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Inhibition of pneumococcal choline-binding proteins and cell growth by esters of bicyclic amines

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Streptococcus pneumoniae is one of the major pathogens worldwide. The use of currently available antibiotics to treat pneumococcal diseases is hampered by increasing resistance levels; also, capsular polysaccharide-based vaccination is of limited efficacy. Therefore, it is desirable to find targets for the development of new antimicrobial drugs specifically designed to fight pneumococcal infections. Choline-binding proteins are a family of polypeptides, found in all *S. pneumoniae* strains, that take part in important physiologic processes of this bacterium. Among them are several murein hydrolases whose enzymatic activity is usually inhibited by an excess of choline. Using a simple chromatographic procedure, we have identified several choline analogs able to strongly interact with the choline-binding module (C-LytA) of the major autolysin of *S. pneumoniae*. Two of these compounds (atropine and ipratropium) display a higher binding affinity to C-LytA than choline, and also increase the stability of the protein. CD and fluorescence spectroscopy analyses revealed that the conformational changes of C-LytA upon binding of these alkaloids are different to those induced by choline, suggesting a different mode of binding. *In vitro* inhibition assays of three pneumococcal, choline-dependent cell wall lytic enzymes also demonstrated a greater inhibitory efficiency of those molecules. Moreover, atropine and ipratropium strongly inhibited *in vitro* pneumococcal growth, altering cell morphology and reducing cell viability, a very different response than that observed upon addition of an excess of choline. These results may open up the possibility of the development of bicyclic amines as new antimicrobials for use against pneumococcal pathologies.

Streptococcus pneumoniae is currently a leading infectious agent worldwide. This Gram-positive bacterium is one of the most common causes of severe diseases, such as pneumonia, otitis media, septicemia, and meningitis [1]. The morbidity and mortality of infections caused by *S. pneumoniae* remain high, despite the availability of antimicrobial agents [2]. Young children are especially susceptible to this microorganism, and pneumococcal pneumonia and meningitis are responsible for 800 000 to 1 million child deaths worldwide every year [3]. Classically, penicillin and its derivatives have been the drugs of choice for the treatment of pneumococcal infections. Also, attempts to provide protective immunity against pneumococcal disease

Abbreviations
CBM, choline-binding module; CBP, choline-binding protein; CBR, choline-binding repeat; DEAE, diethylaminoethanol; MIC, minimal inhibitory concentration; t_m, midpoint of thermal transition.

have concentrated on vaccines that target the antiphagocytic capsular polysaccharides that surround most clinical isolates and that are the major virulence factors of this pathogen. However, the efficacy of the available vaccines is limited, and treating pneumococcal infections through generalized use of antibiotics is unrealistic in the long term because of the genetic plasticity of *S. pneumoniae*, which results in either capsular type shifting or in the rapid appearance and spread of antibiotic-resistant isolates and antibiotic resistance determinants [4]. Therefore, new alternative therapies are needed. Studies must take into account virulence factors common to all pneumococcal isolates, which might be the targets of effective and selective treatment. In this sense, choline-binding proteins (CBPs) are a special class of pneumococcal polypeptides anchored to the cell surface through noncovalent interactions with the choline residues of teichoic acids [5]. These proteins are present in all pneumococcal isolates, have several important physiologic roles, and are related to virulence [6,7]. All the CBPs display a modular organization, with a biologically active module and a highly conserved choline-binding module (CBM) that allows the binding to phosphorylcholine residues. The CBMs are built up of several tandem repeats (choline-binding repeats, CBRs), each consisting of about 20 highly conserved amino acids [7] (see Pfam ID code PF01473: http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF01473).

The LytA amidase, the major murein hydrolase from *S. pneumoniae*, is a CBP that catalyzes the cleavage of the *N*-acetylmuramoyl-L-alanine bond of the peptidoglycan backbone [8]. It is involved in the separation of the daughter cells at the end of cell division and in cellular autolysis [9], where it mediates the release of toxins that damage the host tissues and allows the entry of pneumococcal cells into the blood vessels [10–12]. Other well-known *S. pneumoniae* cell wall hydrolases include the LytB glucosaminidase, the LytC lysozyme, and the Pce phosphorylcholinesterase [7]. PcsB [13] and CbpD [14] have also been described as possible hydrolases, although definitive biochemical data are still lacking.

The C-terminal module of LytA (C-LytA) is the major representative of the CBM family. The elucidation of its crystal structure complexed with choline revealed a novel left-handed ββ-3-solenoid fold formed by the stacking of six loop-β-hairpin structures, corresponding to the CBRs, into an elongated, left-handed superhelix [15,16]. Up to four choline molecules bind to hydrophobic pockets composed of aromatic residues supplied by two consecutive CBRs. NMR has not been useful to date for determining the structures of both the ligated and unligated forms of C-LytA, due to the insolubility of the protein at the required concentrations. The recently solved structures of the phage Cpl-1 lysozyme [17], and Pce [18], together with the modeling of LytC [19], strongly suggest that the cited arrangement of CBRs has been universally adopted by all CBPs. Calorimetric and spectroscopic analyses have demonstrated the presence in C-LytA of low-affinity and high-affinity choline-binding sites [20,21]. Binding of choline promotes dimerization through the stacking of CBR6 [15] and confers stability to C-LytA against thermal [21] and chemical [22] denaturation. Free choline is an inhibitor of the activity of LytA [23,24] and other CBPs [25]. Moreover, addition of an excess of choline to culture media inhibits daughter cell separation and induces the formation of long chains [24]. The finding that C-LytA displays affinity for other tertiary and quaternary alkylamines allowed the development of a single-step purification system for CBPs and CBM-containing fusion proteins ([26], and C-LYTAG Purification System User's Manual from Biomedal, http://www.biomedal.es), and gave support to the hypothesis that choline analogs might also act as inhibitors of the attachment to the cell wall and therefore as potential drugs against *S. pneumoniae*. It has been recently reported that ofloxacin-type quinolones inhibit the activity of some CBPs [27].

In this study, we tested the ability of several water-soluble, choline structural analogs to strongly interact with C-LytA. We found that esters of bicyclic amines such as atropine and ipratropium are more efficient binders and inhibitors of pneumococcal CBPs than is choline, and are also capable of arresting cell growth in liquid cultures. These results may open up the possibility of a new, effective therapy against pneumococcal diseases.

Results

Selection and testing of choline analogs

The minimum structural requirement for choline analogs to specifically bind to the LytA amidase is that of a tertiary alkylamine [26]. This allowed the set-up of an affinity chromatography method for the single-step purification of pneumococcal CBPs and recombinant hybrid proteins containing a CBM, using chromatographic supports derivatized with these analogs, such as 2,2-diethylaminoethanol (DEAE) [26,28]. The standard procedure involves the attachment of the protein to the column, washing with a high ionic strength solution (1.5 M NaCl), and specific elution with 140 mM choline. Compounds able to elute the

Table 1. Compounds tested for their ability to elute C-LytA from a DEAE-cellulose column. Elution is displayed in terms of percentage of protein recovered in the first two column volumes with respect to total load of protein. Experiments were performed in duplicate or triplicate. Conditions are as described in the text.

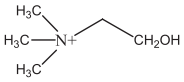
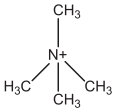
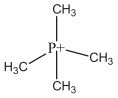
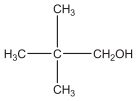
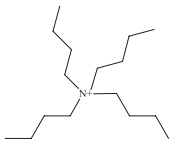
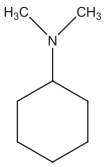
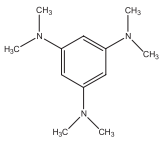
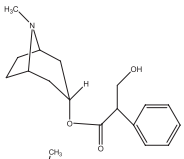
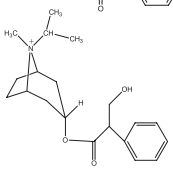
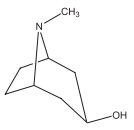
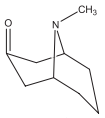
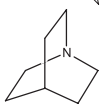
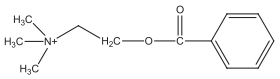
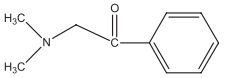
Compound	Chemical formula	Elution at 10 mM (%)	Elution at 140 mM (%)
Choline		< 0.1	> 80
Tetramethylamonium		< 0.1	> 80
Tetramethylphosphonium		< 0.1	> 80
2,2-Dimethyl-1-propanol		< 0.1	< 0.1
Tetrabutylammonium		< 0.1	> 80
1-Methylpyrrolidine		< 0.1	> 80
N,N-Dimethylcyclohexylamine		< 0.1	> 80
2,4,6-Tris(dimethylaminoethyl)phenol		< 0.1	> 80
Atropine		> 80	> 80
Ipratropium		> 80	> 80
Tropine		< 0.1	> 80

Table 1. (Continued).

Compound	Chemical formula	Elution at 10 mM (%)	Elution at 140 mM (%)
Pseudopelletierine		< 0.1	> 80
Quinuclidine		< 0.1	> 80
Benzoylcholine		< 0.1	> 80
3-(Dimethylamino)propiophenone		< 0.1	> 80

protein at the same or lower concentration than choline might therefore specifically inhibit CBPs by competing with choline residues attached to teichoic acid in the cell wall. To determine the minimum concentration of choline capable of eluting C-LytA from a DEAE-cellulose column, 1 mg of C-LytA samples were adsorbed onto 1 mL of resin and subjected to washes with different concentrations of choline. We found that 30 mM was the lowest concentration of choline that caused low but detectable elution of the protein (data not shown). Therefore, we chose 10 mM as a threshold concentration that would be tested in order to select those analogs that were clearly more efficient than choline. In order to establish the types of compound to be examined, and to reduce the number of an otherwise vast set of candidates, we took into account: (a) commercial availability; (b) water solubility at concentrations around 140 mM (so that appropriate biophysical studies could be performed); and (c) difference from choline in a moderate number of groups (i.e. nitrogen substituents, nitrogen atom itself, and hydroxyl substituents), so that we might unambiguously identify individual interaction determinants. Table 1 shows the molecules that were finally selected, together with their ability to elute C-LytA at two concentrations (10 mM and 140 mM), using the experimental procedure described above. Most of the ligands displayed an elution efficiency similar to that of choline, corroborating the broad range of specificity of the protein [26]. In accordance with the lack of observed interactions between the hydroxyl group of choline and C-LytA [15], tetramethylammonium behaved similarly to choline. There was also no difference with tetramethylphosphonium, which is larger than tetramethylammonium but retains the positive charge. In contrast, 2,2-dimethyl-1-propanol, an uncharged analog of choline, failed to elute C-LytA at any concentration, reinforcing the hypothesis that cation–π interactions with the aromatic residues in the binding sites are critical [15]. On the other hand, N-substituents with long linear (tetrabutylammonium), cyclic aliphatic (1-methylpyrrolidine; *N,N*-dimethylcyclohexylamine) or aromatic [2,4,6-tris(dimethylaminoethyl)phenol] chains, that might in principle better fill the hydrophobic binding pockets in C-LytA, did not improve or worsen the elution process, indicating that N-substitution is a minor determinant in the protein–ligand interaction, provided that the substituent is hydrophobic [26]. On the other hand, when testing bicyclic amines, we found that both atropine and ipratropium were completely efficient at 10 mM (Table 1). Both alkaloids are esters of tropic acid and a bicyclic amine. Nevertheless, the effect of nonesterified bicyclic amines such as tropine (the alcohol moiety of the atropine ester), pseudopelletierine or quinuclidine was indistinguishable from that of the other tertiary amines checked. In addition, benzoylcholine and 3-(dimethylamino)propiophenone, both with aromatic chains located away from the nitrogen atom, also failed to improve the elution process in comparison to choline. Therefore, the linkage of a bicyclic amine with an aromatic group, such as tropic acid, seems to result in a synergic combination of properties, and we decided to study the effect of atropine and ipratropium on the structure of the protein in detail.

Spectroscopic features of the conformational change induced by ligands

The influence of ipratropium and atropine on the structure of C-LytA was first analyzed by near-UV CD, as the far-UV CD signal is not recordable, due to the high absorption of these compounds. It should be pointed out that, according to the calculated dimerization constants of C-LytA [21], although some specific dimerization of the protein may take place at neutral pH even in the absence of choline, the amount of this is reduced at the concentrations used in our experiments. Figure 1A depicts the near-UV CD spectrum of C-LytA at 20 °C and pH 7.0, showing two maxima at 265 nm and 290 nm. Upon addition of 20 mM choline (a saturating concentration of ligand), two minima at 284 nm and 294 nm appear, whereas the 265 nm maximum remains essentially unaltered. These spectral changes have been described before, and have been ascribed to conformational changes affecting the spatial arrangement of tryptophan residues [20,28] that form the choline-binding sites [15]. On the other hand, ipratropium and atropine also induced the appearance of these negative bands, although with reduced intensities and with a much lower concentration of ligand, as the ellipticity change is already stabilized at 2.5 mM, as opposed to 20 mM choline (Fig. 1A). Spectra below 270 nm could not be recorded at concentrations of atropine and ipratropium above 5 mM, due to the high absorbance of these compounds. These results suggested that the interaction of C-LytA with the bicyclic amines was stronger than that of choline, and that the conformational change around the aromatic residues in the binding sites might also be different. To confirm this hypothesis, we registered the intrinsic fluorescence spectra of C-LytA complexed with the same ligands (Fig. 1B). The spectrum of unligated C-LytA upon excitation at 280 nm is dominated by tryptophan emission, with a maximum centered at 333 nm. Addition of choline induced an increase in intensity together with a blue shift to 328 nm. As suggested before [22], this could reflect the burial of tryptophan residues upon binding of the ligand. Ipratropium exerted a similar blue shift, but the quantum yield was clearly lower. Finally, atropine reduced the intensity to levels even below those displayed by the uncomplexed protein (Fig. 1B). These results reinforce the hypothesis that atropine and ipratropium are bound to tryptophan-containing sites through different binding interactions. It should be pointed out that the fluorescence intensities of the bicyclic ligands themselves are negligible compared to that of the protein, despite the presence of aromatic moieties, and that the use of an excitation wavelength of 295 nm, specific for tryptophan residues, yielded the same qualitative results, due to energy transfer [22].

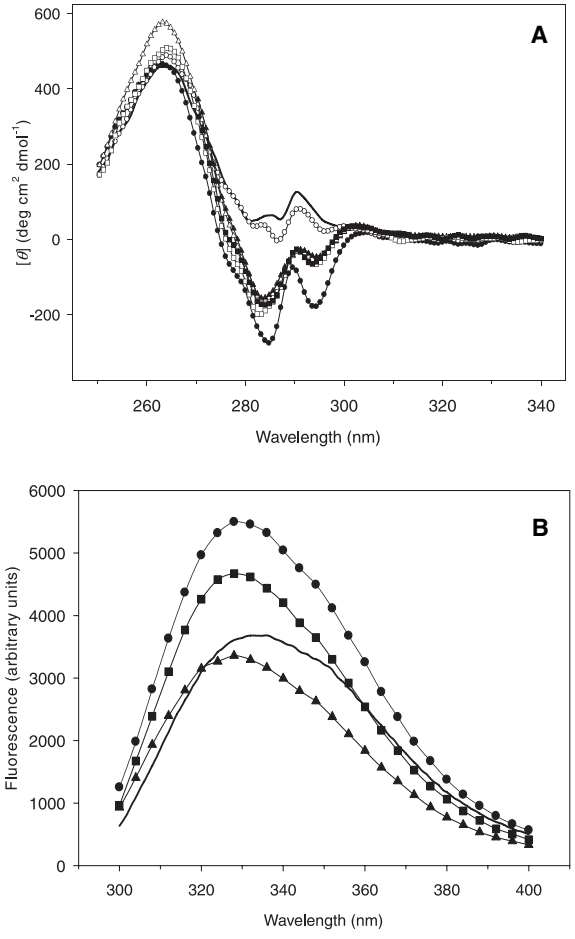


Fig. 1. Spectroscopic analysis of ligand binding to C-LytA. (A) Conformational changes induced by ligands on C-LytA monitored by near-UV CD with no ligand added (—) and upon addition of ligands: ○, ●, 2.5 and 20 mM choline; △, ▲, 2.5 mM and 20 mM atropine; □, ■), 2.5 mM and 20 mM ipratropium. (B) Intrinsic fluorescence spectra upon excitation at 280 nm. Same scheme as in (A).

Equilibrium titrations monitored by CD

A plot of the ellipticity of C-LytA at 295 nm vs. choline concentration displays two well-defined sigmoidal transitions (Fig. 2A), reflecting the presence of high-affinity and low-affinity binding sites in the protein and cooperativity in binding [20]. In contrast to choline, the atropine and ipratropium titration curves present only one transition, which is complete at approximately 2 mM ligand (Fig. 2A). A detailed view reveals a clear overlap with the first choline-induced transition (Fig. 2B), corresponding to the binding to

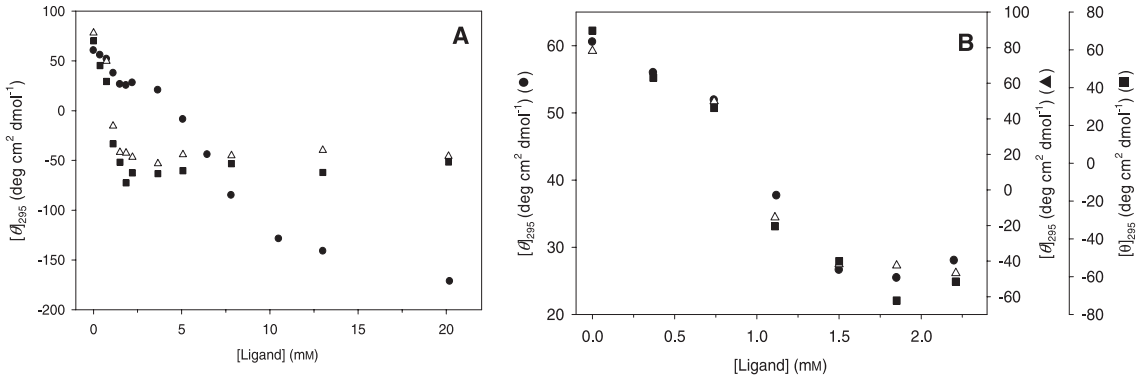


Fig. 2. Titration of the CD signal of C-LytA at 295 nm with ligands. Symbols represent choline (●), atropine (△) and ipratropium (■). (A) Full range of ligand concentration. (B) Detailed view of the 0–2.5 mM range, with normalized axes.

the high-affinity sites. This result suggests that all C-LytA binding sites present the same high-affinity behavior for choline analogs and become saturated at 2 mM (Fig. 2A), and agrees with the higher apparent affinity of ipratropium and atropine than of choline for C-LytA (Table 1, Fig. 1A). Binding of these bicyclic amines turned out to be completely reversible, as simple dialysis restored the near-UV CD spectrum to the signal of unbound protein, although full removal of the analogs took considerably more time than removal of choline. Finally, fluorescence-monitored experiments yielded similar titration curves to those shown in Fig. 2 (data not shown).

It is remarkable that 10 mM free choline is a sufficient concentration for occupying all the binding sites in unligated C-LytA (Fig. 2A), but is unable to elute the protein from a DEAE-cellulose column (see above). Nevertheless, it should be taken into account that, in the elution process, free choline must compete with the DEAE residues in a polidentate matrix. Adsorption to the column is favored entropically, as binding of the first DEAE group brings the C-LytA protein in close proximity to the resin and promotes the subsequent cooperative binding of the rest of the DEAE molecules in a 'zipper-like' fashion. In contrast, binding of free choline means the independent immobilization of five molecules (four of choline and the protein), which is entropically unfavorable with respect to the former situation.

Dimerization of C-LytA

The occupation of high-affinity binding sites by choline triggers the dimerization of C-LytA [21]. The effect of bicyclic amines on C-LytA oligomerization was analyzed by size-exclusion chromatography. Nevertheless, the elongated shape of C-LytA does not allow the precise calculation of the molecular mass of the protein on the basis of its hydrodynamic radius with this method [29]. As shown in Fig. 3, addition of a saturating amount of choline (50 mM) caused a shift to lower elution volumes, in accordance with the formation of a dimer, whereas choline at 1.5 mM only induced a small change in the elution profile, corresponding to partial accumulation of dimers in these conditions [21]. On the other hand, addition of 1.5 mM ipratropium generated a new peak with an elution volume close to that obtained at 50 mM choline (Fig. 3), suggesting substantial accumulation of C-LytA dimer. Finally, the effect of 1.5 mM atropine was intermediate between the effects of choline and ipratropium, showing a profile with two overlapping peaks that suggests the presence of both monomers and dimers in slow equilibrium (Fig. 3).

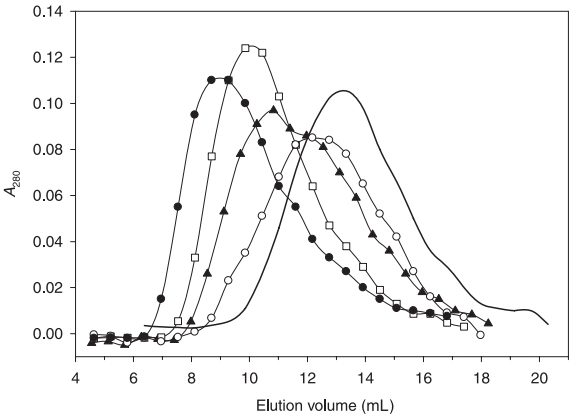


Fig. 3. Size-exclusion chromatography of C-LytA in Sephadex G-75. Solid line, uncomplexed protein; ○, addition of 1.5 mM choline; ●, 50 mM choline; ▲, 1.5 mM atropine; □, 1.5 mM ipratropium.

Thermal unfolding transition in the absence and in the presence of the different ligands

The influence of ligand binding on the thermal stability of C-LytA was studied by monitoring the CD signal at 270 nm (Fig. 4). The temperature scan of a nonligated, freshly purified sample displayed a biphasic transition with temperature midpoints (t_m) of 47.16 ± 0.89 °C and 62.02 ± 0.57 °C (Table 2). These values are in agreement with those obtained in far-UV CD and differential scanning calorimetry experiments, and confirm the accumulation of an intermediate after the first transition [21,22]. Addition of 2.5 mM choline saturates the high-affinity binding sites, induces dimerization, and abolishes the accumulation of the previously mentioned intermediate [21,22], so that thermal denaturation yielded virtually only the second transition, with an increased midpoint temperature (Fig. 4, Table 2).

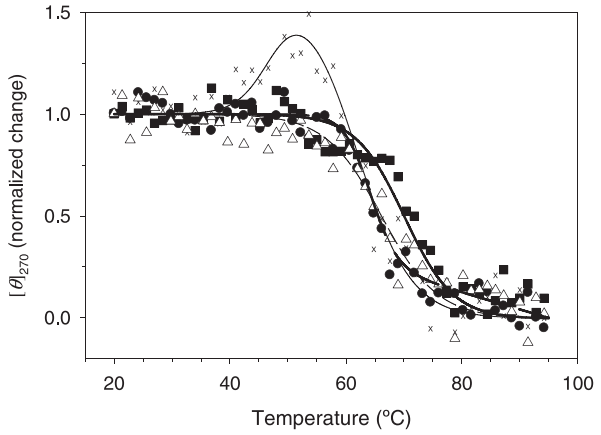


Fig. 4. Thermal denaturation of C-LytA monitored by near-UV CD. Data are normalized with respect to the values at 20 °C and 95 °C for clarity of presentation. ×, absence of ligand; ●, 2.5 mM choline; △, 2.5 mM atropine; ■, 2.5 mM ipratropium. Lines represent single or double (for the nonligated protein) sigmoidal fits to calculate the temperature midpoints. Only one-seventh of the points are shown.

Table 2. Thermal stabilization of C-LytA by ligands.

Ligand added	t_m (°C)
Control (free C-LytA)	62.02 ± 0.57[a]
Choline 2.5 mM	63.97 ± 0.26
Atropine 2.5 mM	66.15 ± 0.30
Ipratropium 2.5 mM	70.07 ± 0.24
Choline 20 mM	69.62 ± 0.28
Atropine 20 mM	71.23 ± 0.41
Ipratropium 20 mM	75.00 ± 0.46
Choline 140 mM	76.01 ± 0.17

[a] Value corresponding to the second thermal transition.

On the other hand, bicyclic amines induced even higher thermal stabilization at the same concentrations, with ipratropium being the most stabilizing ligand (Fig. 4, Table 2). These differences in stabilization were maintained at a ligand concentration of 20 mM (Table 2). It should be noted that protein unfolding was complete in all cases and was reversible at 2.5 mM ligand, whereas, at higher concentrations, full reversibility was only accomplished with choline (data not shown).

Inhibition of pneumococcal murein hydrolases by bicyclic amines

As shown above, tropic esters of bicyclic amines were selected and characterized by biophysical methods as strong ligands that could compete with choline for C-LytA binding. The next step was to determine whether these compounds might also exert an inhibitory effect on the enzymatic activity of full-length choline-binding murein hydrolases. Figure 5A–C shows the inhibitory effect of choline, atropine, and ipratropium on the activity of LytA, LytC and Pce, respectively. In all three cases, the alkaloids turned out to be better inhibitors than choline, ipratropium being the most effective. LytA has been reported to undergo an activation process at low concentrations of choline [24] that is also induced by the two analogs (Fig. 5A). For the LytC lysozyme, such activation is of higher intensity, although only atropine was able to emulate the activating role of choline, whereas ipratropium always acted as a powerful inhibitor at any concentration (Fig. 5B). The reasons for these activation effects are still unknown, although the experimental evidence suggests a significant interaction between the catalytic and choline-binding modules of LytA and LytC [19,21]. It is possible that a low amount of choline could induce a conformational change resulting in module separation and subsequent improvement in the accessibility of the catalytic module to the scissile bond. This effect is clearly enhanced in the case of LytC, probably due to a stronger interaction between modules facilitated by the longer extension of its elongated choline-binding domain (11 repeats).

The Pce phosphorylcholinesterase binds choline not only at its CBM but also at the active site [18]. Therefore, inhibition of the cell wall lytic activity of Pce by atropine and ipratropium could take place by interference with the attachment to the choline-containing teichoic acids and/or direct competition with the phosphorylcholine residues in the active site. In order to distinguish between these possibilities, Pce was assayed with a synthetic, soluble substrate, *p*-nitro-

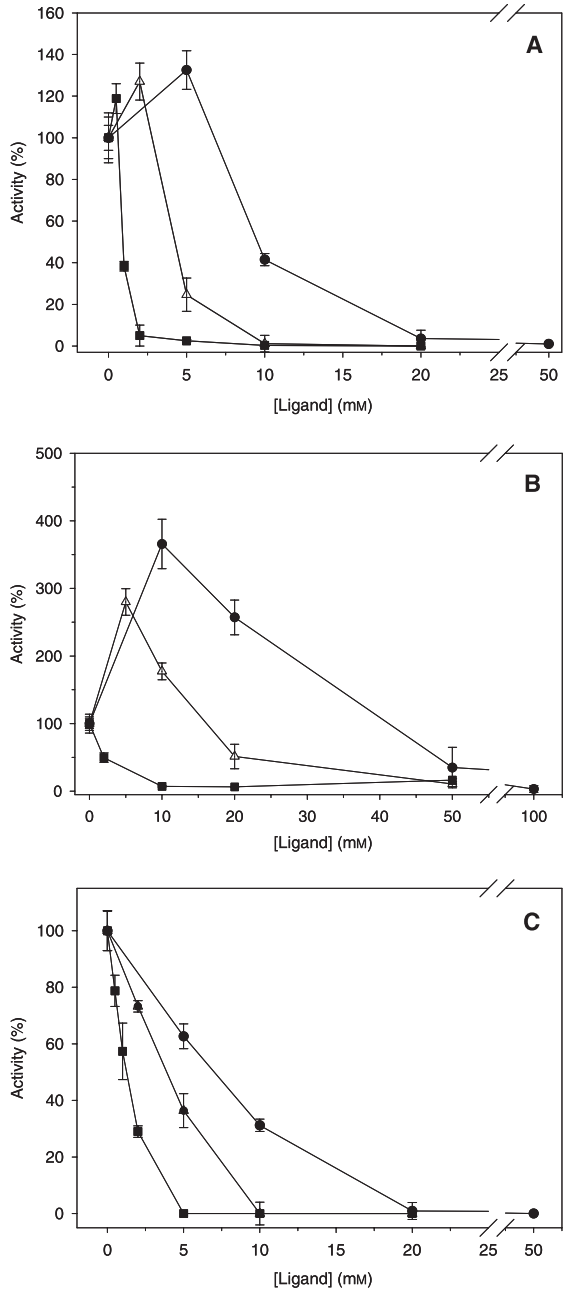


Fig. 5. Effect of choline and analogs on the activity of cell wall lytic enzymes. Data are shown as percentage of activity with respect to nonligated enzyme, and are the average of three independent experiments. (A), LytA; (B), LytC; (C), Pce. Additions: ●, choline; △, atropine; ■, ipratropium. Error bars represent the standard error of the mean.

phenylphosphorylcholine, that makes the role of its CBM unnecessary. As described before [25], choline inhibited the activity of the enzyme in a competitive way. Interestingly, both atropine and ipratropium also showed the same kind of competitive inhibition, although with a higher affinity (inhibition constants of 14.3, 8.0 and 3.5 mM for choline, atropine and ipratropium, respectively) (data not shown). This result demonstrates that the bicyclic amines are also able to bind to the active site of Pce.

Effect of choline analogs on cell growth and viability

It has previously been reported that subjecting pneumococcal cultures to increasing concentrations of choline abolishes daughter cell separation at the end of cell division, causing the formation of long chains of cells with only small effects on cell growth and viability, even at high concentrations of the compound [24]. This effect has been ascribed to the inhibition of the two murein hydrolases involved in the cell separation process, mainly the LytB glucosaminidase and, to a lesser extent, the LytA amidase [30]. As atropine and ipratropium demonstrated a higher capacity to inhibit some CBPs compared to choline (Fig. 5), we checked the effect of these alkaloids on pneumococcal cultures. Figure 6A shows that 4 h after addition to early exponential phase cultures, atropine and ipratropium progressively restrained cell growth. As expected, addition of choline did not modify the bacterial multiplication rate. Remarkably, ipratropium displayed an inhibitory effect even more intense than that of atropine, in accordance with the *in vitro* properties of these compounds. It is worth noting that the extent of the effect of these analogs on the pneumococcal cultures is very dependent on the metabolic state of the cell. As shown in Fig. 6B, addition of these compounds at the beginning of the exponential phase clearly limited growth, but an earlier challenge (in the lag phase) with the same final concentrations produced a total arrest of cell growth. In both cases, the effect of these compounds was different from that of choline (Fig. 6B).

To check whether the toxic effects of atropine and ipratropium might be reversed by addition of excess amounts of choline, most likely by their displacement from the choline-binding sites of CBPs, we added 200 mM choline together with the corresponding analog to the culture medium in the lag phase. However, inhibition of growth by 30 mM atropine was not reversed by this choline concentration, and only a small, although detectable, recovery was noted in the culture with 20 mM ipratropium after several hours of incubation (data not shown).

Although the bicyclic amines did not trigger cell lysis, formation of medium-length chains (6–10 cells on average) and visible alterations, such as cell bulges and

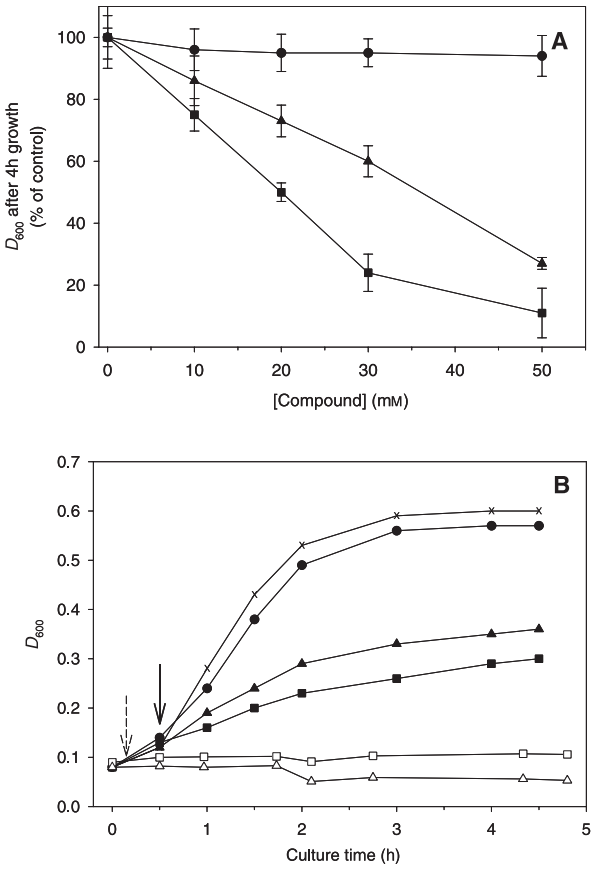


Fig. 6. Effect of choline analogs on *S. pneumoniae* growth. (A) State of cultures after 4 h of incubation of *S. pneumoniae* R6 with compounds (addition at early exponential phase). Data are shown as percentage of the D_{600} of a control culture with no additive. ●, choline; ▲, atropine; ■, ipratropium. Each symbol represents the average of four experiments. Error bars show the standard error of the mean. (B) Growth kinetics. Experiments were repeated four times. A typical experiment is shown: ×, no compound added (control); ●, 50 mM choline added at early exponential phase; △, ▲, 30 mM atropine added at lag and early exponential phases, respectively; □, ■, 20 mM ipratropium added at lag and early exponential phases, respectively. Dashed and solid arrows indicate the addition times corresponding to lag and early exponential phases, respectively.

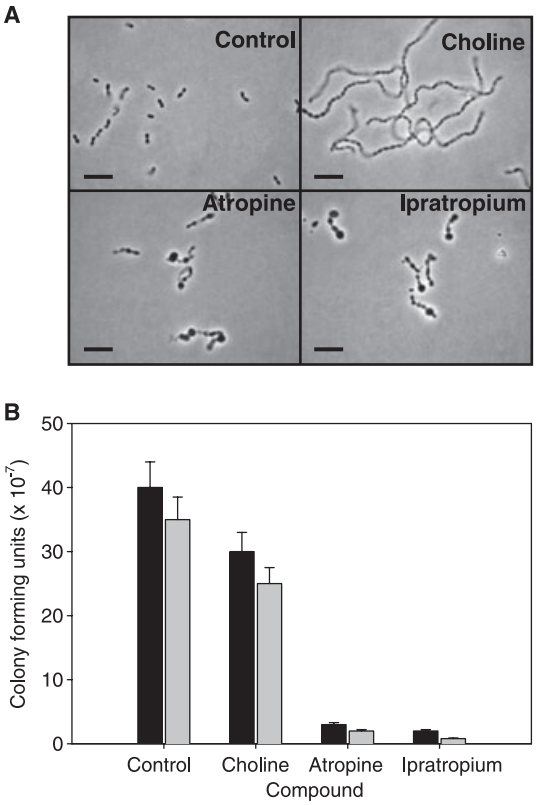


Fig. 7. Morphology and viability of pneumococcal cultures. (A) Phase contrast micrographs of *S. pneumoniae* R6 cultures taken after 4 h of incubation at 37 °C. In clockwise order: untreated control, 50 mM choline, 20 mM ipratropium and 30 mM atropine. Bars represent 5 μm. (B) Cell viabilities of the cultures at 2 and 4 h (black and gray shading, respectively) after the compounds (50 mM choline, 30 mM atropine, and 20 mM ipratropium) were added at the early exponential phase. Each value represents the average of four experiments. Error bars indicate the standard error of the mean.

some cells larger than normal, could be observed (Fig. 7A). The effect of ipratropium was again evident at lower concentrations than those needed for the atropine effect. To gain a deeper insight into the physiologic effect of these compounds, we carried out viability experiments on atropine-challenged and ipratropium-challenged cultures, together with untreated and choline-incubated cultures as controls (Fig. 7B). It has to be pointed out that the apparent small decrease in viability of choline-challenged cells may in part be due to the fact that the ability to form individual colonies upon plating is reduced when the cells belong to long chains instead of being separated diplococci, even though the number of cells should be similar according to the attenuance values. On the other hand, the viable cell numbers in atropine-treated and ipratropium-treated cultures were lowered by $\approx$90% and 95% after 2 h and 4 h of incubation, respectively (Fig. 7B). All these results taken together suggest that these bicyclic amines do not simply affect normal cell growth, but have a toxic effect on pneumococcal cultures that is accompanied by important morphologic alterations and reduced viability. Finally, minimal inhibitory concentrations (MICs) for atropine and ipratropium were calculated following a standard procedure [31] using three different pneumococcal strains, the unencapsulated R6, ATCC 49619 (preceptrol culture), and the encapsulated TIGR4. In all cases,

the MIC values ranged from 12 to 15 mM for both compounds, which correlate rather well with those employed in the cell growth and viability experiments of Fig. 7A,B.

Discussion

CBPs are critical for the life cycle of *S. pneumoniae* [7]. They are ubiquitous in all the pneumococcal isolates tested and are highly related to virulence [6], as maintenance (or lysis) of the cell wall is an essential process for both cell viability and liberation of virulence factors. Inhibition of autolysis by excess choline might, in the first instance, seem to be of therapeutic interest. However, the amount of ligand needed, and the subsequent collateral effects arising from the interaction with muscarinic receptors, make this treatment unfeasible. Therefore, the discovery of choline analogs that are able to inhibit the attachment of the CBPs to the cell wall at lower concentrations may allow the development of new antimicrobial therapies [27] to address the problem of the increasing rates of drug-resistant pneumococcal infections [32,33].

In this work, we searched for choline analogs that interact with the CBM of the LytA amidase (C-LytA) with greater strength than choline. Using the affinity of C-LytA for DEAE-cellulose as a selection tool, we identified two esters of bicyclic amines, namely atropine and ipratropium, which were capable of eluting C-LytA from the column at a lower concentration than is needed for choline to do so. In contrast, the eluting competence of monocyclic and linear alkylamines or nonesterified bicyclic amines was indistinguishable from that of choline. These results suggest that the simultaneous presence of both groups (bicyclic amine and tropic acid substituent) is necessary to explain the stronger binding affinity of these compounds for C-LytA. Titration of the near-UV CD signal of the protein with choline confirmed the presence of high-affinity and low-affinity binding sites [20,21] (Fig. 2). However, only high-affinity binding sites were observed when the protein was challenged with atropine or ipratropium. There are several possible explanations for such behavior. For instance, the bicyclic amines might bind to the same sites as choline, causing a different conformational change that results in switching of all the choline-binding sites to the high-affinity type. This would explain the observation that the three-dimensional environment around tryptophan residues is to some extent different, as deduced from the CD spectra (Fig. 1). On the other hand, they might only bind to high-affinity sites. Finally, the accessibility of the alkaloids to new binding sites cannot be ruled out. In this sense, the analysis of the three-dimensional structure of choline-ligated C-LytA shows that Phe101 and Trp110 are in a suitable conformation to bind a ligand molecule, although they are located in the dimerization interface [15]. It might, in principle, be possible for a molecule of atropine or ipratropium to bind to such an aromatic patch, provided that the dimerization region is not disrupted (Fig. 3).

The amines were also more efficient than choline in inhibiting the *in vitro* activity of LytA, LytC and Pce (Fig. 5). This suggests that these molecules may behave as universal powerful inhibitors of the CBP family in general. The specificity of the interaction with the CBPs is demonstrated by several facts: (a) the ligands specifically elute C-LytA from an affinity chromatography column; (b) like choline, they induce C-LytA dimerization; (c) the only common feature of the three hydrolases tested for their inhibition is the presence of a CBM; (d) LytA amidase is activated by a low concentration of the ligands, an effect that had been previously ascribed only to choline; and (e) the amines show competitive inhibition of Pce on soluble substrates, indicating that they bind to the phosphorylcholine-binding active site.

The bicyclic amines also affected the growth of *S. pneumoniae*, but in a different way than choline (Fig. 6). Instead of forming long chains of cells without the growth rate being affected, atropine-treated or ipratropium-treated cultures showed retardation or complete cessation of growth, clear cell deformation, and significantly decreased cell viability (Figs 6 and 7). These results strongly suggest that the bicyclic amines may induce a different conformational change in one or more CBPs that transforms a simple inhibition of cell wall attachment into a toxic response. However, an alternative explanation is that atropine and ipratropium exert their toxic effect through other targets in addition to, or instead of, the CBPs; this deserves further and thorough investigation.

Despite their structural similarity, atropine and ipratropium showed many functional differences. The only difference between the two molecules is the presence of an additional isopropyl substituent at the nitrogen atom of ipratropium, as, at pH 7.0, both amines must be positively charged. Although their affinities for C-LytA are very similar, they induce different conformational changes, which account for the dissimilarity in their thermal stabilization effects. Nevertheless, the differences between atropine and ipratropium extend to a higher scale than C-LytA. Ipratropium behaves as a more powerful inhibitor of the activity of the three murein hydrolases tested

(LytA, LytC and Pce) (Fig. 5) as well as of cell growth (Fig. 6A). Moreover, ipratropium is not capable of activating LytC at low concentrations, unlike choline and atropine. Solving the three-dimensional structure of C-LytA and other CBPs complexed with atropine and/or ipratropium by X-ray crystallography (work in progress) will help to explain the differential behavior of the various ligands.

Summarizing, our results suggest that esters of bicyclic amines may constitute a promising family of drugs against pneumococcal infections. Atropine is a naturally occurring alkaloid of *Atropa belladonna*, and is used as a sympathetic cholinergic blocking drug in premedication for anesthesia and in ophthalmology. On the other hand, ipratropium is also an anticholinergic agent that has therapeutic uses as an antiasthmatic and a bronchodilatator. Both compounds could be tested for treatment of pneumococcal infections. However, as the concentrations of these amines that are necessary to arrest pneumococcal growth are relatively high, and because of their molecular simplicity, we believe that they should rather be regarded as a starting point for lead optimization by rational design or high-throughput chemistry, yielding new drugs with diminished anticholinergic-derived side-effects and even greater affinity for CBPs, allowing the use of smaller doses.

Experimental procedures

Bacterial strains, growth conditions and viable counts

S. pneumoniae R6 is a derivative of the Rockefeller University strain R36A. Pneumococcal ATCC 49619 and TIGR4 are encapsulated strains used for MIC experiments. The *Escherichia coli* strains harboring recombinant plasmids encoding the different proteins used in this work were as follows: RB791 (pCE17) for C-LytA [34], RB791 (pGL100) for LytA [35], BL21 (DE3) (pLCC14) for LytC [36], and BL21 (DE3) (pRGR12) for Pce [37]. Pneumococcal cultures were grown without aeration in C medium supplemented with 0.08% (w/v) yeast extract (C + Y medium) [38]. Growth was monitored by measuring the attenuance (D) in a Thermo Spectronic spectrophotometer (Waltham, MA, USA). *E. coli* cultures were grown with aeration in LB medium with ampicillin (100 $\mu g \cdot mL^{-1}$). The number of viable pneumococcal cells was determined by counting the number of colonies from appropriate dilutions of culture (in triplicate) spread on the surface of tryptic soy agar plates supplemented with 5% defibrinated sheep blood. Micrographs of samples were obtained with a Nikon Optiphot-2 microscope (Tokyo, Japan).

Protein purification

C-LytA, LytA, LytC and Pce were purified from crude extracts of the corresponding overproducing *E. coli* strains, following the procedure previously described [26,34]. Purification of C-LytA was also optimized using the materials and protocols contained in the C-LYTAG kit (Biomedal, Seville, Spain). Purified proteins were subsequently dialyzed at 20 °C against 20 mM sodium phosphate buffer (pH 7.0), plus 50 mM NaCl, to remove the choline used for elution. The protein concentration was determined spectrophotometrically.

CD spectroscopy

CD experiments were carried out in a Jasco J-810 spectropolarimeter (Tokyo, Japan) equipped with a Peltier PTC-423S system. Isothermal wavelength spectra were acquired at a scan speed of 50 $nm \cdot min^{-1}$ with a response time of 2 s, and averaged over at least six scans at 20 °C. The protein concentration was 19 μM, and the cuvette path length was 1 cm. The buffer was 20 mM sodium phosphate (pH 7.0). For ligand titrations, aliquots from a 150 mM stock solution were added stepwise and incubated for 5 min prior to recording the wavelength spectra. Ellipticities ([θ]) are expressed in units of $deg \cdot cm^2 \cdot (dmol\ of\ protein)^{-1}$. With atropine or ipratropium present at a concentration of over 5 mM, spectra could not be recorded below 270 nm, due to the high absorbance of the sample. For CD-monitored thermal denaturation experiments, the sample was layered with mineral oil to avoid evaporation, and the heating rate was 60 $C^{\circ} \cdot h^{-1}$. When a second scan was required, the heated sample was cooled down in the same cuvette and left for at least 1 h for temperature equilibration.

Fluorescence spectroscopy

Emission scans were performed at 20 °C in a PTI-QuantaMaster fluorimeter (Birmingham, NJ, USA), model QM-62003SE, using a 5 × 5 mm-path-length cuvette and a protein concentration of 19 μM. Tryptophan emission spectra were obtained using an excitation wavelength of 280 nm, with excitation and emission slits of 3 nm, and a scan rate of 60 $nm \cdot min^{-1}$.

Size-exclusion chromatography

Samples of 65 μL containing 200 μg of C-LytA were loaded onto a Sephadex G-75 column (26 × 0.9 cm) (Sigma-Aldrich, St Louis, MO, USA) equilibrated in 20 mM sodium phosphate buffer (pH 7.0) plus 200 mM NaCl and the corresponding amount of ligand. Samples containing ligands were allowed to equilibrate for at least 10 min prior to application. Chromatography was run with the same buffer at a flow rate of 0.5 $mL \cdot min^{-1}$ at 20 °C. Fractions of

500 μL were collected, and their absorbance was measured at 280 nm. Exclusion (6.3 mL) and total (21.0 mL) volumes were determined with Dextran Blue and potassium dichromate, respectively.

In vitro assays of pneumococcal cell wall lytic enzymes

Purified choline-binding enzymes LytA, LytC and Pce were used for cell wall degradation assays, performed basically as previously described [8], using pneumococcal cell walls labeled with [methyl-^{3}H] choline (500 c.p.m.·μL^{-1}, approximately 0.7 μg·μL^{-1}) as substrate, and measuring the amount of radioactivity released into the supernatant, corresponding to solubilized fragments of the cell wall. One unit (U) of activity was defined as the amount of enzyme needed to release 700 c.p.m. of labeled material per 10 min. Experimental conditions depended on the enzyme, and were set as follows: 37 °C and pH 6.9 for LytA; 30 °C and pH 6.0 for LytC; and 30 °C and pH 6.9 for Pce. The specific activities of the enzymes are as follows: LytA, 2.5×10^5 U·mg^{-1} for LytA [36]; LytC, 6×10^3 U·mg^{-1} [36]; and Pce, 2.4×10^3 U·mg^{-1} [37]. The phosphorylcholinesterase activity of Pce was also assayed using the soluble substrate *p*-nitrophenylphosphorylcholine (Sigma-Aldrich), as previously described [37].

Materials

Choline chloride and DEAE-cellulose were obtained from Sigma-Aldrich. Owing to the hygroscopic properties of choline, concentrated stock solutions were always prepared from a freshly opened bottle and stored in aliquots at − 20 °C. All choline analogs were purchased from Fluka (St Louis, MO, USA), except ipratropium bromide (Sigma-Aldrich).

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