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

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

Streptococcus pneumoniae (pneumococcus) causes multiple illnesses in humans, including pneumonia, meningitis, and acute otitis media.[1] It especially affects children under two years of age and the elderly, with an estimated 1.6 million deaths per 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 acid units decorated with phosphocholine groups[3] (Figure 1a). These multivalent architectures serve as attachment sites for a variety of surface-exposed choline-binding proteins (CBPs) that are involved in processes essential 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 1b).

** We would like to thank Xianwen Lou for GPC analysis and Biomedal S.L. for supplying the pALEX2-Ca-GFP plasmid to produce C-LytA-GFP. This work was supported by a VIDI grant from the Netherlands Organisation for Scientific Research (grant 700.56.428), the Escuela Valenciana de Estudios para la Salud (Generalidad Valenciana, Spain, Grant 95/2005) and the Fundación Salvat Inguifarma (Spain). Ciber de Enfermedades Respiratorias (CibeRes) is an initiative of Spanish Instituto de Salud Carlos III. Additional funding was provided by the COMBACT program (S-BIO-0260/2006) of the Comunidad de Madrid. V.M.H.-R. was supported by a PhD fellowship from Spanish Ministry of Education.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200803664.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200803664.

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.[7] Each strand of the homodimer contains six repeats, each composed of a β-hairpin and a large loop that together constitute four choline-binding sites, which are occupied in the crystal structure by three choline residues and a dodecylamine-N,N-dimethyl-N-oxide molecule (gray spheres). The image was prepared using the software package PyMol (Delano Scientific LLC).
ine-binding sites in C-LytA that match the multivalent arrangement of choline groups on the teichoic acid units (Figure 1). The strategy reported herein 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 semisynthetic ligands with high affinity and specificity for biological targets.[13] 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), and they showed 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. Addition of choline dendrimers to C-Lyt-GFP resulted in a substantial decrease in the fluorescence anisotropy of the GFP moiety (Figure 2a), 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 (Supporting Information Figure S3c). Importantly, the apparent affinity was strongly dependent on the dendrimer generation, showing half-maximal effects at 0.1 mM for g5-cho, 1 mM 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, as no changes in anisotropy were observed upon addition of a nonderivatized third-generation dendrimer (g3-NH2) to C-Lyt-GFP (Figure 2a) or in the titration of yellow fluorescent protein alone with choline dendrimers (Figure S3b,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 2b and Figure S4 in the Supporting Information), suggesting a different conformational change in the protein. Again, the effects were observed at 0.05–1 μM concentrations for the higher-generation dendrimers, representing a $10^3$–$10^4$-fold increase of apparent affinity compared to monovalent choline.[6,12]

Next, surface plasmon resonance (SPR) studies were performed. Specific and stable binding to immobilized C-Lyt-GFP was observed for various generations of choline dendrimers, while no binding to immobilized enhanced cyan fluorescent protein (ECFP) was detected (Figure 2c). The binding was unaffected by high salt concentrations (1 M NaCl; data not shown) but was 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 dissociation equilibrium constants ($K_d$) of approximately 0.03 (g5-cho, g3-cho), 0.15 (g2-cho), and 4.1 μM (g1-cho); and a low-affinity site with $K_d$ values of approximately 3 (g5-cho and g3-cho), 23 (g2-cho), and 263 μM (g1-cho; Figure 2d 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 micromolar 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 those obtained using immobilized C-Lyt-GFP (Table S1 and Figure S5b–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.[13] 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 this effect 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 $10^3$–$10^4$-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 second generation most likely do not improve this concurrent binding and only display a more gradual increase in affinity owing to statistical effects that favor binding of the higher-generation dendrimers.

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 17 h incubation, as deduced from the stable 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 3a and Figure S7 in the Supporting Information). At 200 μM, g1-cho did not completely block autolysis, but the attenuation after 17 h was somewhat higher than the control without additives, indicating some residual effect. Addition of 100 μM of the unfunctionalized second-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 3b). Again, a similar inhibition of cell-wall division was observed for free choline only at concentrations of 50 mM.[9] 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 unspecified toxicity of choline dendrimers.

### Table 1: Effects of choline dendrimers on the activity of S. pneumoniae cell-wall-lytic enzymes.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Choline</th>
<th>g1-cho</th>
<th>g2-cho</th>
<th>g3-cho</th>
<th>g5-cho</th>
</tr>
</thead>
<tbody>
<tr>
<td>LytA</td>
<td>9.1 × 10$^3$</td>
<td>10</td>
<td>0.2</td>
<td>0.02</td>
<td>0.006</td>
</tr>
<tr>
<td>LytB</td>
<td>6.3 × 10$^3$</td>
<td>4.7</td>
<td>5.4</td>
<td>3.5</td>
<td>0.9</td>
</tr>
<tr>
<td>LytC</td>
<td>2.0 × 10$^4$</td>
<td>60</td>
<td>2.2</td>
<td>2.9</td>
<td>0.3</td>
</tr>
<tr>
<td>Pce</td>
<td>1.9 × 10$^5$</td>
<td>15</td>
<td>0.8</td>
<td>0.1</td>
<td>0.8</td>
</tr>
</tbody>
</table>

[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 the Supporting Information. Activity in the absence of ligands is taken as 100%.

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 arrange-
ment of the choline-binding repeats in pneumococcal CBMs is probably a key factor to their effectiveness as inhibitors of pneumococcal cell-wall hydrolysis.

Received: July 27, 2008
Revised: October 14, 2008
Published online: December 29, 2008

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