Ledezma et al. (2002) with ajoene and in *T.cruzi* by Engel et al. (1998) after the exposition to cysteine protease inhibitors. Allicin has been also described to inhibit cysteine proteases (Waag et al., 2010) thus suggesting that this mode of action could also be contributing to *Leishmania* cell death. Changes in nuclear chromatin organization with accumulation in the periphery was suggestive of an apoptotic-like cell death process although, as it will further be discussed, no other phenotypic characteristics of apoptosis were found by us in *Leishmania* promastigotes after allicin exposure.

Most outstanding morphological changes took place within the mitochondrion. In allicin treated promastigotes this organelle presented a less electrodense matrix and a significant

swelling, with loss of cristae integrity. Comparable alterations have been described in *L.amazonensis* and *T.cruzi* after treatment with antifungal compounds such as ketoconazole and terbinafine (Lazardi et al., 1990; Vannier-Santos et al., 1995) and after exposition to 2,2-Dipyridyl (Mesquita-Rodrigues et al., 2013). Mitochondrial swelling was also identified in other previous studies (El-On & Messer, 1986; Ledezma et al., 2002; Rodrigues et al., 2002; Machado et al., 2012). It is recognized that mitochondrial dysfunction precedes both programmed cell death and necrosis (Kroemer et al., 1998). The importance of this organelle in the energetic machinery of eukaryotic cells is critical in trypanosomatids such as *Leishmania* promastigotes given the scarce ability of this stage to survive and multiply in anaerobic environments (Hellemond et al., 1997). Moreover, these Protista only have one large mitochondrion, representing ca. 12% of cellular volume containing near one third of the total cell DNA and therefore is the target of many antileishmanial drugs (Monzote-Fidalgo & Gille, 2011).

Our results showed that allicin consistently induced high ROS generation in the cytosolic and mitochondrial compartments, a collapse of mitochondrial membrane potential, diminished levels of ATP, and elevation of intracellular Ca^{2+}. Cells living in aerobic environments generate physiologic levels of ROS these serving to regulate transcription and as endogenous signaling messengers (Zhang et al., 2006). However, excessive ROS production leads to oxidative stress, damage of intracellular organelles, mitochondrial dysfunction and cell death (Duchen, 2000; Zong & Thompson, 2006). In our experiments we found that 30 µM allicin induced an elevation of ROS production similar to that produced by the inhibitor of electron transport antimycin (5 µM). Interestingly there was a dose-dependent production of intracellular ROS as determined by H2DCFDA up to 90 µM allicin, whereas mitochondrial superoxide anion levels reached a plateau with allicin concentrations ≥ 30 µM. Results were obtained after exposition of 3 h to the compound but high levels of ROS induction observed and the reported easy diffusion across membranes of allicin (Miron et al., 2010) suggest that this event probably occurs very early after exposition to the molecule. These results indicate a prooxidant activity of allicin at the concentrations used.

Superoxide dismutase, several peroxidases and peroxyredoxins have been found in *Leishmania* for detoxication of ROS (Dolai et al., 2009). Trypanosomatids are devoid of catalase and classical selenium containing GSH peroxidase. *Leishmania* and other Kinetoplastida lack glutathione reductase (Fairlamb et al., 1985) and therefore the major mechanism to avoid oxidative stress relies on the unique thiol trypanothione (bis-glutathionyl spermidine, $T[SH]_2$) (Fairlamb & Cerami, 1992). Actually, the enzyme reducing trypanothione, TryR, has been shown to be critical for survival of *L.donovani* (Muller et al., 2003). Given the ROS elevation induced by allicin it was relevant to determine the levels of non-protein thiols

and one of the key enzymes involved in *Leishmania* antioxidant defense: TryR. Additionally, it has been reported that allicin interacts rapidly with thiols (Rabinkov et al., 1998). Our results showed that allicin inhibited TryR activity in cell lysates of *L.infantum* promastigotes in a concentration dependent manner. Allicin (30 µM) reduced by 25% the basal activity compared to untreated cells, near 50% of the inhibition induced by clomipramine, a well-known irreversible inhibitor of TryR (Benson et al, 1992). Even though allicin had an effect on TryR activity, the biological significance of this inhibition is not known since our experiments were carried out with parasite lysates and not with the purified enzyme.

Levels of non-protein thiols found were higher than those previously found in wild type but comparable to those determined in arsenic-resistant strains of *L.tarentolae* (Mukhopadhyay et al., 1996) and lower than those reported in antimonium sensitive clones from *L.donovani* (Decuypere et al., 2012). Allicin induced a dose-related intracellular elevation of total non-protein thiols and levels of Cys, GSH and $T[SH]_2$ doubled the basal cellular store when allicin was used at concentrations between 30 and 60 µM. This homeostatic intracellular thiol response has been also found in resistant isolates of *Leishmania* to ROS generated by trivalent antimony (Mandal et al., 2007). At these concentrations of the drug the activity of TryR was diminished in our conditions by 25-50%. Nevertheless, additional experimentation with the purified enzyme to evaluate the effect of allicin in the kinetics of TryR would be necessary to verify the percentage of inhibition and to determine whether it was a reversible or an irreversible process. The related compound ajoene has been described to be an irreversible covalent inhibitor of TryR in *T.cruzi* (Gallwitz et al., 1999), thus, suggesting a probable effect of allicin on TryR enzyme kinetics. In our case, it seems that the allicin-elicited ROS generation did not provoke a collapse of the trypanothione reducing system of *Leishmania* and therefore cell death was not primarily due to this mechanism of action. Moreover, whereas it is recognized that the glutathione/trypanothione system plays a fundamental role to protect kinetoplastids from ROS or other toxic compounds (Fairlamb & Cerami, 1992; Krauth-Siegel & Comini, 2008) no strict correlation has been found between peroxide resistance and the elevation of reduced thiols (trypanothione and glutathione) or increased activity of ornithine decarboxylase, rate-limiting enzyme in trypanothione synthesis (Miller et al., 2000). Additionally, over 90% loss of activity of TryR is required for trypanosomal death (Krieger et al., 2000) most likely because trypanosomatids have redundant mechanisms against oxidative stress.

There is a strong connection between the oxidative stress and the levels of intracellular Ca^{2+} in normal and transformed cells from pluricellular organisms (e.g. Richter, 1993; Camello-Almaraz et al., 2006; Zhang et al., 2006; Zhong & Thompson, 2006) and also in *Leishmania* (e.g. Mukherjee et al., 2002; Das et al., 2008; Dolai et al., 2011). It has been proposed that the

oxidative stress diminishes the intracellular systems of translocation of Ca^{2+} this leading to the uncontrolled elevation of Ca^{2+} in the cytosol (Jewel et al., 1982; Orrenius et al., 1989). Fine tuning of intracellular Ca^{2+} levels is critical for living cells, as Ca^{2+} is an important regulator of many enzyme systems and a paramount cell messenger in all eukaryotic cells including *Leishmania* (Richter, 1993; Benaim & García, 2011). Consequently, the disruption of Ca^{2+} homeostasis in any cell usually drives to lethal effects (Zhivotovsky & Orrenius, 2011).

Ca^{2+} homeostasis is carried out by the regulatory transport from intracellular organelles and from the surrounding medium. In the case of trypanosomatids, including *Leishmania*, normal cytosolic Ca^{2+} concentrations are in the range of 20-50 nM whereas [Ca^{2+}] in the blood are of ca. 2 mM (Benaim & García, 2011) and of ca. 200 nM in the cytosol of mammalian cells. Considering the different stages in the life cycle of *Leishmania* this is indicative of a highly efficient regulatory mechanism. Balance of Ca^{2+} uptake/efflux through the plasma membrane involves a Ca^{2+}-ATPase identified in trypanosomatids capable of pumping out, under normal circumstances, this cation without restriction (Benaim & García, 2011). In addition, intracellular calcium stores include: acidocalcisomes, characteristic organelles of some Protista, including trypanomatids, where the transport is mediated by a Ca^{2+}-ATPase and a Ca^{2+}/H^{+} exchanger mechanism (Docampo & Moreno, 1999; Serrano-Martín et al., 2009); the endoplasmic reticulum (ER), for Ca^{2+} long-term storage (Orrenius et al., 2003); and mitochondria, which play a short-term fundamental role in the cellular physiology in rapid increases in cytoplasmic calcium, thus preventing fatal effects for the cell. Mitochondria are able to transiently store large amounts of Ca^{2+} provided the mitochondrial membrane integrity, as the driving force for Ca^{2+} entry is the electrochemical gradient formed at the inner mitochondrial membrane (Richter, 1993; Benaim & García, 2011). This physiological role is played by energy-dependent Ca^{2+} uptake from the cytosol through a uniporter transporter and cation release by several routes (Orrenius et al, 2003).

In our experiments allicin induced a rapid elevation of cytosolic Ca^{2+}, probably from intracellular stores and particularly mitochondrion. The possibility of extracellular uptake cannot be ruled out since the culture medium contained available Ca^{2+}. By its part the influx of the cation through non-selective cation channels activated under oxidative stress has been proposed as novel mechanism of apoptotic-like death in *L.donovani* promastigotes (Mukherjee et al., 2002). Additional experiments are needed to determine the calcium channels involved and thus the possible effect of ROS on gate opening for the cation in the plasma membrane. However, at the concentrations of allicin causing Ca^{2+} elevation (besides ROS generation) no variations in the plasma membrane permeability were found at least as assessed by SYTOX Green internalization.

Promastigotes of *L.infantum*, exposed to sublethal concentrations of allicin, showed (i) high ROS generation and (ii) increase of cytosolic Ca^{2+}. These alterations were accompanied by (iii) fall of mitochondrial membrane potential (ΔΨm) and (iv) reduced ATP production. The first three alterations are comparable to the observed effects on *Leishmania* of chemical agents inducing oxidative stress (e.g. H_2O_2: Mukherjee et al., 2002; curcumin: Das et al., 2008; 3,3' – Diindoylmethane: Roy et al., 2008; tunicamycin: Dolai et al., 2011). However, the precise sequence of these events is variable depending on the drug and the experimental approaches. Thus, cell death of *Leishmania* induced by some antimicrobial peptides has been related to the direct toxicity of cytosolic Ca^{2+} and its toxic effect on the mitochondrial membrane (e.g. Kulkarni et al., 2009). In our work the high levels of ROS inside the mitochondrion and its apparent saturation (> 30 µM allicin) besides the extensive morphological alterations observed in TEM suggest that this organelle was the primary target of allicin. Thus, allicin-induced ROS generation, not regulated by thiols, provoked the mitochondrial membrane depolarization with fall of ΔΨm, this leading to the decreased ability of the mitochondrion to retain (and regulate) intracellular Ca^{2+} levels and an impaired mitochondrial synthesis of ATP.

It is well-known that ROS can induce mitochondrial dysfunction and ΔΨm dissipation in *Leishmania* (e.g. Fonseca-Silva et al., 2011) and in mammalian cells (e.g. Malik et al., 2007; Lu & Gong, 2009). The maintenance of the ΔΨm is vital for oxidative phosphorylation and subsequent production of ATP. As electrons move through the respiratory chain a proton gradient is generated in the inner mitochondrial membrane that the ATP-synthase uses to power the synthesis of ATP. Moreover in mammals, ROS, intracellular Ca^{2+} levels, ΔΨm depolarization, and a fall in ATP levels can induce mitochondrial permeability transition pore (MPTP) opening (Zorov et al., 2000; Halestrap et al., 2004) that allows the entrance of water and solutes leading to matrix swelling, leakage of mitochondrial proteins into the cytosol and total ΔΨm collapse (Ly et al., 2003). If confirmed, it could explain the mitochondrial ultrastructural alterations observed by TEM (i.e. mitochondrial swelling).

Energy metabolism in trypanosomatids is highly variable in terms of relative importance of organelles (mitochondrion + glycosomes) for ATP production, substrates (amino acids, carbohydrates) and intermediary metabolic products (Tielens & van Hellemond, 2009). Contrary to procyclic trypanosomes (i.e. *Trypanosoma brucei*) the large part of internalized glucose by *Leishmania* promastigotes is fully catabolized to CO_2 in a complete TCA cycle (Saunders et al., 2011). In fact it is estimated that ca. 70% energy requirements of *Leishmania* are provided by oxidative phosphorylation in mitochondria (Manzano et al., 2011).With the exception of Ca^{2+} all other parameters were determined at 3 h; this limitation makes necessary further research. Furthermore, a crosstalk between Ca^{2+} -both intramitochondrial and cytosolic-,

oxidative stress and mitochondrial dysfunction by membrane depolarization ($\Delta\Psi m$) is recognized (e.g. Richter, 1993, Gordeeva et al., 2003) with more than probable feed-back loops (Kroemer et al., 1998). Research to determine the upstream vs. downstream location of events have included the use of inhibitors of oxidative stress, such as N-acetylcysteine –NAC- (e.g. Dolai et al., 2011) although the value of these assays should be carefully taken. Actually NAC, widely used in metazoan cells and *Leishmania*, behaves as a regulator of redox balance and not as antioxidant since many of its effects are related, via membrane-permeant cysteine precursor, to the thiol levels of the cells (Murphy et al., 2011).

Mitochondrial membrane collapse (MMC), and consequently the lack of functionality of mitochondria is an irreversible cellular dysfunction leading depending on the relative weight of bioenergetic catastrophe or protease and endonuclease activation to cell necrosis or apoptosis respectively (Kroemer et al., 1998; Lemasters et al., 1998). It has been shown that allicin is able to induce apoptosis in some cancer cell lines (PC-3, Hep G2) (Arunkumar et al., 2006; Chu et al., 2012). ATP availability, after mitochondrial membrane depolarization, determines the switch from apoptosis (no fall of ATP) to cell necrosis (low ATP) (Richter et al., 1996; Leist et al., 1997; Eguchi et al., 1997; Kim et al., 2003). Apoptosis is an ATP-requiring form of programmed cell death relatively well characterized in cells from metazoans.

The presence of true apoptosis in Protista, such as trypanosomatids, remains a controversial issue on mechanistic (i.e. caspases absence) and evolutionary bases (e.g. selective value of individual programmed cell death in unicellular organisms). Perhaps the use of a term such as apoptosis, as the only form of programmed cell death, should be abandoned since many forms of cell death (autophagy, programmed cell necrosis) cross the strict boundaries of such division and, on occasion, they have been considered a continuum (Zong & Thompson, 2006). In *Leishmania* there are many reports on apoptotic-like death, after the MMC, by the presence of some phenotypic features of metazoan apoptotic cells, namely DNA fragmentation, caspase-like activation, externalization of phosphatidylserine, cell cycle arrest at Sub-G_0/G_1 phase (e.g. Lee et al., 2002; Zangger et al., 2002; Das et al., 2008; Roy et al., 2008; Dolai et al., 2009, 2011; Kulkarni et al., 2009; Mukherjee et al., 2009). For the most part, these programmed cell deaths reported in *Leishmania* present wide variations and hardly exhibit all characteristic markers of apoptosis in cells from pluricellular organisms; probably a reconsideration of death paths in parasitic protozoa should be reevaluated (Proto et al., 2013). Our results did not show any conclusive evidence of apoptotic-like death since DNA fragmentation was not observed, no phosphatidylserine exposure was detected and there was a cell cycle arrest at the G_2/M phase. Consequently, the cell death found in *Leishmania* after treatment with allicin is compatible with cell necrosis.

Taking together our experiments showed that allicin induced high ROS generation, not controlled by intracellular thiols. This impairment of the intra-promastigote redox balance induced the mitochondrial membrane depolarization (ΔΨm) with dysfunction of the TCA and therefore reduction of ATP production, and elevation of intracellular Ca^{2+}. These events, possibly through a feed-back process, provoked the collapse of mitochondrion with loss of integrity and finally a cellular energetic catastrophe leading to the necrotic death of *Leishmania* promastigotes with cell cycle arrest at the premitotic phase (G_2/M) (Figure 11).

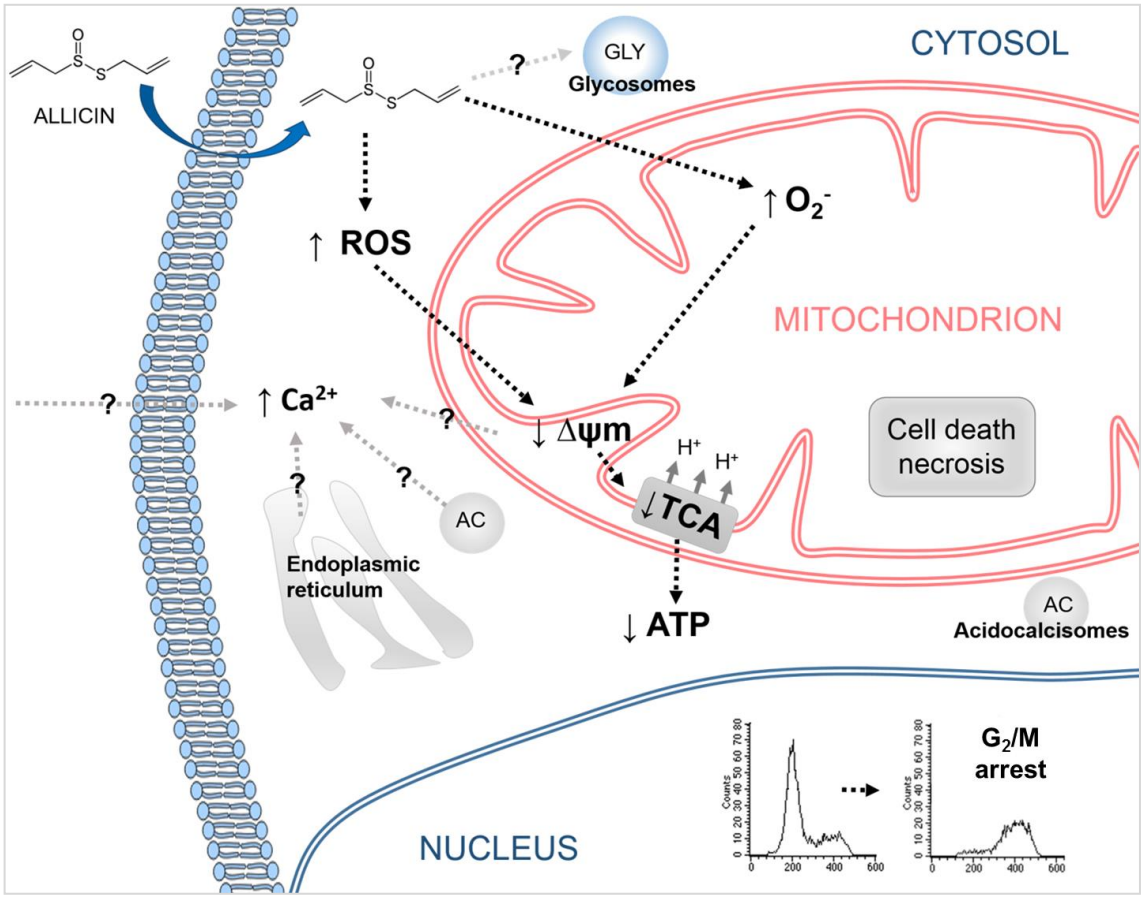


Figure 11. Proposed mechanism of action of allicin in *Leishmania*. Allicin induced oxidative stress not controlled by intracellular thiol homeostasis. Impairment of the redox balance induced ΔΨm depolarization with dysfunction of oxidative phosphorylation and subsequent reduction of ATP synthesis and an increase in cytosolic Ca^{2+} levels. These interconnected events induce –probably through a feed-back process- a mitochondrial collapse with mitochondrial swelling and loss of organelle integrity and, finally, a cellular energetic catastrophe leading to a necrotic cell death of *Leishmania* promastigotes with cell cycle arrest at the premitotic phase (G_2/M). ROS: reactive oxygen species; O_2^-: superoxide anion; ΔΨm: mitochondrial transmembrane potential; Ca^{2+}: calcium cation; ATP: adenosine triphosphate; TCA: tricarboxylic acid cycle or citrate cycle.

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CHAPTER 7

Summarizing Discussion and Conclusions

7.1. Current scenario of chemotherapy of leishmaniasis

The number of entries in the web gives an approximate estimation of the interest on leishmaniasis. Thus "leishmaniasis" and "dog leishmaniasis" are popular items and in a non-selective web engine (i.e. Google) the search for "treatment of leishmaniasis" yields over 840,000 documents and "treatment of canine leishmaniasis" around 200,000 entries. This popularity is not only related to lay contributions (e.g. patients' associations, pet owners, publicity) since using selective data banks widely used by the scientific community (e.g. Pubmed) the term "leishmaniasis" gives back more than 20,000 scientific publications and "dog leishmaniasis" over 2100. With all limitations of these figures it is clear that leishmaniasis and, in particular, treatment of leishmaniasis both in humans and dogs, is an appealing field for the society and also an active area of research for the scientific community.

Be correct this conclusion, it is hardly understandable the scarcity of adequate tools to control the disease (e.g. diagnosis, chemotherapy) and the reported lack of clear criteria for the use of the currently available: *"Prevalence and incidence data for assessing the full impact of (human) leishmaniasis are unreliable. No objective data are available because: i) transmission of the disease occurs in remote rural areas; ii) many cases are not diagnosed because patients do not receive medical care; and (iii) leishmaniasis is notifiable in only 33 of the 88 countries in which it is endemic. Leishmaniasis (human) is one of the most neglected tropical diseases, in terms of the few tools available for control and the lack of clear criteria for methods of control. The most pressing research needs for leishmaniasis control are the search for alternative and cheap medicines for oral, parenteral or topical administration in shorter treatment cycles, and identification of mechanisms to facilitate access to existing control measures, including health-sector reform in some developing countries".* These quotes correspond to the Secretariat Report from the 60th World Health Assembly (WHO) on Control of leishmaniasis (Report A60/10) (22 March 2007). Seven years later leishmaniasis has been declared endemic in 98 countries (Alvar et al., 2012) and, in spite of the worldwide research effort and the intensive labor and resources invested, the pursued goals are essentially the same (WHO, 2010). In the veterinary arena, and particularly in the endemic areas for ZVL by *L.infantum* (=*L.chagasi*), several of the above mentioned needs are also applicable.

Regardless of the continued work on the development of vaccines and the apparent feasibility of antileishmanial vaccine development given the reported resistant status to homologous priming infection in humans; the traditional leishmanization protecting against cutaneous leishmaniasis in the Old World; and the partial protection against the infection elicited in CanL by the recently marketed preparations (e.g. CaniLeish®), no available vaccine

exists for human leishmaniasis and, the existing version for CanL has several drawbacks (e.g. side effects, partial protection). In the absence of efficacious vaccines the main control system of leishmaniasis is chemotherapy.

Unfortunately, current control of leishmaniasis, both human and canine, is inadequate. Presently, liposomal amphotericin b (AmB), paromomycin and miltefosine are considered by WHO (www.who.int/tdr/diseases/leish) as the most promising drugs and, together with antimonials (SbV), constitute the main arsenal for leishmaniasis treatments. Shortcomings from these drugs include the emergence of resistance to the first line treatment SbV (e.g. India, Nepal) (Croft et al., 2006), high toxicity and teratogenicity of some of them, absence of parasitological cure in most cases and unaffordable prices of the less toxic and more efficacious preparations (Sundar & Chaterjee, 2006). Novel short course and highly efficient therapies are needed in the field to face leishmaniasis (Sundar & Chakravarty, 2013).

7.2. Antileishmanial drug discovery requires reliable experimental models

Promastigote cultures as a first step drug screening

Drug discovery process requires the validation of the synthetic or natural products aimed for therapeutic use. A variety of surrogate experimental models have been depicted to test the potential value of a given molecule in the treatment of leishmaniasis. In the first steps of the drug pipeline development, when "go-no go" decisions are taken, no animals models are used but rather non-cellular systems and parasites cultures. Since most targets of currently used drugs (antileishmanial and others) are enzymes the inhibitory activity of the potential drugs on the enzyme target has been employed as a selective criterion. Moreover this strategy can be easily adapted to High Throughput Systems (HTS) of screening. Obviously there is a wide gap between biochemistry, pharmacology and, finally, therapeutics and despite shortcomings phenotypic screening using parasites still is preferred and has been successfully adapted to HTS.

In the case of *Leishmania* the vast majority of screenings to identify hits and leads, even in massive analyses, have been performed using cultured promastigotes (e.g. Siqueira-Neto et al., 2010; Walker et al., 2011). This cultured stage, comparable to the dividing cells within the sandfly vectors, differs (e.g. morphology, biochemistry) from the actual parasitic stage, amastigote, present in the infected host and is not an ideal model for checking potentially useful drugs. However, cultures of intracellular amastigotes are not available to many labs,

promastigotes and amastigotes share many metabolic pathways –promastigotes have been used to monitor clinical emergence of resistance to miltefosine (Kulshrestha et al., 2013)-, and some *Leishmania* spp such as *L.donovani* and, particularly, *L.infantum* have slow multiplication rates. Furthermore, with some exceptions (e.g. prodrugs as SbV), compounds effective against amastigotes exhibit also antiproliferative effect on promastigotes (e.g. AmB, miltefosine) (Sharlow et al., 2009).

Phenotypic screening of drug inhibitory effect on promastigotes by microscopy is time consuming and to circumvent this limitation several indirect methods have been developed such as nephelometry, colorimetry, reporter genes, RT-PCR, 3H-thymidine incorporation, or software development. Among them, cell growth monitoring by the determination of extracellular levels of resorufin, the product of reduction of resazurin, and in particular Alamar Blue, is a single-step, low cost and of negligible toxicity to the cells. Alamar Blue has been extensively used to monitor the multiplication of normal and transformed mammalian cells (O'Brien et al., 2000; Sykes & Avery, 2009).

Preliminary tests carried out in our laboratory, with this method and colorimetry or fluorimetry, yielded inconsistent results when monitoring cell multiplication of *L.infantum* and *L.donovani* promastigotes using standard culture conditions (26ºC, air phase) in different culture media (e.g. RPMI 1640, Schneider). Since more than probably the performance of a redox indicator would be affected by the gas-phase of cultures, particularly oxygen, the possible effect of the promastigotes' atmosphere was explored. Results obtained using a 5% CO2 /95% air and additional buffering with 20 mM Hepes greatly improved the results (linearity, correlation between cell counts and resorufin level) with initial plating cell densities of $2.5x10^5$ promastigotes/mL for both *Leishmania* spp and using spectrophotometry or, better, fluorimetry (Chapter 2). This finding agrees well with the use of a partial CO_2 atmosphere for Alamar Blue in the majority of assays with mammalian cells (expected) and in those with blood stream trypanosomes (Räz et al., 1997; Sykes & Avery, 2009). No adequate explanation, unless it was not stated, for the absence of inconveniences in the previous experiments on *Leishmania* carried out with aerated cultures (de Oliveira-Silva et al., 2008; Shimony & Jaffe, 2008; Vermeersch et al., 2009; Kulshrestha et al., 2013) or in HTS with a 384-well format (Siqueira-Neto et al., 2010). On experiential store it is known that different growth curves, cell density and apparent efficacy of some drugs on *Leishmania* promastigotes can be obtained, depending on the culture media and additives employed. It is thus possible that slight variations in culture conditions could allow them to overcome the inconvenient found by us using aerated atmosphere and Alamar Blue. In any case, experimental conditions described in our contribution showed high repeatability, applicability to both *Leishmania* spp tested, no interference with red phenol, and

resazurin reduction and cell numbers were highly correlated (r^2 =0.9713). This made the modification developed a good tool to screen potential antileishmanial drugs in 96-well format and probably adaptable to HTS. In addition, it stressed the need of standardization of culture conditions and monitoring.

Antileishmanial drug screening with intracellular amastigotes

It is generally assumed that *in vivo* models have superior predictive value than *in vitro* or *ex vivo* models to test antileishmanial drugs and that intracellular amastigotes are more convenient than axenic amastigotes and promastigotes for this purpose (Sereno et al., 2007). However, the systematic use of intracellular amastigotes is hampered by the unavailability of the technique to many laboratories, slow multiplication rates of some leishmanias (see above) and the species-dependent culture requirements. Clearly there are interspecies differences even in those closely related and, actually, the first modified method of infection of macrophages (mouse peritoneal and J774 cell line) with promastigotes of *L.infantum* was published less than 20 years ago (Méndez et al., 1996) when routine culture of *L.donovani* intracellular amastigotes was carried out in many laboratories. Method developed included an overnight low temperature (33 ºC) treatment of macrophages (Mφ) prior to the promastigote-macrophage contact. That infection procedure has been used since then (e.g. Ordóñez-Gutiérrez et al., 2007, 2009; Wert et al., 2011), although infection rate (% of infected Mφ) and amastigote burden (number of intracellular amastigotes/infected Mφ) values were lower than those obtained with *L.donovani*. On these grounds the exploration of a more reliable screening tool for potential antileishmanial drugs on intracellular amastigotes (higher infection rate and parasites' multiplication) was considered by us a necessity to become a routine procedure for the screening of antileishmanial drugs.

An improvement of the available technique for *L.infantum* infection has been developed by combining the previously established method in our laboratory (Méndez et al., 1996), the isolation of metacyclics with a discontinuous Ficoll gradient (Yao et al., 2008), and opsonization with 15% normal mouse serum (Chapter 3). The modification of the infection method was successful increasing the infection rate with both *Leishmania* spp (*L.infantum*: 65-70% of J774 cells, ca. 80% of mouse peritoneal macrophages; *L.donovani*: 70-75% of J774 cells, ca. 95% of mouse peritoneal macrophages) (Figure 1) and intracellular multiplication. The modification performed was useful for the culture of both *Leishmania* spp tested and provide us with a valuable tool for *in vitro* and *ex vivo* drug screening against this more predictive parasite stage. We have not tested the fluorimetric method to determine the viability of intracellular

amastigotes of *Leishmania* (Bilbao-Ramos et al., 2012). Combination of the infection improvement and determination of amastigotes multiplication inside Mφ with fluorimetry would provide us with a powerful protocol to test potentially useful drugs against leishmaniasis.

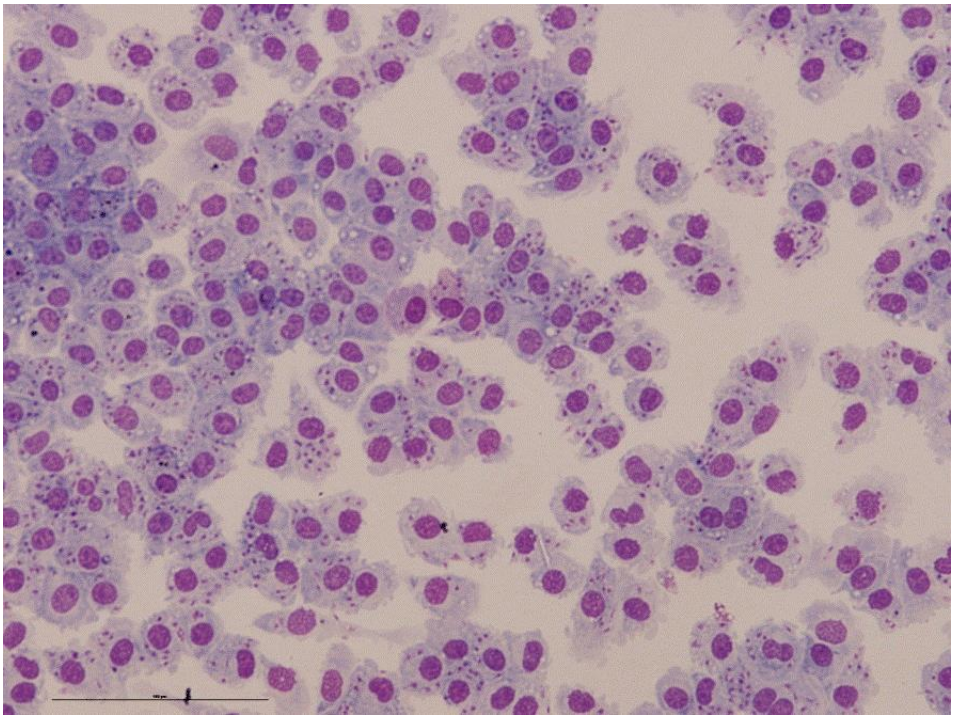

Figure 1. *Ex vivo L. donovani* infection: BALB/c peritoneal macrophages with intracellular amastigotes. Cells were stained with May-Grünwald-Giemsa 24 h after infection. Bar: 100 µm.

7.3. Micromolar concentrations of allicin (diallyl thiosulfinate) inhibit the multiplication of promastigotes and intracellular amastigotes of *Leishmania*

As previously stated the low number of available antileishmanial drugs and the shortcomings from most of them, including the resistance emergence, make the discovery and development of alternative chemotherapeutic agents an urgent need. Strategies to develop new treatments can be grossly divided in short-to-medium term [e.g. new indications for old drugs –a large proportion of present antileishmanial drugs-, combination therapy, incorporation to drug delivery systems (DDS)] and long-term (e.g. discovery of new synthetic or natural agents, immunotherapy) (Pink et al., 2005; Pham et al., 2013).

Allicin (diallyl thiosulfinate = 2-Propene- 1-sulfinothioic acid S-2-propenyl ester), a natural product present in plants of the Family Alliaceae, including garlic, has shown antibacterial activity against bacteria [e.g. *Helicobacter pylori*, *methicillin-resistant Staphylococcus aureus (MRSA)*] (O'Gara et al., 2000; Cutler & Wilson, 2004), antifungal (e.g. *Candida*, *Aspergillus*) (Khodavandi et al., 2011; Cañizares et al., 2004) and different Protista (e.g. *Trypanosoma*, *Leishmania*, *Entamoeba*, *Plasmodium*) (Mirelman et al., 1987; Coppi et al. 2006; Waag et al., 2010). Previous studies involving *Leishmania* were carried out using promastigotes and the information provided was very limited. The absence of information, our experience with of methods for promastigote and amastigote screening, and the availability of stabilized allicin prompted us to test the potential antiproliferative effect of allicin on the two major causative agents of visceral leishmaniasis, *L. donovani* and *L. infantum* (Chapter 3).

A significant inhibitory effect of allicin in the micromolar range on the multiplication of *L.donovani* and *L.infantum* has been found by us. Inhibition was time- and dose-dependent against promastigotes and intracellular amastigotes *in vitro* (J774 cells) and *ex vivo* (BALB/c mouse peritoneal Mφ). The approximate IC_{50} value obtained (10- 30 µM, 24 h exposition) for promastigotes from both species was in the line of the value indicated by Ankri and Mirelman (1999) (30 µg.mL-1) for *L.major* and slightly higher than that reported by McClure et al. (1996) for *L.chagasi* and *L.mexicana*. More importantly, allicin, at these concentrations, effectively inhibited the multiplication of amastigotes. As far as we know this was the first report on the inhibitory effect of allicin against *Leishmania* intracellular amastigotes. In addition, toxicity of allicin in the concentration range used was low and no significant effect on mouse Mφ was observed up to 40 µM as estimated by MTT method and the absence of morphological alteration of macrophages up to 60 µM in both J774 cells and BALB/c peritoneal Mφ.

Administration of allicin every 24 h greatly increased the anti-leishmanial activity of the compound. This finding suggested the degradation of the diallyl thiosulfinate at 26º and 37 ºC (McClure et al. 1996; Cañizares et al. 2004). Allicin is a highly reactive thiosulfinate which is rapidly transformed to other types of organosulfur compounds, particularly diallyl disulfides (DADS), diallyl sulphides (DAS), ajoene and vinyldithiins (Amagase, 2006). The degradation of the allicin preparation employed was confirmed by mass spectrometry (MS) since sulphides and vinyldithiins were present but neither ajoene (E or Z) nor allicin were found in any of the analysis. The absence of allicin and ajoene could be due to their short half-life at the culture temperatures (26 ºC and 37 ºC) (i.e. ajoene, less than 1 min) and the analytical method employed. It has been indicated that thermal sensitivity of allicin at physiological temperature as well as its rapid transformation to other types of organosulphur compounds (Amagase, 2006) could rule out its therapeutic use. However, it has been shown that decline of allicin is

accompanied by its transformation into other compounds (i.e. ajoene) (Fujisawa et al. 2008) with strong anti-leishmanial activity (Ledezma et al. 2002). Whether or not the actual antileishmanial agent in our studies is allicin or some other degradation product requires further experimentation. Moreover, the metabolism of allicin, and therefore its bioavailability, is unknown for the most part. Value of IC_{50} found by us *in vitro* and *ex vivo* (5-10 µM) against intracellular amastigotes possibly excludes allicin as monotherapy against leishmanial infections. Nonetheless, low cost and scarce toxicity of this compound would allow its use in combined therapy with other well established anti-leishmanial drugs.

7.4. Synergistic combination of allicin and amphotericin B in the micromolar range on *Leishmania* promastigotes and intracellular amastigotes

To increase the lifespan of existing antileishmanials and face the reported resistance or unresponsiveness to some currently used drugs (e.g. miltefosine, SbV) an alternative approach to monotherapy is drug combination using well established chemotherapeutic agents (Olliaro, 2010). The notable activity of allicin and its low toxicity for mammalian cells (J774, peritoneal mouse Mφ) prompted us to explore the possibility of using this molecule in combination with amphotericin B (AmB). This antibiotic, once a second line antileishmanial treatment, has transformed, after the emergence of resistance to SbV in some areas (Croft & Coombs, 2003; Croft et al., 2006) particularly formulated in liposomes, as the choice drug for (human) leishmaniasis control. Moreover, no significant resistance against this molecule has been reported in *Leishmania* in spite of its long time (over 50 years) of use in clinical practice (Durand et al., 1998; Gray et al., 2012). The use of this antibiotic with extraordinary antileishmanial efficacy has been restricted by the side effects induced and long periods of treatment with clinical monitorization.

On these grounds our approach was the exploration of the potential synergistic or additive antileishmanial effect of the combination (low micromolar concentrations of AmB + micromolar allicin) thus avoiding the toxic concentrations needed with AmB in monotherapy. Data obtained in many combination studies using checkerboard approaches with two-fold dilutions of two drugs lack in significance due to the absence of further analysis. Therefore, for promastigotes screening, combinations of allicin and AmB at a non-constant ratio were used following the robust Chou-Talalay method (Chou, 2006; Chou, 2008; Chou & Hayball, 1996) for drug combination analysis. Results obtained showed that the combination of both molecules

ranged from moderately synergic to synergic at low concentrations against both promastigotes (0.07 µM AmB + 35.45 µM allicin induced a 95% growth inhibition) and amastigotes (ca. 45% reduction with 0.05 µM AmB +10 µM allicin); this represented near a two-fold reduction in the IC_{50} value of the antibiotic added alone (Vermeersch et al., 2009). The observed differences between promastigotes, with a 7 to 11-fold reduction of AmB, and amastigotes, with ca. two-fold reduction, support the interest of using intracellular amastigotes in drug screening (Sereno et al., 2007; Seifert et al., 2011) (Chapter 4).

Leishmania has considerable amounts of ergosterol on the plasma membrane. Apparently allicin produces an enhancement of fungicidal activity of AmB by inhibiting the ergosterol trafficking from plasma membrane to vacuole membrane in Candida (Fungi) (Ogita et al., 2009). Whether or not this enhancement of AmB effect in the presence of allicin is responsible of the synergism found by us in amastigotes and particularly promastigotes of *Leishmania* needs further experimentation. Data obtained with drug combinations *in vitro* and *ex vivo* have many limitations in spite of the dose-effect relationship analysis carried out (i.e. unforeseen interactions, pharmacokinetics) (Chou, 2008). However, the two-fold reduction in the required dose of AmB against intracellular amastigotes and the possibility of using higher non-toxic doses of allicin (>10 µM) suggested the interest of the exploration of this combination *in vivo*.

7.5. Allicin is effective against experimental leishmaniasis in hamster and reduces the required amphotericin effective dose

In face of the shortage of effective and affordable treatments for leishmaniasis, combination therapy has emerged as one of the most favored and readily implemented strategies. A variety of drugs and schedules have been tested (Chunge et al., 1985; Murray et al., 2003; Sundar & Chakravarty, 2013). The rationale behind this approach relates to the potential advantages of the combinations (e.g. delay or prevention of the development of resistance; shortening of treatment regimens) (Seifert & Croft, 2006; Griensven et al., 2010) although resistance can also develop at least under laboratory conditions (García-Hernández et al., 2012).

In our case we tested the combination of the newly recognized antileishmanial molecule allicin *in vitro* and *ex vivo*, and AmB. This antibiotic probably is the best existing drug against leishmanias with cure rates exceeding 95% (Alvar et al., 2006; Paila et al., 2010) and negligible levels of resistance after being employed as the reference drug against systemic fungal infections for more than 50 years (Durand et al., 1998; Gray et al., 2012). It is generally considered that AmB binding to ergosterol from *Leishmania* membrane is the main event

causing the cell destruction. The differential affinity of AmB by cholesterol (in mammalian cells) and ergosterol (in *Leishmania* membranes) would account for the differential toxicity for hosts and parasites. Actually the residual toxicity, by unspecific binding to cholesterol, is the main shortcoming from AmB (Laniado-Laborín & Cabrales-Vargas, 2009; Hamill, 2013).

Our results showed that (i) allicin exhibited a very low toxicity for mammalian cells besides significantly inhibiting the multiplication of *Leishmania* inside Mφ (Chapter 3), and (ii) a synergistic antileishmanial activity of AmB and allicin *in vitro* and *ex vivo*, on intracellular amastigotes of *Leishmania* (Chapter 4). Unfortunately, although intracellular amastigotes from *Leishmania* are a good *in vitro* or *ex vivo* model to test potentially useful antileishmanial drugs (Sereno et al., 2007), there is a wide gap between biochemistry, pharmacology and therapeutics. Therefore we planned to evaluate the efficacy of allicin and also its combination with low concentrations of AmB in a recognized experimental model of visceral leishmaniasis: hamster infections.

The final aim of the combination strategy would be the reduction (below toxic levels) of the necessary AmB doses to effectively control the infection in hamsters (Chapter 5). As expected the experimental model employed (*L.infantum* and hamster) produced consistent infections with significantly higher parasite burdens in the spleen and altered blood urea nitrogen thus confirming its suitability for *in vivo* testing of antileishmanial agents (Binzahim et al., 1993; Requena et al., 2000; Melby et al., 2001; Dea-Ayuela et al., 2007; Moreira et al., 2012). Groups of infected hamsters were treated from day 45th post infection onwards [AmB (1 or 5 mg/Kg/day), allicin (5 mg/Kg/day) or a combination of AmB (1 mg/Kg/day) + allicin (5 mg/Kg/day) for 5 days] and no clinical signs or biopathological alterations were observed; therefore, indicating the lack of toxicity of AmB and allicin or their combination at the doses administered.

Treatment, *in vivo* follow up and *Leishmania* burden determined by the limiting dilution assay, showed that allicin (5 mg/Kg/day, 5 days) had a clear antileishmanial effect in hamsters since 2 out of 6 animals were cleared of *L.infantum* in both spleen and liver. As far as we know this is the first evidence of the antileishmanial activity of allicin *in vivo* without apparent toxicity for infected and treated animals. By its part the combination of the lower dose of AmB + allicin reduced over 95% the *L.infantum* burden, this level of efficacy being actually comparable (p<0.05) to the standard AmB treatment (5 mg/Kg/day).These results confirmed *in vivo* the synergistic effect reported *in vitro* and *ex vivo* for this combination (Chapter 4) and suggests the possibility of reducing 5 times the required dose of AmB in monotherapy without loss of efficacy.

Actual basis of the antileishmanial activity of the combination is not known and should be explored since, apparently, allicin improved the accumulation of the antibiotic in the target organs and in the treated hamsters a comparatively higher specific IgG_2 response was observed perhaps related to the enhancement of a pro-inflammatory response induced by allicin (Feng et al., 2012). More research is needed in this predictive model of human and canine leishmaniasis (e.g. refined dosage, time schedule, administration via) but our results point towards the interest of this combination, reducing the needed dose of AmB and thus its toxicity, in the chemotherapy of leishmaniasis.

7.6. Mechanistic basis of the antileishmanial activity of allicin: bioenergetic catastrophe and cell necrosis

Despite the reported value of allicin and related compounds to inhibit the multiplication of unicellular eukaryotic organisms (e.g. *Candida*, *Trypanosoma, and Leishmania*) and transformed cells from metazoans (Chapters 1 & 3) its mechanism of action is virtually an unexplored research field. It has been indicated that, given its highly reactive nature, allicin rapidly interacts with thiols, thiol-dependent enzymatic systems, cysteine proteinases, microtubules (Ankri & Mirelman, 1999; Prager-Khoutorsky et al., 2007; Miron et al., 2010; Waag et al., 2010). Additionally, it has been reported to induce cell apoptosis (Oomen et al., 2004). Nevertheless, the actual intracellular targets responsible of the cytocidal/cytostatic effect of the molecule are largely unknown. Availability of a stabilized allicin formulation and the suggestive results obtained *in vitro*, *ex vivo* and *in vivo* against *Leishmania* prompted us to investigate its possible antileishmanial mechanism of action.

Transmission electron microscopy (TEM) studies carried out showed that the most prominent alteration observed in promastigotes of *L.infantum* and *L.donovani* treated with this molecule was the loss of integrity of mitochondrial and kinetoplast membrane (Chapter 1 & 3).This finding was most indicative of the involvement of energetic cell machinery on the activity of allicin. Primarily, because microscopy is a golden standard to determine intracellular targets of toxic agents and also the type of cell death; and in second place, because trypanosomatids, including *Leishmania*, only have a large mitochondrion representing ca. 12% of cellular volume (Monzote-Fidalgo & Gille, 2011). Kinetoplastids strongly rely on this organelle given their scarce capacity to survive and multiply in anaerobic environments (Hellemond et al., 1997; Manzano et al., 2012).

Our results showed that allicin, at sublethal concentrations, induced an elevation of the production of reactive oxygen species (ROS). Although ROS are present in normal cells playing a significant role as signaling messengers (Zhang et al., 2006) the overproduction is linked to oxidative stress, mitochondrial dysfunction and cell death (Duchen, 2000; Zong & Thompson, 2006). Since allicin can easily cross cell membranes (Miron et al., 2010) this event should take place very rapidly after exposition to the molecule. To cope with the damaging oxidative stress *Leishmania* relies mainly on trypanothione (=bis glutathionyl spermidine) (Fairlamb & Cerami, 1992) since these Kinetoplastida are devoid of catalase and classical selenium containing GSH peroxidase and no glutathione reductase is present (Fairlamb et al., 1985). Our results showed that trypanothione reductase (TryR) activity was only moderately inhibited and, expectedly, thiols (cysteine, glutathione and trypanothione) levels increased. These findings, besides confirming the interaction of allicin with thiols (Rabinkov et al., 1998), suggested that the molecule did not provoke a collapse of the trypanothione reducing system and therefore cell death of allicin-treated *Leishmania* was not primarily related to this mechanism of action.

There is a tight connection between oxidative stress and intracellular Ca^{2+} in all organisms including *Leishmania* (Mukherjee et al., 2002; Das et al., 2008; Dolai et al., 2011). The fine tuning of intracellular Ca^{2+} level is critical for cell homeostasis (Richter, 1993) and oxidative stress can disrupt the intracellular calcium translocation (Orrenius et al., 1989) among calcium stores (e.g. mitochondrion, acidocalcisomes, endoplasmic reticulum). Our experiments showed that allicin induced a rapid elevation of intracellular Ca^{2+}, probably from intracellular stores and particularly mitochondrion, since no variations in the plasma membrane permeability were found at least as assessed by SYTOX Green internalization. ROS generation and increase of cytosolic Ca^{2+} in *Leishmania* exposed to sublethal allicin concentrations were accompanied by the fall of mitochondrial membrane potential ($\Delta\Psi m$) and reduced ATP production. The high levels of superoxide anion (O_2^-) inside the mitochondrion and its apparent saturation (> 30 µM allicin) besides the extensive morphological alterations observed in TEM (Chapter 1 & 3) suggested that this organelle was the primary target of allicin. Thus, we propose that allicin-induced ROS generation, not regulated by thiols, provoked the mitochondrial membrane depolarization with fall of $\Delta\Psi m$, this leading to the decreased ability of the mitochondrion to retain (and regulate) intracellular Ca^{2+} levels and therefore causing uncoupling and impairment of mitochondrial ATP synthesis. This is critical since an estimated 70% of all energy requirements in *Leishmania* are fulfilled by oxidative phosphorylation in mitochondria (Manzano et al., 2012). Nonetheless, there are still open questions and further investigation is needed to validate this proposed mechanism of action. Increase in cytosolic Ca^{2+} can be due either to the transport from the extracellular milieu or can be extruded from intracellular calcium

stores (i.e. acidocalcisomes, mitochondrion, endoplasmic reticulum). Calcium origin should be addressed. Additionally, it is difficult to assess if calcium elevation or ROS generation is the initial event triggering *Leishmania* cell death. Further experimentation using calcium chelating agents and antioxidants prior to or in conjunction with the allicin treatment is needed. Other important energetic organelles in *Leishmania* are glycosomes. Evaluation of the role of this organelle in the mechanism of action of allicin should also be considered.

Mitochondrial membrane collapse (MMC), and consequently the lack of functionality of mitochondria, is an irreversible cellular dysfunction leading, depending on the relative weight of bioenergetic catastrophe/protease and endonuclease activation, to cell necrosis or apoptosis respectively (Kroemer et al., 1998). Apparently allicin is able to induce apoptosis in some cancer cell lines (PC-3, Hep G2) (Arunkumar et al., 2006; Chu et al., 2012). ATP availability, after mitochondrial membrane depolarization, determines the switch from apoptosis (no fall of ATP) to cell necrosis (low ATP) (Leist et al., 1997; Eguchi et al., 1997; Kim et al., 2003). In our case there was a net fall of ATP production. Despite the variations found in programmed cell death our results did not show any conclusive evidence of apoptotic-like death since DNA fragmentation was not observed (TUNEL assay), no phosphatidylserine (Annexin V/PI staining) was exposed and there was a cell cycle arrest at G_2/M phase (PI staining). On these grounds, the death type found in *Leishmania* treated with allicin is compatible with cell necrosis.

Additional experiments would be required to precisely know the downstream-upstream sequence of events but available data point towards the early induction of high ROS generation in *Leishmania* by exposure to allicin. The impairment of the intra-promastigote redox balance induced the mitochondrial membrane depolarization ($\Delta\Psi m$) with dysfunction of the TCA and therefore reduction of ATP production, and elevation of intracellular Ca^{2+}. These events, possibly through a feed-back process, lead to the collapse of mitochondrion with loss of integrity and finally a cellular energetic catastrophe leading to the necrotic death of *Leishmania* promastigotes with cell arrest at the premitotic phase (G_2/M).

7.7. Concluding remarks

Current chemotherapy of leishmaniasis is not satisfactory in terms of administration via and length of treatments, high price of the most effective and safe drugs (e.g. liposomal AmB), serious side effects (e.g. teratogenicity of miltefosine) and toxicity. Nevertheless, as stated by WHO (2010) and the routine human and veterinary chemotherapeutic indications, the most effective drugs against this infection have, on average, more than 50 years and no new chemical

entities (NEC) are presently foreseen. Moreover no obvious reasons can be invoked for this low efficacy in antileishmanial drug discovery (Pink et al., 2005). Generally speaking this unexpected crisis of innovation in the pharmaceutical research is not restricted to leishmaniasis or even parasitic diseases and perhaps is related to the internal structure and expense distribution of pharmaceutical companies, with strict division between financial and R&D departments (Cuatrecasas, 2006) and the anachronism of patent system (e.g. panda's thumb: a long history of evolution but not suitable for present times) (Moon et al., 2012). In the particular case of leishmaniasis, besides these components, drug development has followed opportunistic strategies (e.g. piggy-back development) and low returns are expected from potential users.

The analysis of this situation transcends the purpose of our present work although it deserves a deep evaluation. The enormous advances in basic biology of *Leishmania* and the available technologies (e.g. molecular biology, genomic, proteomics, transcriptomics, metabolomics, robotics, miniaturization, and bioinformatics) have not resulted in a substantial improvement of the development of antileishmanial drugs (Koehn & Carter, 2005). Drug discovery processes quickly integrated the advances from biomedical sciences, particularly molecular biology and engineering in the 1980s. It was assumed that the use of the powerful techniques (target selection, HTS screening) would reduce costs and time invested to obtain drugs safer, affordable, in a shorter discovery time-line (Koehn & Carter, 2005) and profitable besides providing a rational approach to drug discovery. Combinatorial chemistry (directed through molecular docking and *in silico* design) or undirected, with favorable ADMET properties (absorption, distribution, metabolism, excretion, toxicity) would put on the bench an unprecedented number of potentially useful drugs. Innovation is linked to the inherent risk of failure, and failure in companies is synonym of expensive. Pharma industry under the mantra "*fail fast, fail cheap*" tried to increase success rates but results point towards that maybe they are asking the wrong questions. Up to date attrition rate is very high and although many molecules are being tested, in laboratory and field conditions, no new antileishmanial drug has been developed. Certainly there are no single solutions to these problems although some comments could be made on the prevailing drug development system. Since the end of the 1980s many pharmaceutical companies stopped the work on natural products in spite of the high rate of success with that source of molecules (Newman, 2008). It was assumed that the HTS-CombiChem tandem would produce a full range of drugs. To date this approach, while labor-saving, has not yielded the expected results after checking large chemical libraries for hit and lead identification and subsequent lead optimization to produce new drug candidates.

Chemical synthesis has followed in many cases the feasibility of chemical reactions using a limited handful of structures. On the contrary, scaffolds identified from natural sources have an

unsurpassed variety and complexity, much higher than those obtained in synthetic products (Lee & Schneider, 2001; Feher & Schmidt, 2003). With the present shortage of new drugs, the high attrition rate of the synthetic chemistry-based drug development and the innovation crisis, the role of natural or natural-derived molecules as an importance source of potentially effective drugs against leishmaniasis (and other, particularly parasitic diseases) in our opinion should be reevaluated. Present technological tools, including molecular biology, molecular docking and HTS, and the improvements in chemical synthesis by mimicking natural molecules (Feher & Schmidt, 2003) could overcome the traditional drawbacks from natural molecules (e.g. low yield, limited supply, complex structures). Moreover, bioguiding of the process with well-established and predictive models *in vitro*, *ex vivo* and *in vivo* could result in more efficient way of developing new drugs or combinations.

Our experimental work showed that a natural molecule, allicin, displayed a significant antileishmanial effect *in vitro* and *in vivo* and, perhaps more importantly, its concurrent administration in the micromolar range allowed to reduce the needed amount of AmB to effectively control experimental leishmaniasis in hamsters. These results are encouraging and support the interest of exploring molecules from natural sources and take advantage of the technologies presently available.

7.8. Conclusions

i. The modification developed for the Alamar Blue assay, with promastigote cultures with 5% CO_2 atmosphere, 20 mM Hepes buffer and fluorimetry readings, increased reproducibility and sensitivity of the method and allows its routine use in drug screening for the identification of potential antileishmanial molecules.

ii. The combination of the thermal pretreatment and isolation of an enriched population of metacyclic promastigotes of *Leishmania infantum* and *L.donovani* increased internalization and intracellular multiplication in macrophages. High infection rates (% of infected Mφ), number of intracellular amastigotes and reproducibility of the results make the modified infection procedure a good tool for drug screening and characterization of virulence in *Leishmania* isolates.

iii. The antiproliferative effect of diallyl thiosulphinate (=allicin), in the micromolar range, on promastigotes and intracellular amastigotes of *L.donovani* and *L.infantum* has been demonstrated for the first time. The IC_{50} determined for the intracellular stage (10-30 μM) probably excludes this molecule for the monotherapy of leishmaniasis.

iv. The robust methodology employed for the analysis of the interaction between allicin and amphotericin B (AmB) showed from moderate synergy to synergy between both molecules against both stages of *Leishmania*, allowing a 50% reduction of the required dose of AmB. These results point towards the interest of this combination for the treatment of leishmaniasis.

v. Allicin, administered intraperitoneally to hamsters (*Mesocricetus auratus*) experimentally infected with *L.infantum* (5 mg/Kg/day, 5 days), exhibited an important antiparasitic activity without toxicity as shown by the absence of clinical signs or physiological abnormalities.

vi. The combination of allicin (5 mg/Kg/day, 5 days) and AmB (1 mg/Kg/day, 5 days) showed antiparasitic efficacy *in vivo* (reduction > 95% of the parasite burden) comparable to that shown by the standard AmB dose (5 mg/Kg/day, 5 days). The reduction of the AmB dose required points towards the interest of this combination for the treatment of leishmaniasis.

vii. The differences observed in the IC_{50} values between promastigotes and amastigotes for the combination allicin + AmB and the apparent synergistic effect found with intracellular amastigotes, confirmed by *in vivo* studies, suggest the need of using intracellular amastigotes of *Leishmania* for drug screening for their higher predictive value.

viii. Apparently, allicin targets the mitochondrion of *Leishmania*. Diallyl thiosulphinate induces increased ROS generation in the cytosol and mitochondrion. The redox imbalance provokes the mitochondrial membrane depolarization, rise of cytoplasmic calcium without alteration of permeability of the plasma membrane and fall of ATP production. These events induce a mitochondrial collapse with loss of integrity, energetic cellular catastrophe and necrotic cell death with cell cycle arrest in the premitotic phase (G_2/M).

7.9. References

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Summary

Summary

Antileishmanial activity of diallyl thiosulphinate (=allicin), alone or in combination with amphotericin B (AmB), has been tested *in vitro*, *ex vivo* and *in vivo* on *Leishmania* promastigotes and intracellular amastigotes. In addition, a resazurin-based fluorimetric method to estimate the promastigotes' proliferation has been improved and the mechanism of action of allicin on *Leishmania* has been explored.

The value of resazurin-based Alamar Blue redox indicator to determine multiplication of *Leishmania* promastigotes in 96-well microtiter plates was examined. In addition, assay was validated with AmB and allicin. The method was tested on *L.donovani* and *L.infantum* promastigotes under different culture conditions (variable air-phase, presence of phenol red, initial cell density, incubation time, use of Hepes buffer). Results showed that the gas-phase of promastigote cultures was critical. The method yielded consistent results with initial plating cell densities of 2.5 x 10^5 promastigotes/well, up to 72 h incubation and 5% CO_2 atmosphere or reduced air availability (sealed plastic bags, film-sealed microplates). Detection of low numbers of promastigotes and earlier results could be obtained using fluorimetry instead of spectrophotometry. The addition of 20 mM Hepes improved the results. Fluorescence intensity correlated to promastigotes number in both *Leishmania* spp. Inhibitory concentration (IC_{50}) values for AmB and allicin using cell counting and fluorimetry were comparable. Under these conditions this one-step, low-cost redox indicator can be used in drug sensitivity assays and studies of differential proliferation rates of *Leishmania* isolates or strains in a 96-well format.

Anti-leishmanial activity of allicin has been tested *in vitro* against promastigotes and intracellular amastigotes of *L.donovani* and *L.infantum*. Macrophage infections have been carried out *in vitro* in the murine cell line J774 and *ex vivo* with peritoneal macrophages (Mφ) from BALB/c mice with a modified method to isolate metacyclic promastigotes. The compound has shown a significant *in vitro* effect on the multiplication of promastigotes of *L.donovani* and *L.infantum* in a time- and dose-dependent manner. It has been shown for the first time the inhibition of multiplication of intracellular amastigotes of *Leishmania* by allicin. Inhibitory concentrations of the compound were in the micromolar range (10-30 µM) for both *Leishmania* species. Antileishmanial effect of allicin apparently was not related to products of degradation of the molecule as assessed by mass spectrometry analysis. Inhibitory activity of allicin against promastigotes and intracellular amastigotes increased when the compound was added to the

cultures every 24 h. Two administrations of 5 μM allicin inhibited by ca. 50% the proliferation of *Leishmania* amastigotes. Low toxicity for mammalian cells of this compound suggests the interest of exploring the value of allicin in combined therapy against leishmanial infections.

Current monotherapy against visceral leishmaniasis has serious side effects and resistant *Leishmania* strains have been identified. The antibiotic AmB has shown an extraordinary anti-leishmanial efficacy without emergence of resistance; however toxicity has limited its general use. Results obtained showed, using a non-fixed ratio analysis, that the combination of diallyl thiosulfinate (allicin) and AmB ranged from moderately synergic to synergic at low concentrations (0.07 μM AmB + 35.45 μM allicin induced a 95% growth inhibition). None of the treatments, alone or in combination, had noticeable adverse effect on Mφ in the concentration range examined (Allicin: 0.5, 1, 5 and 10 μM; AmB: 0.05, 0.075, 0.1 μM). Allicin, AmB or the combination did not affect the infection rate (% of infected Mφ) of *Leishmania*. Allicin enhanced the activity of AmB on intracellular amastigotes of *L.donovani* and *L.infantum* (ca. 45% reduction on amastigote burden with 0.05 μM AmB +10 μM allicin) this representing near a twofold reduction in the IC_{50} value of the antibiotic added alone. Results point towards the interest of testing this combination *in vivo* thus reducing the toxicity associated to the monotherapy with AmB.

Results obtained *in vitro* and *ex vivo* with the combination of AmB and allicin prompted us to evaluate the efficacy of the combination of allicin and amphotericin deoxycholate (AmB) in the chemotherapy of *L.infantum* infection with the final aim of reducing the dose of AmB in the chemotherapy of visceral leishmaniasis. For this purpose hamsters were infected intraperitoneally (ip) with *L.infantum* (10^7 stationary phase promastigotes). On day 45 post-infection animals were treated ip with AmB (1 or 5 mg/kg/day), allicin (5 mg/kg/day) or a combination of AmB (1 mg/kg/day)+allicin (5 mg/kg/day) for 5 days. Animals were clinically and biopathologically monitored and the antibody response (IgG, IgG_1, IgG_2) was determined. Parasite burdens were estimated by limiting dilution and AmB biodistribution was determined by HPLC in plasma, kidney, spleen and liver. No clinical signs or liver and kidney alterations were observed. AmB (1 mg/kg/day) did not clear the *Leishmania* infection and no parasites were detected in two animals treated with 5 mg/kg/day allicin. Combination therapy (5 mg/kg allicin+1 mg/kg AmB) reduced the *L.infantum* burden by >95%. Antileishmanial activity of the combination was comparable ($p<0.05$) to the standard AmB treatment (5 mg/kg/day).

Antimicrobial activity of allicin has been extensively studied although the precise mechanism of action underlying its antileishmanial effect has been virtually unexplored. Transmission electron microscopy showed that allicin induced relevant morphological changes,

including mitochondrial swelling, invaginations in the inner mitochondrial membrane and loss of internal integrity. Other important alterations observed involved swelling of the nuclear membrane and an intense vacuolization of the cytoplasm. Exposure of the parasites to allicin led to high production of intracellular and mitochondrial reactive oxygen species (ROS) which was not controlled by thiol homeostasis, collapse of the mitochondrial membrane potential, reduced production of ATP and elevation of cytosolic calcium. The incubation of the promastigotes with SYTOX Green revealed that decrease of ATP was not associated with plasma membrane permeabilization. Annexin V and propidium iodide (PI) staining indicated that allicin did not induce phosphatidylserine exposure on the plasma membrane. Moreover, TUNEL analysis demonstrated that allicin did not provoke DNA fragmentation. These results point towards a necrotic type of cell death in *Leishmania* after exposure to diallyl thiosulphinate. In addition, analysis of the cell cycle with PI staining showed that allicin induced cell cycle arrest in the G_2/M phase. We conclude that allicin primarily targets mitochondria and induces oxidative stress, uncontrolled by the antioxidant defence of the cell, which leads to mitochondrial dysfunction, increase of cytosolic calcium levels and a bioenergetic catastrophe leading to cell necrosis and cell cycle arrest in the premitotic phase.

Resumen

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Escenario actual de la quimioterapia de la leishmaniosis

El informe de la 60ª Asamblea Mundial de la Salud (OMS) para el Control de la leishmaniosis (Informe A60/10) (22 de marzo de 2007) concluyó que, en el ámbito de la medicina humana, los datos disponibles sobre la prevalencia e incidencia de la leishmaniosis no eran fiables debido al carácter rural de muchos de los procesos, falta de diagnóstico y notificación al no ser una enfermedad de declaración obligatoria (EDO) en todos los países en los que estaba presente. Asimismo se señaló, entre las necesidades de investigación más urgentes para el control de la enfermedad, la exploración de nuevos fármacos económicos alternativos y con ciclos de tratamiento más cortos y, por ello, de más fácil implementación. En 2014, la situación, a pesar de los esfuerzos de investigación y desarrollo (I+D) es similar. En el ámbito veterinario, y en particular en las áreas endémicas para ZVL por *L.infantum* (= *L.chagasi*), varias de las necesidades mencionadas anteriormente también son aplicables.

El desarrollo de una respuesta protectora en el hombre frente a una reinfección homóloga por *Leishmania* así como la inmunización tradicional frente a la leishmaniosis cutánea, mediante leishmanización, sugiere que el desarrollo de una vacuna eficaz frente a la leishmaniosis es factible. Sin embargo, a pesar del profundo conocimiento de la biología, bioquímica y biología molecular de *Leishmania* spp, y de la inmunología de las leishmaniosis, no existe ninguna vacuna registrada frente a la leishmaniosis humana. La protección lograda con algunas de las preparaciones comerciales frente a la leishmaniosis canina (e.g. CaniLeish®) es solo parcial (no impide la infección aunque mejora el cuadro clínico del paciente), de escasa duración (revacunación anual) además de provocar efectos secundarios de consideración.

En estas circunstancias el principal sistema de control de la leishmaniosis es la quimioterapia. La anfotericina B liposomada (AmB), paromomicina y la miltefosina han sido considerados por la OMS (www.who.int/tdr/diseases/leish) como los fármacos más prometedores y, junto con los antimoniales (SbV), constituyen el principal arsenal para el control terapéutica de la leishmaniosis. Estos fármacos, sin embargo, presentan importantes inconvenientes. Así, existen resistencias de *L.donovani* a algunos de ellos (e.g. SbV, miltefosina) (Croft et al., 2006), otros son muy tóxicos o teratogénicos, ninguno de ellos logra una curación parasitológica y las preparaciones menos tóxicas y eficaces (e.g. AmB liposomada) tienen un precio elevado que las hace inaccesibles a la población humana de las

áreas endémicas (Sundar & Chaterjee, 2006; Sundar & Chakravarty, 2013) e irrelevantes en un contexto veterinario.

El descubrimiento de fármacos frente a *Leishmania* requiere modelos experimentales fiables. Cribado de fármacos con promastigotes.

Se han empleado diferentes modelos experimentales para evaluar el potencial terapéutico de una molécula o combinación frente a la leishmaniosis. En las primeras fases de la exploración de una molécula de potencial interés se emplean modelos celulares. Teniendo en cuenta que la mayoría de los compuestos empleados en la terapéutica (frente a la leishmaniosis y otros procesos) tienen un mecanismo de acción basado en la inhibición de enzimas, la actividad de estos ha sido empleada como criterio selectivo de fácil adaptación al cribado (dirigido o no) mediante sistemas de cribado de alto rendimiento ("High Throughput Systems", HTS). Sin embargo, la distancia existente entre bioquímica (inhibición de un enzima), farmacología y terapéutica (farmacocinética, farmacodinamia) hace que sean preferibles el empleo de cribados fenotípicos empleando cultivos de *Leishmania*. Además, algunos de ellos han sido adaptados con éxito a cribados HTS. Con el objetivo de alcanzar un mayor éxito en el cribado y que este sea además menos costoso deben favorecerse los cribados selectivos o dirigidos (e.g. familia de compuestos de estructura o acción similar, compuestos con dianas terapéuticas conocidas, propiedades farmacocinéticas favorables).

La gran mayoría de cribados, incluso en los análisis masivos, se han realizado utilizando promastigotes cultivados (e.g. Siqueira-Neto et al, 2010; Walker et al, 2011). Esta fase, comparable a las células que se dividen dentro de los vectores (Diptera, Psychodidae), difiere (por ejemplo, en su morfología y su bioquímica) de la fase parasitaria, el amastigote, presente en los vertebrados infectados. Al no ser la fase responsable de la infección, el promastigote no es un modelo ideal para la identificación de fármacos potencialmente útiles. No obstante, promastigotes y amastigotes comparten muchas rutas metabólicas y por lo tanto, con algunas excepciones (e.g. profármacos como SbV), las moléculas eficaces frente a promastigotes lo son también frente a amastigotes (e.g. AmB, miltefosina) (Sharlow et al., 2009). El estadio extracelular también ha sido empleado para determinar la resistencia clínica frente a algunos fármacos, como la miltefosina (Kulshrestha et al, 2013). Por otra parte, los ensayos con amastigotes intracelulares –a pesar de su mayor interés- son más laboriosos y no están presentes en todos los laboratorios.

El cribado fenotípico en promastigotes de fármacos leishmanicidas/leishmaniostáticos mediante microscopía requiere una gran inversión de tiempo y personal por lo que se han desarrollado métodos indirectos tales como la nefelometría, colorimetría, PCR cuantitativa, citometría de flujo, incorporación de 3H-timidina o tratamiento de imágenes (e.g. ensayos con "*reporter genes*") entre otros. Un método de cuantificación colorimétrica, Alamar Blue, es de bajo coste, fácil ejecución y toxicidad insignificante para las células. Este método, basado en la determinación de resorrufina, producto de la reducción de resazurina, se ha empleado con frecuencia para determinar la multiplicación de células de mamífero, normales y transformadas (O'Brien et al., 2000; Sykes & Avery, 2009).

Las pruebas preliminares realizadas en nuestro laboratorio mostraron resultados inconsistentes en el seguimiento de la multiplicación de promastigotes de *L.infantum* y *L.donovani*, en condiciones estándar (26ºC, aireación) y con diferentes medios de cultivo (e.g. RPMI 1640, Schneider). Ya que probablemente el rendimiento de un indicador redox se vería afectado por la fase gaseosa de los cultivos, en particular por el oxígeno y el dióxido de carbono, se exploró y cuantificó su posible efecto en el ensayo de Alamar Blue. Los resultados obtenidos con una atmósfera de 5% CO_2/95% aire sobre cultivos tamponados con 20 mM HEPES mejoraron de forma notable los resultados (linealidad y correlación entre el recuento de células y el nivel de resorrufina detectado) cuando los cultivos se iniciaron con un inóculo de 2,5 x 10^5 promastigotes de *Leishmania* spp y la reducción de resazurina fue valorada mediante fluorometría (Capítulo 2). Nuestros resultados fueron consistentes con el empleo de una atmósfera de CO_2 habitual en la mayoría de los ensayos con células de mamíferos, los efectuados sobre tripanosomas de torrente circulatorio y aquellos realizados en amastigotes axénicos de *Leishmania* (Räz et al., 1997; Sykes & Avery, 2009). No disponemos de explicación adecuada a la ausencia de inconvenientes en experimentos anteriores con *Leishmania* spp sin CO_2 (de Oliveira-Silva et al, 2008; Shimony & Jaffe, 2008; Vermeersch et al, 2009; Kulshrestha et al, 2013) o en HTS con un formato de 384 pocillos (Siqueira-Neto et al., 2010) salvo que no fuera explícitamente indicado o debido al diferente comportamiento de los promastigotes de *Leishmania* relacionado con modificaciones de los medios de cultivo (e.g. aditivos, concentración, suero fetal bovino).

Las condiciones experimentales descritas en nuestro trabajo han permitido obtener una alta repetibilidad y reproducibilidad de los resultados con las dos especies ensayadas (*L.infantum* y *L.donovani*). Además no existió interferencia con el rojo fenol presente en el medio de cultivo y la reducción de resazurina estuvo estrechamente relacionada con el nº de células (determinado mediante recuento en cámara de Neubauer) (r^2= 0,9713). La modificación desarrollada, además de constituir una herramienta adecuada para el cribado de fármacos de interés frente a la

leishmaniosis de posible empleo en formatos adaptables a HTS, apunta a la necesidad de estandarización de las condiciones de cultivo de *Leishmania* y la cuantificación de la multiplicación de promastigotes.

Evaluación de fármacos frente a amastigotes intracelulares de *Leishmania*

De forma general se considera que los modelos *in vivo* de cribado de fármacos tienen un valor predictivo superior que los modelos *in vitro* o *ex vivo*, y que los amastigotes intracelulares son más convenientes para este propósito que los promastigotes o los amastigotes axénicos (Sereno et al., 2007). Sin embargo, el uso sistemático de amastigotes intracelulares está menos extendido debido a los requerimientos técnicos superiores –respecto a promastigotes-, tasa de multiplicación lenta en algunas especies de *Leishmania* (e.g. *L.infantum vs L.donovani*), así como los requerimientos específicos de algunas especies. Estos requerimientos singulares explican la diferente eficacia de las infecciones en dependencia de la especie de *Leishmania* implicada, incluso cuando están estrechamente emparentadas. De hecho, en el caso de *L.infantum* el primer método de infección de macrófagos (Mφ) peritoneales de ratón (y la línea celular murina J774) con promastigotes fue publicado hace menos de 20 años (Méndez et al., 1996), mucho después que dichos cultivos fuesen rutinarios con *L.donovani*. Dicho método modificado incluía que la temperatura en el periodo de contacto (incubación *overnight*) de los Mφ con los promastigotes de *L.infantum* fuera de 33°C, temperatura es más cercana a la de la piel donde se produce la infección.

Este procedimiento ha sido utilizado desde entonces (e.g. Ordóñez-Gutiérrez et al, 2007, 2009; Wert et al, 2011) aunque la tasa de infección (% de Mφ infectados) y la carga parasitaria (número de amastigotes intracelulares/Mφ infectado) eran inferiores a los valores obtenidos en infecciones paralelas con *L.donovani*. Consideramos, por ello, explorar la posibilidad obtener infecciones más eficaces (superior carga parasitaria, mayor % de infección) y consistentes con *L.infantum* como paso previo a su utilización en el cribado de moléculas de potencial interés. Se ha desarrollado una modificación del método de infección mediante la combinación del método establecido previamente en nuestro laboratorio (Méndez et al., 1996), el aislamiento de metacíclicos con un gradiente de Ficoll discontinuo (Yao et al., 2008), y la opsonización de los promastigotes con 15% de suero de ratón (Capítulo 3). La modificación del método de infección ha permitido obtener elevaciones notables de la tasa de infección de ambas especies (*L.infantum*: 65-70% en células J774, ca. 80% en Mφ peritoneales de BALB/c; *L. donovani*: 70-75% en J774, ca. 95% en Mφ peritoneales de ratón) y de la multiplicación intracelular, particularmente de *L.infantum*. El empleo conjunto del método de infección mejorado y el

método fluorométrico para determinar la viabilidad de amastigotes intracelulares de *Leishmania* (Bilbao-Ramos et al., 2012), no ensayado por nosotros, podría constituir una potente plataforma para el ensayo de fármacos leishmanicidas/leishmaniostáticos y ser adaptado a HTS.

Dialil tiosulfinato (alicina) inhibe a concentraciones micromolares (μM) la multiplicación de promastigotes y amastigotes de *Leishmania*.

Las deficiencias actuales del control quimioterápico de la leishmaniosis (reducido arsenal terapéutico, resistencias, toxicidad, ausencia de curación parasitológica) hacen del I+D de nuevos agentes una necesidad urgente. Las estrategias a seguir para lograr este objetivo pueden ser a corto-medio plazo [e.g. nuevas indicaciones para fármacos antiguos –muchos de los tratamientos actuales de la leishmaniosis-, terapia combinada, incorporación de sistemas de administración de fármacos (DDS)] o a largo plazo (e.g. inmunoterapia, identificación de nuevas moléculas sintéticas o naturales) (Pink et al., 2005; Pham et al, 2013).

La alicina (dialil tiosulfinato), producto natural presente en plantas de la familia Alliaceae, ha mostrado actividad antibacteriana [e.g. *Helicobacter pylori*, cepas de *Staphylococcus aureus* resistentes a la meticilina (MRSA)] (O'Gara et al., 2000; Cutler & Wilson, 2004), antifúngica (i.e. *Candida*) (Cañizares et al., 2004; Khodavandi et al, 2011) y actividad antiproliferativa frente a distintos Protista (e.g. *Trypanosoma*, *Leishmania*, *Entamoeba*, *Plasmodium*) (Mirelman et al, 1987; Coppi et al 2006; Waag et al, 2010). La información existente sobre la actividad de esta molécula sobre *Leishmania* era muy reducida y solo basada en los estudios efectuados sobre promastigotes. Esta falta de información, el desarrollo del modelo de infección de Mφ con promastigotes de *Leishmania* y la disponibilidad de alicina estabilizada nos llevó a probar el potencial efecto antiproliferativo de alicina frente a los dos principales agentes causantes de la leishmaniosis visceral, *L.donovani* y *L.infantum* (Capítulo 3).

Los resultados obtenidos han mostrado un efecto inhibitorio significativo de alicina a concentraciones micromolares sobre la multiplicación de *L.donovani* y *L.infantum*. La inhibición fue tiempo y dosis dependiente y tanto frente a promastigotes como amastigotes intracelulares *in vitro* (células J774) y *ex vivo* (Mφ peritoneales de ratón BALB/c). La IC_{50} obtenida (ca. 10-30 μM, 24 h de exposición) para los promastigotes de ambas especies estaba en la línea del valor indicado por Ankri & Mirelman (1999) (30 μg/mL) para *L.major* y ligeramente superior que la señalada por McClure et al. (1996) para *L.chagasi* y *L.mexicana*. Más relevante fue la observación de que, la alicina, a estas concentraciones, inhibió la multiplicación de amastigotes, hecho no señalado anteriormente. Además, la toxicidad de

alicina a las concentraciones empleadas fue baja y no mostró toxicidad en células de mamífero (método MTT, ausencia de alteraciones morfológicas en células J774 y Mφ murinos).

La administración de alicina en dosis repetidas cada 24 h incrementó significativamente su eficacia antiproliferativa. Este hallazgo sugería la degradación del dialil tiosulfinato a 26ºC y 37ºC (McClure et al 1996; Cañizares et al., 2004). Esta molécula es altamente reactiva y es rápidamente transformada a otros tipos de compuestos orgánicos de azufre, en particular dialitrisulfuro (DATS), dialildisulfuro (DADS), dialilsulfuro (DAS), ajoeno y vinilditinas (Amagase, 2006).

La degradación de la preparación de alicina utilizada tras una exposición de 24 h a dichas temperaturas se confirmó mediante microextracción en fase sólida, cromatografía de gases y espectrometría de masas (SPME/GC/MS). Las muestras tratadas mostraron sulfuros y vinilditinas pero no ajoeno (E o Z) o alicina. La ausencia de alicina y ajoeno podría ser debida a su corta vida media a las temperaturas de cultivo (26ºC y 37ºC) (ajoeno < 1 min) o bien al método analítico empleado. En este sentido se ha indicado que la sensibilidad térmica de alicina a temperatura fisiológica, así como su rápida transformación en otros tipos de compuestos orgánicos de azufre (Amagase, 2006) podrían descartar su uso terapéutico. Sin embargo, se ha demostrado que la disminución de la alicina es acompañada por su transformación en otros compuestos (i.e. ajoeno) (Fujisawa et al. 2008) que presenta una importante actividad anti-*Leishmania* (Ledezma et al. 2002). Además, otros subproductos de la degradación de alicina (e.g. DATS, DADS, DAS) han demostrado tener también potente actividad antimicrobiana (e.g. DAS y DADS frente a *Helicobacter pylori*; DATS comercializados en China frente a *Giardia*; DADS y ajoeno han demostrado actividad antiviral) (Corzo-García et al., 2007) y no debe descartarse su posible efecto frente a *Leishmania*. La determinación de la molécula responsable de la actividad frente a *Leishmania* observada en nuestro estudio, alicina o algún producto de degradación, requiere experimentación adicional. Por otra parte el metabolismo de la alicina, y por lo tanto su biodisponibilidad, son en su mayor parte desconocidos (Lawson, 1998; Lawson & Wang, 2005).

El valor de IC_{50} determinado *in vitro* y *ex vivo* (ca. 5-10 µM en dosis repetidas) frente a amastigotes intracelulares posiblemente excluye a la alicina como monoterapia frente a las leishmaniosis. Sin embargo, su coste reducido y su escasa toxicidad podrían permitir el uso de alicina en terapia combinada con otros fármacos anti-*Leishmania* bien establecidos.

Interacción de alicina y anfotericina B, a concentraciones micromolares, frente a promastigotes y amastigotes intracelulares de *Leishmania*

Entre las estrategias empleadas para hacer frente a la aparición de resistencias y a la reducción de la eficacia de algunos de los fármacos empleados en la monoterapia de la leishmaniosis (e.g. SbV, miltefosina) se encuentra la utilización de combinaciones de fármacos de conocida actividad leishmanicida/ leishmaniostática (Olliaro, 2010; Sundar & Chakravarty, 2013). La notable actividad anti-*Leishmania* de alicina y la reducida toxicidad para células de mamífero encontradas, impulsaron la exploración de la combinación de esta molécula con Anfotericina B (AmB).

Este compuesto, de ser un tratamiento de segunda línea para la leishmaniosis, se ha convertido, como consecuencia de la aparición de resistencias, en el fármaco de elección en muchas zonas endémicas de la enfermedad (Croft & Coombs, 2003; Croft et al, 2006; Sundar & Chakravarty, 2013). En la actualidad la AmB, especialmente la formulada en liposomas, es la primera línea terapéutica para el control en el hombre de esta enfermedad, en particular de las leishmaniosis viscerales (OMS, 2010). Además, a pesar de su utilización en la práctica clínica (leishmaniosis, micosis sistémicas) durante más de 50 años, la aparición de resistencias es anecdótica (Durand et al., 1998; Gray et al., 2012; Purkait et al., 2012). Las restricciones al uso de este antibiótico han estado relacionadas con los efectos secundarios (toxicidad) y larga duración de los tratamientos con monitorización clínica necesaria. Nuestra experimentación pretendía conocer el posible efecto sinérgico, aditivo o antagónico de alicina y AmB a concentraciones micromolares (atóxica), *in vitro* y *ex vivo*, con el objetivo de reducir la dosis necesaria de AmB en monoterapia y, por ello, minimizar su toxicidad.

La mayor parte de los estudios experimentales llevados a cabo sobre combinación de fármacos frente a *Leishmania* han empleado combinaciones de diluciones de dos moléculas sin análisis ulterior. Sin embargo, existen metodologías de mayor significación que la aproximación "*checkerboard*" para determinar la interacción real de dos compuestos (e.g. anergia, sinergia, antagonismo) (Odds, 2003). Por ello, en nuestro estudio sobre el efecto anti-*Leishmania* de la combinación AmB + alicina se utilizó la robusta metodología de Chou-Talalay y el software CalcuSyn desarrollado por dichos autores (Chou, 2006; Chou, 2008; Chou & Hayball, 1996). Los resultados mostraron que la combinación de ambas moléculas fue de moderadamente sinérgica a sinérgica con concentraciones bajas de ambos compuestos tanto frente a promastigotes (i.e. la combinación de 0,07 µM AmB + 35,45 µM alicina indujo una inhibición del crecimiento del 95%) como amastigotes (i.e. reducción del ~ 45% con 0,05 µM AmB + 10 µM alicina). Estas concentraciones representaban una reducción cercana al 50% en el valor de

la IC_{50} del antibiótico cuando era empleado como único fármaco (Vermeersch et al., 2009; Maia et al., 2013). Por otra parte las diferencias observadas entre los resultados obtenidos con promastigotes (con una reducción de 7-11 veces de la concentración necesaria de AmB) y amastigotes (con una reducción próxima al 50%) subrayan la necesidad de utilizar amastigotes intracelulares en la identificación de moléculas de interés frente a *Leishmania* (Sereno et al, 2007; Seifert et al., 2011) (Capítulo 4).

Al igual que ocurre en hongos, el principal esterol presente en la membrana plasmática de *Leishmania* es el ergosterol. Aparentemente, alicina es capaz de incrementar la actividad fungicida de la AmB mediante la inhibición del tráfico de ergosterol desde la membrana celular a la membrana de las vacuolas originadas por AmB en *Candida albicans* (Fungi) (Ogita et al., 2009). En nuestro caso, la base de esta acción sinérgica frente a *Leishmania* requiere experimentación adicional. Los datos obtenidos con combinaciones de fármacos *in vitro* y *ex vivo* presentan limitaciones (e.g. interacciones imprevistas, modificaciones en los perfiles de PK/PD) a pesar del análisis de relación dosis-efecto llevado a cabo (Chou, 2008). A todos los efectos, una combinación de los fármacos A+B puede comportarse como un tercer fármaco, C. No obstante, la reducción en la dosis necesaria de AmB frente a amastigotes intracelulares de *Leishmania* y la posibilidad de utilizar dosis no tóxicas de alicina (>10 μM) sugirieron el interés de la exploración de esta combinación *in vivo*.

Alicina es eficaz contra la leishmaniosis visceral experimental en hámster y reduce la dosis efectiva requerida de anfotericina B

La terapia combinatoria ha sido una de las estrategias empleadas, fomentada por la OMS (2010), para hacer frente a la aparición de fallos terapéuticos y resistencias en *Leishmania* (Chunge et al., 1985; Murray et al., 2003). Experimentalmente se ha logrado generar resistencia frente a combinaciones en *L.donovani* (García-Hernández et al., 2012), por lo que se debe tener cautela en su implementación. No obstante, el empleo de fármacos en combinación para el tratamiento de la LV presenta importantes ventajas entre las que se incluyen el aumento de la eficacia y la tolerancia del tratamiento, la reducción de la toxicidad, duración y coste del mismo, y la limitación o prevención de la aparición de resistencias aumentando así la vida útil de los medicamentos que tenemos a nuestra disposición (Seifert & Croft, 2006; Griensven et al., 2010).

AmB probablemente es el mejor medicamento existente frente a la leishmaniosis con tasas de curación superiores al 95% (Alvar et al., 2006; Paila et al., 2010; Sundar & Chakravarty,

2013) y niveles insignificantes de resistencia después de ser empleado como fármaco de referencia contra las infecciones fúngicas sistémicas durante más de 50 años (Durand et al, 1998; Gray et al, 2012).

En general se considera que la unión de AmB al ergosterol de la membrana celular de *Leishmania* es la principal causa de su actividad leishmanicida (Purkait et al., 2012). La afinidad diferencial de AmB por el colesterol (abundante en células de mamífero) y ergosterol (en las membranas de *Leishmania*) explicaría la toxicidad diferencial para los hospedadores y los agentes parasitarios. En realidad, la toxicidad residual de AmB, por unión inespecífica al colesterol, se considera el principal inconveniente para su uso (Laniado-Laborín & Cabrales-Vargas, 2009; Hamill, 2013).

Nuestros resultados mostraron que (i) alicina exhibió una toxicidad muy baja para células de mamífero además de ser capaz de inhibir significativamente la multiplicación de amastigotes intracelulares de *Leishmania* (Capítulo 3), y (ii) la actividad *in vitro* y *ex vivo* frente al parásito resultante de la interacción de AmB + alicina en combinación resultó sinérgica (Capítulo 4). Los amastigotes intracelulares de *Leishmania* son un buen modelo para evaluar fármacos de potencial valor (Sereno et al., 2007) pero no permiten anticipar el valor terapéutico del fármaco o combinación. Por ello, se evaluó la eficacia terapéutica de la alicina, así como de su combinación con AmB a bajas dosis, en un modelo experimental de leishmaniosis visceral.

El objetivo final de la estrategia de combinación sería la reducción (por debajo de los niveles tóxicos) de las dosis de AmB necesarias para controlar eficazmente la infección crónica por *L.infantum* en criceto (*Mesocricetus auratus*) (Capítulo 5). El modelo experimental permitió obtener infecciones consistentes, con cargas parasitarias significativamente más altas en bazo –respecto a hígado- y una alteración de los niveles hemáticos de urea, confirmando así su idoneidad para los ensayos *in vivo* de moléculas leishmanicidas/leishmaniostáticas (Binzahim et al., 1993; Requena et al, 2000; Melby et al., 2001; Dea-Ayuela et al., 2007; Moreira et al, 2012). Los grupos de cricetos infectados fueron tratados desde el día 45 post-infección mediante administración intraperitoneal de los compuestos [AmB (1 o 5 mg/Kg/día), alicina (5 mg/Kg/día) o una combinación de AmB (1 mg/Kg/día) + alicina (5 mg/Kg/día) durante 5 días consecutivos]. Ninguno de los compuestos ni la combinación indujeron signos clínicos en los animales o alteraciones fisiológicas lo que confirmó la ausencia de toxicidad de AmB, alicina o la combinación con la pauta terapéutica empleada. El tratamiento con alicina (5 mg/Kg/día, 5 días) eliminó la infección por *L.infantum* en 2 de los 6 animales tratados, tanto en bazo como en hígado. Estos resultados han supuesto la primera evidencia de la actividad de alicina *in vivo* frente a las infecciones por *Leishmania*.

La combinación de AmB (1 mg/Kg/día) + alicina (5 mg/Kg/día) redujo más del 95% de la carga parasitaria de *L. infantum*, nivel de eficacia comparable (p<0,05) con los resultados obtenidos con la dosificación estándar de AmB (5 mg/Kg/día). Dichos resultados confirman *in vivo* la sinergia observada *in vitro* y *ex vivo* (Capítulo 4) y sugiere la posibilidad de reducir cinco veces la dosis requerida de AmB en monoterapia sin pérdida de eficacia. El mecanismo responsable de la interacción sinérgica o aditiva hallada no es conocido y debe ser explorado. Aparentemente, alicina mejoró la acumulación del antibiótico en los órganos diana y en los hámsteres tratados se observó una respuesta específica IgG_2 comparativamente mayor, quizás relacionada con la respuesta proinflamatoria inducida por alicina (Feng et al., 2012). Se necesita más investigación en este modelo predictivo de leishmaniosis humana y canina (e.g. posología, vía de administración, naturaleza de la interacción) pero nuestros resultados apuntan hacia el interés de esta combinación, con la posibilidad de reducir la dosis necesaria de AmB, y por lo tanto su potencial toxicidad, en la quimioterapia de leishmaniosis.

Mecanismo de acción de alicina: catástrofe bioenergética y necrosis celular en *Leishmania*

A pesar de la eficacia de alicina y compuestos relacionados para inhibir la multiplicación de organismos eucariotas unicelulares (e.g. *Candida, Trypanosoma, Leishmania*) y de células cancerosas (Capítulos 1 y 3) su mecanismo de acción es en su mayor parte desconocido. Se ha indicado que, dada su naturaleza altamente reactiva, probablemente reacciona rápidamente con tioles, sistemas enzimáticos tiol-dependientes, cisteín preoteasas y microtúbulos (Ankri & Mirelman, 1999; Prager-Khoutorsky et al., 2007; Miron et al., 2010; Waag et al., 2010). Además, se ha descrito su capacidad para inducir apoptosis en células tumorales (Oomen et al., 2004). Sin embargo, las dianas intracelulares responsables del efecto citocida/citostático de la molécula son en gran parte desconocidas. La disponibilidad de una formulación estabilizada de alicina y los resultados obtenidos *in vitro, ex vivo* e *in vivo* frente a *Leishmania* nos llevaron a investigar su posible mecanismo de acción leishmanicida/leishmaniostático.

Mediante microscopía electrónica de transmisión (MET) la alteración más notable observada en promastigotes de *L. infantum* y *L.donovani* tratados con alicina fue la pérdida de integridad de la membrana mitocondrial y la alteración a nivel del kinetoplasto (Capítulo 3 & 5). La microscopía es una notable herramienta para determinar las dianas intracelulares así como el tipo de muerte celular. Por otra parte, los Trypanosomatidae, incluyendo *Leishmania*, solo tienen una gran mitocondria que representa ca. 12% del volumen celular (Monzote-Fidalgo & Gille, 2011) y dependen en gran medida de esta organela dada su escasa capacidad para

sobrevivir y multiplicarse en ambientes anaeróbicos (Hellemond et al., 1997; Manzano et al, 2012).

Nuestros resultados mostraron que alicina, a concentraciones subletales, indujo una elevación de la producción de especies reactivas de oxígeno (ROS). Aunque ROS están presentes en células normales y desempeñan un papel significativo como mensajeros (Zhang et al, 2006) la elevación de sus niveles está relacionada con el estrés oxidativo, la disfunción mitocondrial y la muerte celular (Duchen, 2000; Zong & Thompson, 2006). Ya que la alicina atraviesa membranas celulares con facilidad (Miron et al., 2010), dicha elevación debe producirse muy rápidamente. La principal defensa antioxidante de *Leishmania* depende principalmente de tripanotion (=bis-glutationil espermidina) (Fairlamb & Cerami, 1992), debido a que los Kinetoplastida carecen de catalasa, glutation peroxidasa y de glutation reductasa (Fairlamb et al., 1985). Nuestros resultados mostraron que alicina solo inhibió de forma moderada la actividad de la tripanotion reductasa y existió una elevación de los niveles totales de tioles (i.e. cisteína, glutation y tripanotion) en las células tratadas. Estos resultados, además de confirmar la interacción de alicina con tioles (Rabinkov et al., 1998), sugerían que la muerte celular de *Leishmania* no estaba relacionada principalmente con este mecanismo de acción de alicina.

Existe una estrecha relación entre el estrés oxidativo y el Ca^{2+} intracelular en todos los organismos, incluyendo *Leishmania* (Mukherjee et al, 2002; Das et al, 2008; Dolai et al, 2011). Una fina regulación de los niveles de Ca^{2+} intracelulares resulta crítica para la homeostasis y supervivencia celular (Richter, 1993). Se ha demostrado que el estrés oxidativo puede interrumpir la translocación de calcio intracelular (Orrenius et al., 1989) en las diferentes reservas celulares de calcio (e.g. mitocondria, acidocalcisomas, retículo endoplasmático). Nuestros experimentos mostraron que la alicina indujo una rápida elevación del Ca^{2+} citoplasmático. Este incremento probablemente esté relacionado con el transporte desde los depósitos intracelulares y, en particular desde la mitocondria, ya que no se encontraron variaciones en la permeabilidad de la membrana plasmática. No obstante, se requiere experimentación adicional para comprobar el origen extracelular (i.e. apertura de canales iónicos en la membrana plasmática) o intracelular (i.e. movilización de depósitos) del catión. La generación de ROS y el aumento de Ca^{2+} citosólico en *Leishmania* expuestas a concentraciones subletales de alicina fueron acompañados por la caída del potencial transmembrana mitocondrial ($\Delta\Psi m$) y por la reducción de la producción de ATP. Los elevados niveles de ROS intramitocondriales y su aparente saturación (> 30 µM alicina) además de las alteraciones morfológicas observadas en MET (Capítulo 3 & 5) apuntan a la mitocondria como diana primaria de alicina en *Leishmania*.

Así, la generación de ROS inducida por alicina, no compensada por tioles, provocaría la despolarización de la membrana mitocondrial con caída del ΔΨm; ello reduciría la capacidad de la mitocondria para retener (y regular) los niveles intracelulares de Ca^{2+} junto con la disfunción de la fosforilación oxidativa y derivada caída de la producción de ATP. Esta caída es crítica ya que se estima que el 70% de todas las necesidades energéticas de *Leishmania* dependen de la fosforilación oxidativa en mitocondria (Manzano et al., 2012).

El colapso de la membrana mitocondrial (CMM) y, por consiguiente, la disfuncionalidad de la mitocondria, es un proceso irreversible que culmina con la muerte celular ya sea por necrosis o bien por apoptosis (Kroemer et al., 1998). Aparentemente alicina es capaz de inducir apoptosis en algunas líneas celulares cancerosas (PC-3, Hep G2) (Arunkumar et al, 2006; Chu et al., 2012.). La disponibilidad de ATP, tras la despolarización de la membrana mitocondrial, determina si la muerte se producirá mediante apoptosis (sin caída de ATP) o necrosis (bajos niveles de ATP) (Leist et al., 1997; Eguchi et al., 1997; Kim et al., 2003). En nuestro caso existió una caída neta de la producción de ATP. A pesar de las variaciones encontradas en los tipos de muerte celular programada nuestros resultados no mostraron ninguna evidencia concluyente de muerte apoptótica ya que no se observó fragmentación de ADN (mediante TUNEL), no se halló exposición de fosfatidilserina [mediante tinción con Anexina V y yoduro de propidio (IP)] y el ciclo celular se detuvo en la fase G_2/M (análisis con IP). Por tanto, nuestros resultados apuntan a que alicina induce la muerte de *Leishmania* mediante un proceso de necrosis celular.

Serían necesarios más experimentos para determinar la secuencia precisa de eventos pero los datos disponibles apuntan hacia la rápida generación de ROS –intracelular e intramitocondrial- en *Leishmania* como consecuencia de la exposición a alicina. El desequilibrio redox provocaría la despolarización de la membrana mitocondrial (ΔΨm) con disfunción del ciclo del ácido tricarboxílico (CAT) y, por lo tanto, la reducción de la producción de ATP acompañada de una elevación del Ca^{2+} citosólico. Estos procesos, posiblemente a través de un proceso de retroalimentación, llevarían al colapso de la mitocondria con pérdida de su integridad y, finalmente, a una catástrofe energética celular que de forma irreversible conduciría a la muerte, por necrosis, de los promastigotes de *Leishmania* con detención del ciclo celular en la fase premitótica (G_2/M).

Observaciones finales y conclusiones

La quimioterapia actual de la leishmaniosis no es satisfactoria teniendo en cuenta vía de administración y la duración de los tratamientos, el precio de los fármacos más eficaces y seguros (e.g. AmB liposomada), los efectos secundarios (e.g. teratogenicidad de miltefosina) y la toxicidad de la mayoría de compuestos. Sin embargo, los medicamentos más eficaces contra esta infección tienen, en general, más de 50 años y no se anticipa el lanzamiento de nuevas moléculas eficaces. La falta de alternativas terapéuticas para el control de la leishmaniosis y otras enfermedades parasitarias no tiene razones obvias derivadas de una dificultad específica inherente a estos agentes etiológicos (Pink et al., 2005). Se ha señalado que esta crisis de innovación por parte de la industria farmacéutica –no solo restringida a este grupo de enfermedades- está más relacionada con factores como la estructura interna de la industria, con separación estricta de los departamentos financieros y de I+D (Cuatrecasas, 2006) y al anacronismo del actual sistema de patentes (Moon et al., 2012). A estos factores, en el caso particular de la leishmaniosis, habría que añadir los escasos retornos esperables en términos económicos y el uso de estrategias oportunistas seguidas en numerosas ocasiones para la identificación de nuevas moléculas con actividad antiparasitaria frente a esta enfermedad (e.g. indicaciones nuevas para fármacos antiguos; nuevas presentaciones).

Es relevante señalar que los enormes progresos de la biología básica de *Leishmania* y las tecnologías disponibles (e.g. genómica, proteómica, transcriptómica, metabolómica, robótica, miniaturización, bioinformática) no han dado lugar a una mejora sustancial del desarrollo de fármacos leishmanicidas/leishmaniostáticos. En la década de los 80 del siglo pasado se anticipó que los avances de la biología molecular, además de poder ser integrados con facilidad en el descubrimiento de fármacos (e.g. identificación de dianas, sistemas de cribado de alta capacidad), reducirían los tiempos de desarrollo de fármacos seguros y asequibles permitiendo reducir costes, humanos y materiales (Koehn & Carter, 2005). En efecto, el cribado sistemático, dirigido o no, de bibliotecas químicas permitiría identificar moléculas con potencial terapéutico en cortos periodos de tiempo. Por otra parte, el tándem "cribado de alta capacidad/química combinatoria" pondría a disposición de la comunidad científica un número de moléculas sin precedentes. Sin embargo, la tasa de fracaso ha sido muy alta y, aunque existen numerosas moléculas en evaluación en condiciones de laboratorio y de campo, no se ha desarrollado ningún nuevo fármaco frente a *Leishmania*.

La escasez actual de fármacos eficaces frente a la leishmaniosis junto con la escasa eficiencia del desarrollo de moléculas de interés obtenidas mediante la síntesis química y la crisis de innovación de la industria farmacéutica aconsejan, en nuestra opinión, una

reevaluación del potencial terapéutico de moléculas de origen natural o sus derivados (). La investigación sobre productos naturales fue abandonada por la industria de forma simultánea a la utilización sistemática de la síntesis química, a pesar de los éxitos obtenidos a partir de productos naturales (Newman, 1980) y su papel en la terapéutica actual de numerosas enfermedades. Las moléculas naturales presentan una extraordinaria variedad y complejidad estructural muy superior a las sintéticas (Lee & Schneider, 2001; Feher & Schmidt, 2003) mientras que en la química de síntesis solo se utiliza un número reducido de estructuras.

La riqueza y complejidad en término de número de moléculas presentes en un extracto de origen natural constituyó una importante limitación para el estudio de nuevos principios activos derivados de plantas u otros organismos (e.g. hongos, organismos marinos). En particular, la mayor dificultad era lograr identificar, aislar y conseguir cantidades suficientes de la molécula con actividad biológica presente en una mezcla compleja como es un extracto. En la actualidad se dispone de potentes herramientas (e.g. espectrometría de masas, resonancia magnética nulcaer, diversas técnicas cromatográficas, técnicas de biología molecular, sistemas de cribado de alta capacidad) y más información (e.g. relación estructura-función) que podrían subsanar los inconvenientes inherentes al estudio de productos naturales (Feher & Schmidt, 2003; Koehn & Carter, 2005). Además, el análisis "bioguiado" del proceso de identificación, con modelos bien establecidos y predictivos *in vitro*, *ex vivo* e *in vivo* puede resultar en una forma más eficiente de desarrollar nuevos fármacos y combinaciones.

Nuestro trabajo experimental ha mostrado que una molécula natural, alicina, exhibe un efecto significativo frente a *Leishmania in vitro* e *in vivo* y, quizás de forma más notable, su administración a concentraciones micromolares atóxicas ha permitido reducir la dosis necesaria de AmB para controlar eficazmente la leishmaniosis experimental. Estos resultados sugieren el interés de la exploración de nuevas moléculas de origen natural con las tecnologías disponibles en la actualidad.

Conclusiones

i. La modificación desarrollada del método Alamar Blue, en cultivos con atmósfera de 5% CO_2, tamponado adicional (20 mM Hepes) y fluorimetría, incrementó la sensibilidad y precisión de la técnica y permite su empleo rutinario en el cribado de moléculas con potencial actividad leishmanicida/leishmaniostática.

ii. La combinación de tratamiento térmico y separación de promastigotes metacíclicos de *Leishmania infantum* y *L. donovani* incrementó la internalización y multiplicación intracelular en macrófagos. Los elevados niveles de infección (% Mφ infectados), número de amastigotes producidos y repetibilidad de los resultados convierten a este método en una buena herramienta tanto para el cribado de fármacos como para la caracterización de la virulencia de aislados de *Leishmania*.

iii. Se ha demostrado, por vez primera, la inhibición de la multiplicación de promastigotes y amastigotes intracelulares de *L.donovani* y *L.infantum* por dialil tiosulfinato (=alicina) a concentraciones micromolares. El valor IC_{50} hallado para la forma intracelular (10-30 µM) probablemente excluye a esta molécula como monoterapia en el tratamiento de la leishmaniosis.

iv. El empleo de un método robusto de análisis de la interacción entre alicina y anfotericina B (AmB) mostró la existencia de sinergia moderada a sinergia entre ambas moléculas frente a los dos estadios de desarrollo de *Leishmania* permitiendo reducir un 50% la concentración requerida del antibiótico. Estos resultados apuntan al interés de esta combinación en el tratamiento de la leishmaniosis.

v. Alicina, administrada a cricetos (*Mesocricetus auratus*) infectados experimentalmente con *L.infantum* (5 mg/Kg/día, 5 días) mostró una notable actividad antiparasitaria y ausencia de toxicidad, evidenciada por la ausencia de signos clínicos o alteraciones fisiológicas.

vi. La combinación de alicina (5 mg/Kg/día, 5 días) y AmB (1 mg/Kg/día, 5 días) mostró *in vivo* una eficacia antiparasitaria (reducción > 95% de la carga parasitaria) comparable a la medicación estándar con AmB (5 mg/Kg/día, 5 días). La reducción de las dosis de AmB necesarias, en presencia de alicina, apunta al interés de esta combinación en la terapéutica de la leishmaniosis.

vii. La diferencia en los valores de IC_{50} hallados para la combinación alicina + AmB en promastigotes y amastigotes, así como la aparente sinergia observada en el modelo de amastigotes intracelulares, confirmada *in vivo*, sugieren la necesidad de realizar cribados de fármacos frente a *Leishmania* en amastigotes, por su superior valor predictivo.

viii. La mitocondria de *Leishmania* aparentemente es la organela diana de alicina. Dialil tiosulfinato induciría la generación de niveles elevados de ROS en el citosol y la mitocondria. La alteración del equilibrio redox provoca la despolarización de la membrana mitocondrial, elevación de calcio citosólico sin alteración de la permeabilidad de la membrana plasmática y caída de la producción de ATP. Estos eventos provocarían un colapso de la mitocondria con pérdida de su integridad, catástrofe bionergética y muerte por necrosis con detención del ciclo celular en fase premitótica (G_2/M).

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Annex

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Improvement of 96-well microplate assay for estimation of cell growth and inhibition of *Leishmania* with Alamar Blue

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ABSTRACT

The value of resazurin-based Alamar Blue redox indicator to determine multiplication of *Leishmania* promastigotes in 96-well microtiter plates was examined. In addition, assay was validated with amphotericin B (AmB) and allicin. The method was tested on *L.donovani* and *L.infantum* promastigotes under different culture conditions (variable air-phase, presence of phenol red, initial cell density, incubation time, use of Hepes buffer). Results showed that the gas-phase of promastigote cultures was critical. The method yielded consistent results with initial plating cell densities of 2.5×10^5 promastigotes/well, up to 72 h incubation and 5% CO_2 atmosphere or reduced air availability (sealed plastic bags, film-sealed microplates). Detection of low numbers of promastigotes and earlier results could be obtained using fluorimetry instead of spectrophotometry. The addition of 20 mM Hepes improved the results. Fluorescence intensity correlated to promastigotes number in both *Leishmania* spp. Inhibitory concentration (IC_{50}) values for AmB and allicin using cell counting and fluorimetry were comparable. Under these conditions this one-step, low-cost redox indicator can be used in drug sensitivity assays and studies of differential proliferation rates of *Leishmania* isolates or strains in a 96-well format.

1. Introduction

Visceral leishmaniasis is a parasitic disease caused by *Leishmania donovani* and *L. infantum* (= *L.chagasi*) (Kinetoplastida). The infection affects both humans and dogs in large areas of the world (i.e. India, Mediterranean Basin, and South America) and it is fatal unless treated. Current first-line chemotherapy of leishmaniasis relies on a rather limited arsenal of drugs, most of which have serious side-effects including nephro- and hepatotoxicity and teratogenicity. Therefore, the identification of new molecules or formulations is an urgent need and has been recognised by WHO as one of the research areas where a sustained effort has to be made (Alvar et al., 2006).

It is assumed that in vivo models have superior predictive value than in vitro models, and that screening using intracellular amastigotes are more convenient than axenic amastigotes and promastigotes (Sereno et al., 2007; Vermeersch et al., 2009; De Muylder et al., 2011; Gupta and Shakya, 2011). In spite of the limitations the promastigote stage is currently used and screening with this parasitic stage has been exploited as a first-step to identify "hit" and "lead" anti-leishmanial compounds in undirected massive screening [High Throughput Screening (HTS)] of chemical libraries (Sharlow et al., 2009; Siqueira-Neto et al., 2010; Walker et al., 2011).

There are several in vitro systems available to determine promastigotes proliferation of *Leishmania* spp (i.e. reporter gene assays, enzymatic determinations, H^3 -thymidine incorporation, colorimetric methods). Among colorimetric methods, resazurin-based Alamar Blue entails several advantages. First, it is simple to use as it requires only a one-step procedure. Other benefits reported are its low cost, environmentally friendly composition and transferability to field sites if necessary (Räz et al., 1997). Unlike other assays, this redox indicator is relatively non-toxic to cells and can be used with long incubation periods (up to 72 h) (Fumarola et al., 2004). This indicator has been extensively used in the related genus *Trypanosoma* (Räz et al., 1997; Rolón et al., 2006; Sykes and Avery, 2009) and in some *Leishmania* spp (Mikus and Steverding, 2000; de Oliveira-Silva et al., 2008; Shimony and Jaffe, 2008; Kulshrestha et al., 2013). Recently, this method has been adapted using HTS with *Leishmania* and two different platforms and a 384-well format (Sharlow et al., 2009; Siqueira-Neto et al., 2010).

Our laboratory has been engaged on the study of the anti-leishmanial antiproliferative effect of different molecules. While Alamar Blue could be easily employed to determine the cytotoxicity for the murine cell line J774 (Wert et al., 2011), results obtained with *Leishmania* promastigotes were inconsistent, since resazurin reduction did not correlate with cell counts. Given the lack of experimental details given in the available literature dealing with *Leishmania*, our aim was to examine the value of Alamar Blue to determine the multiplication and growth inhibition of *L. donovani* and *L. infantum* promastigotes under different culture conditions (variable air-phase, cell density and incubation time). Results showed that optimal conditions of Alamar Blue assay with promastigotes in 96-well microtiter plates included a 5% CO_2 atmosphere, the presence

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on 20 mM Hepes in the culture medium and an initial concentration of promastigotes of ca. 2.5×10^5/mL. With these conditions, reduced resazurin (resorufin) measured by fluorimetry provided an accurate estimation of promastigotes multiplication and could be used for drug screening and IC_{50} estimation.

2. Material and methods

2.1. Parasites

An autochthonous isolate of *L. infantum* (UCM 9), obtained from affected dogs in the area of Madrid (Spain) by the Clinical Service of the Department of Animal Health, Faculty of Veterinary Medicine (Universidad Complutense), and Khartoum 1246 isolate from *L. donovani*, provided by Dr. Toraño (Department of Immunology, Instituto de Salud Carlos III, Madrid) were routinely maintained as promastigotes in RPMI 1640 medium (Lonza Group, Basel, Switzerland) at 26 °C supplemented with heat inactivated (30 min, 56 °C) foetal bovine serum (FBS) (Sera Laboratories International, Horsted Keynes, UK) and 100 U/mL penicillin + 100 μg/mL streptomycin (BioWhittaker, Verviers, Belgium) in 25 mL culture flasks.

2.2. Chemicals

Alamar Blue was purchased from AbD Serotec (Oxford, UK). Allicin (2-Propene-1-sulfinothioic acid S-2-propenyl ester) was obtained as liquid Allisure® from Allicin International Ltd (Rye, East Sussex, UK) at a concentration of 5000 ppm and kept at a temperature of −80 °C until used. Amphotericin B (AmB) was obtained as fungizone (Sigma, St. Louis, USA).

2.3. Promastigote assays

Depending on the experiment promastigotes were cultured in flat-bottomed 96-well cell culture microtiter plates with lid (Costar, Corning, NY, USA), in microtiter plates wrapped with Parafilm®, or in plates sealed with Thermal adhesive film for PCR plates (Simport, Beloeil, Canada). For comparative purposes promastigote cultures were also done in 1.5 mL eppendorf ® tubes. Cultures were carried out at 26 °C in aerated culture chamber or incubated in a 95% air/5% CO_2 humidified atmosphere. Culture media (RPMI 1640) with and without additional 20 mM Hepes were employed depending on the experiment.

2.4. Alamar Blue assay

Concentration of resorufin, the product of reduction of resazurin, in the *Leishmania* cultures was determined following the manufacturer's recommendations by reading the absorbance (A) at 570 and 600 nm, and fluorescence (550 nm excitation wavelength, 590 nm emission wavelength) in a FLUOstar Omega (BMG Labtech, Ortenberg, Germany) fluorimeter. Fluorescence intensity was expressed as arbitrary units (A.U.). Briefly, mid-log phase promastigotes were added to the wells of microtiter plates or eppendorf tubes up to a volume of 200 μL/well. After 24 h, 20 μL Alamar Blue (10% v/v) was added and the cultures were kept for 24, 48 or 72 additional hours. Absorbance and fluorescence intensity were determined every 24 h. Promastigote counts were carried out in Neubauer improved chambers and cell viability was assessed by trypan blue exclusion staining. Untreated cultures, wells without cells and the maximal concentration of the drugs, and wells with culture medium and Alamar Blue (10% v/v) were included as controls. All experiments were performed at least in triplicate.

2.5. Statistical analysis

Results were expressed as means ± standard deviation. Data were compared by analysis of variance (one- and two-ways ANOVA) and GLM analysis using GraphPad Prism5. Differences were considered significant when $p < 0.05$. Figures were also prepared with GraphPad Prism5.

3. Results

3.1. Determination of optimal cell density

Different concentrations (10^4, 2.5 x10^4, 5×10^4, 7.5×10^4, 10^5, 2.5×10^5, 5×10^5, 7.5×10^5, 10^6) of mid-log phase promastigotes of *L.donovani* and *L.infantum* were added in a final volume of 200 μL/well in 96-microtiter plates. Cultures were carried out at 26 °C in plates with lid under a 5% CO_2 atmosphere or in film-sealed plates. After 24 h incubation, Alamar Blue was added and the plates were kept for 24, 48 or 72 h. For each time determination a plate was used. Resazurin was effectively reduced to resorufin in the medium, evidenced by the higher levels of absorbance and, particularly, fluorescence related both to the initial promastigotes density and the time of culture. Absorbance determinations, especially with low initial promastigote concentration ($<10^5$ promastigotes/well) were more variable (not shown). This variability was not observed when cultures were kept in the CO_2 atmosphere. Results were more consistent when resorufin concentration was determined by fluorimetry. Fluorescence was significantly higher (ca. 2 times) when cultures were exposed to 5% CO_2 (Fig. 1A) as compared to those performed in film-sealed plates (Fig. 1B). Highest levels of fluorescence were obtained with initial inoculums of 2.5 to 5×10^5 promastigotes/well from both *Leishmania* species, after 72 h and exposition to CO_2.

3.2. Correlation between Alamar Blue reduction and promastigotes multiplication

Preliminary results obtained in our laboratory allowed the use of the redox indicator to determine the proliferation of *Leishmania* promastigotes in culture tubes. However, as shown above, for a given initial number of promastigotes and time of incubation, significant differences ($p < 0.05$) were found in the concentration of resorufin estimated by absorbance and, particularly, fluorimetry depending on the exposition to CO_2 or to a limited air phase in the sealed microtiter plates. To rule out the possibility of resazurin reduction being an inaccurate estimation of *Leishmania* multiplication, promastigotes (2.5×10^5/well) were cultured in 96-well plates under a CO_2 atmosphere or under air phase. For comparative purposes parallel cultures were done in eppendorf tubes. In all cases cell multiplication was estimated by fluorimetry and cell counting of viable *Leishmania* in Neubauer chamber. A set of cultures was employed for each time determination (24, 48, 72 and 96 h).

Fig. 2 shows that no significant differences ($p > 0.05$) were found between cultures in the experiment irrespective of the exposition to 5% CO_2 or to an unlimited air phase. After 96 h, all cultures reached values ca. 2.7×10^6 promastigotes/mL in both *Leishmania* spp (Fig. 2A). However, resazurin concentration determined by fluorimetry was strongly dependent on the culture conditions (Fig. 2B). Highest levels of fluorescence were seen in cultures exposed to CO_2 and the lowest values were present using standard culture conditions with air atmosphere. Cultures in eppendorf tubes, with a limited amount of air, displayed intermediate values. These results were consistent with the absence of colour change in the standard microtiter plates in spite of the active multiplication. These results suggested the importance of the air phase of the cultures in the usefulness of Alamar Blue method to determine *Leishmania* proliferation and also the need of standardization of the assay.

3.3. Effect of atmosphere and buffer on resazurin reduction by promastigotes of Leishmania

Promastigotes of *L.infantum* and *L.donovani* were cultured in 96-well standard microtiter plates exposed to 5% CO_2, in standard microplates, in microplates wrapped with Parafilm, in microplates in sealed plastic

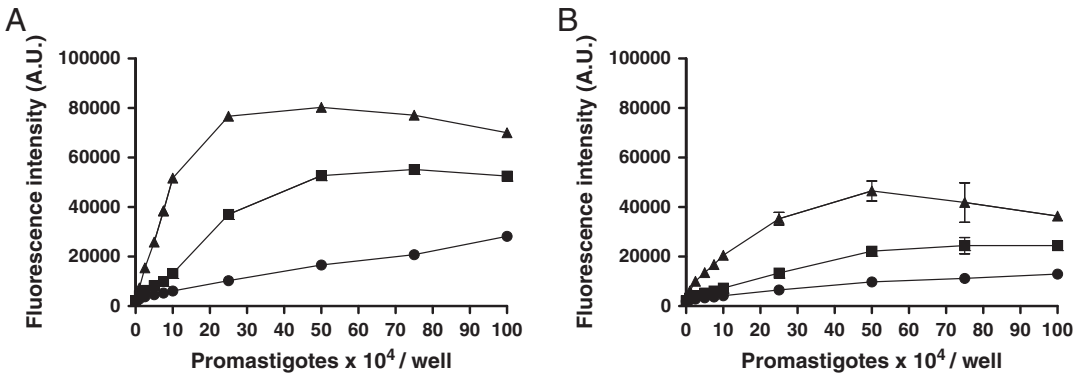

Fig. 1. Effect of incubation time with Alamar Blue on the fluorescence curves with promastigotes of *L.donovani*. Increasing initial plating concentrations of promastigotes were incubated with Alamar Blue (10% v/v) and the fluorescence intensity determined (arbitrary units, A.U.) (excitation wavelength 560 nm, emission wavelength 590 nm). A: In the presence of 5% CO_2. B: Film-sealed microplates. ●: 24 h; ■: 48 h and ▲: 72 h. Results are means ± standard deviations of 3 determinations.

bags or film-sealed microplates for PCR. Moreover parallel cultures were done in eppendorf tubes to monitor cell viability (trypan Blue exclusion dye) and multiplication (counting in Neubauer chamber). Cultures with standard RPMI medium and medium with 20 mM Hepes were employed. Absorbance and fluorescence were determined 24, 48 and 72 h after Alamar Blue addition.

As expected time-dependent fluorescence increases were observed in all culture conditions and both *Leishmania* species earlier than absorbance variations (not shown) in the determinations carried out. Fig. 3 shows the results obtained with *L. infantum* and similar values were obtained for *L.donovani*. It was found that the presence of 5% CO_2 or limited air availability was critical to get the highest fluorescence values. Microplates with lid exposed to air had the lowest levels, comparable to those found in Parafilm wrapped plates. By its part, 96-well plates cultured in a CO_2 incubator displayed the highest resorufin concentrations in all time determinations carried out. In cultures done in eppendorf tubes, film-sealed microplates and plastic-sealed microplates intermediate values of absorbance and intensity of fluorescence were obtained. Moreover, the presence of additional 20 mM Hepes improved the resazurin reduction in all culture conditions, particularly when film-sealed microtiter plates and eppendorf tubes were employed ($p < 0.01 - p < 0.001$).

3.4. Value of resazurin reduction measured by fluorimetry for drug screening in Leishmania

Value of resazurin transformation by fluorimetry for drug screening in 96-well microtiter plates was tested with two compounds with antileishmanial activity, namely allicin and AmB. In all cases 2.5×10^5 promastigotes/well for both species were used. In the case of allicin, 10, 30, 60 and 120 μM concentrations were used and microplates in CO_2 or aerated incubator, and with and without additional 20 mM Hepes in the medium, were used. Parallel counts were done in Neubauer haemocytometer with cultures in eppendorf tubes exposed to the same allicin concentrations. Cultures were treated for 24 h with allicin and absorbance and fluorescence were monitored at 24, 48 and 72 h after exposure to the drug. A range of AmB (0, 0.001, 0.002, 0.003, 0.004, 0.005, 0.006, 0.007, 0.008, 0.009, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.5, 1 and 10 μM) was tested with the optimal assay conditions found (5% CO_2, 20 mM Hepes). Fluorescence and absorbance were measured 72 h after exposure to the antibiotic.

Fig. 4A shows the relationship between the allicin concentration and the growth inhibition of *L.donovani* promastigotes determined by cell counting and fluorescence of the cultures. Addition of 20 mM Hepes improved the detection of fluorescence in aerated microplates whereas no correlation between growth inhibition and resorufin levels in the cultures was observed without Hepes in this case. Our results clearly showed that Alamar Blue reduction closely correlated to microscopic cell counts and viability, in the allicin concentration range examined, provided that a 5% CO_2 atmosphere and 20 mM Hepes were present (Fig. 4B). Comparable results were obtained for *L.infantum*. Inhibition of multiplication of *Leishmania* promastigotes in the presence of AmB confirmed the value of the resorufin level with fluorimetry using the assay conditions described in the screening and IC_{50} determination of this antileishmanial drug. Actually, for the particular *L.infantum* isolate

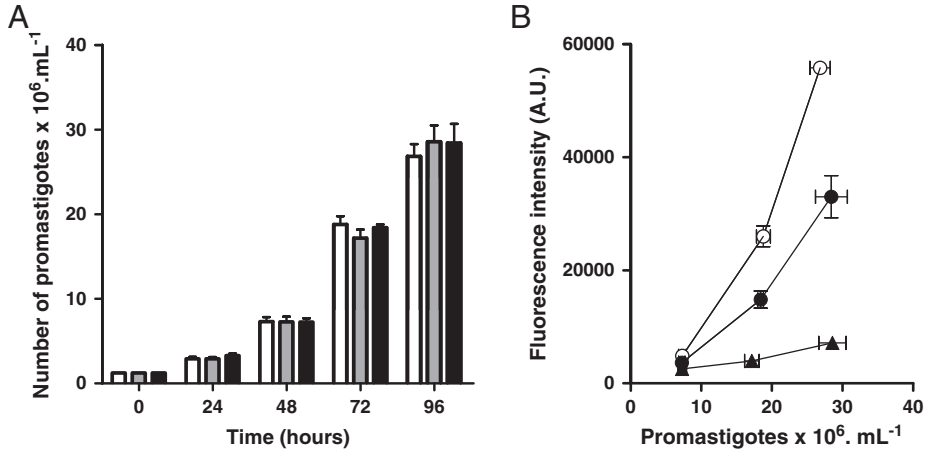

Fig. 2. A: Proliferation of *Leishmania* promastigotes under different culture conditions [White bars: CO_2 atmosphere; grey bars: aerated cultures; solid bars: eppendorf tubes]. Promastigotes were seeded (2.5×10^5 promastigotes/well) on day 0. Alamar Blue was added 24 h later. B: Relationship between fluorescence intensity (A.U.) and promastigote counts at different times and culture conditions ○: 5% CO_2 atmopshere; ●:aerated microplates; ▲:eppendorf tubes. Results are means ± standard deviations of 3 determinations.

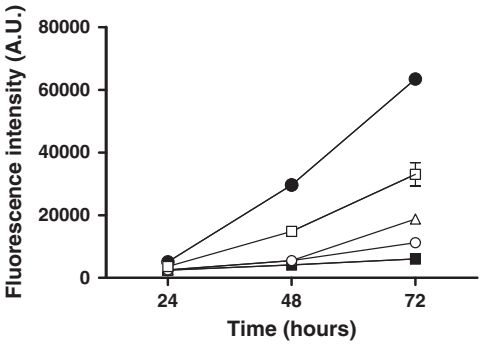

Fig. 3. Fluorescence intensity (A.U.) of promastigote cultures of *L.donovani* with additional buffer (20 mM Hepes) at different times of incubation. All cultures except □ were carried out in 96-well microplates. ●: 5% CO_2 atmosphere; ■:aerated microplates; ▲: Parafilm wrapped plates; ○: plastic bag-sealed microplates; Δ: Film-sealed microplates; □: eppendorf tubes. Results are the means ± standard deviations of 3 experiments.

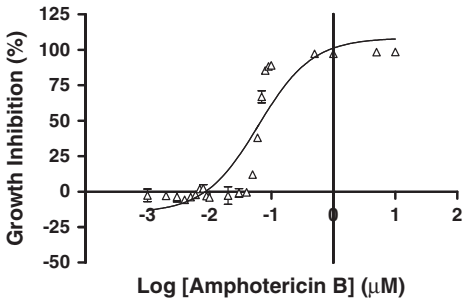

Fig. 5. Determination of IC_{50} of amphotericin B for *L.donovani* promastigotes using Alamar Blue reagent. Experimental conditions included an initial plating concentration of 2.5×10^5 promastigotes/well, 5% CO_2 atmosphere and 20 mM Hepes. Results are the means ± standard deviations of 3 experiments.

employed an approximate IC_{50} of 0.07 μM was obtained by cell counting >whereas using the best-fit values with fluorescence determinations the value obtained was 0.0618 μM (Fig. 5). Fig. 6 shows a representative result of the effect of CO_2 and 20 mM Hepes on the reduction of resazurin.

4. Discussion

Present results, with the optimal conditions described, show that Alamar Blue, and presumably other resazurin-based methods, is an accurate assay to determine the multiplication of *Leishmania* promastigotes and growth inhibition elicited by antileishmanial drugs. Assay conditions examined in a checkerboard manner showed that several factors were relevant to obtain consistent results, namely an initial plating density of promastigotes ca. 2.5×10^5, besides the presence of a 5% CO_2 atmosphere and additional buffering of the medium with 20 mM Hepes. Our results on the optimal cell density were comparable to the findings by Mikus and Steverding (2000) with *L.major* and Shimony and Jaffe (2008) with *L.donovani* and support the lower reduction rate of *Leishmania* compared to *T. cruzi* epimastigotes (Rolón et al., 2006) and bloodstream trypanosomes (Räz et al., 1997). A linear fluorescence response was observed with lower plating densities of promastigotes and therefore the method could probably be used in 72 h experiments in the range of 5×10^4 to 10^5 promastigotes/well. The plateau observed with higher cell densities and incubation time are probably related to the conversion of resorufin to colourless and non-fluorescent hydroresorufin (O'Brien et al., 2000).

Alamar Blue method has been successfully employed to monitor the growth of mammalian cells (Ansar Ahmed et al., 1994; Nakayama et al., 1997; O'Brien et al., 2000; Sykes and Avery, 2009). Most of the assays have been performed in a 95 % air/5 % CO_2 atmosphere, including those carried out in African trypanosomes (Räz et al., 1997; Sykes and Avery, 2009), or in plastic sealed bags (Martin et al., 2003). More than probably the performance of a redox indicator added to a culture medium would be affected by the gas-phase although this factor apparently has not been considered in the previous contributions on *Leishmania* (de Oliveira-Silva et al., 2008; Shimony and Jaffe, 2008; Vermeersch et al., 2009; Kulshrestha et al., 2013) and *T. cruzi* epimastigotes (Rolón et al., 2006) using 96-well microtiter plates or, more recently with the HTS with 384-well format, using *L.major* (Siqueira-Neto et al., 2010). Our results showed that, using the 96-well plate format incubation in a 5 % CO_2 gas phase yielded accurate results of cell growth and inhibition in *Leishmania* ($r^2 = 0.9713$). The carbon dioxide dissolved in the medium allowed the efficient reduction of resazurin to the fluorescent resorufin. Actually, O'Brien et al. (2000) highlighted the need of CO_2 in the medium when reduction is occurring in order to capture electrons. In the absence of 5% CO_2 additional buffering with Hepes could circumvent some of the limitations provided that a limited volume of gas phase was present (capped eppendorf tubes, film sealed plates, sealed plastic bags). Earlier and more sensitive estimation of resorufin was obtained by fluorimetry and thus should be preferred to spectrophotometry. The significantly higher sensitivity of fluorimetry allowed the

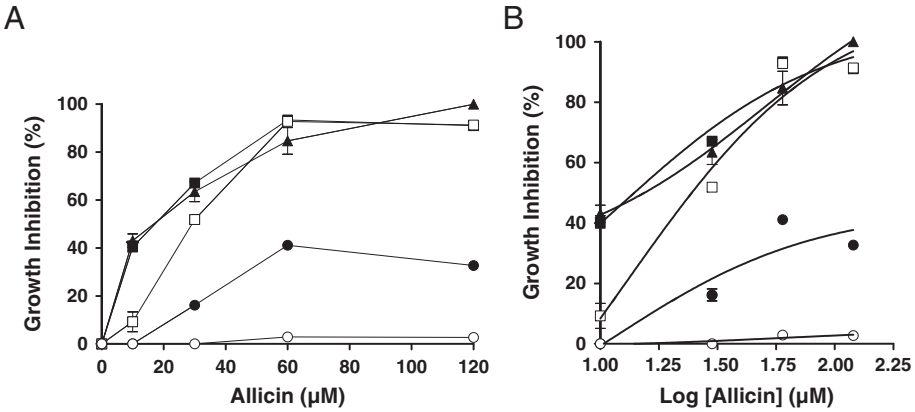

Fig. 4. A: Growth inhibition of *L.donovani* promastigotes in the presence of allicin (24 h) estimated by cell counts and fluorescence intensity (A.U.). ▲: cell counts; ■: 5% CO_2 atmosphere and 20 mM Hepes; □: 5% CO_2 atmosphere; ●: aerated microplates and 20 mM Hepes; ○: aerated microplates. B: Best-fit representation of the effect of allicin on growth inhibition of *L.donovani* promastigotes, determined by fluorescence and cell countings. ▲: cell counts; ■: 5% CO_2 atmosphere and 20 mM Hepes; □: 5% CO_2 atmosphere; ●: aerated microplates and 20 mM Hepes; ○: aerated microplates. Results are means ± standard deviations of 3 experiments.

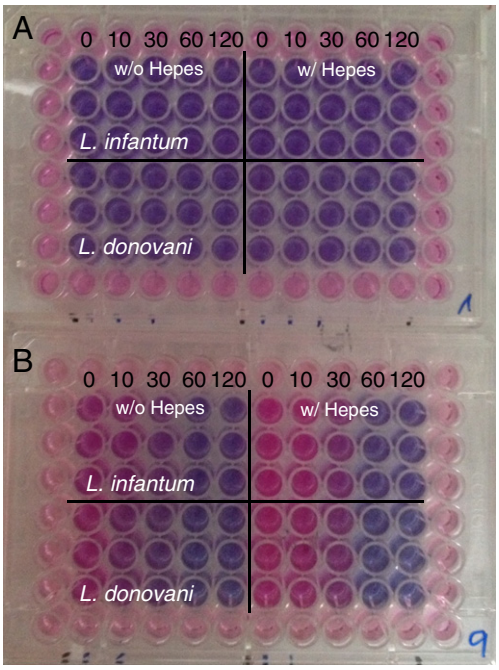

Fig. 6. Representative image of *L.infantum* and *L.donovani* promastigote cultures exposed to different concentrations of allicin (0, 10, 30, 60 and 120 μM), with and without 20 mM Hepes, in the presence of 5% CO_2 atmosphere (B) or in air exposed 96-well microplates (A). Marginal wells were not used to avoid edge effect.

earlier identification of anti-leishmanial activity of allicin. Promastigote multiplication and inhibition could be easily detected by fluorescence after 48 h whereas observation of significant absorbance variations needed 72 h as observed by Mikus and Steverding (2000).

Visceral leishmaniasis, both human and animal, caused by *L.donovani* and *L.infantum* (=*L.chagasi*) continues being a challenge for medical doctors and veterinary clinicians in many regions of the world. Available drugs have important shortcomings (nephrotoxicity, hepatotoxicity, gastrointestinal disturbances) and, moreover, in some areas *Leishmania* strains resistant to first line drugs have been described (Croft et al., 2006). Identification of new potentially effective drugs or preparations requires robust and reliable screening methods. In vivo tests of efficacy must be reduced at a minimum by both ethical and economic reasons and therefore in vitro screening of potentially useful compounds is the first step to identify "hits and leads". Obviously, the best in vitro model to test antileishmanial activity of compounds is done on the intracellular amastigote within macrophages (Sereno et al., 2007). However, intracellular amastigotes screening is only available to some laboratories, species such as *L. donovani*, and specially *L.infantum*, have a slow rate of division (Gupta and Shakya, 2011) and some *Leishmania* isolates from clinical cases do not infect the macrophage cell lines employed. By its part, promastigotes are easy to culture and they share with amastigotes many metabolic pathways and thus some of the most commonly used antileishmanial agents, such as AmB or miltefosine, are effective against both parasite stages. Therefore, promastigote screening is useful (Sharlow et al., 2009) before testing in intracellular amastigotes, and in vivo, and can be employed to monitor susceptibility of clinical isolates to some drugs (i.e. Miltefosine, Kulshrestha et al., 2013).

Among the available methods (^{3}H-thymidine incorporation, microscopic counting, quantitative PCR, enzymatic and colorimetric methods) to determine proliferation of *Leishmania* promastigotes, resazurin-based assays such as Alamar Blue can be carried out in a single step, are unaffected by the red phenol from the culture medium and have the advantage of low cost and negligible toxicity. Validation with *L.donovani* and *L.infantum* promastigotes showed that this method could be advantageously used in trials of cell proliferation and drug screening, including the determination of approximate IC_{50} values against promastigotes. Conditions described for *Leishmania* included the presence of 5% CO_2, or reduced air availability, to allow the efficient reduction of resazurin to resorufin, in a promastigote-density related manner. The method was improved by fluorimetry although spectrophotometry could also be employed. Given the accuracy (fluorescence vs. promastigotes density), the low number of promastigotes needed and results obtained in the determination of IC_{50} for AmB and allicin as compared to the results with microscopic counting (i.e. Corral-Caridad et al., 2012), this 96-well microplate could be adapted for a 384-well format.

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Effect of allicin on promastigotes and intracellular amastigotes of *Leishmania donovani* and *L. infantum*

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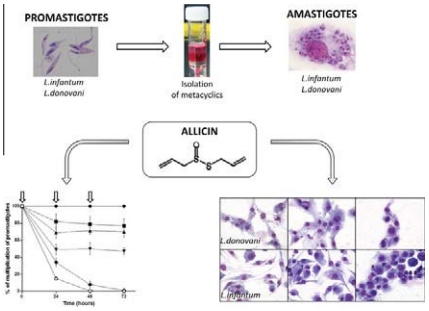
HIGHLIGHTS

- Allicin inhibits in vitro the proliferation of promastigotes of *Leishmania*.
- No significant toxicity for mammalian cells was found up to 40 μM allicin.
- Low micromolar allicin (10 μM) reduced the multiplication of *Leishmania* amastigotes.
- Serial treatment every 24 h with the compound improved its antileishmanial effect.

GRAPHICAL ABSTRACT



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ABSTRACT

Anti-leishmanial activity of allicin (=diallyl thiosulphinate) has been tested in vitro against promastigotes and intracellular amastigotes of *Leishmania donovani* and *Leishmania infantum*. Macrophage infections have been carried out in vitro in the murine cell line J774 and ex vivo with peritoneal macrophages from BALB/c mice with a modified method to isolate metacyclic promastigotes. The compound has shown a significant in vitro effect on the multiplication of promastigotes of *L. donovani* and *L. infantum* in a time- and dose-dependent manner. It has been shown for the first time the inhibition of multiplication of intracellular amastigotes of *Leishmania* by allicin. Inhibitory concentrations of the compound were in the micromolar range (10–30 μM) for both *Leishmania* species. Antileishmanial effect of allicin apparently was not related to products of degradation of the molecule as assessed by mass spectrometry analysis. Inhibitory activity of allicin against promastigotes and intracellular amastigotes increased when the compound was added to the cultures every 24 h. Two administrations of 5 μM allicin inhibited by ca. 50% the proliferation of *Leishmania* amastigotes. Low toxicity for mammalian cells of this compound suggests the interest of exploring the value of allicin in combined therapy against leishmanial infections.

1. Introduction

Human *Leishmania* infections are present in all inhabited continents, with around 12 million people infected, 2 million new cases every year and 350 million people living in areas at risk (WHO, 2000). Leishmaniasis is a spectral infection with a range of clinical presentations, from self healing dermal infections to visceral fatal processes. Visceral leishmaniasis caused by *Leishmania donovani* and *Leishmania infantum* = *Leishmania chagasi* is a severe disease whose distribution has expanded frequently associated to HIV infection (Desjeux, 2004). In addition to human cases, canine leishmaniasis by *L. infantum* (Mediterranean Basin) and *L. chagasi* (South America) constitutes a first order pathology in veterinary clinics besides its zoonotical importance as reservoirs. The main control system of leishmaniasis in both humans and dogs is chemotherapy. Liposomal amphotericin (AmB), paromomycin and miltefosine were considered the most promising drug for

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chemotherapy of leishmaniasis (www.who.int/tdr/diseases/leish). However, these drugs have some important shortcomings, including high toxicity and teratogenicity of some of them, absence of parasitological cure in most cases and unaffordable prices for some of the compounds (Sundar and Chaterjee, 2006). Moreover, in some areas resistant *Leishmania* strains to commonly used drugs have emerged (Croft and Coombs, 2003; Croft et al., 2006). Therefore, new drugs or combinations are needed (Alvar et al., 2006; Chappuis et al., 2007; Mishra et al., 2007). This scenario favors the exploration of alternative drugs. Among them, allicin (diallyl thiosulfinate = 2-Propene-1-sulfinothioic acid S-2-propenyl ester), a natural product present in plants of the Family Alliaceae, including garlic, has shown antibacterial activity against *Helicobacter pylori* (O'Gara et al., 2000; Cañizares et al., 2004) and methicillin-resistant *Staphylococcus aureus* (MRSA) (Cutler and Wilson, 2004) besides a full range of medicinal and antimicrobial effects (Lawson, 1998; Ankri and Mirelman, 1999). Reports on the antifungal (*Candida*) (i.e. Khodavandi et al., 2011) and antiprotozoal (Mirelman et al., 1987: *Entamoeba*; Coppi et al., 2006: *Plasmodium*; Waag et al., 2010: *Plasmodium* and *Trypanosoma*) activity of this molecule have been published. Ankri and Mirelman (1999) referred to unpublished results on the inhibition of *Leishmania major* growth by 30 μM allicin. Moreover, McClure et al. (1996) observed that allicin, besides other garlic extracts, was effective against the in vitro growth of *Leishmania mexicana* and *L. chagasi* promastigotes but no further testing was carried out. On these grounds we have tested the potential antiproliferative effect of allicin on promastigotes and amastigotes of the two major causative agents of visceral leishmaniasis, *L. donovani* and *L. infantum*. Our results showed that micromolar concentrations of allicin are able to inhibit the proliferation of both stages and species.

2. Material and methods

2.1. Parasites

An autochthonous isolate of *L. infantum* (UCM 9), obtained from affected dogs in the area of Madrid (Spain) by the Clinical Service of the Department of Animal Health, Faculty of Veterinary Medicine (UCM), and "Khartoum 1246" isolate from *L. donovani*, provided by the Department of Immunology, Instituto de Salud Carlos III, Madrid, were routinely maintained as promastigotes in RPMI 1640 medium (Lonza Group, Switzerland) at 26 °C supplemented with heat inactivated (30 min at 56 °C) fetal bovine serum (FBS) (Sera Laboratories International, Horsted Keynes, UK), 100 U/mL penicillin + 100 μg/mL streptomycin (BioWhittaker, Verviers, Belgium) and 1% L-glutamine in 25 ml culture flasks.

2.2. Promastigote assay

Promastigotes were cultured in 10 mL sterile polystyrene tubes. Allicin was obtained as liquid Allisure® from Allicin International Ltd. (Rye, East Sussex, UK). Dilutions of the compound were added up to 2 mL final volume. After 24, 48 and 72 h at 26 °C, live promastigotes were counted in an improved Neubauer chamber. Results from treated cultures were expressed as % of the multiplication obtained in untreated control cultures. Cultures were performed at least in triplicate and cultures treated with 0.1 μM amphotericin B (AmB) were included as control.

2.3. Cell cytotoxicity assay

2.3.1. MTT assay

MTT assay was employed to determine the toxicity of allicin for both J774 cells and mouse peritoneal macrophages. Peritoneal macrophages were isolated from female BALB/c mice. Cell suspension with RPMI-1640 medium (supplemented with 10% heat-inactivated FCS, 1% penicillin/streptomycin, 1% L-Glutamine) were seeded in 96 well flat-bottomed cell culture plates (Corning, New York, USA). Plates were incubated overnight in a humidified atmosphere 5% CO_2/air atmosphere at 37 °C to ensure cell adherence. After 24 h, cells (4×10^6 peritoneal macrophages/mL; 5×10^3/mL J774 cells) were treated with increasing concentrations of allicin (1–120 μM). Non-treated cells were included as a negative control. At the end of the drug exposure period (24 h), the medium from all the wells was removed and the cells were fed with 200 μL of fresh medium, and plates were incubated for another 24 h. The viability of cells was determined by the MTT reduction assay as described before (Plumb, 2004). Briefly, 200 μL of fresh medium and 50 μL of the MTT (Sigma, St. Louis, USA) solution (5 mg/mL in PBS) were added to all wells; plates were incubated for 4 h protected from light in a humidified atmosphere at 37 °C. The remaining formazan crystals were dissolved by adding 200 μL of DMSO (Sigma, St. Louis, USA) and 25 μL/well of Sorensen's glycine buffer were added to stop the reaction. Absorbance was read at 570 nm. The approximate IC_{50} concentration was determined as the allicin concentration required to reduce the absorbance to half that of the untreated control wells (considered as 100% viable). Three independent experiments, with eight replicates, were carried out for assessing the cytotoxicity of allicin with this method.

2.4. Amastigote assay

In the amastigote assay, infection was carried out using a modification of the methods developed by us (Méndez et al., 1996; Wert et al., 2011) and the procedure described by Yao et al. (2008) to isolate metacyclic promastigotes. Depending on the experiment, infections with *Leishmania* were carried out in vitro, using the murine macrophage cell line J774, and ex vivo, with peritoneal macrophages from BALB/c mice. Stationary phase *Leishmania* promastigotes (day 7) were centrifuged without brake (365*g*, 10 min) through a Ficoll gradient (0, 10% in Medium 199, and 30% in sterile PBS). Parasites recovered from the interphase between 0% and 10% Ficoll were washed with fresh medium and opsonised with 15% normal mouse serum in a solution 1:1 (v/v) RPMI medium and HBSS, 0.15 mM $CaCl_2$ and 1 mM $MgCl_2$ for 30 min at 37 °C. Murine J774 cells (2.5×10^4 cells/well) were cultured in 8-well Lab-Tek chambers (Nunc, Roskilde, Denmark), for 16 h with mitomycin C (Calbiochem, Merck, USA) at 1 μg/mL was to avoid cell proliferation. The washed parasites were added to the macrophage cultures (parasite: macrophage ratio 10:1) and maintained at 33 °C in 5% CO_2 overnight without mitomycin. Non-internalized promastigotes were eliminated by thorough washing three times with fresh medium. Compounds were added at appropriate concentrations and maintained for 24 or 48 h depending on the experiment carried out.

In the ex vivo experiments, peritoneal macrophages were obtained from female BALB/c mice and cultured as described above except for the absence of mitomycin treatment. Macrophage concentration was adjusted to 2.5×10^5/well up to a final volume of 200 μL/well. Metacyclic promastigotes were isolated and opsonised as previously described and parasite: macrophage ratio was 5:1. After a contact period overnight, Labtek plates were washed with RPMI 1640 and the compounds were added at different concentrations and times, and kept at 37 °C for 24, 48 or 72 h depending on the experiment. Slides were fixed and stained (May- Grünwald-Giemsa, Merck Darmstadt, Germany) and the number of amastigotes/100 cells and % of infected cells was determined. Cultures were performed at least in triplicate.

2.5. Solid phase microextraction (SPME) and gas chromatography/mass spectrometry (GC/MS)

The SPME technique was carried out using a 85 μm Light Blue Carboxen/PDMS fiber coated with polydimethylsiloxane. Samples

(100 μL) from the experimental cultures were prepared in extraction vial of 20 mL. RPMI, PBS and internal standards (1 μL) were included as control samples. Extraction was performed at 40 °C during 60 min under agitation (250 rpm) using an automatic injector Multifiber Change CTC Analysis model Mps2xl. Thermal desorption of analytes from the fiber was carried out in the chromatograph injector port at 220 °C in splitless mode. GC was performed with an Agilent Technologies model 5975C gas chromatograph, at a constant flow of 1 mL/min for 5 min using a VA624 column (60 m × 0.25 mm; 1.4 μm). Oven temperature programme employed included an initial isothermal time at 35 °C for 5 min, followed by a 5 °C/min ramp up to 240 °C setting during 30 min in isothermal mode. MS was performed with Agilent Technologies model 7890A mass analyzer without solvent delay in scan mode and in the 35–650 *m*/*z* mass range. Analysis was performed at the MS Unit-UCM.

2.6. Transmission electron microscopy (TEM)

The specimens were fixed for 3 h in a solution of glutaraldehyde 2.5% + paraformaldehyde 4% in PBS at 4 °C followed by 2% osmium tetroxide + 3% potassium ferrocyanide for 1 h, room temperature, darkness. Samples were dehydrated with increasing concentrations of acetone in water and soaked in resin SPURR (Fedelco Madrid, Spain). The resin was polymerized at 70 °C for 72 h. Thin slices, 0.5 μm thick, were dyed with Richardson's methylene blue and observed by light microscopy. Ultra thin slices, 70 nm thick, were treated with 2% uranyl acetate, as well as Reynold's lead citrate solution and examined with a Zeiss EM 902, transmission electron microscope (Carl Zeiss, Germany) at the SME-UCM.

2.7. Statistical analysis

Statistical significance of differences between mean values obtained in the different groups was performed by one-way and two-way ANOVA, repeated measures analysis and Bonferroni test. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Allicin inhibits the multiplication of Leishmania promastigotes

Micromolar concentrations of allicin effectively inhibited the growth of promastigotes of *L. infantum* and *L. donovani* in a dose- and time-dependent manner ($p < 0.0001$) (Fig. 1a and b). Only a moderate anti-leishmanial activity (ca. 30–40% reduction as compared to control) was observed with the lowest concentration (30 μM) whereas 60 μM and 120 μM provoked a reduction over 80% of the initial population of promastigotes after 24 h ($p < 0.05$). Addition of 120 μM allicin completely eliminated the promastigotes after 72 h. There was a certain recovery of *Leishmania* cultures treated with 30 μM allicin from day 1 onwards. Microscopical observation showed active cell division with this concentration (not shown) and the motility of promastigotes was higher after 72 h than in similarly treated cells during 48 h. As expected, 0.1 μM amphotericin provoked a reduction bewteen 80% and 90% of the promastigote number after 24 h. Treatment with allicin (60 and 120 μM) provoked important ultrastructural alterations of *Leishmania* promastigotes (Fig. 2). Cellular swelling with reduction of electrodense material in the cell cytoplasm and generalized

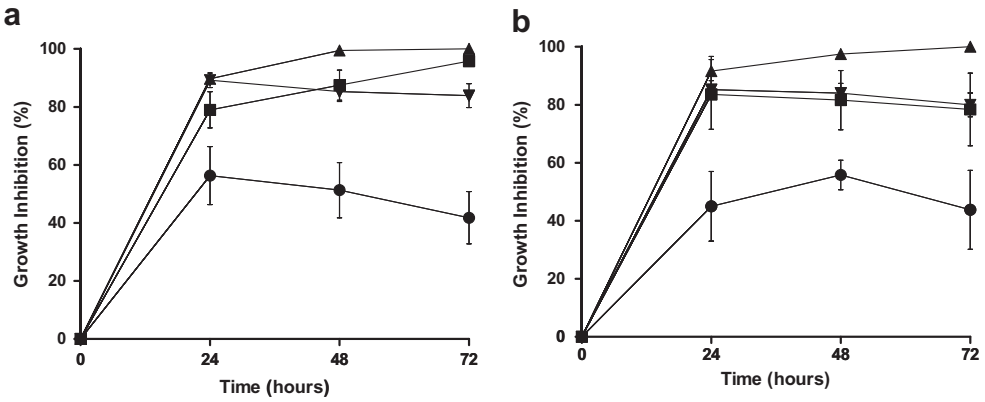

Fig. 1. Effect of allicin on the proliferation of *Leishmania infantum* (Fig. 1a) and *Leishmania donovani* promastigotes (Fig. 1b). Compounds were added at time 0. ●: 30 μM; ■: 60 μM; ▲: 120 μM; ▼: amphotericin (0.1 μM). Results are the means of three experiments ± standard deviation.

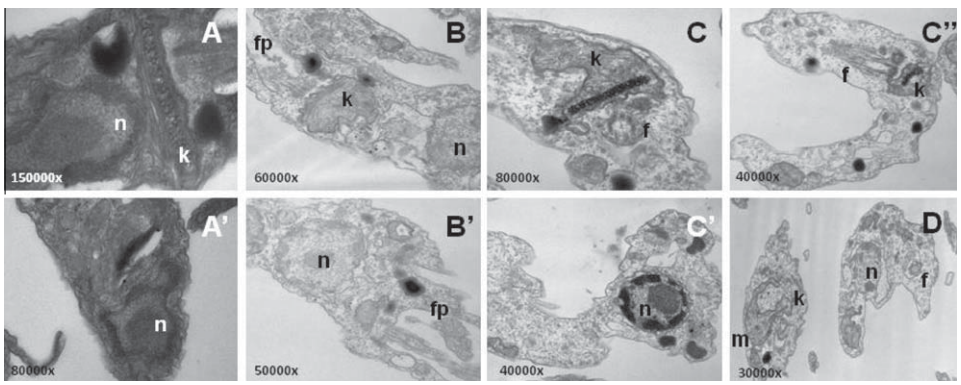

Fig. 2. Transmission electron micrographs of promastigotes of *Leishmania infantum* treated with 60 μM (B and B′), 90 μM (D) and 120 μM (C, C′, C″) as compared to untreated control cultures (A and A′). Treated promastigotes showed generalized vacuolization, reduction of electrodense material in the cytoplasm, cellular swelling(C″), loss of integrity of mitochondrial and kinetoplast membranes (B and C), and chromatin condensation in the nucleus (C′) and kDNA (C). n: nucleus, k: kinetoplast, fp: flagellar pocket, f: flagellum, m: mitochondrion. Magnifications are given on each micrograph.

vacuolisation were observed. In addition, there was deformation and loss of integrity of membranes from both mitochondrion and kinetoplast, and chromatin condensation in the nucleus and kDNA.

3.2. Toxicity of allicin for mammalian cells

Toxicity of a range of allicin concentrations on the murine macrophage cell line J774 and mouse peritoneal macrophages was examined (Fig. 3) with MTT method. Apparently, all concentrations of allicin affected cell viability ($p < 0.0001$) and 10 μM almost completely arrested cell proliferation of J774 after 24 h (Fig. 3a). However, results obtained with MTT did not correlate with the microscopical examination of May-Grünwald-Giemsa stained cultures. Cultures treated up to 60 μM allicin did not show significant microscopic alterations as can be observed in Fig. 4(c and c′). The apparent discrepancy could be related to the lack of discrimination between cytotoxic and cytostatic effects from these colorimetric methods. On these grounds toxicity was tested in non-dividing primary cells, mouse peritoneal macrophages (Fig. 3b). Results showed that allicin did not induce any significant ($p < 0.0001$) toxicity up to a concentration of 60 μM and the cell viability was reduced only by 10% after exposition of macrophages to this concentration.

3.3. Allicin also inhibits the multiplication of intracellular amastigotes of Leishmania

The apparent low toxicity prompted us to examine the anti-leishmanial activity of allicin on intracellular amastigotes of *Leishmania*. Given our initial experimental design, the effect of allicin was tested on previously infected J774 cells. The modification of the infection procedure developed was highly successful since ca. 70% of the J774 cells were infected with *Leishmania* amastigotes. Results obtained showed that the compound was effective against *Leishmania* amastigotes multiplication after 24 h incubation. This anti-leishmanial effect was observed in both *L. donovani* and *L. infantum* in spite of the higher amastigote burden in *L. donovani* infections (Fig. 4). Apparently the compound behaved as leishmaniostatic since no notable reduction in the infection rate was appreciated in any of the species tested whereas a significant ($p < 0.05$) reduction in the amastigote burden was induced. Antileishmanial effect of allicin was dose-dependent (Fig. 5) and dose–response curves yielded an approximate IC_{50} value of 10 μM for both species.

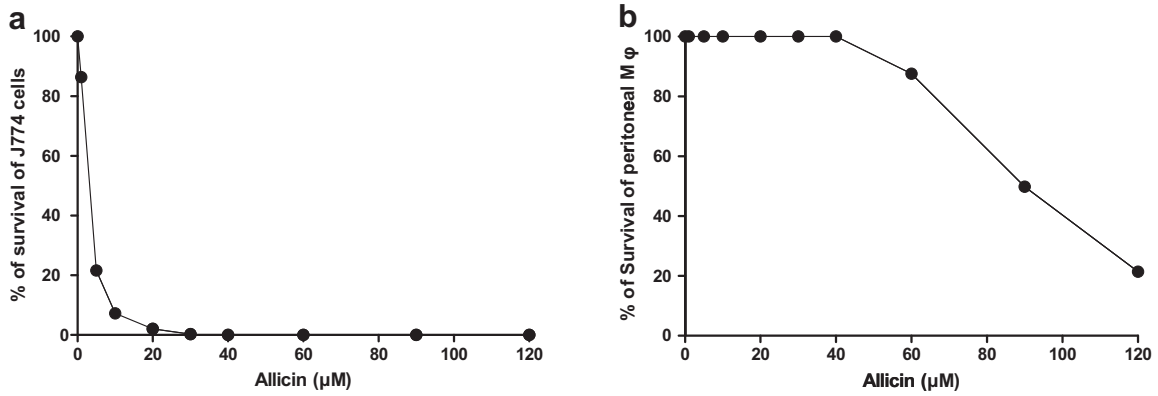

Fig. 3. Effect of different concentrations of allicin on the viability of J774 cells (a) and BALB/c mice peritoneal macrophages (b). Cytotoxicity was determined by MTT assay. Results are means of eight determinations.

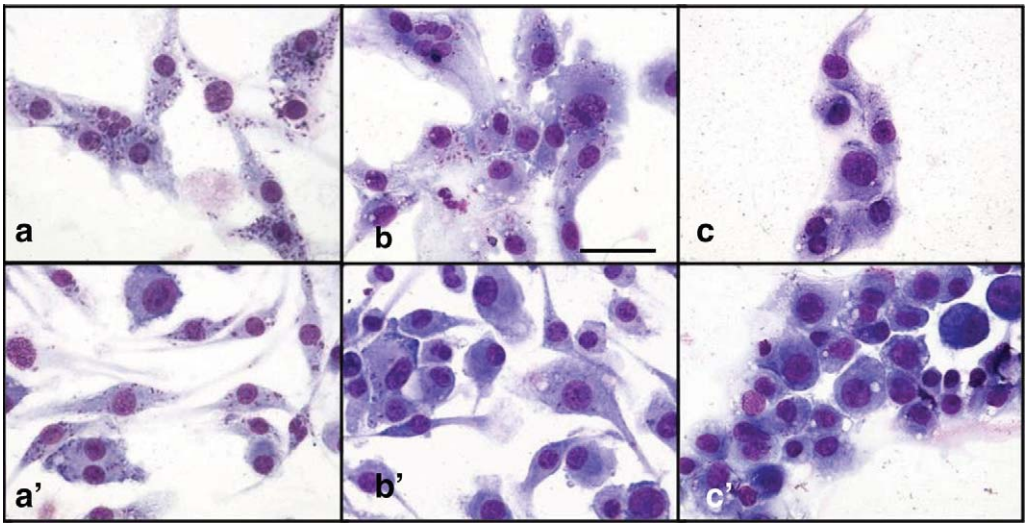

Fig. 4. Effect of allicin on the infection of J774 macrophages with *Leishmania donovani* (upper row) and *Leishmania infantum* (lower row): a and a′: infected and untreated control cultures; b and b′: cultures treated with 30 μM allicin; c and c′: cultures treated with 60 μM allicin. Micrographs are representative images stained with May-Grünwald-Giemsa of the triplicate cultures. Bar: 50 μm.

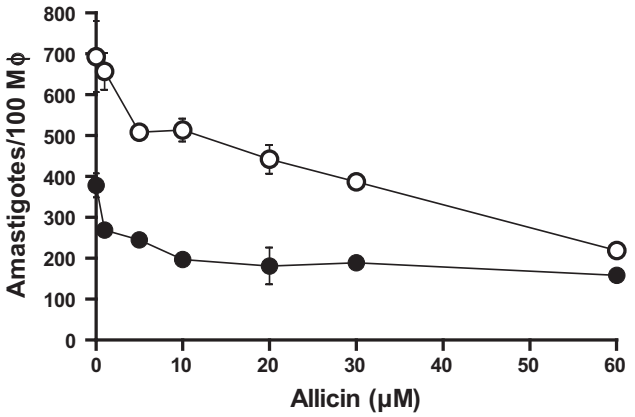

Fig. 5. Effect of the single administration of different concentrations of allicin on the intracellular amastigote burden of J774 cells infected with *Leishmania donovani* (○) and *Leishmania infantum* (●). Results are the mean ± standard deviation of three experiments.

3.4. Thermal degradation of allicin

The apparent recovery of promastigotes cultures of *L. donovani* and *L. infantum* treated with 30 μM allicin (see Fig. 1) suggested the possibility of degradation of the compound in the experimental conditions established. Mass spectrometry analysis of allicin in RPMI 1640 medium, exposed for 24 h at the culture temperatures for promastigotes and amastigotes, 26 and 37 °C respectively, showed that the molecule was degraded at both temperatures (Fig. 6). Our technique did allow us to detect 19 peaks in fresh untreated allicin preparation (Fig. 6a) whereas only 12 peaks were found after treatment at 37 °C for 24 h (not shown). Thermal degradation products included diallyl sulfides (DAS), diallyl disulphides (DADS) and a vinyldithiin although no evidence of allicin or ajoene was obtained. Significantly simpler chromatograms were observed in cultures containing allicin (120 μM) treated at 26 °C. Only DADS (Fig. 6b) and 2-Butoxyethyl acetate and Benzaldehyde, 3–5 dimethyl were found; the last two compounds corresponded to the culture medium, RPMI 1640 (Fig. 1c). Consistently with the degradation of allicin, anti-leishmanial activity of the preparation was greatly improved when this product was freshly added every 24 h. Fig. 7 shows the results obtained with *L. donovani* and comparable results were obtained with *L. infantum* (not shown). All concentrations induced a reduction in the number of promastigotes, after 24 h exposition to the drug, in a concentration related manner ($p < 0.05$) and no recovery of the cultures could be appreciated along the experiment. A different pattern of inhibition was observed onwards (48 and 72 h) depending on the concentration of allicin. Cultures with the lower concentrations tested (1, 5 and 10 μM) did not multiply for the duration of the experiment. By its part, the antileishmanial effect of 30 and 60 μM, added every 24 h, provoked a strong reduction of the promastigote number and, eventually, promastigote clearance with the highest concentration tested after 48 h. These results suggested a leishmaniostatic effect of allicin at low concentrations whereas concentrations over 30 μM behaved as leishmanicidal for promastigotes under our conditions.

The effect of the addition of allicin every 24 h on the actual stage causing the leishmanial infection in mammals, amastigotes, was evaluated in an ex vivo assay with mouse peritoneal macrophages infected with *L. donovani* (Table 1).The modification of the infection procedure was very efficient since over 90% of the macrophages were infected with amastigotes after 24 h. Infection rate was only slightly affected by both treatment with allicin, and time. The effect of the compound on the number of macrophages infected was moderate and actually with the highest concentration used (30 μM), after 72 h infection rate was 60% ± 6.5% as compared to 86% ± 2.6% in the untreated cultures ($p < 0.05$). More relevant was the effect of allicin on the intracellular amastigote burden. Untreated control cultures displayed active multiplication along the experiment, reaching 965.7 ± 59.9 amastigotes/100 macrophages from the initial burden of 489 ± 24.2 after 24 h. Antiproliferative effect of allicin was concentration and time dependent ($p < 0.0001$, $F = 12.64$). Both amastigote burden (number of amastigotes/100 macrophages) and relative parasite growth inhibition were significantly reduced with all concentrations of allicin ($p < 0.05$–0.01). After 24 h, 10 μM allicin reduced by ca. 40% the number of amastigotes as compared to the untreated control cultures and over 67% after 72 h ($p < 0.05$), with three administrations of allicin. More importantly, 5 μM allicin significantly ($p = 0.0014$) reduced by ca. 50% the number of intracellular *Leishmania* amastigotes after 48 h.

4. Discussion

A significant inhibitory effect of allicin on the multiplication of both *L. donovani* and *L. infantum* has been found by us. Inhibition was time- and dose-dependent against promastigotes and intracellular amastigotes in vitro (J774 cells) and ex vivo (mouse peritoneal macrophages). Very few reports have been published on the effect of allicin against *Leishmania* promastigotes. The approximate IC_{50} value obtained (10–30 μM, 24 h exposition) by us for promastigotes from both species is in the line of the value indicated by Ankri and Mirelman (1999) (30 μg mL^{-1}) for promastigotes of *L. major* and slightly higher than that reported by McClure et al. (1996) for *L. chagasi* and *L. mexicana*. Allicin, in the micromolar range, effectively inhibited the multiplication of amastigotes within J774 murine line cells and peritoneal macrophages. As far as we know this is the first report on the inhibitory effect of allicin against intracellular amastigotes. Mechanism of action of allicin has been related to the rapid reaction with thiol groups (Rabinkov et al., 1998) but the actual intracellular targets responsible of the cytostatic/cytocidal effects are largely unknown. It has been reported that allicin induces apoptosis (Oomen et al., 2004) and our TEM results showed condensation of nuclear chromatin and kDNA and loss of integrity of mitochondrial and kinetoplast membrane. Ledezma et al. (2002) also observed nuclear alterations in *L. amazonensis* promastigotes treated with the related compound ajoene. Antioxidant systems of glutathione and trypanothione/trypanothione reductase participate in the protection of *Leishmania* against the toxic effect of nitrogen-derived reactive species from macrophages (Romão et al., 2006). Interference of allicin with these thiol-redox proteins could lead to amastigote destruction and this possibility should be explored. Besides this interaction other cell targets (i.e. other thiol-dependent enzymatic systems, cysteine proteinases, microtubule disruption) (Ankri and Mirelman, 1999; Prager-Khoutorsky et al., 2007; Miron et al., 2010; Waag et al., 2010) should be involved since comparable IC_{50} values for allicin, administered as a single dose, were found by us in intracellular amastigotes in J774 cells and extracellular promastigotes.

The modified method employed in the macrophage infection with *L. infantum* metacyclic promastigotes (Ficoll gradient + opsonisation with 15% mouse serum) allowed to obtain higher yields than those achieved with previously used methods (Méndez et al., 1996; Wert et al., 2011). Comparative advantage of the methodology was particularly evident in the ex vivo experiments with mouse peritoneal macrophages and provides us with a valuable tool for in vitro drug screening against intracellular amastigotes of this species. Under our conditions, toxicity of allicin in the concentration range used was low and no significant effect on mouse macrophages was observed up to 40 μM as estimated by MTT method. Moreover, the absence of substantial toxicity of the compound was confirmed by microscopical examination without any noticeable cell

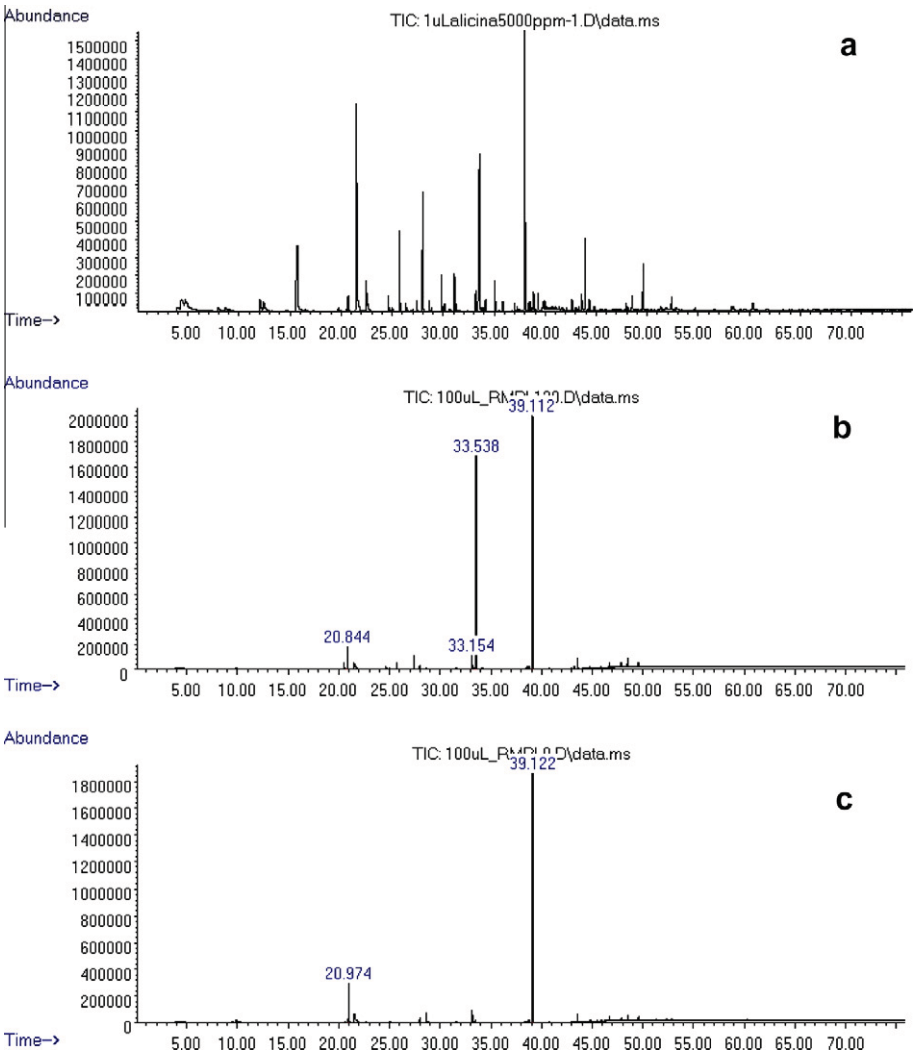

Fig. 6. Mass spectrometry representative chromatograms of 120 μM allicin + RPMI 1640 medium treated for 24 h at 26 °C (b). For comparative purposes fresh untreated allicin (5000 ppm) (a) and RPMI 1640 medium treated for 24 h at 26 °C (c) chromatograms are included.

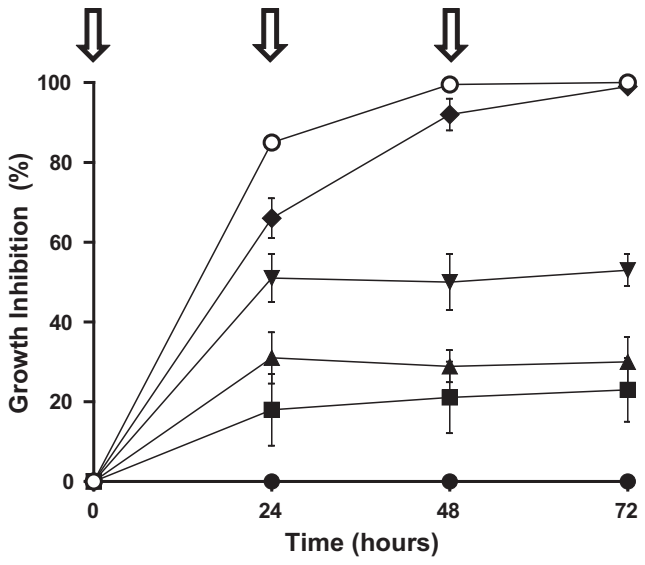

Fig. 7. Effect of different concentrations of allicin added every 24 h on the multiplication of *Leishmania donovani* promastigotes. Arrows: days of treatment with allicin. ●: untreated control cultures; ■: 1 μM; ▲: 5 μM; ▼: 10 μM; ♦: 30 μM; ○: 60 μM. Results are means ± standard deviation of three experiments.

alteration up to 60 μM in both J774 cells and BALB/c peritoneal macrophages. A lack of correlation between the results of toxicity obtained with the colorimetric method in J774 cells and the findings in peritoneal macrophages was found. This apparent inconsistency could be related to the limitations of colorimetric methods to distinguish between cytostatic and cytotoxic effects of a drug (Plumb, 2004). Our results showed that allicin is a potent cytostatic agent but of scarce toxicity for mammalian cells as confirmed by the results obtained in non-dividing peritoneal macrophages. In addition it stresses the need of performing direct microscopic observation of the cells besides indirect methods when testing potentially useful drugs against intracellular parasites.

Administration of allicin every 24 h greatly increased the antileishmanial activity of the compound. This finding suggested the degradation of the diallyl thiosulfinate at 26 and 37 °C and is consistent with the reported thermo-sensitivity of the molecule (McClure et al., 1996; Cañizares et al., 2004). The degradation of the allicin preparation employed by us after 24 h at 26 and 37 °C was confirmed by mass spectrometry. Allicin is a highly reactive thiosulfinate which is rapidly transformed to other types of organosulfur compounds, particularly diallyl disulfides (DADS), diallyl sulfides (DAS), ajoene and vinyldithiins (Amagase, 2006). Our results showed the presence of sulfides and vinyldithiins but not ajoene (E or Z) and allicin were found in any of the analysis. Simpler

Table 1
Effect of allicin replaced every 24 h on the multiplication of intracellular amastigotes of *Leishmania donovani* in peritoneal macrophages. Values are means ± (standard deviation).

Allicin (μM)	Amastigotes/ 100 Mφ	% of infection	% Parasite growth inhibition
24 h			
0(a)	489.0 (24.2)^{bcde}	91.7 (3.5)	0.00
1(b)	418.7 (28.5)^{ed}	91.0 (5.5)	14.38 (5.84)^{cde}
5(c)	346.7 (32.5)^{ae}	81.7 (2.5)*	29.11 (6.65)^{bde}
10(d)	292.0 (16.7)^{ab}	74.0 (4.0)*	40.29 (3.41)^{bce}
30(e)	177.3 (6.8)^{abc}	70.0 (1.0)*	63.74 (1.39)^{bcd}
48 h			
0 (a)	781.0 (64.9)^{bcde}	79.0 (7.5)	0.00
1(b)	632.0 (58.5)^{acde}	77.0 (6.0)	19.08 (7.49)^{cde}
5(c)	402.0 (21.3)^{abe}	70.0 (4.5)	48.53 (2.73)^{be}
10(d)	373.3 (61.2)^{abe}	79.3 (3.7)	52.20 (7.84)^{be}
30(e)	228.3 (22.0)^{abcd}	70.7 (2.5)	70.76(2.81)^{bcd}
72 h			
0(a)	965.7 (59.9)^{bcde}	86.0 (2.6)	0.00
1(b)	670.7 (56.7)^{acde}	82.7 (4.0)	30.55 (5.87)^{cde}
5(c)	436.3 (39.5)^{abde}	72.0 (5.5)*	54.82 (4.09)^{bde}
10(d)	317.7 (7.5)^{abc}	65.0 (1.0)*	67.10 (0.77)^{bc}
30(e)	267.7 (29.6)^{abc}	60.0 (6.5)*	72.28 (3.07)^{bc}

Asterisks (*) represent values significantly different ($p < 0.05$) to the untreated control cultures. Different superscripts within a column for each time (24, 48, 72) represent differences ($p < 0.05$–0.01) between cultures treated with allicin.

chromatograms of culture samples are probably related to the lower concentrations of allicin (<120 μM) as compared to the standard sample. The absence of allicin and ajoene could be due to their short half life at the culture temperatures (26 and 37 °C) (i.e. ajoene, less than 1 min) and the analytical method employed. It is possible that less stringent analysis (HPLC) (Lanzotti, 2006) would allow the detection of these compounds. It is noteworthy to indicate that most studies on the degradation of allicin have been carried out with free allicin whereas our experiments were performed with a stabilized form of this molecule. Whether or not the actual antileishmanial agent in our studies is allicin or some other degradation product requires further experimentation. It has been indicated that thermal sensitivity of allicin at physiological temperature as well as its rapid transformation to other types of organosulphur compounds (Amagase, 2006) could rule out its therapeutic use. However, it has been shown that decline of allicin is accompanied by its transformation into other compounds (i.e. ajoene) (Fujisawa et al., 2008) with strong anti-leishmanial activity (Ledezma et al., 2002). Moreover, the metabolism of allicin, and therefore its bioavailability, is unknown for the most part. Recently, oral and peritoneal administration of methanolic extracts of garlic significantly reduced the *L. donovani* burden in spleens of hamster infected with *L. donovani* (Wabwoba et al., 2010). Chemotherapy of leishmaniasis is far from ideal (Alvar et al., 2006; Chappuis et al., 2007) and new drugs and combinations are required. Value of IC_{50} found by us in vitro and ex vivo (5–10 μM) against intracellular amastigotes possibly excludes allicin as monotherapy against leishmanial infections. However, low cost and scarce toxicity of this compound would allow its use in combined therapy with other well established anti-leishmanial drugs.

In conclusion, allicin (=diallyl thiosulphinate) has shown a significant in vitro effect on the multiplication of promastigotes of *L. donovani* and *L. infantum*. Allicin exhibited a scarce toxicity for J774 cells and mouse macrophages. Moreover, it has been shown for the first time the inhibition of multiplication of intracellular amastigotes of *Leishmania*. Consistent infections of macrophages (J774 cells, mouse peritoneal) with *L. infantum* have been obtained with a modified method to isolate metacyclic promastigotes. Inhibitory concentrations of allicin were in the micromolar range for both *Leishmania* species and stages. More stable galenic preparations are probably needed. However, low toxicity for mammalian cells of this compound suggests the interest of testing this molecule in vivo in combined therapy against leishmanial infections.

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Antimicrobial Agents and Chemotherapy

In Vitro Synergistic Effect of Amphotericin B and Allicin on Leishmania donovani and L. infantum

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In Vitro Synergistic Effect of Amphotericin B and Allicin on *Leishmania donovani* and *L. infantum*

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Current monotherapy against visceral leishmaniasis has serious side effects, and resistant *Leishmania* strains have been identified. Amphotericin B (AmB) has shown an extraordinary antileishmanial efficacy without emergence of resistance; however, toxicity has limited its general use. Results obtained showed, using a fixed-ratio analysis, that the combination of diallyl thiosulfinate (allicin) and AmB ranged from moderately synergic to synergic at low concentrations (0.07 μM AmB plus 35.45 μM allicin induced 95% growth inhibition). None of the treatments, alone or in combination, had noticeable adverse effects on macrophages (Mϕ) in the concentration range examined (allicin, 0.5, 1, 5 and 10 μM; AmB, 0.05, 0.075, and 0.1 μM). Allicin, AmB, or the combination did not affect the infection rate (percentage of infected Mϕ) of *Leishmania*. Allicin enhanced the activity of AmB on intracellular amastigotes of *Leishmania donovani* and *L. infantum* (ca. 45% reduction of amastigote burden with 0.05 μM AmB plus 10 μM allicin); this represented nearly a 2-fold reduction in the 50% inhibitory concentration (IC_{50}) of the antibiotic added alone. Results point toward the possible utility of testing this combination *in vivo* to reduce the toxicity associated with monotherapy with AmB.

Human *Leishmania* infections are present in all inhabited continents, with around 12 million people infected, 2 million new cases every year, and estimates of around 350 million people living in areas at risk (http://who.int/health-topics/leishmaniasis.htm). Leishmaniasis is a spectral infection with a range of clinical presentations, from self-healing dermal infections to deadly processes. Cutaneous leishmaniasis is by large the most common disease, but visceral leishmaniasis (caused by *Leishmania donovani* and *L. infantum* [=*L. chagasi*]) is also present in all continents, and the infections produce severe disease. The distribution of these infections has greatly expanded, and coinfection of *Leishmania* and HIV is very frequent (1). In addition to human cases, canine leishmaniasis caused by *L. infantum* (Mediterranean Basin) and *L. chagasi* (South America) constitutes a first-order pathology in veterinary clinics besides the zoonotical importance of dogs as reservoirs. The main control system of leishmaniasis in both humans and dogs is chemotherapy. However, antileishmanial drugs have some important shortcomings, including high toxicity and teratogenicity in some cases, absence of parasitological cure in most cases, and unaffordable prices for some of the compounds and presentations. Moreover, in some areas, *Leishmania* strains resistant to commonly used drugs, particularly antimonials, have emerged (2). Liposomal amphotericin (AmB), paromomycin, and miltefosine were considered the most promising drugs for chemotherapy of leishmaniasis (http://www.who.int/tdr/diseases-topics/leishmaniasis/en/). However, for the large part, these drugs were introduced over 40 years ago, and new active compounds or combinations against *Leishmania* are needed (3, 4).

The pharmaceutical industry has experienced a contraction during recent years, resulting in very few companies being present in the market. It is anticipated that investment and intercompany competition and consequently the launch of new antiparasitic agents will be reduced (5). One of the alternative chemotherapeutic approaches is the use of combinations of effective existing drugs whose toxicity precluded them from being widely used with chemically unrelated compounds of reduced toxicity. The antibiotic AmB, besides being the standard treatment for systemic fungal infections, has shown an extraordinary antileishmanial efficacy. Its main mechanism of action, binding to ergosterol-containing membranes of *Leishmania* (6), probably explains the lack of significant emergence of resistance to the compound. However, toxicity has limited its general use, and different low-toxicity preparations have been developed (i.e., liposomes), but their high price limits their standard use (7). Some low-cost vehicles for the antibiotic have been tested *in vitro* and *in vivo* (i.e., albumin microspheres [8] and polylactic-co-glycolic acid [PLGA] [9, 10]), although without further development. For its part, allicin (diallyl thiosulfinate = 2-propene-1-sulfinothioic acid *S*-2-propenyl ester), a natural product present in plants of the family Alliaceae, with antibacterial (11), antifungal (*Candida*) (12), and antiprotozoal (*Plasmodium* and *Leishmania* promastigotes) (13, 14) activities, has been found to exhibit a notable antileishmanial effect on the intracellular stages of *L. donovani* and *L. infantum* without substantial cytotoxicity for mammalian cells (15).

On these grounds, our approach was the exploration of the potential synergistic or additive antileishmanial effect of the combination of AmB and allicin (low micromolar concentrations of AmB plus micromolar allicin), thus avoiding the toxic concentrations needed with AmB in monotherapy. Results obtained *in vitro* against both promastigotes and intracellular amastigotes of *L. donovani* and *L. infantum* showed that allicin significantly enhanced the leishmanicidal activity of AmB and therefore reduced the required amount of AmB to eliminate intracellular infection of Mϕ by *Leishmania*.

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MATERIALS AND METHODS

Parasites. *L. infantum* (MCAN/ES/2001/UCM-9) is an autochthonous isolate obtained at the Clinical Service of the Department of Animal Health, Faculty of Veterinary Medicine (UCM), from a naturally infected dog (Madrid, Spain). *L. donovani* isolate Khartoum 1246 (MHOM/SD/43/124) was provided by A. Toraño (Department of Immunology, Instituto de Salud Carlos III, Madrid, Spain). Both species were routinely maintained as promastigotes in 25-ml culture flasks at 26°C in RPMI 1640 medium (Lonza Group, Switzerland) supplemented with 10% heat-inactivated (30 min at 56°C) fetal bovine serum (FBS; Sera Laboratories International, Horsted Keynes, United Kingdom) and 100 U/ml of penicillin plus 100 μg/ml of streptomycin (BioWhittaker, Verviers, Belgium).

Drugs. Fungizone (deoxycholate-dispersed amphotericin B) was a gift from Bristol-Myers Squibb (France). Stabilized allicin was obtained as liquid Allisure (5,000 ppm) from Allicin International Ltd. (Rye, East Sussex, United Kingdom). For the *in vitro* experiments, 10 mM stock solutions of amphotericin B deoxycholate (AmB) in dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO) were prepared. Further dilutions were freshly made in RPMI 1640 medium. The final DMSO concentration never exceeded 0.5% in the culture medium and had no effect on cell growth. Dilutions of allicin were directly performed in culture medium.

Promastigote assay. (i) Cell counts. Preliminary tests to determine the 50% inhibitory concentrations (IC_{50s}) of the individual drugs were performed. For the combination assay, the IC_{50}s of the single drugs, previously determined by cell counts, were combined. *Leishmania* log-phase promastigotes were cultured in 10-ml sterile polystyrene tubes (initial concentration, 10^6 parasites/ml; 2 ml/tube) and treated with 0.07 μM AmB or 30 μM allicin or the combination of both (0.07 μM AmB plus 30 μM allicin). Cultures were counted 24, 48, and 72 h posttreatment in a Neubauer chamber, and the viability was determined with trypan blue exclusion dye (0.4% in phosphate-buffered saline [PBS]). Experiments were performed in triplicate.

(ii) alamarBlue assay for promastigote proliferation and synergism of the drug combination. Mid-log-phase promastigotes were seeded in 96-well culture plates (Corning, New York, NY) at an initial plating density of 2.5×10^5 cells/well (200 μl/well). Cultures were exposed for 24 h to various concentrations of the drugs and their combinations. Promastigote proliferation and inhibition were determined with alamarBlue reagent (AbDSerotec Ltd., United Kingdom) (10%, vol/vol) by following the manufacturer's recommendations by reading fluorescence intensity (550-nm excitation wavelength and 590-nm emission wavelength) in a FLUOstar Omega (BMG Labtech) fluorimeter after 72 h of incubation with the dye. Untreated cultures, wells without cells and the maximal concentration from each compound and their combinations, and wells with culture medium and alamarBlue (10%, vol/vol) were included as controls. Results for growth inhibition were expressed as percentage of growth in untreated control cultures. Cultures were performed at least in triplicate.

alamarBlue assays with increasing concentrations of the single drugs were performed first, and appropriate drug combination ratios were tested according to the results obtained. Drugs were combined at constant ratios (0.01:5, 0.01:10, and 0.01:20 [μM AmB/μM allicin]) by following a modified fixed-ratio method (16), and their dose-effect relationships were assessed by the Chou-Talalay combination index (CI) method using CalcuSyn software (17, 18). This method is based on the multiple-drug effect equation derived from the median-effect principle and the mass action law. Results provide a quantitative determination for synergism ($CI < 1$), additivity ($CI = 1$), and antagonism ($CI > 1$). The software allows the calculation of the dose reduction index (DRI), a parameter which indicates the fold dose reduction allowed in a drug combination to reach a given degree of inhibition compared to the drug as a single agent. It provides other dose-effect-related parameters such as the potency (D_m) of the drugs alone or in combination, this representing the concentration which inhibits cell growth by 50%, and the shape of the dose-effect curve (m), where m of 1, >1, and <1 indicate hyperbolic, sigmoidal, and flat sigmoidal curves, respectively. The linear correlation coefficient (r) of the median-effect plot represents the conformity of the data to the mass action law.

Cytotoxicity assay for mammalian cells. Toxicity for mammalian cells was determined *ex vivo* in murine (female BALB/c mice; Harlan Barcelona, Spain) peritoneal Mϕ isolated by peritoneal lavage, resuspended in RPMI 1640 medium supplemented with 10% FBS, and seeded in 96-well culture plates (4×10^6 cells/ml; 200 μl/well). Plates were incubated overnight to allow cell adherence at 37°C in a 5% CO_2–95% air mixture. Different concentrations of allicin (10, 30, and 40 μM) plus AmB (0.1 and 0.5 μM) were added to the cultures. At the end of the drug exposure period (24 h), the medium was removed; wells were replenished with fresh medium and incubated for another 24 h. The viability of cells was determined by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay as described previously (19). Briefly, 50 μl of MTT (Sigma) solution (5 mg/ml in sterile PBS) was added to the wells, and the plates were kept for 4 h at 37°C in a humidified atmosphere protected from light. Formazan crystals were dissolved with 200 μl of DMSO and 25 μl/well of Sorensen's glycine buffer (0.1 M glycine and 0.1 M NaCl, adjusted to pH 10.5 with 1 M NaOH) and added to stop the reaction. Absorbance was read at 570 nm in a microplate reader. Untreated cells and wells without cells and the maximal concentration from each compound were included. Three independent experiments were carried out.

Amastigote assay and determination of the synergism of the drug combination. Infection of Mϕ was carried out with the modification developed by us (15, 20). In brief, 2.5×10^4 J774 cells/well (200 μl/well) were cultured in 8-well Lab-Tek chambers (Nunc, Roskilde, Denmark) for 16 h to ensure adherence. Stationary-phase *Leishmania* promastigotes (day 7 to 10) were centrifuged (370 × *g*, 10 min, without brake) in a 5810R centrifuge (Eppendorf, Germany) through a Ficoll gradient (0, 10% in medium 199, and 30% in sterile PBS). Metacyclic parasites recovered were washed and opsonized with 15% normal mouse serum (Jackson ImmunoResearch, United Kingdom) in a solution (1/1, vol/vol) of RPMI medium and Hanks' balanced salt solution (HBSS; Sigma), 0.15 mM $CaCl_2$, and 1 mM $MgCl_2$ for 30 min at 37°C and 5% CO_2. Infection was carried out overnight at 33°C at a parasite/Mϕ ratio of 10:1 in 5% CO_2. Noninternalized promastigotes were removed by thorough washing, and fresh media containing the appropriate compounds dilutions were added to the wells. Cultures were treated with AmB (0.05, 0.075, and 0.1 μM) and allicin (0.05, 1, 5, and 10 μM) administered alone and in combination for 48 h. Unless otherwise stated, mitomycin C (Calbiochem, Merck, USA) at 1 μg/ml was added to avoid cell line proliferation. Untreated cultures, cultures treated with mitomycin C, and cultures treated with 0.5% DMSO were included as negative controls. Slides were fixed and stained (May-Grünwald Giemsa; Merck Darmstadt, Germany), and the number of amastigotes/100 cells and percent infected cells were determined. Drug activity was expressed as percentage of growth inhibition compared to that in untreated cultures. Three independent experiments were performed at least in triplicate.

Statistical analysis. Graphs were generated and statistical analysis was performed using GraphPad Prism 5. Statistical significance of differences between mean values obtained in the different groups was determined by repeated-measures generalized linear model analysis, one-way analysis of variance (ANOVA), and Bonferroni test. Differences were considered significant at a P value of <0.05.

RESULTS

Figure 1 shows the results obtained by cell counting in the preliminary test on the effect of the combination of AmB and allicin. Similar results were obtained with both species, *L. donovani* (Fig. 1A) and *L. infantum* (Fig. 1B). Both compounds, AmB (0.07 μM) and allicin (30 μM), reduced by more than 50% the *Leishmania*

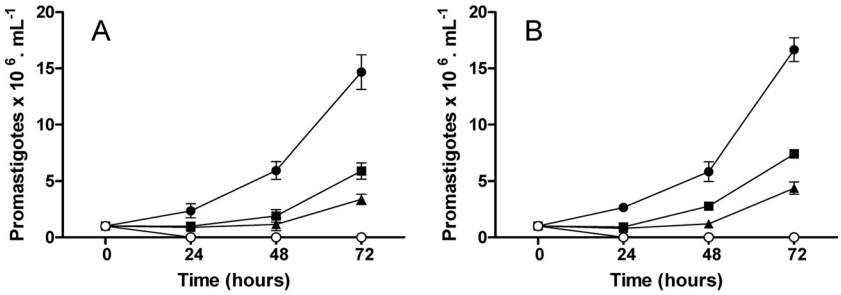

FIG 1 Effects of 30 μM allicin (■), 0.07 μM AmB (▲), and the combination (30 μM allicin plus 0.07 μM AmB) (○) on the multiplication of *L. donovani* (A) and *L. infantum* (B) promastigotes after 24, 48, and 72 h. ●, untreated control cultures. Data are means ± standard deviations of an experiment in triplicate.

growth after 24 h of exposure, although a certain recovery of cell multiplication was observed on the 48-h and 72-h samplings. Combination of AmB (0.07 μM) and allicin (30 μM), however, provoked the cell death of the majority of the leishmanial population, and actually, after 72 h, *L. infantum* counts reached a mere 3×10^3 ml^{-1}, this representing a reduction of more than 99% compared to the untreated cultures. These results were suggestive of a positive interaction (additive or synergic) between the compounds. To determine the effect of the combination, drugs were tested at constant micromolar ratios (0.01:5, 0.01:10, and 0.01:20) (AmB/allicin); growth inhibition was estimated with the alamarBlue fluorimetric method. The two *Leishmania* species behaved similarly; Table 1 shows the results obtained for *L. infantum* promastigotes. In all cases, data obtained agreed with mass law action (r) and inhibition followed a sigmoid curve ($m > 1$). D_m values, corresponding to IC_{50}s, obtained for the individual drugs tested were in the range of the previously estimated concentrations (22.1 μM for allicin and 0.09 μM for AmB). Growth inhibition results showed the significantly higher effect of the combination with allicin even with 0.01 μM AmB. Thus, whereas AmB administered alone did not induce at this concentration any inhibition of promastigotes' multiplication, the combination with 20 μM allicin provoked a 37.5% growth reduction. This effect of allicin was more evident with 0.02 μM AmB, since when the antibiotic was combined with 20 μM allicin, 50% inhibition was reached (IC_{50}), and with 40 μM allicin, 89% (IC_{90}) of the multiplication was stopped. Similarly, an AmB concentration of 0.08 μM, close to the IC_{50} of this compound, with 40 μM allicin reduced almost completely the multiplication of the promastigotes. It is noteworthy that microscopic examination of the promastigotes subjected to

TABLE 1 Effects of AmB and allicin and their combination on promastigotes of *Leishmania infantum* determined by alamarBlue assay[a]

D_1-D_2 combination ratio	AmB concn (μM)	Allicin concn (μM)	Growth Inhibition (%)[b]	*m*	*Dm* (μM)	*r*
0.01:5	0.0025	1.25	3.5 ± 0.59			
	0.005	2.5	3.4 ± 0.98			
	0.01	5	3.5 ± 0.45			
	0.02	10	3.6 ± 2.84			
	0.04	20	15.0 ± 1.32			
	0.08	40	99.0 ± 0.03			
	0.16	80	99.0 ± 0.02	2.655	11.71	0.90
0.01:10	0.0025	2.5	3.1 ± 1.54			
	0.005	5	3.3 ± 1.49			
	0.01	10	4.0 ± 2.02			
	0.02	20	52.0 ± 2.22			
	0.04	40	97.0 ± 1.65			
	0.08	80	99.0 ± 0.11			
	0.16	160	99.0 ± 0.12	1.998	21.96	0.91
0.01:20	0.0025	5	1.42 ± 1.05			
	0.005	10	2.09 ± 1.13			
	0.01	20	37.5 ± 1.52			
	0.02	40	89.0 ± 2.31			
	0.04	80	99.0 ± 0.07			
	0.08	160	99.0 ± 0.04			
	0.16	320	99.0 ± 0.02	2.478	26.30	0.94

[a] Drugs were combined at constant ratios (0.01:5, 0.01:10, and 0.01:20) and their dose-effect parameters were assessed by the Chou-Talalay method (17) using CalcuSyn software. D_m (median-effect dose) signifies the potency and it is the concentration which inhibits cell growth by 50%; m is the shape of the dose-effect curve, where m values of 1, >1, and <1 indicate hyperbolic, sigmoidal, and flat sigmoidal curves, respectively; r represents the linear correlation coefficient of the median-effect plot (conformity of the data to the mass action law principle). The m, D_m (μM), and r values for AmB (drug 1 [D_1]) were 1.451, 0.09, and 0.86, respectively, and those for allicin (D_2) were 2.066, 22.13, and 0.90, respectively.

[b] Results are expressed as the means ± standard deviations of three independent experiments.

TABLE 2 Dose-effect relationships of the combination of AmB and allicin on *Leishmania infantum* promastigotes[a]

D_1-D_2 combination ratio	% growth inhibition (ED_n)	CI	DRI		Dose required (μM)	
			AmB	Allicin	AmB	Allicin
0.01:5	50	**0.79 (moderate synergism)**	3.89	1.89	0.023	11.69
	75	**0.65 (synergism)**	5.48	2.13	0.035	17.69
	90	**0.54 (synergism)**	7.73	2.39	0.053	26.75
	95	**0.49 (synergism)**	11.52	2.74	0.071	35.45
0.01:10	50	1.23 (moderate antagonism)	4.14	1.01	0.022	21.94
	75	1.16 (slight antagonism)	5.31	1.03	0.037	36.49
	90	1.09 (nearly additive)	6.81	1.05	0.061	60.71
	95	1.05 (nearly additive)	8.06	1.07	0.085	85.82
0.01:20	50	1.33 (moderate antagonism)	6.92	0.84	0.015	31.22
	75	1.19 (slight antagonism)	9.47	0.91	0.023	47.48
	90	1.07 (nearly additive)	12.96	1.01	0.036	72.23
	95	0.99 (nearly additive)	16.05	1.06	0.048	96.07

[a] Drugs were combined at constant ratios (0.01:5, 0.01:10, and 0.01:20), and their dose-effect relationships were assessed by the Chou-Talalay method (17) using CalcuSyn software. CI was calculated by the combination index equation. CI of <1, 1, and >1 indicate synergism, additive effect, and antagonism, respectively, at different effective doses (ED_{50}, ED_{75}, ED_{90}, and ED_{95}). Synergistic effects (CI < 1) are in bold. DRI indicates the fold dose reduction allowed in a drug combination to reach a given degree of inhibition compared to the drug as a single agent. Computer-simulated dose-required values of each drug in combination to reach a given effect level are included.

the combinations showed that they were not viable and that no recovery of the cultures was found (data not shown). The combined effect of the two compounds in terms of synergism-antagonism was complex, as shown at Table 2. Generally speaking, all combinations allowed reduction of the concentrations required for AmB (DRI), and synergism (CI < 1) was evident only with the fixed ratio (0.01 μM AmB plus 5 μM allicin) (Fig. 2). Combination allowed a 7- to 10-fold reduction of the AmB dose required to provoke a 90 to 95% reduction in the promastigotes' multiplication.

Given the results obtained with the extracellular stage of *Leishmania*, interaction was tested on intracellular amastigotes. Toxicity of the combination of AmB plus allicin was assayed on peritoneal Mϕ from BALB/c mice. None of the concentrations of allicin employed (10 through 40 μM) in the combination induced any significant toxicity as assessed by MTT assay (Fig. 3). However, 0.5 μM AmB, irrespective of the allicin concentration used, significantly ($P < 0.001$) inhibited the viability of Mϕ, more than 60%. As expected, no toxicity for mammalian cells was found with 0.1 μM AmB. This value is in the range of reported IC_{50}s for *Leishmania* and supported the exploration of the combined treatment with allicin on the multiplication of intracellular amastigotes of *Leishmania* given the apparent absence of toxicity of the dialylsulfide up to 40 μM. Only concentrations below 0.1 μM AmB and 10 μM allicin were tested (Table 3). For comparative purposes, the effect of both compounds administered alone was also included. Since antileishmanial effect was determined by cell counting, drugs were combined at a nonconstant ratio in a checkerboard manner. As expected, AmB alone showed an approximate IC_{50} of 0.1 μM, and the maximal concentration of allicin (10 μM) reduced by ca. 30% the multiplication of amastigotes. Tested concentrations of the drugs, added alone or in combination, did not significantly affect the viability and infection rate of Mϕ under our experimental conditions; infection rates ranged from 47.3% ± 3.8% to 67.0% ± 3.5%, and the results obtained with the two *Leishmania* species (*L. infantum* and *L. donovani*) were similar (data not shown). An interaction between the compounds was found from moderately synergistic with the lowest concentrations to antagonistic with the highest concentration used (0.1 μM AmB plus 10 μM allicin) (Fig. 4A). In spite of this deduced interaction (CI), the most significant finding of the experiment was that 10 μM allicin could induce ca. 50% reduction of amastigotes' multiplication when administered with 0.05 μM AmB. It should be pointed out that for the particular *L. infantum*

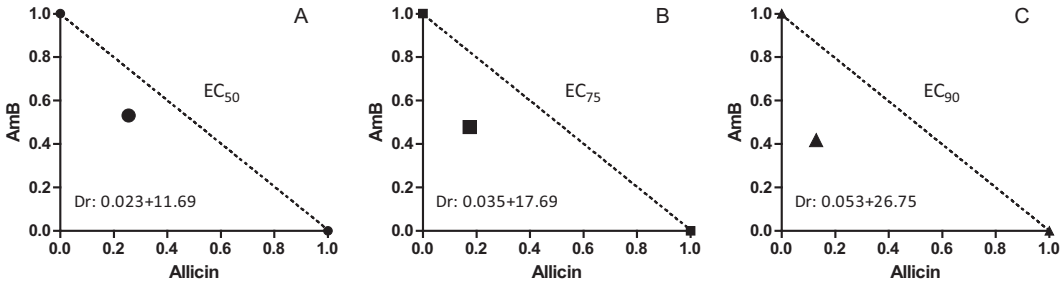

FIG 2 Representative normalized isobolograms of the interaction of AmB with allicin at a fixed ratio (0.01 μM AmB to 5 μM allicin). Lines intersect at the *x* and *y* axes at the normalized concentrations corresponding to the EC_{50} (●) (A), EC_{75} (■) (B), and EC_{90} (▲) (C) when the compounds were administered alone. The same symbols are used for the concentrations found with the AmB-plus-allicin combination to elicit the same effect as the drugs added alone (EC_{50}, EC_{75}, and EC_{90}). Dr, dose required (μM AmB + μM allicin); EC, effective concentration.

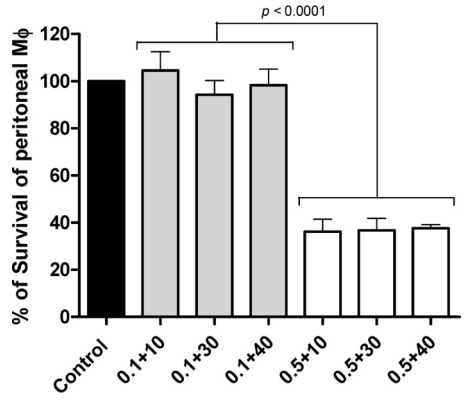

FIG 3 Effect of the combination of AmB and allicin on the *ex vivo* survival of mouse peritoneal macrophages (Mϕ). Numbers on the *x* axis represent the concentration (μM) of AmB plus concentration (μM) of allicin. Solid bar, untreated control Mϕ; gray bars, Mϕ cultures treated with 0.1 μM AmB; white bars, Mϕ cultures treated with 0.5 μM AmB. Results are the means ± standard deviations of three independent experiments.

isolate employed, this AmB concentration administered alone had almost no effect (7.3% ± 5.5% growth inhibition) on the multiplication of intracellular amastigotes. This represents a 2-fold reduction in the dose required for the antibiotic.

DISCUSSION

The aim of this work was the evaluation of the *in vitro* antileishmanial effect of the combination of AmB and allicin with the final purpose of reducing the required doses of the antibiotic and therefore the toxicity associated with its use. Results showed, using a fixed-ratio analysis with the extracellular stage (17, 18) and a checkerboard analysis with amastigotes, that the combination of the two compounds was from moderately synergic to synergic at low concentrations against both promastigotes (0.07 μM AmB plus 35.45 μM allicin induced 95% growth inhibition) and amastigotes (ca. 45% reduction with 0.05 μM AmB plus 10 μM allicin); this represents nearly a 2-fold reduction in the IC_{50} of the antibiotic added alone (15, 21). The differences observed between promastigotes, with a 7- to 11-fold reduction of AmB, and amastigotes, with a ca. 2-fold reduction, support an interest in using intracellular amastigotes in drug screening (9, 21, 22). It is clear that *in vitro* and *ex vivo* tests measure only the antiparasitic effect and that no direct prediction of the effect *in vivo* can be drawn from the results (23). Analysis showed that some drug combinations were classified as antagonist in spite of reaching a highly inhibitory effect in amastigotes and particularly promastigotes. This finding, previously observed in anti-*Plasmodium* screening (24, 25), suggests that even drugs associated with an indifferent effect *in vitro* could be useful therapeutic partners *in vivo*. In addition, differential interaction between AmB and allicin found in promastigotes and amastigotes of *Leishmania* could give us some clues on the mechanism of action of the drugs used. The main mechanism of action of AmB against *Leishmania* is related to the preferential binding to ergosterol (6), which impairs the permeability of membranes, resulting in loss of small cations and causing cell death (26, 27). This mechanism is also responsible for the

TABLE 3 Effects of AmB, allicin, and their combination on the multiplication of intracellular amastigotes of *Leishmania infantum* in J774 cells[a]

Drug combination nonconstant ratio (μM)					DRI	
AmB	Allicin	% infection[b]	% growth inhibition[b]	CI	AmB	Allicin
0	0	64.7 ± 2.5	0			
0.05		67.0 ± 3.5	7.3 ± 5.5			
0.075		64.7 ± 4.5	25.8 ± 5.1			
0.1		58.3 ± 2.1	50.1 ± 5.5			
	0.05	60.3 ± 2.1	1.3 ± 4.2			
	1	61.7 ± 5.7	15.7 ± 4.6			
	5	56.7 ± 3.1	24.9 ± 1.8			
	10	63.0 ± 4.4	32.8 ± 3.9			
0.05	0.05	62.7 ± 3.1	26.1 ± 3.1**	**0.75 (moderate synergism)**	1.51	11.28
	1	60.0 ± 4.0	28.5 ± 4.4**	**0.80 (moderate synergism)**	1.55	6.04
	5	56.7 ± 1.5	34.2 ± 6.3***	1.30 (moderate antagonism)	1.67	1.42
	10	56.3 ± 1.5	45.6 ± 8.5***	1.58 (antagonism)	1.91	0.94
0.075	0.05	56.0 ± 9.5	47.4 ± 4.5***	**0.82 (moderate synergism)**	1.29	19.68
	1	54.0 ± 8.2	55.8 ± 10.9***	**0.79 (moderate synergism)**	1.42	11.96
	5	59.0 ± 4.4	54.8 ± 2.0***	1.13 (nearly additive)	1.41	2.34
	10	59.0 ± 2.6	54.0 ± 3.6***	1.58 (antagonism)	1.39	1.15
0.1	0.05	47.3 ± 3.8	61.4 ± 3.8*	**0.90 (slight synergism)**	1.13	27.58
	1	53.3 ± 3.8	61.5 ± 3.3*	**0.95 (nearly additive)**	1.13	13.86
	5	53.0 ± 3.6	64.5 ± 5.0**	1.18 (slight antagonism)	1.17	3.00
	10	47.7 ± 1.5	64.9 ± 0.9**	1.50 (antagonism)	1.18	1.51

[a] Drugs were combined at a nonconstant ratio and their dose-effect relationships assessed by the Chou-Talalay method (17) using CalcuSyn software. CI was calculated by the combination index equation. CI of <1, 1, and >1 indicate synergism, additive effect, and antagonism, respectively, at the different drug combination doses. Synergistic effects (CI < 1) are in bold. DRI indicates the fold dose reduction allowed in a drug combination at the different data points compared with each drug alone. Statistical differences between the effects of AmB alone and the combination of AmB plus allicin are indicated as follows: *, $P < 0.05$; **, $P < 0.001$; and ***, $P < 0.0001$.
[b] Values are means ± standard deviations.

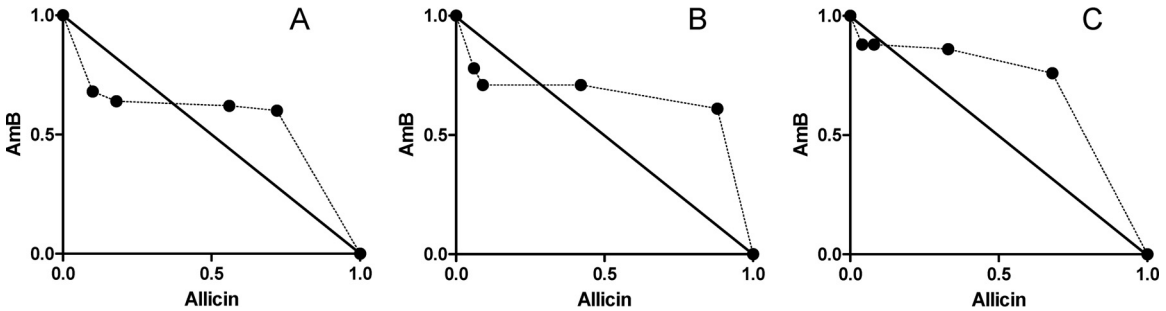

FIG 4 Representative isobolograms of the *in vitro* interaction between AmB and allicin on intracellular amastigotes of *L. donovani*. Results correspond to the nonconstant-ratio experiments using 0.05 μM (A), 0.075 μM (B), and 0.1 μM (C) AmB and 0.05, 1, 5, and 10 μM allicin. Black circles indicate the IC_{50} of the combination at the concentrations on the *x* and *y* axes.

fungicidal activity of the antibiotic. The part mechanistic basis of the antiproliferative, and in particular the leishmanial, activity of allicin (15, 19) is not clearly understood, although several cellular targets have been incriminated (28). Apparently allicin produces an enhancement of fungicidal activity of AmB by inhibiting ergosterol trafficking from the plasma membrane to the vacuole membrane in *Candida* (a member of the Fungi) (29). *Leishmania* also has considerable amounts of ergosterol on the plasma membrane. Resistance to AmB in clinical isolates of *L. donovani* has been associated, besides the presence of cholesta-5,7, 24-trien-3β-ol instead of ergosterol, with the upregulation of the thiol metabolic pathway (30). No thiol contents have been yet determined by us in allicin-treated *Leishmania*, but interference with thiols has been considered the main mechanism of action for allicin (28, 31). The synergy observed could be related to the impairment of the thiol metabolic pathway provoked by allicin, thus enhancing the effect of AmB. Whether or not this enhancement of AmB effect in the presence of allicin is responsible for the synergism found by us in amastigotes and, particularly, promastigotes of *Leishmania* needs further experimentation, which is under way.

Chemotherapy of leishmaniasis, both human and animal, is not yet solved in spite of the substantial efforts made on basic aspects of *Leishmania* biology. An alternative approach to monotherapy is the combination of drugs using well-established chemotherapeutic agents (i.e., miltefosine plus amphotericin B or antimonials plus amphotericin B) (32); in addition, these drug combinations have been common in veterinary clinical practice. Our results showed the apparently synergistic effect of allicin and AmB in the micromolar range. Data obtained with drug combinations *in vitro* have many limitations in spite of the dose-effect relationship analysis carried out (i.e., unforeseen interactions and pharmacokinetics) (18). However, the 2-fold reduction in the required dose of AmB against intracellular amastigotes and the possibility of using higher nontoxic doses of allicin (>10 μM) suggest exploration of this combination *in vivo*.

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Efficacy of low doses of amphotericin B plus allicin against experimental visceral leishmaniasis

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Objectives: To evaluate the efficacy of the combination of allicin and amphotericin deoxycholate (AmB) in the chemotherapy of *Leishmania infantum* infection with the final aim of reducing the dose of AmB in the chemotherapy of visceral leishmaniasis.

Methods: Hamsters were intraperitoneally (ip) infected with *L. infantum* (10^7 stationary phase promastigotes). On day 45 post-infection animals were treated ip with AmB (1 or 5 mg/kg/day), allicin (5 mg/kg/day) or a combination of AmB (1 mg/kg/day)+allicin (5 mg/kg/day) for 5 days. Animals were clinically and biopathologically monitored and the antibody response (IgG, IgG_1, IgG_2) was determined. Parasite burdens were estimated by limiting dilution and AmB biodistribution was determined by HPLC in plasma, kidney, spleen and liver.

Results: No clinical signs or liver and kidney alterations were observed. AmB (1 mg/kg/day) did not clear the *Leishmania* infection and no parasites were detected in two animals treated with 5 mg/kg/day allicin. Combination therapy (5 mg/kg allicin+1 mg/kg AmB) reduced the *L. infantum* burden by >95%. Antileishmanial activity of the combination was comparable ($P<0.05$) to the standard AmB treatment (5 mg/kg).

Conclusions: Allicin alone (5 mg/kg/day for 5 days) significantly reduced the *Leishmania* burden in spleen and liver of infected hamsters. Co-administration of allicin (5 mg/kg/day for 5 days) and AmB (1 mg/kg/day for 5 days) showed a partial additive effect on the reduction of leishmanial burden in both target organs.

Keywords: *Leishmania*, hamster, amphotericin, allicin, visceral leishmaniasis

Introduction

Visceral leishmaniasis, a disease that is fatal unless treated, is caused by *Leishmania donovani* and *Leishmania infantum* (=*L. chagasi*) and is responsible for ~40000 human deaths per year.[1] As such, this infection is second only to malaria among human parasitic diseases. Moreover, *L. infantum* infection is a first-order pathology in veterinary medicine since in endemic regions infection reaches a prevalence sometimes exceeding 25% of the total dog population, besides its role as a reservoir for the human population. Visceral leishmaniasis is among the frequent coinfections found in immunodepressed patients (e.g. HIV+ or solid organ transplant recipients). No available vaccine for humans has been developed and the present immunoprophylaxis of dog infections has important drawbacks (e.g. annual revaccination, adverse effects). Chemotherapy, both of humans and dogs, is imperfect and relies on the use of drugs discovered >50 years ago. In addition, important resistances have been described against some of the first-line drugs (i.e. antimonials) employed in endemic areas.[2] The long half-life of some of the alternative treatments (e.g. miltefosine) and easy selection of resistant lines of *L. donovani* to others (e.g. paromomycin)[3] suggests that emergence of resistance has to be expected when used in monotherapy.

Amphotericin B (AmB) has been extensively used against systemic fungal infections and as a second-line treatment against leishmaniasis. It is a very effective antileishmanial drug and the main mechanism of action that has been described, binding to ergosterol-containing *Leishmania* membranes, does not favour the appearance of resistance.[4,5] The main inconvenience of this drug has been the toxicity associated with its use as the deoxycholate,[6,7] but this limitation has been partially circumvented by the use of low-toxicity vehicles (e.g. lipid carriers).[8] However, the high price of liposomal preparations makes the treatment unaffordable in most endemic areas for human use and simply

irrelevant for veterinary purposes. Among the possible strategies to manage the problems with the currently available antileishmanial compounds—inefficacy, price, toxicity and induction of resistance—combination therapy and the use of new molecules have been indicated as research areas worth exploration.[9] Allicin (2-propene-1-sulfinothioic acid S-2-penyl ester; dialylsulphur) has shown activity against some protozoa, including *Leishmania*.[10,11] The main mechanism of action of allicin is related to its high permeability through cell membranes and interaction with thiols.[12] Stabilized allicin has shown a significant antiproliferative effect, in the micromolar range, against amastigotes (the stage present in the infected hosts) of *L. donovani* and *L. infantum* without evidence of toxicity for mammalian cells.[13] Apparently allicin enhances the antifungal (*Candida*) activity of AmB by inhibiting ergosterol trafficking.[14] Recently, a synergistic effect of low concentrations of allicin and AmB has been reported against these species using an intracellular *ex vivo* model (mouse macrophages and amastigotes).[15] We have tested the effect of the combination of AmB at a 5-fold reduced dose with allicin on experimental infections of hamsters with *L. infantum*. The final aim of the research is the reduction of the required dose of AmB and therefore the toxicity associated with the antibiotic if used in monotherapy of leishmaniasis.

Materials and methods

Leishmania culture media and drugs

L. infantum (MCAN/ES/97/10.445) zymodeme MON-1 was obtained from a naturally infected dog and has been routinely maintained by subpassage in hamsters and as promastigotes in RPMI 1640 medium (Lonza) in 25 mL culture flasks at 26°C supplemented with 10% heat-inactivated (30 min, 56°C) fetal bovine serum (FBS; TDI), 1% L-glutamine (Lonza) and 100 U/mL penicillin + 100 μg/mL streptomycin (Lonza). Fungizone® (deoxycholate-dispersed amphotericin B) was from Bristol-Myers Squibb and stabilized allicin was obtained as liquid Allisure® (15000 ppm) from Allicin International Ltd.

Experimental infection of hamsters and follow-up

Female hamsters were purchased from Janvier SAS. At the time of their arrival their weight ranged from 80 to 90 g. After a quarantine period of 30 days, animals were randomly allocated to six matched groups of six hamsters each except group 1 (five animals). Group 1 was the uninfected control group; group 2 was infected but untreated; group 3 was treated with 5 mg/kg/day AmB for 5 days; group 4 was treated with 1 mg/kg/day AmB for 5 days; group 5 was treated with 5 mg/kg/day allicin for 5 days; and group 6 was treated with 1 mg/kg/day AmB plus 5 mg/kg/day allicin for 5 days. Dilutions of the compounds were made with 5% dextrose solution to a final volume of 500 μL. All treatments were administered by the intraperitoneal (ip) route. Animals, except those in group 1, were infected with 10^7 stationary phase promastigotes in 500 μL by ip injection on day 0 of the experiment. From day 45 post-infection (p.i.) onwards, the hamsters were treated with the appropriate drug or drug combination. The uninfected control group received the same volume (200 μL) of dextrose 5% solution. On day 30 p.i. one of the animals in the infected, untreated group (group 2) was euthanized to assess the infection. Ten days after the last treatment animals were anaesthetized with isoflurane, bled by intracardiac puncture and sacrificed by anaesthetic overdose. Experimental animals were dissected and the liver, kidneys and spleen were removed, weighed and used to estimate the parasite burden (liver and spleen) and to perform the pharmacological determinations.

During the entire experiment the animals were housed at the facilities of the Instituto de Salud Carlos III, Majadahonda, Madrid (ISCIII) with a 12 h light/dark cycle, fed with pellets and given water *ad libitum*. Animals were observed daily for undesired reactions to the infection and treatments and body weight was determined weekly. Blood samples (serum and plasma) were obtained at the end of the experiment to determine the levels of renal and liver function markers [urea, creatinine, glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and alkaline phosphatase (ALP)] by standard clinical laboratory techniques (DLV Laboratorio Veterinario, Madrid). Experimental design and procedures were approved by the ethics committee of the ISCIII and followed the recommendations of EC Directive 2010/63/EU.

ELISA

The peripheral specific antileishmanial antibody response (IgG, IgG_1 and IgG_2) was determined by ELISA using serum samples from all experimental animals. Microtitre 96-well plates (Nunc) were coated with 25 μg/mL (50 μL/well) of soluble *L. infantum* extract or fixed promastigotes (10^8 promastigotes/mL; 50 μL/well) overnight at 4°C. For the latter, mid-log-phase promastigotes were fixed with 0.025% formaldehyde solution (Panreac) for 2 h at room temperature (RT) and washed three times with PBS. To ensure cell adhesion, prior to blocking, promastigote-coated plates were centrifuged (500 **g**, 10 min, RT). Plates were blocked with 2% BSA (100 μL/well) at 37°C for 1 h. Animal sera were used at dilution 1/50 (50 μL/well), as established by prior ELISA chequerboard titrations (not shown), and incubated for 2 h at 37°C. After washing, secondary antibodies (50 μL/well) were added and the plates were incubated for 1 h at RT. Antibodies were diluted in PBS–Tween at 1/2000 dilution for total IgG [Goat Anti-Hamster IgG(H+L)-HRP, Southern Biotech] and at 1/1000 dilution for biotinylated mouse anti-Armenian hamster IgG_1 (Abcam) and mouse anti-Armenian hamster IgG_2 (Abcam), both of which react with heavy chains of Syrian hamster IgG isotypes. After washing, IgG_1 and IgG_2 plates were further incubated with 50 μL/well HRP-conjugated streptavidin (Southern Biotech) at a dilution of 1/2000 and incubated for 30 min at RT. Finally, a 1 mg/mL solution of *O*-phenylenediamine (Sigma) and H_2O_2 (1/1000) were added (100 μL/well). The reaction was stopped with 50 μL of 3 N H_2SO_4 and absorbance was read at 492 nm.

Efficacy of treatments

Besides clinical observation of the experimental hamsters, at the end of the experiment the antileishmanial efficacy of drugs and combinations was estimated by determining the parasite burden by limiting dilution assay as previously described.[16] Briefly, livers and spleens were removed, weighed and homogenized in Schneider's medium (Sigma) supplemented with 20% heat-inactivated FBS, 2% sterile human urine, 20 mM HEPES (Lonza), 1% L-glutamine (Lonza) and 100 U/mL penicillin + 100 μg/mL streptomycin (Lonza). Organ homogenates were filtered through a cell strainer to ensure a single-cell suspension. Further dilutions of the homogenate were done in the same medium to a final concentration of 10 mg/mL; 200 μL/well of this cell suspension was added to the first well of a 96-well culture plate (Corning) and serial 4-fold dilutions were made across the plate. Plates were incubated for 2 weeks at 26°C and the presence of parasites was assessed by microscopy. The parasite burden (number of parasites/g of organ) was calculated as described by Buffet *et al.* (1995).[17]

Amphotericin B biodistribution in treated hamsters

Blood samples were obtained at the end of the experiment from group 3 (hamsters treated with 5 mg/kg AmB), group 4 (treated with 1 mg/kg AmB) and group 6 (treated with 1 mg/kg AmB + 5 mg/kg allicin). Plasma was obtained and samples were stored at −20°C until analyses were

performed. Plasma samples (100 μL) were spiked with meloxicam (Fagrón SL) as internal standard at a final concentration of 10 μg/mL. Two extractions were carried out with methanol (300 μL each) and a third extraction with acetonitrile (300 μL). After every extraction, the mixture was vortexed and centrifuged at 9000 rpm, 10 min, 4°C. The supernatants were collected (300 μL×3) and evaporated off in a concentrator (Savant, SpeedVac®) at 35°C. Samples were reconstituted with 200 μL of a 1:1 mixture of methanol: mobile phase (acetonitrile:acetic acid: water) (52:4.3:43.7, v/v/v). The reconstituted samples were centrifuged (9000 rpm, 5 min) and the supernatants were analysed by the validated HPLC method described below.

HPLC was equipped with a Jasco PU-1580 pump, a Jasco AS-2050 Plus autosampler and a Jasco UV-1575 UV–visible detector. Integration of the peaks was performed with the program Borwin 1.5 for PC (JMBS Developments). AmB was isocratically eluted using a Thermo Hypersil BDS C18 reverse-phase column (200×4.6 mm, 5 μm) and a mobile phase as above with a flow rate of 1 mL/min. Absorbance was monitored at 406 nm and the injection volume was set at 100 μL. Under these conditions, the relative retention times of AmB and the internal standard were 7.9 and 5.1 min, respectively. Plasma AmB concentrations were calculated from linear regression calibration curves of the AmB/internal standard peak height ratio. The linear range in plasma was 0.01 to 10 μg/mL ($y=0.2191x-0.0026$; $R^2=0.9986$).

Liver samples (0.5 g) were spiked with meloxicam (10 μL at 200 μg/mL) as internal standard. Samples were homogenized with PBS (pH 7.4, 0.25 mL). Then, AmB was extracted with methanol (1 mL). The mixture was vortexed and then centrifuged (5000 rpm, 20 min). The supernatants were filtered through a 0.45 μm filter (Millipore® Millex PVDF) and then analysed by the HPLC method previously described. Liver AmB concentrations were calculated from linear regression calibration curves of the AmB/internal standard peak height ratio. Similarly, kidney samples (0.15 g) and spleen samples (0.075 g) were analysed. Spleen samples were pooled (three animals/group) to obtain the required amount of tissue.

Statistical analysis

Graphs and statistical analysis were performed using GraphPad Prism 5. The statistical significance of differences between mean values obtained in the different groups was determined by one-way analysis of variance followed by Tukey's test. In all cases a P value of $P<0.05$ was considered significant.

Results

Clinical follow-up

No clinical signs were noted during the experiment as a consequence of either infection with *L. infantum* alone (group 2) or infection and treatments administered. Similarly, no important alterations were found in any of the biochemical parameters determined in the experimental hamsters (Figure 1) since renal (urea, creatinine) and liver function markers (ALP and GOT/GPT ratio) were comparable in all experimental groups irrespective of infection and the treatments administered. The only exception was the blood urea nitrogen level in infected, untreated hamsters (group 2), which was significantly higher than levels in uninfected control animals and animals receiving AmB (5 mg/kg), allicin (5 mg/kg) or the allicin+AmB combination. However, creatinine values were in all cases <0.2 mg/dL. ALP levels were in the range of 214–251 U/L and the GOT/GPT index was <1.0 in all groups. These results suggest that allicin, AmB or the combination (allicin+mB) with the doses and treatment schedule followed in our experiment did not induce any significant toxicity in the infected animals.

Antibody response

Comparable results were obtained with the two antigens, although higher OD values were obtained with the cell-based assay. *L. infantum* infection elicited a significant rise in anti-*Leishmania* IgG against the soluble extract of the parasite (Figure 2) and fixed promastigotes (Figure 3) as assessed by the results observed in infected+untreated hamsters (group 2). Using fixed promastigotes of *Leishmania*, animals treated with the lowest AmB dose (group 4) displayed values not significantly different from those found in infected, untreated hamsters (group 2) (Figure 3a). However, animals treated with allicin alone (group 5) or together with a low dose of AmB (group 6) showed significantly lower antibody values ($P<0.05$). Values in animals treated with the combination were not different from those in animals treated with standard dosage of AmB (group 3; 5 mg/kg/day, 5 days) ($P>0.05$) or the uninfected control group (group 1). The pattern found for the specific IgG_2 response was more complex (Figures 2b and 3b). Excluding the uninfected control hamsters, the lowest antibody values were found in animals receiving 5 mg/kg/day AmB (group 3) and the highest IgG_2 levels in hamsters treated with allicin alone (group 5). Moreover, group 3 hamsters were significantly different ($P<0.05$) from the group treated with allicin alone (group 5) but not from the infected control group (group 2) or those treated with the lowest AmB dose (group 4) or the combination of allicin+AmB (group 6). The IgG_1 response was very low in all groups and no significant differences between them were found (Figures 2c and 3c).

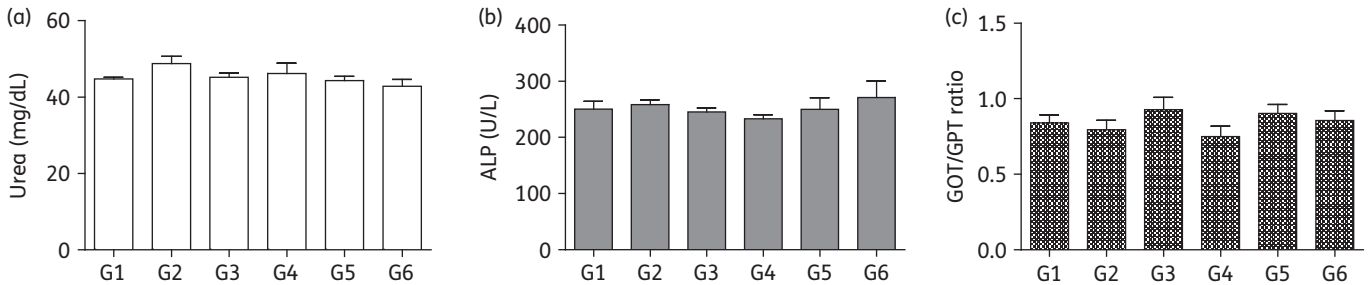

Figure 1. Values of renal and kidney function markers at the end of the experiment. G1, uninfected control; G2, infected with *Leishmania infantum*; G3, infected and treated with 5 mg/kg/day AmB; G4, infected and treated with 1 mg/kg/day AmB; G5, infected and treated with 5 mg/kg/day allicin; G6, infected and treated with 1 mg/kg/day AmB +5 mg/kg/day allicin. ALP, alkaline phosphatase; GPT, serum glutamate pyruvate transaminase; GOT, serum glutamic oxaloacetic transaminase. Data are means±SEM.

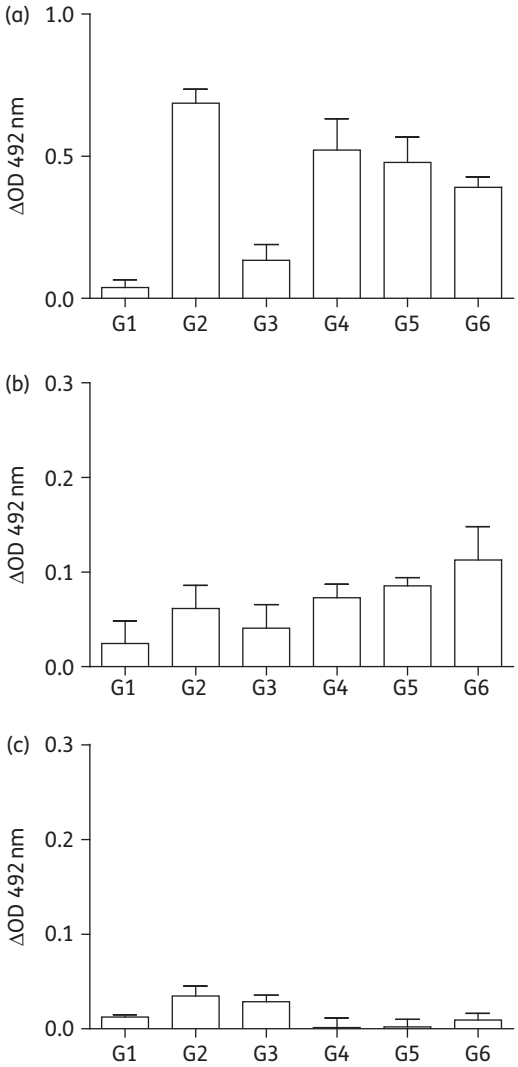

Figure 2. Serum specific antibody responses against soluble *Leishmania infantum* antigen (SLA) estimated by ELISA on day 60 of the experiment. (a) Total IgG (H+L). (b) IgG_2. (c) IgG_1. G1–G6, experimental groups as in Figure 1. ΔOD, increase in OD. Values are means ± SEM.

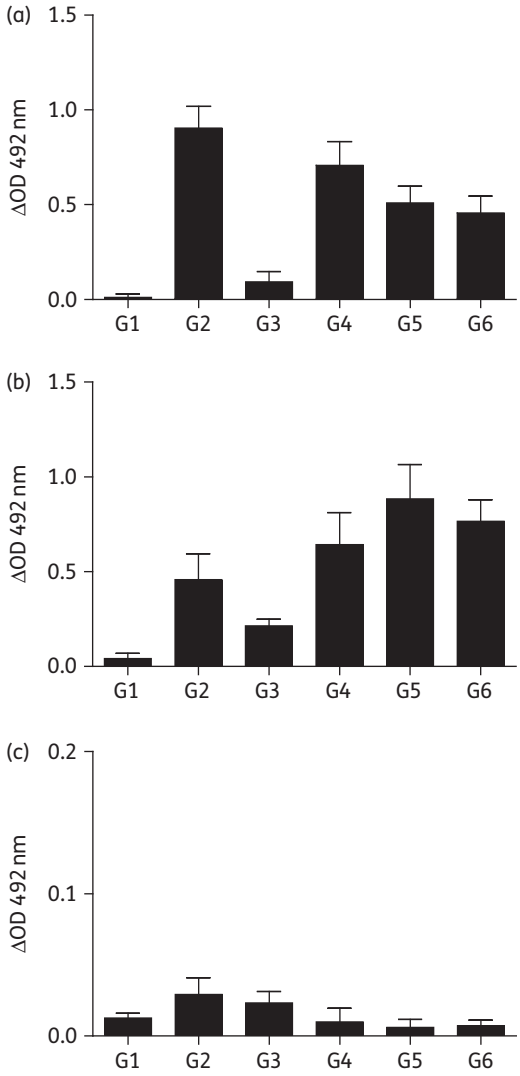

Figure 3. Serum specific antibody response to fixed promastigotes of *Leishmania infantum* estimated by ELISA on day 60 of the experiment. (a) Total IgG (H+L). (b) IgG_2. (c) IgG_1. G1–G6, experimental groups as in Figure 1. ΔOD, increase in OD. Values are means ± SEM.

Liver and spleen Leishmania burden

Figure 4 shows the results obtained in the determination of *L. infantum* burden in liver and spleen. The infection procedure (ip administration) was efficient and all infected control hamsters (group 2) had detectable infections. Spleen burdens of *Leishmania* (~6.0 $\log_{10}$ units) were, in general, significantly higher than those found in the liver (~3.0 $\log_{10}$ units). The negative control group (group 1) did not show any parasites. The curative effect of AmB was dependent on the dose administered. With the highest dose (5 mg/kg AmB; group 3) no parasites were detected in two animals and the average reduction of *L. infantum* burden was 94.5% in the spleen and 90.6% in the liver. However, none of the hamsters treated with 1 mg/kg AmB (group 4) was free of infection, although the parasite burden was reduced by ~70% in the spleen and a similar reduction was found in the liver. Allicin, administered alone (group 5), cleared *Leishmania* infection in two of the hamsters and both liver and spleen parasite burdens were significantly lower than those found in the infected, untreated group ($P<0.05$). Results with the combination of 1 mg/kg AmB and 5 mg/kg allicin) (group 6) showed a partially additive effect. The reduction in *Leishmania* burden was significantly ($P<0.05$) higher than that obtained with 1 mg/kg AmB and slightly better than that obtained with the higher dose of allicin (5 mg/kg). In three out of six hamsters in group 6 no *Leishmania* were recovered from the liver and in two out of six no parasites were detected in the spleen. Results in terms of organ clearance were similar to those found with the standard AmB treatment (5 mg/kg AmB).

Amphotericin B pharmacokinetics

Very low AmB plasma concentrations were found in all experimental animals (Figure 5). However, AmB was detected in all

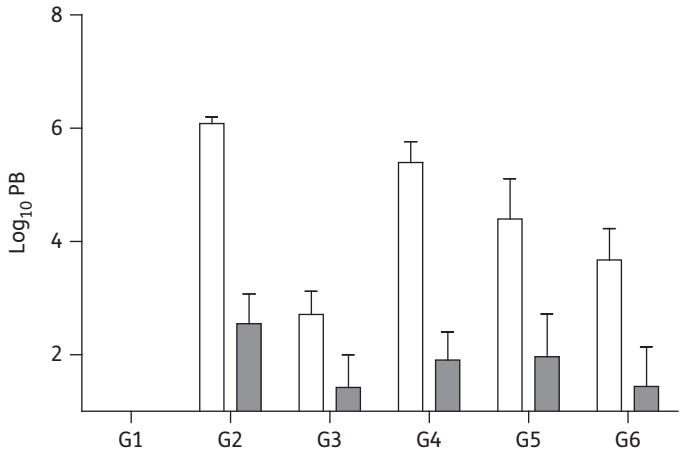

Figure 4. *Leishmania infantum* burden (parasite burden, PB) determined by limiting dilution. G1–G6, experimental groups as in Figure 1. White bars, spleen burden; grey bars, liver burden. Values are means ± SEM.

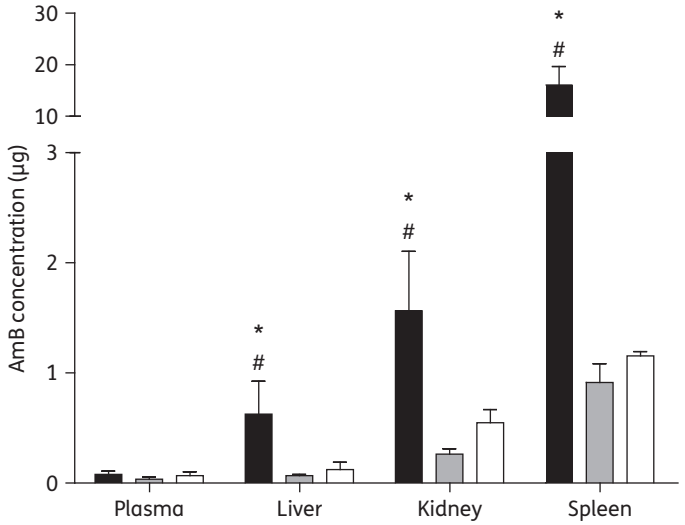

Figure 5. Amphotericin B (AmB) concentrations in plasma, liver, kidney and spleen of hamsters infected and treated with 5 mg/kg/day AmB (black bars) (group 3), 1 mg/kg/day AmB (grey bars) (group 4) and 1 mg/kg/day AmB + 5 mg/kg/day allicin (white bars) (group 6). Values are means ± SD. Statistically significant differences ($P < 0.0001$) were found between groups 3 and 4 (*) and between groups 3 and 6 (#).

organs analysed (kidney, liver and spleen). Animals in group 3 (5 mg/kg AmB) exhibited significantly higher AmB levels in liver, kidney and spleen than animals in groups 4 (1 mg/kg AmB) and 6 (1 mg/kg AmB + 5 mg/kg allicin) but no differences were observed in plasma among groups. AmB levels in group 6 hamsters were higher than those in group 4, although the difference was not statistically significant.

Discussion

Successful chemotherapy of leishmaniasis remains a challenge in spite of the research that has been done on the fundamental biology of the aetiological agents. In practice only a handful drugs are currently used and clinical isolates of *L. donovani* have shown notable resistance to the pentavalent antimonials.[18] Moreover, given the relatively easy selection of resistant *Leishmania* lines to paromomycin[3] and miltefosine,[4] together with the oral administration of the latter and risks associated with self-medication, clinical resistance after monotherapy with these drugs will probably appear.[19] AmB is probably the best existing drug against leishmaniasis, with cure rates exceeding 95%[9,20] and negligible levels of resistance after being employed as the reference drug against systemic fungal infections for >50 years.[5] The main mechanism of action of AmB is still controversial,[21–23] although it is generally considered that binding of AmB to ergosterol in the *Leishmania* membrane is the first or the unique event[5] causing cell destruction. The few reports of clinical resistance in *Leishmania* are related to a modification of ergosterol to the closely related lipid cholesta-5,7,24-trien-3β-ol.[24] To cope with the shortage of available drugs against leishmaniasis, combination therapy with a variety of drugs and schedules[25,26] has some potential advantages, such as delaying or preventing the development of resistance and shortening of treatment regimens.[27] Since the main shortcoming of AmB is toxicity,[6,7] our approach used a combination of allicin with the antibiotic AmB with the aim of reducing the amount of AmB required and therefore the potential toxicity of the antibiotic. The experimental model employed (*L. infantum* and hamster) produced consistent infections with significantly higher parasite burdens in the spleen than in the liver, thus confirming its value[28–32] and its suitability for *in vivo* testing of antileishmanial agents. Moreover, the limiting dilution assay allowed the detection of the parasite 2 months after infection in both the spleen and the comparatively less parasitized liver. No clinical signs or biopathological alterations were observed in any of the infected or infected + treated animals, indicating the lack of toxicity of AmB and allicin at the doses administered. The levels of ALP found in our experiment were higher than the reference range of 50–186 U/L. This could be related to the age of the hamsters, since immature animals have higher ALP levels than adults due to release of the enzyme from growing bone.[33] The alteration in blood urea nitrogen level in group 2 hamsters provided evidence of renal compromise in infected animals. Moreover, higher values of urea, observed in all groups compared with the reference range (12–26 mg/dL), were probably due to diet (i.e. high protein content) and sex (i.e. female).[33] In our experiment, 5 mg/kg allicin administered by the ip route, to avoid the potential degradation induced by the conditions in the stomach, elicited a clear antileishmanial effect in hamsters since two out of six animals were cleared of *L. infantum* in both spleen and liver. As far as we know this is the first evidence of the antileishmanial activity of allicin *in vivo* without apparent toxicity in infected, treated animals. The combination of allicin + AmB significantly improved the efficacy of low-dose AmB (group 4), and was slightly more efficacious than allicin at 5 mg/kg. The low-dose combination of AmB + allicin (group 6) cleared the leishmanial infection in three out of six hamsters. Moreover, the reduction in the *Leishmania* spleen burden was almost as great as that obtained with standard AmB chemotherapy in this model (5 mg/kg, 5 days, ip) (96.57% versus 94.5%).[34] These results suggest a partial additive antileishmanial effect of the combination *in vivo*. Results obtained with either drug administered alone to hamsters support the predictive value of the *in vitro* and *ex vivo* model

(*Leishmania*-infected macrophages) for drug screening of antileishmanial molecules.[35] No information on the mechanism of action of the combination against *Leishmania* is available. However, in fungi (e.g. *Candida albicans*) the enhanced fungicidal activity of this combination[36] has been related to the inhibition of ergosterol trafficking by allicin in the presence of non-lethal concentrations of AmB.[14,37] AmB levels in treated hamsters suggested, but did not demonstrate conclusively, that allicin improves the accumulation of the antibiotic in the target organs, and this should be explored. Resolution of *Leishmania* infection after vaccination or successful therapy seems to be associated with a Th2-to-Th1 switch with a dominant IgG_2 response.[30,38–41] Our results showed a degree of correlation between total IgG (H+L) and *Leishmania* burden.[29] However, hamsters treated with 5 mg/kg AmB (group 3) had lower levels of specific IgG_2 than animals treated with 1 mg/kg AmB. This suggests that the relationship of the IgG_1 and IgG_2 pattern to the outcome of leishmanial infections in hamsters is not clear. In fact, hamsters treated with AmB showed antibody levels comparable to those of infected, untreated hamsters.[42] In addition, slightly different patterns were obtained with fixed promastigotes and soluble extracts of *Leishmania*. These variations are probably related to the different sets of antigens recognized by the sera of infected hamsters. Allicin apparently enhances proinflammatory responses (IFN-γ, TNF and IL-12p70) in mice,[43] which could explain the relatively high IgG_2 response in animals treated with allicin or allicin+AmB. Whether or not the role of these cytokines is similar in mice and hamsters or, alternatively, allicin has immunostimulating properties should be clarified. More research is needed (refining of the dosage and time schedule), but our results point to the interest of this combination as a means of reducing the dose of AmB needed in the chemotherapy of leishmaniasis.

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None to declare.

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