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Pharmacological inhibitors of extracellular signal-regulated protein kinases attenuate the apoptotic action of cisplatin in human myeloid leukemia cells via glutathione-independent reduction in intracellular drug accumulation

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Abstract

It has been reported that inhibition of extracellular signal-regulated protein kinases (ERKs) attenuates the toxicity cisplatin (*cis*-platinum (II)-diammine dichloride) in some cell types. This response was here investigated using human myeloid leukemia cells. Cisplatin stimulated ERK1/2 phosphorylation and caused apoptosis in U-937 promonocytic cells, an effect which was attenuated by the MEK/ERK inhibitors PD98059 and U0126. While ERK1/2 activation was a general phenomenon, irrespective of the used cell type or antitumour drug, the MEK/ERK inhibitors only reduced cisplatin toxicity in human myeloid cells (THP-1, HL-60 and NB-4), but not in RAW 264.7 mouse macrophages and NRK-52E rat renal tubular cells; and failed to reduce the toxicity etoposide, camptothecin, melphalan and arsenic trioxide, in U-937 cells. U0126 attenuated cisplatin–DNA binding and intracellular peroxide accumulation, which are important regulators of cisplatin toxicity. Although cisplatin decreased the intracellular glutathione (GSH) content, which was restored by U0126, treatments with GSH-ethyl ester and DL-buthionine-(*S,R*)-sulfoximine revealed that GSH does not regulate cisplatin toxicity in the present experimental conditions. In spite of it, PD98059 and U0126 reduced the intracellular accumulation of cisplatin. These results suggest that GSH-independent modulation of drug transport is a major mechanism explaining the anti-apoptotic action of MEK/ERK inhibitors in cisplatin-treated myeloid cells.

Keywords: Cisplatin; Apoptosis; ERK inhibitor; Drug accumulation; Glutathione; Myeloid cell

1. Introduction

While the execution of apoptosis follows a basic set of well-characterized and in general well-conserved morphological and biochemical traits [see Ref. 1, as a historical perspective], the mechanisms responsible for the death signalling and regulation are less known, and are greatly dependent on both the apoptotic stimulus and cell type. One of the most relevant aspects in the regulation of apoptosis is the signalling by mitogen-activated protein kinases (MAPKs), a family of serine/threonine kinases which mediate intracellular signal transduction in res-

Abbreviations: Ac-DEVD-*p*NA, *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroaniline; BHA, butylated hydroxyanisole; BSO, DL-buthionine-(*S,R*)-sulfoximine; CDDP, cisplatin, *cis*-platinum (II)-diammine dichloride; DAPI, 4,6-diamino-2-phenylindole; ERK, extracellular signal-regulated kinase; FI-ICP-MS, flow injection-inductively coupled plasma mass spectrometry; GSH, reduced glutathione; GSH-OEt, reduced glutathione ethyl ester; H_2DCFDA, dichlorodihydrofluorescein diacetate; JNK, c-Jun NH_2-terminal kinase, stress-activated protein kinase 1; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PI, propidium iodide; PKC, calcium-dependent protein kinase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate

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ponse to different stimuli [2]. Three major MAPKs have been identified, namely c-Jun NH_2-terminal kinases (JNK, stress-activated protein kinase 1), p38 (stress-activated protein kinase 2), and extracellular-signal regulated protein kinases (ERK1/2, p44/p42). As a general rule, JNK and p38 are mainly activated by cytotoxic insults and are associated to apoptosis induction. By contrast, ERKs are mainly (but not exclusively) activated by growth factors, stimulate cell proliferation, and as such prevent apoptosis [2,3]. However, this rule is subjected to multiple exceptions.

An apparent example of non-canonical role of MAPKs could be given by the protective action of ERK inhibitors against cisplatin toxicity in some cell types. Cisplatin is a DNA alkylating agent with proved efficacy in the treatment of a wide range of solid tumours and some forms of leukemia [4,5, and references therein]. This drug causes apoptotic and necrotic cell death by mechanisms primary related to the formation of DNA adducts, although other factors (e.g., intracellular oxidation, mitochondrial dysfunction and cytoskeleton disruption) may also play a decisive role [5–7]. Treatment of ovarian carcinoma cells [8,9] and the C8161 (but not other) melanoma cell line [10] with cisplatin caused ERK activation and cell death, and this later effect was potentiated by ERK inhibitors, indicating that ERKs behave as survival-inducing kinases in these cells. By contrast, blockade of ERK activation, either by pharmacological inhibitors or gene transfer procedures, reduced the cisplatin toxicity in cervical carcinoma [11,12], hepatoblastoma [12], osteosarcoma and neuroblastoma [13] cell lines. With few exceptions—e.g., the work of Yeh et al. [12], which investigated the regulatory role of NFκB—no clear explanation was given for the protective action of ERK inhibitors.

We earlier reported that cisplatin normally induces apoptosis in U-937 human promonocytic leukemia cells, but that the mode of death could be changed to necrosis after severe glutathione (GSH) depletion [6,14]. In fact, cisplatin may behave as a GSH-sensitive drug, in the sense that its toxicity is dependent on the intracellular GSH content [7,15, and references therein]. In addition, we recently observed that protein kinase C activators increased the toxicity of arsenic trioxide (another GSH-sensitive agent) in U-937 cells via ERK-mediated GSH depletion, and that MEK/ERK inhibitors reduced apoptosis by restoring the GSH level [16]. These observations led us to analyze the behaviour of ERKs, the effects of MEK/ERK inhibitors, and the possible influence of GSH, during cisplatin-induced apoptosis in promonocytic cells. The obtained results indicated that pharmacological MEK/ERK inhibitors attenuate the apoptotic action of cisplatin in human myeloid leukemia cells by reducing the intracellular drug accumulation. However, this effect is regulated in a GSH-independent manner.

2. Materials and methods

2.1. Materials

All components for cell culture were obtained from Invitrogen S.A. (Barcelona, Spain). 4,6-Diamino-2-phenylindole (DAPI) was from Serva (Heidelberg, Germany). Monochlorobimane, dichlorodihydrofluorescein diacetate (H_2DCFDA), and rhodamine 123 were obtained from Molecular Probes (Eugene, OR, USA). The calcium-dependent protein kinase (PKC) inhibitor GF190203X was obtained from Biomol Research Laboratories (Plymouth Meeting, PA, USA). The MEK/ERK inhibitors PD98059 and U0126, and the caspase 3-specific substrate *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (Ac-DEVD-*p*NA), were obtained from Calbiochem (Darmstadt, Germany). Rabbit polyclonal antibodies against human p44/42 MAPK, phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}), p38 MAPK, phospho-p38 MAPK (Thr^{180}/Tyr^{182}), SAPK/JNK, and phospho-SAPK/JNK (Thr^{183}/Tyr^{185}) were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-rabbit peroxidase-conjugated antibody was from DAKO Diagnósticos, S.A. (Barcelona, Spain). Platinum, arsenic and germanium and iridium standard solutions (1 mg/ml) were obtained from Merck (Darmstadt, Germany). All other reagents were from Sigma (Madrid, Spain).

2.2. Cells and treatments

U-937 [17] and THP-1 [18] human promonocytic, HL-60 human promyelocytic [19], NB-4 human acute myeloid [20] leukemia cells, and RAW 264.7 mouse macrophages [21] were routinely grown in RPMI 1640, and NRK-52E rat renal tubular cells [22] in Dulbecco's modified Eagle's medium, supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 0.2% sodium bicarbonate and antibiotics in a humidified 5% CO_2atmosphere at 37 °C. U-937, THP-1, HL-60 and NB-4 cells were seeded at 2×10^5 cells/ml 18 h before the initiation of the experiments, while treatments with RAW 264.7 and NRK-52E cells were initiated at semi-confluence. For pulse-treatment experiments, the U-937 cells were incubated for 1–3 h with cisplatin, then washed twice with pre-warmed (37 °C) RPMI 1640 medium, and allowed to recover for the desired time periods in drug-free complete culture medium. Stock solutions of 12-*O*-tetradecanoylphorbol 13-acetate (TPA, 2 mM), etoposide (20 mM), camptothecin (10 mM), GF109203X (2.42 mM), PD98059 (20 mM), U0126 (2.63 mM), monochlorobimane (200 mM), and Ac-DEVD-*p*NA (5 mM) were prepared in dimethyl sulfoxide; butylated hydroxyanisole (BHA, 0.5 M) and H_2DCFDA (5 mM) in ethanol; and melphalan (164 mM) in a mixture of ethanol/HCl (40:1, v/v). All these solution were stored at −20 °C. Stock solutions of cisplatin (3.3 mM) and arsenic trioxide (100 mM) were prepared in distilled water; and stock solutions of DAPI (10 μg/ml), propidium iodide

(PI, 1 mg/ml), and rhodamine 123 (1 mg/ml), in phosphate-buffered saline (PBS). These solutions were stored at 4 °C. Solutions of reduced glutathione ethyl ester (GSH-OEt, 50 mM) and DL-buthionine-(*S*,*R*)-sulfoximine (BSO, 50 mM) were freshly prepared in PBS and distilled water, respectively, before application.

2.3. Determination of apoptosis, necrosis, and caspase activity

Distinctive characteristics of apoptotic cells were the presence of chromatin condensation/fragmentation and the acquisition of sub-G_1 DNA content. To examine chromatin structure, cells were fixed with ethanol, stained with DAPI, and examined by fluorescence microscopy. To measure DNA content, cells were permeabilized, stained with PI, and examined by flow cytometry. Distinctive characteristics of primary necrosis were the sharp increase in cell size and the free massive influx of dyes in non-permabilized cells. This was determined by incubating non-permeabilized cells with trypan blue or PI, and examination by microscopy or flow cytometry, respectively. All these procedures were described in detail elsewhere [6].

Caspase-3 activity was determined in vitro by measuring the capacity of total cell extracts to cleave the caspase-3-specific substrate Ac-DEVD-*p*NA, following the earlier described procedure [14].

2.4. Measurement of peroxide accumulation and mitochondrial transmembrane potential

The intracellular accumulation of peroxides was determined using the fluorescent probe H_2DCFDA [23]. With this aim, the cells were grown in red phenol-lacking culture medium, and 1 h prior to treatment with cisplatin the cells were loaded with 5 μM H_2DCFDA. After treatment, the cells were centrifuged and resuspended in PBS, and the fluorescence measured by flow cytometry. Control cells were subjected to the same manipulation, except for treatment with cisplatin.

To measure the mitochondrial transmembrane potential ($\Delta\Psi_m$), cells were washed with PBS and then incubated for 20 min at room temperature with PBS containing 1 μg/ml rhodamine 123. After two washes and final resuspension in PBS, the fluorescence was measured by flow cytometry.

2.5. Measurement of intracellular GSH content

The total cellular GSH content was determined by fluorometry after cell loading with monochlorobimane, following the previously described procedure [6].

2.6. Immunoblot assays

To obtain total cellular extracts, cells were collected by centrifugation, washed with PBS, and lysed by 5 min heating at 100 °C in Laemmli's buffer [24] containing a protease inhibitor cocktail, 10 mM sodium fluoride and 1 mM sodium orthovanadate. After centrifugation (5 min at 14,000×*g*, 4 °C), the supernatants (10 μg protein per lane) were analyzed by SDS-polyacrylamide gel electrophoresis, blotted onto membranes, and immunodetected, as previously described [25].

2.7. FI-ICP-MS determinations

For determination of total intracellular platinum and arsenic content, total cellular extracts were prepared as indicated above. For determination DNA-bound platinum, samples of 10^7 cells were collected by centrifugation and washed with PBS, and the DNA was extracted using a QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany), following the procedure described by the manufacturer. Concentrated HCl and HNO_3 solutions were purified by sub-distillation in a polytetrafluor ethylen distiller BSR-939IR (Berghof/America, Coral Springs, FL, USA). The flow injection (FI) manifold consisted of a peristaltic pump and one rotatory injection valve for sample injection (50 μl) into the 0.24 M HCl carrier (for platinum) or 0.30 M HNO_3 (for arsenic) streams at 1 ml/min. This manifold was connected directly to the ICP-MS via the nebulizer inlet tubing. A Quadropole-ICP-MS (HP-4500, Agilent Technologies, Yokogawa Analytical System, Tokyo, Japan), equipped with a Babington-type nebulizer, a Fassel torch and a double-pass Scott-type spray chamber cooler by a Peltier system, was employed for the measurement. Peak areas were measured for the isotopes ^{195}Pt and ^{191}Ir (internal standard for platinum), and ^{75}As and ^{72}Ge (internal standard for arsenic). Analysis was carried out by external calibration. Arsenic determination was not interfered with by the formation of $ArCl^+$ since the chloride concentration in the cells did not exceed the minimum interferent level. The backgrounds obtained using extracts from control (cisplatin- and arsenic trioxide-untreated) cells were routinely subtracted from all values.

3. Results

3.1. Apoptosis induction

First, we measured the frequency of apoptosis in U-937 cell cultures treated with cisplatin under different experimental conditions. In a first group of experiments, cisplatin was applied at concentrations ranging from 35 to 250 μM for short time-periods (from 1 to 8 h). This was the experimental design commonly used by us and other researchers to analyze apoptosis induction by DNA-damaging drugs (including cisplatin) in myeloid leukemia cells [6, and other works]. Cisplatin caused a concentration-dependent (Fig. 1A) and time-dependent (Fig. 1B) increase in the frequency of cells with fragmented chromatin, which is a

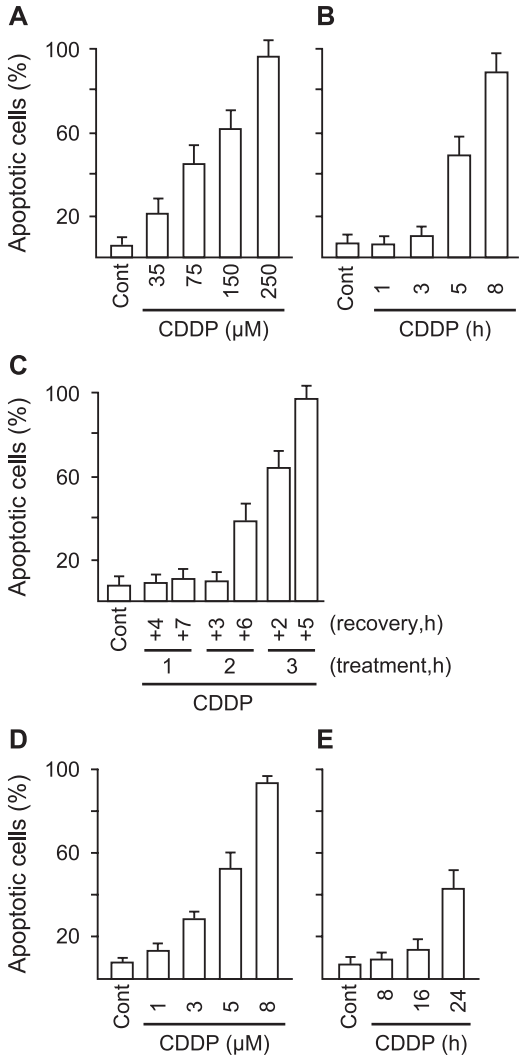

Fig. 1. Generation of apoptosis by cisplatin in U-937 cell cultures. The panels show the frequency of cells with fragmented chromatin, as determined by chromatin condensation/fragmentation, in untreated (Cont) and cisplatin (CDDP)-treated cultures. (A) Continuous treatment for 5 h with the indicated concentrations of cisplatin. (B) Continuous treatment for the indicated time-periods with 75 μM cisplatin. (C) Pulse-treatment with 75 μM cisplatin for 1, 2 and 3 h, followed by recovery for the indicated time-periods in drug-free medium. (D) Continuous treatment for 24 h with the indicated concentrations of cisplatin. (E) Continuous treatment for the indicated time-periods with 5 μM cisplatin. The results are the mean±S.D. of at least three determinations.

characteristic of apoptosis. At the concentration of 75 μM cisplatin, approximately 50% of the cells were already apoptotic at 5 h of treatment (Fig. 1B). The frequency of apoptosis increased thereafter, but "secondary" (apoptosis-derived) necrosis and cell destruction started to be observed. Hence, 75 μM cisplatin and 5 h were the concentration and maximum time period adopted to analyze regulatory mechanisms with this experimental design. Pulse-treatment experiments indicated that a minimum of 3 h exposure to cisplatin was required for the drug to be fully effective—i.e., to cause similar toxicity as in the continuous treatment (compare 3+2 and 3+5 h in Fig. 1C, with 5 and 8 h, respectively, in Fig. 1B). At all assayed conditions, the frequency of "primary" necrotic cells was negligible (approximately 4%, as in untreated cultures). A more detailed examination of apoptotic and necrotic markers was carried out in preceding works [6,14], and hence is omitted here.

For comparison with the conditions used with other cell models [9–12], experiments were also carried out in which lower cisplatin concentrations (1–8 μM) were applied for longer time periods (16–24 h). We also observed a concentration- and time-dependent apoptosis induction, reaching 40–50% at 24-h treatment with 5 μM cisplatin (Fig. 1D and E). However, this type of treatment also elicited other cellular responses, such as cell blockade at G_2/M, cell hypertrophy, and expression of differentiation markers (Ref. [26], and results not shown). For this reason, this experimental design was only occasionally used for confirmatory purposes.

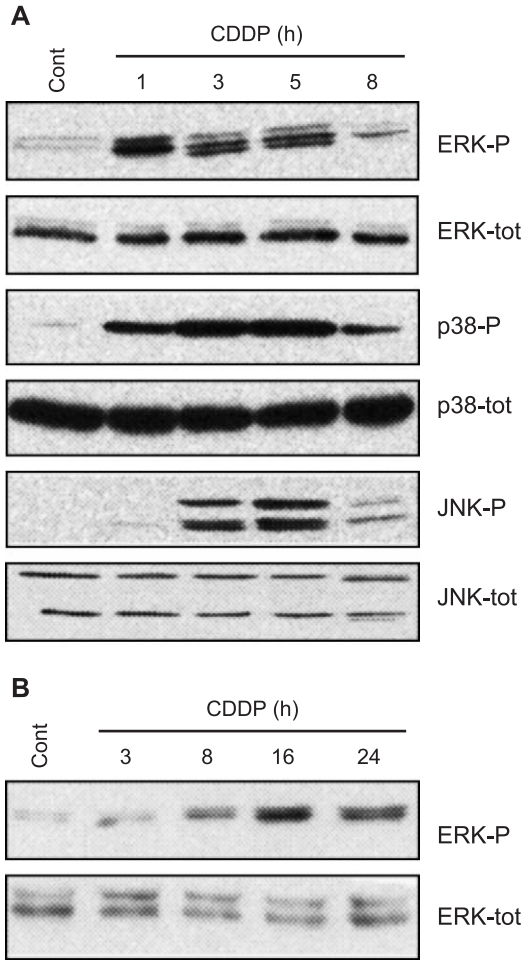

Fig. 2. MAPK activation by cisplatin. (A) Relative levels of total (-tot) and phosphorylated (-P) ERK1/2, p38, and JNK, in untreated U-937 cells (Cont) and cells treated for the indicated time-periods with 75 μM cisplatin, as measured by immunoblot using the appropriate antibodies. (B) Relative level of total and phosphorylated ERK1/2 in cells treated for the indicated time-periods with 5 μM cisplatin.

3.2. ERK activation and effect of ERK inhibitors

Next, we analyzed the capacity of cisplatin to cause ERK activation, as measured by the increase in the phosphorylation state. As indicated in Fig. 2A, 75 μM cisplatin stimulated ERK1/2 phosphorylation, which was already detected at 1 h of treatment. Similar responses were observed with the other major MAPKs, which were here analyzed for comparison—namely increased p38 phosphorylation at 1 h of treatment, and increased JNK phosphorylation at 3 h of treatment (Fig. 2A). Treatment with 5 μM cisplatin (adopted for the long-term experiments) also stimulated ERK phosphorylation, albeit at later times (Fig. 2B).

The possible role of ERKs as regulators of cisplatin-induced apoptosis was then investigated using appropriate

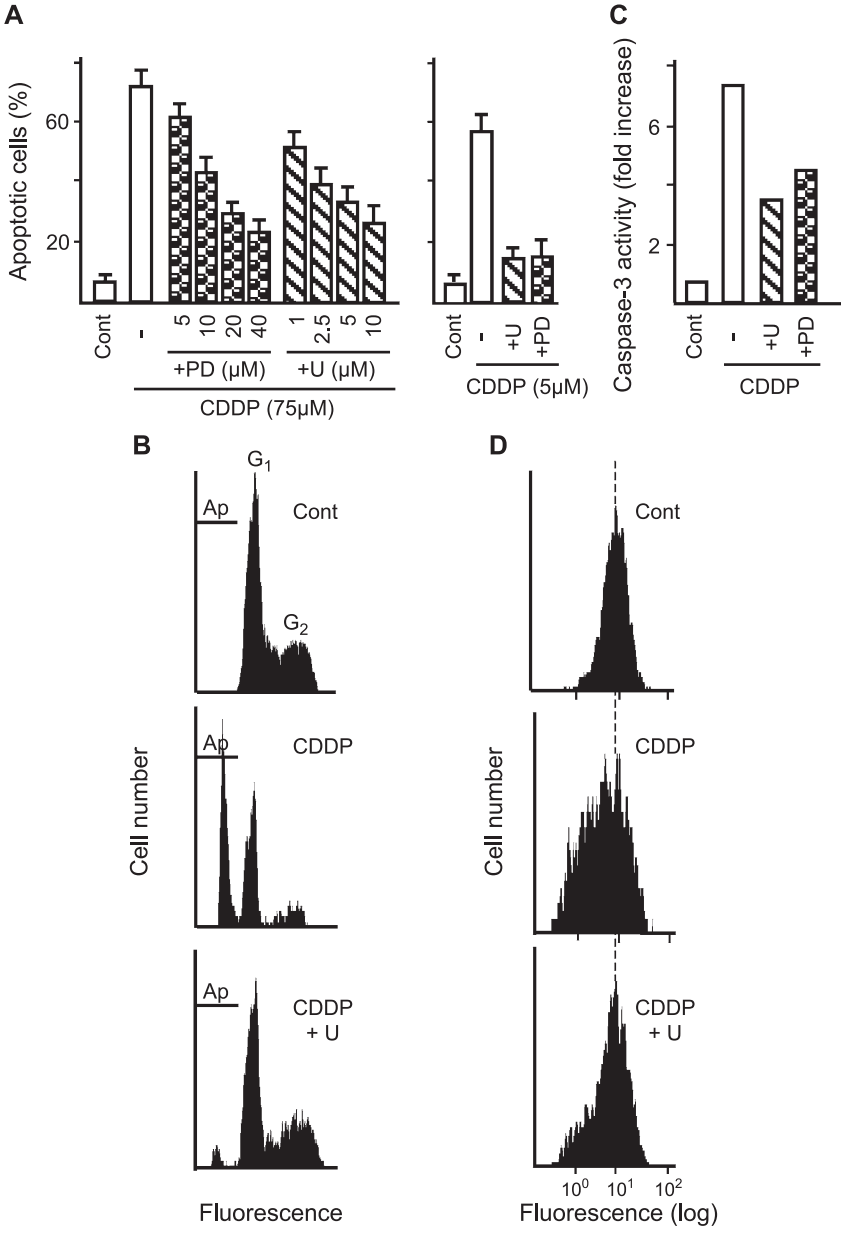

Fig. 3. Effect of ERK inhibitors on cisplatin-induced apoptosis, caspase-3 activity and mitochondrial transmembrane potential ($\Delta\Psi_m$). (A) Left panel, frequency of U-937 cells with fragmented chromatin in untreated cultures (Cont) and cultures treated for 5 h with 75 μM cisplatin in the absence (−) or presence of the indicated concentrations of PD98059 (+PD) and U0126 (+U). Right panel, similar determinations, using 5 μM cisplatin, 20 μM PD98059 and 2.5 μM U0126, for 24 h. (B) Cell distribution according to their DNA content, as determined by cell permeabilization, PI staining and flow cytometry. Cells with sub-G_1 DNA content are considered as apoptotic (Ap). (C) Caspase-3 activity, as determined by in vitro assays using total cellular extracts and DEVD-*p*NA as substrate. (D) Disruption of $\Delta\Psi_m$, as determined by the changes in fluorescence upon rhodamine 123 loading. The vertical dotted line in each profile represents the main fluorescence value in the control, to better discern the displacement caused by the treatments. In B–D, cisplatin was applied at 75 μM for 5 h, and PD98059 and U0126 were used at 40 and 10 μM, respectively. The kinase inhibitors were always added 30 min before cisplatin. All other conditions were as in Fig. 1.

pharmacological inhibitors of the MEK/ERK pathway, namely PD98059 and U0126 [27,28]. More direct approaches could not be employed, due to the poor efficacy and/or excessive toxicity of the commonly used transfection procedures in U-937 and other myeloid cells (results not shown). It was observed that both PD98059 (in the range of 5 to 40 μM) and U0126 (in the range of 1 to 10 μM) caused a concentration-dependent attenuation of apoptosis induction by 75 μM cisplatin (Fig. 3A). On the basis of these observations—and also of our earlier results which demonstrated effective inhibition of ERK phosphorylation [16,25]—the concentrations of 40 μM PD98059 and 10 μM U0126 were adopted for further experiments. The MEK/ERK inhibitors also reduced apoptosis induction in the long-term experiments (24 h with 5 μM cisplatin: Fig. 3A). The protective action of the inhibitors was corroborated by measuring their capacity to decrease the frequency of cells with sub-G_1 DNA content, which is also a marker of apoptosis (Fig. 3B); and to attenuate the activity of caspase-3, which is required for apoptosis execution (Fig. 3C). In addition, the inhibitors reduced the dissipation of mitochondrial transmembrane potential ($\Delta\Psi_m$) (Fig. 3D, and results not shown), a phenomenon which normally accompanies, and may also regulate, apoptosis induction [29].

To analyze whether the protective action of MEK/ERK inhibitors was a cell line-specific effect, THP-1 human promonocytic, HL-60 human myelomonocytic, and NB-4 human acute promyelocytic leukemia cells were treated with 150, 40 and 100 μM cisplatin, respectively. These concentrations were adopted after performing dose-response assays (not shown), since they caused approximately 40–60% apoptosis at 5 h of treatment. It was observed that PD98059 and U0126 attenuated apoptosis in those cell lines, as measured by chromatin fragmentation (Fig. 4A). By contrast, the MEK/ERK inhibitors failed to attenuate apoptosis in RAW 264.7 mouse macrophages and NRK-52E rat renal tubular cells treated with 75 and 100 μM cisplatin, respectively, here used as additional controls (Fig. 4A). These results were corroborated by measuring the frequency of cells with sub-G_1 DNA (Fig. 4B). Adequate controls indicated that in all assayed cell lines cisplatin stimulated ERK phosphorylation, which was reduced by the MEK/ERK inhibitors (results not shown).

To analyze whether the protective action of MEK/ERK inhibitors is specific for cisplatin, U-937 cells were treated

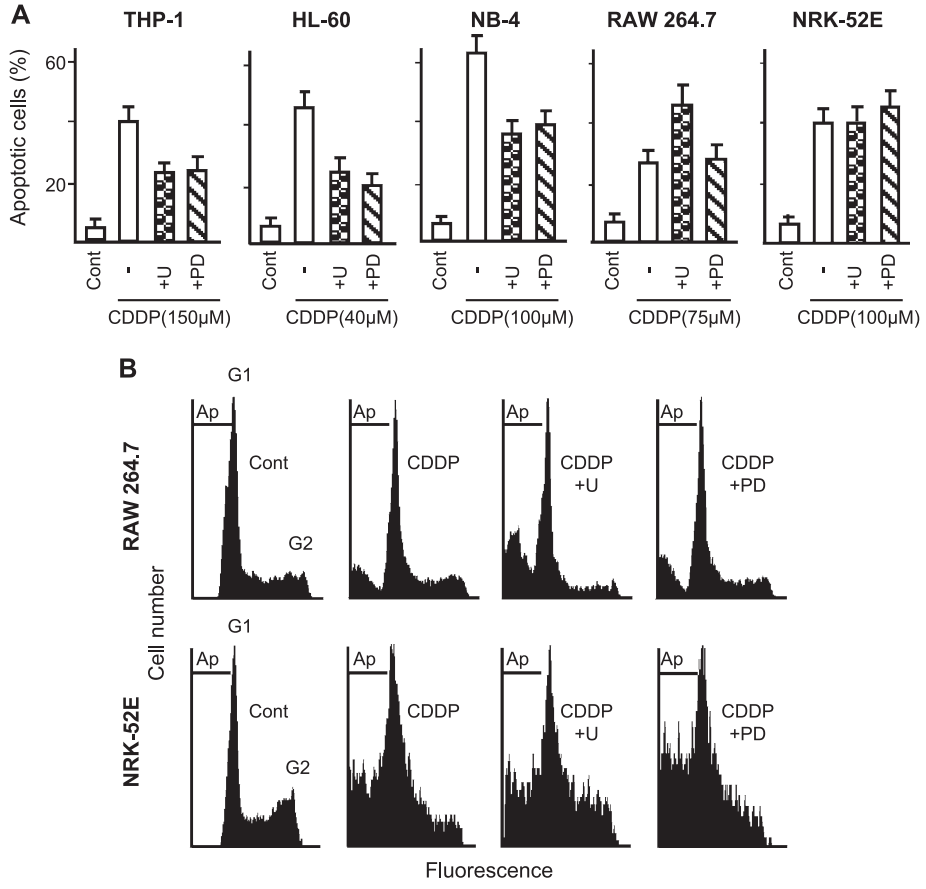

Fig. 4. Modulation of cisplatin-induced apoptosis by ERK inhibitors in different cell lines. (A) Frequency of apoptotic cells, as determined by chromatin fragmentation, in THP-1, HL-60, NB-4 and RAW 264.7 cell cultures treated for 5 h, and NRK-52E treated for 24 h with the indicated concentrations of cisplatin, in the absence (−) or presence of 10 μM U0126 and 40 μM PD98059. (B) Cell distribution according to their DNA content in RAW 264.7 and NRK-52E cells, using the same conditions as above. All other conditions were as in Figs. 1 and 3.

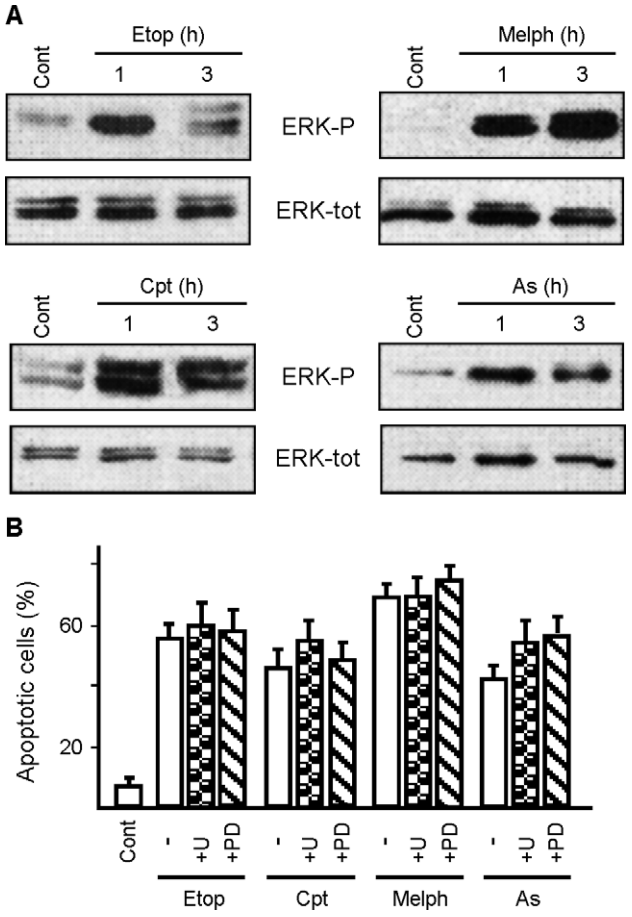

Fig. 5. ERK activation and apoptosis induction by different antitumour drugs. (A) Relative levels of total and phosphorylated ERKs in untreated U-937 cells (Cont) and cells treated for the indicated time periods with 10 μM etoposide (Etop), 0.4 μM camptothecin (Cpt), 40 μM melphalan (Melph) and 35 μM arsenic trioxide (As). (B) Frequency of apoptotic cells upon treatment for 5 h with the indicated drugs, in the absence (−) or presence of 10 μM U0126 (+U) and 40 μM PD98059 (+PD). All other conditions were as in Figs. 1 and 3.

with other antitumour drugs, namely the DNA topoisomerase II inhibitor etoposide (10 μM), the DNA topoisomerase I inhibitor camptothecin (0.4 μM), the DNA alkylating drug melphalan (40 μM), and the anti-leukemic agent arsenic trioxide (35 μM). These concentrations were adopted from earlier studies [6] and preliminary dose-response studies (result not shown). These agents rapidly caused ERK activation (Fig. 5A), as in the case of cisplatin. Nonetheless, the MEK/ERK inhibitors failed to prevent the generation of apoptosis, as measured by chromatin fragmentation (Fig. 5B). These observations were corroborated by measuring the frequency of cells with sub-G_1 DNA content (result not shown).

It was earlier indicated that the activation of the MEK/ERK pathway by cisplatin may be mediated by PKC activation [8]. For this reason, additional determinations were carried out using GF109203X, a broad-spectrum PKC inhibitor [30]. Control assays indicated that 2 μM GF109203X prevented the ERK activation caused by 20 nM TPA, a typical PKC activator (Fig. 6A). However, GF109203X did not affect ERK activation (Fig. 6A) and apoptosis induction (Fig. 6B) by cisplatin, indicating that these effects are independent of PKC in this experimental system.

3.3. DNA platination, intracellular oxidation and effect of antioxidants

It is generally accepted that the toxicity of cisplatin is primarily the consequence of its capacity to bind genomic DNA [5,7]. Nevertheless, we have demonstrated that cisplatin causes intracellular oxidation, which may also be important for apoptosis [6]. For these reasons, experiments were carried out to measure DNA platination, as an indication of cisplatin–DNA binding, as well as intracellular accumulation of peroxides, as an indication of oxidation. It was observed that cisplatin caused a time-dependent increase in the amount of DNA-bound platinum, which was reduced by U0126 (Fig. 7A). The maximum degree of reduction was attained at 3 h, the critical period to trigger apoptosis (see Fig. 1C), and decreased at 5 h, when apoptosis is already under execution (see Fig. 1B). Measurements at 1 h gave very low, unreliable values, and hence are omitted. In addition, cisplatin increased peroxide accumulation, and the increase was also reduced by U0126 (Fig. 7B). Noteworthy, experiments using antioxidant agents revealed that BHA (a nonspecific antioxidant) and catalase (specific against hydrogen peroxide) attenuated the cispla-

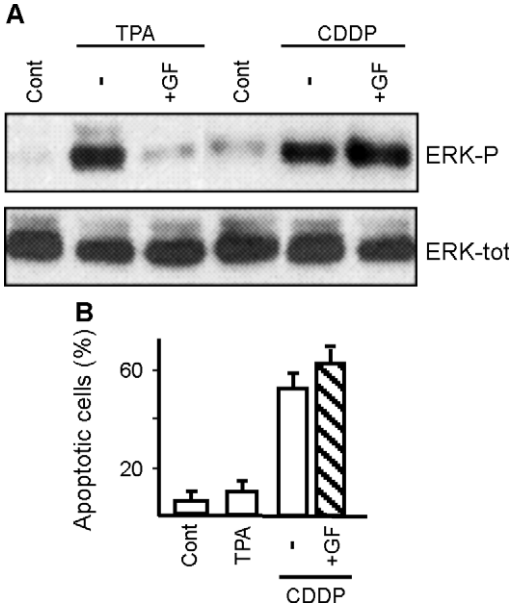

Fig. 6. Effect of PKC inhibition on ERK activation and apoptosis. (A) Frequency of total and phosphorylated ERKs upon treatment of U-937 cells for 3 h with 20 nM TPA or 75 μM CDDP, in the absence (−) or presence of 2 μM GF109203X (+GF). (B) Frequency of apoptosis in cell cultures subjected for 5 h to the same treatments. All other conditions were as in Figs. 1 and 3.

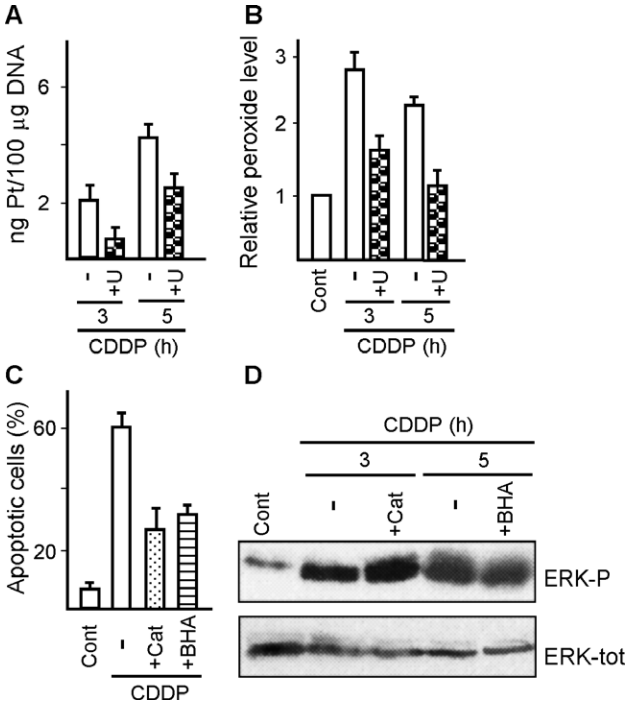

Fig. 7. DNA platination, intracellular oxidation, and effect of antioxidants. (A) Amounts of DNA-bound platinum, as determined by FI-ICP-MS, in U-937 cells treated for the indicated time periods with cisplatin, in the absence (−) or presence of U0126 (+U). (B) Changes in intracellular peroxide accumulation, as determined by H_2DCFDA-derived fluorescence, in U-937 cells treated for the indicated time periods with cisplatin, with and without U0126. The results are expressed in relation to untreated cells (Cont), which received the arbitrary value of one. (C) Frequency of apoptosis upon treatment for 5 h with cisplatin, either in the absence (−) or presence of 500 U/ml catalase (+Cat) or 200 μM BHA (+BHA). (D) Relative levels of total and phosphorylated ERKs upon treatment for the indicated time periods with cisplatin, with and without catalase and BHA. Cisplatin was always used at 75 μM, and U0126 at 10 μM. All other conditions were as in Figs. 1 and 3.

tin-provoked apoptosis (Fig. 7C), but failed to reduce ERK phosphorylation (Fig. 7D). Taken together, these results indicate that ERK inhibitors exert their protective action at some point upstream from the trigger of intracellular oxidation.

3.4. Intracellular platinum content

The observation that U0126 inhibited apparently unrelated effects of cisplatin, such as DNA platination and intracellular oxidation, might indicate that the ERK inhibitor affects the drug availability. For this reason, experiments were carried out to measure the intracellular platinum content, as an indication of cisplatin accumulation, in the absence and presence of MEK/ERK inhibitors. The results are indicated in Fig. 8A. It was observed that treatment for 1–5 h with 75 μM cisplatin caused a slow, progressive increase in intracellular platinum content, and that the increase was reduced by U0126 and PD98059. As in the case of DNA-bound platinum, the maximum degree of reduction was attained at 3 h, the critical regulatory period prior to apoptosis execution. By contrast, intracellular platinum was not reduced by the PKC inhibitor GF109203X in U-937 cells (Fig. 8A), or by PD98059 in RAW 264.7 macrophages (Fig. 8B), two situations in which cell death was not affected (see Figs. 6 and 4, respectively). Moreover, control experiments using arsenic trioxide revealed a rapid intracellular arsenic accumulation, which was not reduced and was even slightly increased by PD98059 (Fig. 8C), a result which parallels the effect of the inhibitor on arsenic trioxide-provoked apoptosis (see Fig. 5B). Taken together, these observations strongly suggest that reduction of intracellular drug accumulation is a mechanism by which MEK/ERK inhibitors attenuate the toxicity of cisplatin in U-937 cells.

3.5. GSH levels

It is known that cisplatin is detoxified by a mechanism which includes conjugation with GSH, followed by

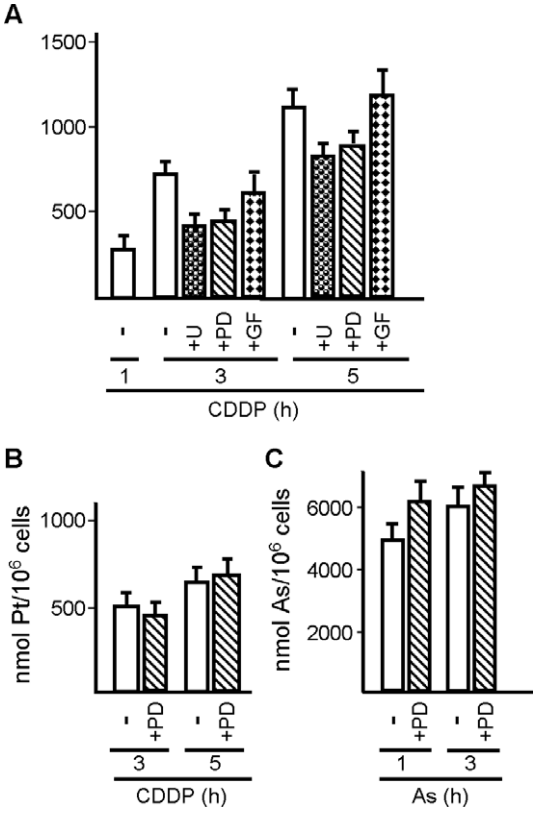

Fig. 8. Intracellular platinum and arsenic content. (A) Intracellular platinum content in U-937 cells treated for the indicated time periods with cisplatin in the absence (−) or presence of U0126, PD98059, or GF109203X. (B) Intracellular platinum content in RAW 264.7 macrophages treated with cisplatin in the absence (−) or presence of PD98059. (C) Intracellular arsenic content in U-937 cells treated with arsenic trioxide (As) in the absence (−) or presence of PD98059. Cisplatin and arsenic trioxide were used at 75 and 35 μM, respectively; and U0126 (+U), PD98059 (+PD) and GF109203X (+GF), at 10, 40 and 2 μM, respectively. The determinations were carried out by FI-ICP-MS, using total cellular extracts. The results represent the mean±S.D. of four (A) or three (B, C) determinations.

extrusion from cell [7,15, and references therein]. For this reason, we asked whether the reduction in intracellular cisplatin accumulation and attenuation of apoptosis by MEK/ERK inhibitors could be a consequence of alterations in GSH content. As indicated in Fig. 9A, treatment with 75 μM cisplatin decreased the GSH level, and the decrease was prevented by U0126. Nevertheless, the importance of GSH reduction as a regulatory event was unclear. In fact, GSH depletion was only clearly observed at 5 h of treatment, a time at which apoptosis is already under execution (see Fig. 1B); and U0126 did not modify GSH content at 3 h, a time at which the intracellular cisplatin accumulation was greatly decreased (see Fig. 8A). Hence, additional determinations were carried out using GSH-OEt, a cell-permeable compound earlier used to augment the intracellular GSH content in U-937 cells [31]. Since cisplatin could directly bind GSH in solution, in these experiments the drugs were separately administered—i.e., a 3-h pulse-treatment with cisplatin alone, followed by recovery with GSH-OEt alone. As indicated in Fig. 9B, GSH-OEt failed to reduce the toxicity of cisplatin.

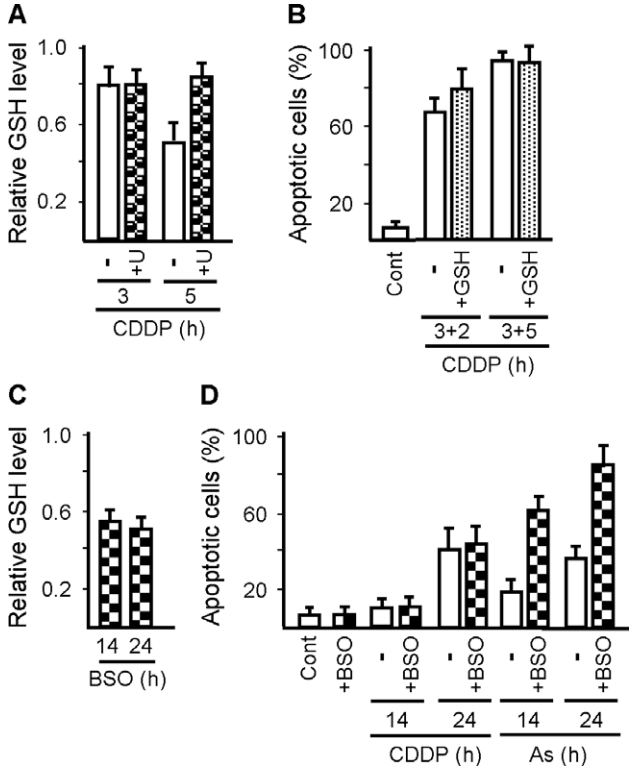

Fig. 9. GSH levels and effects of GSH-OEt and BSO. (A) Changes in intracellular GSH content, as determined by cell loading with monochlorobimane and fluorometry, in U-937 cells treated for the indicated time periods with 75 μM cisplatin, in the absence (−) or presence of U0126 (+U). The values are expressed in relation to untreated cells (Cont), which received the arbitrary value of one. Approximate GSH content in controls: 9.5 nmol/10^6 cells. (B) Frequency of apoptotic cells in cultures pulse-labelled for 3 h with cisplatin alone, and then allowed to recover for 2 (3+2) or 5 (3+5) h in cisplatin-free medium in the absence (−) or presence of 5 mM GSH-OEt (+GSH). (C) Changes in intracellular GSH levels at the indicated time-periods of treatment with 1 mM BSO. (C) Frequency of apoptotic cells in cultures treated for 24 h with 1 mM BSO alone, and for the indicated time-periods with 5 μM cisplatin or 4 μM arsenic trioxide, either with (+BSO) or without (−) 24-h pre-incubation with BSO. All other conditions were as in Figs. 1 and 3.

The relationship between GSH content and cisplatin toxicity was also examined in the long-term experiments using BSO, a specific inhibitor of the activity of γ-glutamylcysteine synthetase, the rate-limiting enzyme for GSH biosynthesis [32]. Although 1 mM BSO caused an approximately 50–60% reduction in intracellular GSH content at 14–24 h (Fig. 9C), co-treatment with BSO did not modify the cisplatin toxicity (Fig. 9D). This contrasts with the response of arsenic trioxide, the toxicity of which was potentiated by co-treatment with BSO (Fig. 9D). Taken together, these results indicate that GSH is not a regulatory factor for cisplatin toxicity under the here used experimental conditions.

4. Discussion

The results in this work corroborate our previous observations indicating that cisplatin causes apoptosis in U-937 promonocytic cells [6,14]. Pulse-treatment experiments revealed that a relatively prolonged exposure to the drug (3 h) is required for cisplatin to efficaciously trigger cell death. This contrasts with the rapid action of other antitumour drugs, such as arsenic trioxide or the DNA-damaging agents etoposide and camptothecin, which require shorter pulse-treatments (0.5–1 h) to trigger apoptosis (results not shown). The delayed effect of cisplatin on cell viability is consistent with, and may be a consequence of, its slow kinetics of intracellular accumulation and DNA binding (see Figs. 7A and 8A). Our results also show a correlation between ERK activation and apoptosis induction, as judged by the capacity of cisplatin to cause ERK phosphorylation, and the capacity of PD98059 and U0126 to attenuate cell death. Although this later effect was equally observed in all assayed human myeloid cell lines (U-937, THP-1, HL-60 and NB-4), it was not obtained with other cell types (RAW 264.7, NRK-52E), confirming the notion that the protective action of MEK/ERK inhibitors is a cell type-specific response [8–13]. On the other hand, the attenuation of cisplatin toxicity was a drug-specific response, which could not be reproduced with other antitumour drugs.

It must be pointed out that, although 1-h treatment period with cisplatin was insufficient to cause apoptosis, it sufficed to cause ERK activation (see Fig. 2), suggesting that this effect requires minimal levels, or is even is independent of the intracellular drug content. A possible explanation is that cisplatin triggers kinase activation by direct interaction with plasma membrane components. In this regard, it has been demonstrated that cisplatin binds membrane phospholipids, causing perturbations in the bilayer structure which might affect the membrane functioning [33,34]. More specifically, it has been indicated that ERK activation and apoptosis

induction by cisplatin and other DNA-damaging agents are mediated by growth factor receptor activation—although the exact mechanism by which the drugs may affect growth factor receptors is unclear [11,35, and references therein]. The observations that cisplatin behaved as an oxidant agent, and that the antioxidant *N*-acetyl cysteine attenuated epidermal growth factor receptor activation, ERK activation and apoptosis, suggested that intracellular oxidation may be an upstream mediator of cisplatin effects [11,35]. Nevertheless, this interpretation must be considered with caution, since *N*-acetyl-L-cysteine is a thiol-containing molecule, which could protect the cells by direct binding and inactivation of cisplatin, rather than because of its antioxidant properties. Actually, in our experiments the antioxidant agents BHA and catalase attenuated cell death without reducing ERK phosphorylation in U-937 cells. Instead, U0126 reduced peroxide accumulation, which is consistent with earlier results indicating that MAP kinases may mediate the trigger of oxidation in myeloid cells [25, and references therein]. On the other hand, the possibility that PKC could mediate the action of cisplatin in U-937 cells may be excluded, due to the inability of the PKC inhibitor GF109203X to prevent ERK phosphorylation and apoptosis in this cell line.

Finally, our results showed that the MEK/ERK inhibitors reduce the intracellular cisplatin accumulation in U-937 cells, which correlates with their capacity to attenuate apoptosis. The possibility that the protective action of the inhibitors could be a trivial consequence of cisplatin scavenging via direct drug–drug interaction in the extracellular milieu may be excluded, since no protection was observed in RAW 264.7 and NRK-52E cells, when used under similar experimental conditions as in U-937 cells. Hence, we may reasonably conclude that the MEK/ERK inhibitors somewhat affect the overall cisplatin transport system(s) in human myeloid cells. The identification of the exact target(s) of regulation is a difficult task, due to the great complexity and still incomplete knowledge of the cisplatin import/export machinery (reviewed in Ref. [7]). We focussed the attention on the intracellular GSH content, since (i) as indicated above, GSH depletion was apparently the immediate reason by which TPA-provoked ERK activation increased the toxicity of arsenic trioxide, a GSH-sensitive drug, in U-937 cells [16]; and (ii) the level of intracellular GSH was reported to be the rate-limiting step of the glutathione-based detoxification machinery in HepG2 cells, which leads to cisplatin extrusion from the cells [15]. However, intracellular GSH may not play a significant regulatory role in the present experimental conditions. In fact, treatments with the GSH elevating agent GSH-OEt and the GSH depleting agent BSO failed to affect the cisplatin-induced apoptosis (see Fig. 9B and D). Moreover, the decrease in intracellular cisplatin accumulation caused by MEK/ERK inhibitors at 3 h of treatment (see Fig. 8A) was not accompanied by a change in GSH level (see Fig. 9A). Hence, the late GSH decrease at 5 h of treatment with 75 μM cisplatin (concomitant with the execution of apoptosis) and its reversal by U0126 are probably a mere consequence of cell death. Of course, the exclusion of GSH as a critical factor does not invalidate the hypothesis that cisplatin transport (either drug uptake or efflux) is susceptible to regulation by protein kinases. For instance, PKC activation by phorbol ester was reported to potentiate cisplatin accumulation in HeLa cells by stimulating the rate of drug uptake [36]. On the other hand, the capacity of MEK/ERK inhibitors to provide a late protection against cisplatin in osteosarcoma and neuroblastoma cells [13] might indirectly argue for an increased rate of drug efflux in these cell types.

In summary, the present results represent the first direct demonstration that MEK/ERK inhibitors reduce cisplatin accumulation in human myeloid cells, and as such also provide a good explanation for the protective (anti-apoptotic) action of these inhibitors in these cells. Whether the same mechanism may account for the protective effect of ERK inhibition in other cell types remains to be investigated. Of course, this explanation does not exclude an additional contribution of other regulatory mechanisms—e.g., the modulation of critical transcription factor activities, as earlier reported [12].

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