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Crystal structures of CbpF complexed with atropine and ipratropium reveal clues for the design of novel antimicrobials against *Streptococcus pneumoniae*

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ABSTRACT

Background: Streptococcus pneumoniae is a major pathogen responsible of important diseases worldwide such as pneumonia and meningitis. An increasing resistance level hampers the use of currently available antibiotics to treat pneumococcal diseases. Consequently, it is desirable to find new targets for the development of novel antimicrobial drugs to treat pneumococcal infections. Surface choline-binding proteins (CBPs) are essential in bacterial physiology and infectivity. In this sense, esters of bicyclic amines (EBAs) such as atropine and ipratropium have been previously described to act as choline analogs and effectively compete with teichoic acids on binding to CBPs, consequently preventing *in vitro* pneumococcal growth, altering cell morphology and reducing cell viability.
Methods: With the aim of gaining a deeper insight into the structural determinants of the strong interaction between CBPs and EBAs, the three-dimensional structures of choline-binding protein F (CbpF), one of the most abundant proteins in the pneumococcal cell wall, complexed with atropine and ipratropium, have been obtained.
Results: The choline analogs bound both to the carboxy-terminal module, involved in cell wall binding, and, unexpectedly, also to the amino-terminal module, that possesses a regulatory role in pneumococcal autolysis.
Conclusions: Analysis of the complexes confirmed the importance of the tropic acid moiety of the EBAs on the strength of the binding, through π–π interactions with aromatic residues in the binding site.
General significance: These results represent the first example describing the molecular basis of the inhibition of CBPs by EBA molecules and pave the way for the development of new generations of antipneumococcal drugs.

1. Introduction

Streptococcus pneumoniae (pneumococcus), a Gram-positive encapsulated diplococcus bacterium, is a major cause of invasive infections (meningitis, bacteremia) and diseases affecting the upper (otitis media and sinusitis) and lower (pneumonia) respiratory tracts, among others [1]. Pneumococcal diseases are widespread both in developed and developing countries with more than 1.6 million deaths per year according to the World Health Organization [2], half of them in children under five [3]. Nowadays, vaccination based on the polysaccharidic capsule, together with treatment with β-lactam antibiotics, currently constitutes the major choices to tackle pneumococcal diseases [1]. However, the wide diversity of *S. pneumoniae* (more than 90 different serotypes) and the increasing emergence of antibiotic-resistant strains make it necessary to search for new methods to fight this microorganism. Therefore, new effective and selective strategies are needed that take into account novel targets common to most pneumococcal isolates.

A characteristic feature of the pneumococcal cell wall is the presence of teichoic acid units decorated with phosphorylcholine residues [4] acting as attachment ligands for the so-called choline-binding proteins (CBPs). The CBP family does not only include proteins from *S. pneumoniae* but also from several of its bacteriophages, as well as from a few other related bacterial species [5–7]. The pneumococcal CBPs are present in all isolates and are essential for the viability and virulence of this microorganism, being involved in processes such as cell-wall division, release of bacterial toxins and adhesion to the host [6–8]. All CBPs share the presence of choline-binding modules (CBMs) (http://pfam.sanger.ac.uk//family/PF01473), which are in turn built up from a variable number of choline-binding repeats (CBRs) of

Abbreviations: CBM, choline-binding module; CBP, choline-binding protein; CBR, choline-binding repeat; CbpF, choline-binding protein F; EBAs, esters of bicyclic amines; DEAE, diethylaminoethyl

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approximately 20 residues, very rich in aromatic amino acids. So far, the three-dimensional structure of four pneumococcal CBPs has been elucidated, namely Cpl-1 [9], CbpE (also named Pce) [10], CbpF [11] and LytC [12], as well as the isolated CBM modules from the LytA amidase (C-LytA protein) [13] and the Spr1274 protein [14]. The basic CBR is comprised of a 12–14-residue β-hairpin followed by an 8–10-residue loop, conforming a ββ-3-solenoid superhelix. Choline-binding sites in CBPs involve the participation of two aromatic amino acids from one hairpin and another one from the next, configuring a hydrophobic pocket that accommodates the choline methyl groups and establish cation–π interactions with the positive charge of the ligand. Furthermore, a variety of choline analogs such as diethylaminoethanol (DEAE) efficiently emulates the role of choline [15], a characteristic that is commonly used for the single-step purification of CBM-tagged fusion proteins [16,17].

Since CBPs are common to all serotypes, they are attractive drug targets for the treatment of pneumococcal diseases, as their selective inhibition might constitute a promising way for new therapies. In this sense, exogenously added choline and choline analogs have been shown to 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, and inducing instead the formation of long chains [15,16,18–20]. These effects are also 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. Remarkably, esters of bicyclic amines (EBAs) such as atropine and ipratropium (Fig. 1) have proved to be stronger binders than choline and more deleterious at lower concentrations, inducing morphological changes in the cell surface and, more importantly, preventing cell growth in liquid media and decreasing cell viability in more than 90% [21]. Atropine is an alkaloid from *Atropa belladonna*, that is used as a cholinergic blocking drug in premedication for anesthesia and in ophthalmology, and ipratropium is another anticholinergic agent that is employed as antiasthmatic and bronchodilatator. While these EBAs could be thought as potential antimicrobials *per se*, their anticholinergic side-effects may constitute a serious drawback. Instead, they should be considered as head compounds from which new variants can be rationally designed, and to do so, exhaustive, structural information on their interaction with CBPs is needed. In this article we study the structure of crystal complexes between EBAs and CbpF, one of the most abundant choline-binding proteins in the surface of *S. pneumoniae*, and that has been proved to inhibit *in vivo* the autolytic LytC muramidase and to play a direct role in the regulation of pneumococcal autolysis. The results highlight the structural importance of the aromatic rings in the ligands and give clues for the development of new EBA derivatives with antipneumococcal properties.

2. Materials and methods

2.1. Materials

Choline chloride, ipratropium bromide, atropine sulphate and DEAE-cellulose were from Sigma-Aldrich.

2.2. Protein purification

Choline-bound CbpF was purified and crystallized as described earlier [22], obtaining a protein final concentration of 2.7 mg/ml in 20 mM Tris–HCl pH 8.0 plus 140 mM choline chloride.

2.3. Evaluation of binding strength of choline and choline analogs to CbpF

A 120-microliter aliquot from a sonicated extract overproducing the CbpF protein [22] was incubated with 60 μl of fluidMAG DEAE-starch magnetic nanoparticles (200 nm diameter, approx. 5.5×10^{12} particles/ml) (Chemicell, Germany) for 15 min. These nanoparticles act as an affinity matrix for CbpF and other CBPs (manuscript in preparation). Non-bound proteins were separated by the application of an external magnet (Chemicell) and the particles were first washed with 1 M NaCl and then incubated with 120 μl of 20 mM sodium phosphate buffer, pH 7.0, containing the corresponding concentrations of choline or choline analogs. Due to the interference of atropine and ipratropium in the UV region, precluding quantification by absorption spectroscopy, the eluted protein was quantified using the Bio-Rad Protein Assay using hen egg-white lysozyme as the calibration standard.

2.4. Crystallization and data collection

Crystallization of CbpF in the presence of choline was accomplished as described [22]. In order to obtain complexes with drugs, two techniques were used: (i) fast soaking, in which the CbpF crystals were transferred for 10 s to a drop of reservoir solution with ipratropium at 500 mM and (ii) co-crystallization, in which the protein solution was incubated overnight with 10 mM of atropine, and then mixed with the reservoir solution. The reservoir solutions contained 0.2 M ammonium sulphate, 30% PEG 8000 and 0.1 M sodium cacodylate buffer pH 6.5. Crystals up to $0.7 \times 0.4 \times 0.3$ mm were grown in one week. Crystals were cryoprotected by a quick soak in reservoir solution containing 15% (v/v) glycerol and flash-cooled in liquid nitrogen, maintained at 100 K during data collection. CbpF:ipratropium complex was measured on an ADSC Quantum 210 image plate detector at beamline BM16 of the ESRF (European Synchrotron Radiation Facility, Grenoble, France). CbpF:atropine complex was measured on a MAR 345 image-plate detector using Cu Kα X-Rays generated by an in-house Enraf-Nonius rotating-anode generator equipped with a double-mirror focusing system and operated at 40 kV and 90 mA. All data sets collected at ESRF were processed using *XDS* [23] and scaled with *SCALA* from the *CCP4* package [24]. Data set collected with Cu Kα radiation was integrated using *MOSFLM* [25] and scaled with *SCALA*. A summary of data-collection parameters and processing statistics is shown in Table 1.

2.5. Structure determination and refinement

The three-dimensional structure of CbpF complexes was solved by the molecular replacement method with *MOLREP* program [26] from the CCP4 package. The CbpF native structure from *S. pneumoniae* (PDB

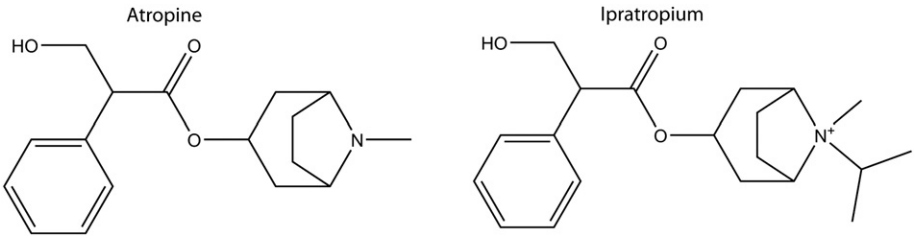

Fig. 1. A 2D representation of atropine and ipratropium, the esters of bicyclic amines used in the complex with CbpF. Drawings were rendered with ChemDraw 10.0 (CambridgeSoft).