Multivalent Choline Dendrimers as Potent Inhibitors of Pneumococcal Cell Wall Hydrolysis**

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Streptococcus pneumoniae (pneumococcus) causes multiple illnesses in humans, including pneumonia, meningitis and acute otitis media.[1] It especially affects children under 2 years of age and the elderly, with an estimation of 1.6 million deaths a year.[2] The fight against this pathogen is hindered by the increase of antibiotic resistance and the limited efficacy of current vaccines.[1] A characteristic feature of the pneumococcal cell wall is the presence of teichoic acids decorated with phosphocholine groups[3] (Figure 1 a). These multivalent architectures serve as attachment sites for a variety of surface-exposed choline-binding proteins (CBPs) that are involved in essential processes for virulence such as cell-wall division, the release of bacterial toxins and adhesion to the host.[4] All CBPs contain a characteristic choline-binding module (CBM) consisting of several choline binding repeats, sequences of about 20 amino acids with a loop-β-hairpin structure, that are arranged into a left-handed superhelix.[5,6] Two consecutive repeats configure a choline binding site by means of three aromatic residues. The best characterized member of these choline-binding modules, the C-terminal moiety of the LytA amidase (C-LytA),[7,8] contains six of these repeat sequences that together form four choline-binding sites[5] (Figure 1 b). Because CBPs are common to all serotypes, they are attractive drug targets for the treatment of pneumococcal diseases. Exogenously added choline and choline analogues competitively inhibit the binding of CBPs to the cell wall, blocking cell separation and the characteristic autolysis of *S. pneumoniae* at the end of the stationary phase of growth, inducing instead the formation of long chains,[9] or even preventing growth[10]. These effects are thought to reduce bacterial virulence by preventing the release of toxins upon cell autolysis and limiting the dissemination of the bacteria on the host tissue during infection[11]. However, the required concentrations of these compounds (in the millimolar range) are much too high for its therapeutic use as antimicrobials in vivo.

The weak interaction between choline and a single choline binding site[10,12] explains the need for tandem choline binding sites in C-LytA that match the multivalent arrangement of choline groups on the teichoic acids (Figure 1). The strategy reported here was to develop strong and highly specific inhibitors by mimicking this characteristic presentation of choline residues on the cell wall. Multivalency has been

recognized as an important strategy to develop semi-synthetic ligands with high affinity and specificity for biological targets.[13] Here, dendrimers were chosen as attractive scaffolds that can provide well-defined multivalent ligands with enough inherent flexibility to adjust to the valency and spacing of choline binding sites.[14]

Choline-functionalized poly(propylene imine) dendrimers containing 4, 8, 16 or 64 choline end groups (**g1-cho, g2-cho**, **g3-cho**, and **g5-cho**, respectively) were readily obtained by reaction of the amine end groups of poly(propylene imine) dendrimers with activated choline (Scheme 1; Figure S1 in the Supporting Information), showing well-defined structures after synthesis and workup (Figure S2 in the Supporting Information). A fusion protein of C-LytA with green fluorescent protein (C-Lyt-GFP) was used to provide initial evidence for the interaction of these choline dendrimers with CBMs in solution. Titration of choline dendrimers to C-Lyt-GFP resulted in a substantial decrease in the fluorescence anisotropy of the GFP moiety (Figure 2 a), most likely as a result of energy transfer between two close GFP fluorophores assembled around a single dendrimer particle (Figure S3 in the Supporting Information). Addition of choline to C-Lyt-GFP, which promotes specific dimerization through the C-LytA module[5,7], also induced similar changes in anisotropy (Figure S3 c). Importantly, the apparent affinity was strongly dependent on the dendrimer generation, showing half-maximal effects at 0.1 μM for **g5-cho**, 1 μM for **g3-cho** and **g2-cho**, and 100 μM for **g1-cho**. The change in anisotropy was specific for the interaction between choline dendrimers and C-LytA, since no changes in anisotropy were observed upon addition of a non-derivatized 3rd generation dendrimer (**g3-NH_2**) to C-Lyt-GFP (Figure 2 a), or the titration of yellow fluorescent protein alone with choline dendrimers (Figures S3 b,d in the Supporting Information). At concentrations of dendrimers above 100 μM visible aggregates appeared that could be resolubilized by the addition of free choline, suggesting that such aggregates may comprise a network of choline dendrimers and protein cross-linked through specific, multiple choline-dependent interactions.

The far-UV circular dichroism (CD) spectrum of C-LytA is dominated by the contribution of aromatic residues in the choline binding sites.[12] In contrast to the binding of free choline, a decrease in the CD signal was observed upon addition of choline dendrimers. (Figure 2 b and Figure S4 in the Supporting

Information) suggesting a different conformational change in the protein. Again, the effects were observed at 0.1-1 μM concentrations for the higher generation dendrimers, representing a 10^3-10^4 fold increase of apparent affinity compared to monovalent choline.[8,12]

Next, surface plasmon resonance (SPR) studies were performed. Specific and stable binding was observed for various generations of choline dendrimers to immobilized C-Lyt-GFP, while no binding was detected to immobilized enhanced cyan fluorescent protein (ECFP) (Figure 2 c). The binding was unaffected by high salt concentrations (1 M NaCl; data not shown), but efficiently disrupted by 1 M choline which is consistent with specific binding of choline dendrimers to the C-LytA moiety. Analysis of titration experiments indicated the presence of two binding modes, a high affinity site with K_d's of ~0.03 μM (**g5-cho**, **g3- cho**), 0.15 μM (**g2-cho**) and 4.1 μM (**g1-cho**), and a low affinity site with K_d's of ~3 μM (**g5-cho** and **g3-cho**), 23 μM (**g2-cho**), and 263 μM (**g1-cho**) (Figure 2 d and Table S1 in the Supporting Information). To exclude possible effects of surface multivalency and protein immobilization, the interaction between CBMs and choline dendrimers was also studied using biotin-labeled **g3-cho** and **g5-cho** immobilized on streptavidin-functionalized SPR chips. Again, strong and specific binding was observed for CBM-containing proteins (C-LytA and C-Lyt-GFP) at low μM concentrations, while no relevant binding was detected for GFP (Figure S5 in the Supporting Information). The presence of two binding modes was observed with K_d values that were similar to the ones obtained using immobilized C-Lyt-GFP (Table S1 and Figures S5 b-e in the Supporting Information).

To test whether choline dendrimers can effectively block the binding of CBPs to the pneumococcal cell wall, their inhibitory potency was determined in vitro for the LytA amidase, LytB β-*N*-acetylglucosaminidase, LytC lysozyme and Pce phosphorylcholinesterase (Table 1 and Figure S6 in the Supporting Information**)**. These enzymes are involved in essential virulent processes such as regulation of the availability of choline residues in the cell wall, separation of daughter cells at the end of cell division and autolysis at the end of the stationary phase[15]. Similar inhibitory profiles by dendrimers are observed for enzymes with totally different catalytic domains, confirming that the inhibition results from specific

binding to their common CBMs. A peculiar activation of LytA and LytC is achieved at low choline concentrations, a phenomenon that has been described before[10], but that is not detected with choline dendrimers. Excellent agreement was observed between the IC_{50} values obtained for full length LytA and the apparent affinities obtained for its isolated choline-binding module in the *in vitro* binding experiments, showing a ~10^4-10^5 fold increased potency of **g2-cho**, **g3-cho** and **g5-cho** compared to free choline (Table 1). The most pronounced increases in affinity were observed between free choline and **g1-cho**, and between **g1-cho** and **g2-cho**, probably reflecting an increased ability to form multiple, simultaneous interactions within the same CBM. Dendrimers beyond the 2nd generation most likely do not improve this concurrent binding and only display a more gradual increase in affinity due to statistical effects that favor binding of the higher generation dendrimers.

In order to evaluate the effects of choline dendrimers on *S. pneumoniae* growth, we checked their effect on cell separation and autolysis in the stationary phase of a cell culture. Addition of choline dendrimers to the medium at the beginning of the exponential growth phase effectively blocked autolysis of *S. pneumoniae* R6 cultures after a 17 h incubation, as deduced from the maintenance of the optical density (attenuance) values, which are directly related to the density of bacterial cells. A concentration of around 10 μM for **g5-cho** and **g3-cho** and 100 μM for **g2-cho,** exerted a similar effect as 50 mM choline (Figure 3 a and Figure S7 in the Supporting Information). At 200 μM, **g1-cho** did not completely block autolysis, but the attenuance after 17 h was somewhat higher than the control without additives, indicating some residual effect. Addition of 100 μM of the unfunctionalized 2nd generation amine dendrimer (**g2-NH_2**) did not induce any effect on growth, which confirms the specificity of the choline dendrimers. Microscopic analysis showed the presence of long chains of cells after 4 h of culture with 100 μM **g2-cho**, **g3-cho** and **g5-cho**, but not with 100 μM **g1-cho** (Figure 3 b). Again, as described,[9] a similar inhibition of cell wall division was observed for free choline only at 50 mM concentrations. Because no other morphological modifications were detected and cell viability remained unaffected (data not shown), these effects on growth are clearly not due to an unspecific toxicity of choline dendrimers.

In conclusion, choline dendrimers were developed that bind to CBMs with high affinity and specificity, yielding an attractive new class of potential antimicrobial compounds. Important advantages of these choline dendrimers over other polymer based inhibitors of cell-protein interactions include their monodispersity and their efficacy at low valency and molecular weight. The strong complementarity between the multivalent dendritic architecture and the tandem arrangement of the choline binding repeats in pneumococcal CBMs is probably a key factor to their effectiveness as inhibitors of pneumococcal cell wall hydrolysis.

Keywords: dendrimers · multivalency · inhibitors · antimicrobial · medicinal chemistry

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Dendritic cell wall mimics

Víctor M. Hernández-Rocamora, Beatriz Maestro, Bas de Waal, María Morales, Pedro García, E.W. Meijer, Maarten Merkx*, Jesús M. Sanz* _ **Page – Page**

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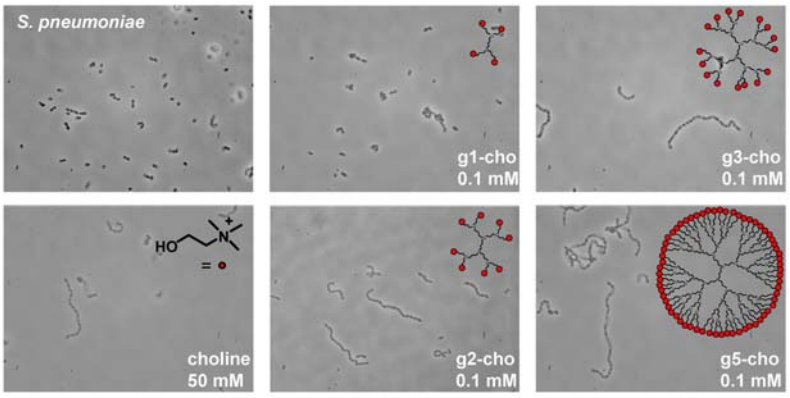


Dendritic cell wall mimics: Choline-binding proteins from *Streptococcus pneumoniae* recognize distinctive multivalent choline architectures on the bacterial cell wall. Choline-functionalized dendrimers are reported as potent inhibitors of these essential enzymes, with a 10^3-10^4 fold increase in apparent affinity compared to free choline, resulting in inhibition of autolysis and cell separation in bacterial cultures at low micromolar concentrations.

Figure Captions:

Figure 1. a) Schematic representation of the *S. pneumoniae* cell wall structure showing the multivalent arrangement of phosphocholine groups. b) X-ray structure of the choline binding module of the LytA amidase (C-LytA) based on PDB 1HCX.[5] Each strand of the homodimer contains six repeats, each one composed of a β-hairpin and a large loop, that together constitute four choline binding sites, occupied in the crystal structure by three choline residues and a dodecylamine-*N*,*N*-dimethyl-*N*-oxide molecule (grey spheres). The image was prepared using the software package PyMol (Delano Scientific LLC).

Figure 2. *In vitro* characterization of the interaction between choline dendrimers and CBPs. a) Binding of choline dendrimers to C-Lyt-GFP studied using fluorescence anisotropy. Various generations of choline dendrimers were titrated to 1 μM C-Lyt-GFP monitoring fluorescence anisotropy at 550 nm using excitation at 480 nm. b) Binding of choline dendrimers to 3 μM C-LytA monitored using CD at 220 nm. c) SPR analysis of interaction between different generations of choline dendrimers and immobilized CLyt-GFP (2000 RU). The arrow represents a 30-sec injection of 1 M choline to the **g5-cho** experiment. The binding of 10 μM **g5-cho** to 2000 RU of immobilized ECFP is shown for comparison. d) Representative SPR equilibrium binding curves of choline dendrimers to C-Lyt-GFP obtained by the titration of a surface with 2000 RU of immobilized C-Lyt-GFP with the indicated choline dendrimers. Solid lines in d) represent the best fit of the data to a model for two binding sites (see the Supporting information). The data displayed in d) were normalized according to the experimental total R_{MAX} (the sum of the R_{MAX} parameters of each binding site) obtained from the fit results, which are shown on Table S1 in the Supporting Information. All the experiments shown in this figure were performed at 20 °C in 100 mM PBS at pH 7 with 500 mM NaCl. High ionic strength was used to avoid non-specific ionic interactions of the positively-charged dendrimers with the proteins.

Figure 3. Effect of choline dendrimers on *S. pneumoniae* growth. a) Relative attenuance (D) of *S. pneumoniae* R6 cultures after 17 h, grown in the presence or absence of the indicated compounds. Data are the average of two independent experiments. b) Phase contrast micrographies of cultures taken after 4 h of growth in the presence or absence of 100 μM of dendrimers or 50 mM choline. Bars represent 5 μm.

Scheme 1. Chemical structures of the choline-functionalized poly(propylene imine) dendrimers used in this study.

Figure 1

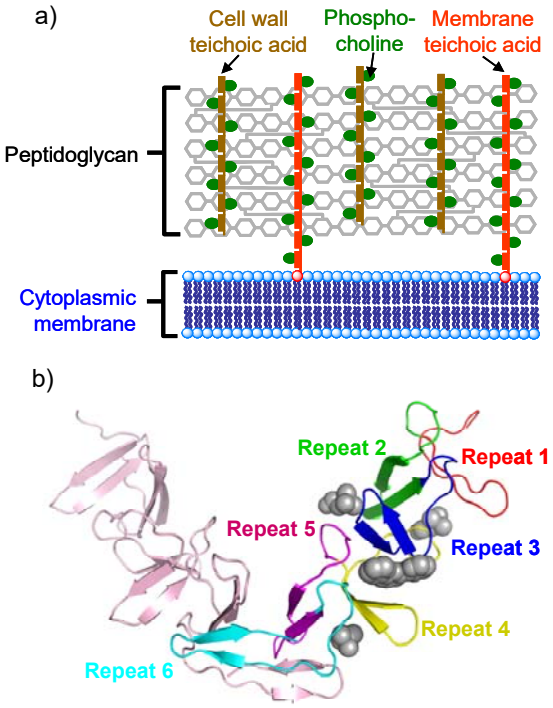

a)
Cell wall teichoic acid
Phospho-choline
Membrane teichoic acid
Peptidoglycan
Cytoplasmic membrane
b)
Repeat 2
Repeat 1
Repeat 5
Repeat 3
Repeat 4
Repeat 6

Figure 2

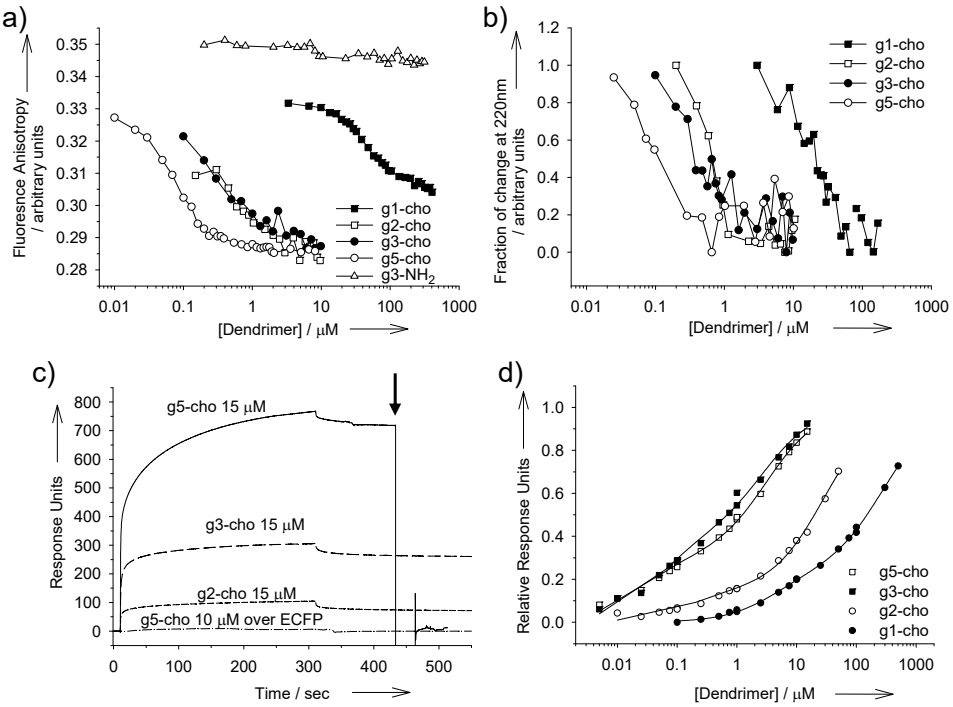
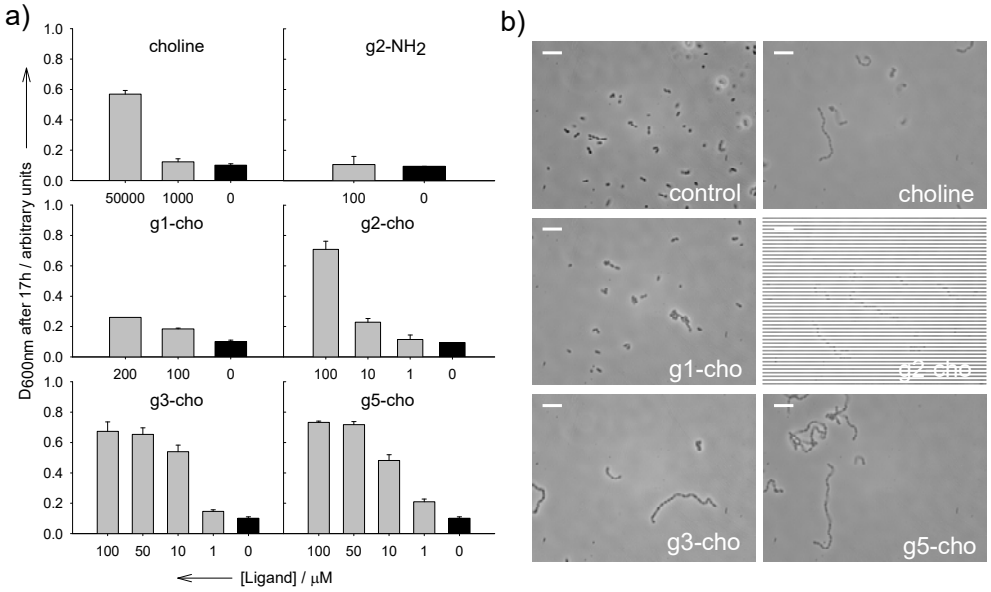

a)
Fluoresnce Anisotropy / arbitrary units
[Dendrimer] / μM
g1-cho
g2-cho
g3-cho
g5-cho
g3-NH2
b)
Fraction of change at 220nm / arbitrary units
[Dendrimer] / μM
g1-cho
g2-cho
g3-cho
g5-cho
c)
Response Units
Time / sec
g5-cho 15 μM
g3-cho 15 μM
g2-cho 15 μM
g5-cho 10 μM over ECFP
d)
Relative Response Units
[Dendrimer] / μM
g5-cho
g3-cho
g2-cho
g1-cho

Figure 3


a)
b)
choline
g2-NH2
g1-cho
g2-cho
g3-cho
g5-cho
D600nm after 17h / arbitrary units
[Ligand] / µM
control
choline
g1-cho
g3-cho
g5-cho

Scheme 1

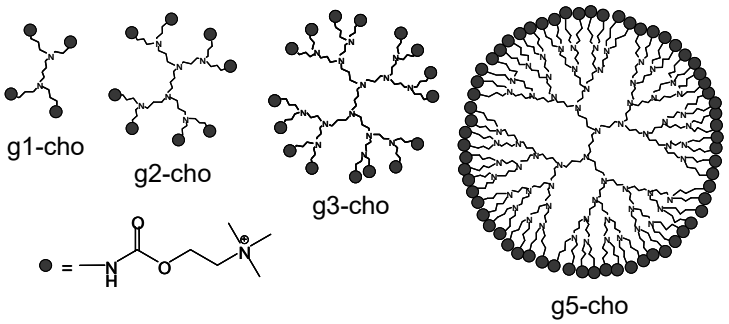


Table 1

Effects of choline dendrimers on the activity of *S. pneumoniae* cell wall lytic enzymes

	IC_{50}[a]				
Enzyme	Choline	g1-cho	g2-cho	g3-cho	g5-cho
LytA	$9.1 \cdot 10^3$	10	0.2	0.02	0.006
LytB	$6.3 \cdot 10^3$	4.7	5.4	3.5	0.9
LytC	$2.0 \cdot 10^3$	60	2.2	2.9	0.3
Pce	$1.9 \cdot 10^3$	15	0.8	0.1	0.8

[a] Concentration of choline and choline dendrimers (IC_{50}) that causes 50% inhibition of enzyme activity for the cell wall lytic enzymes LytA, LytB, LytC and Pce, calculated by interpolation from the curves displayed in Figure S6 in Supporting Information. Activity in the absence of ligands is taken as 100 %. Data in μM.