In Vitro Bactericidal Activity of the Antiprotozoal Drug Miltefosine against *Streptococcus pneumoniae* and Other Pathogenic Streptococci

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Miltefosine (hexadecylphosphocholine), the first oral drug against visceral leishmaniasis, triggered pneumococcal autolysis at concentrations higher than 2.5 μM. Bactericidal activity was also observed in cultures of other streptococci, although these failed to undergo lysis. The autolysis elicited by miltefosine can be attributed to triggering of the pneumococcal autolysin LytA.

The alarming rise in antibiotic resistance worldwide has fostered the search for new antibacterial drugs. *Streptococcus pneumoniae*, the main bacterium responsible for community-acquired pneumonia, meningitis, bacteremia, and otitis media, besides showing ever-increasing resistance to penicillin, is also acquiring resistance to other antimicrobial classes such as macrolides, tetracyclines, and sulfonamides. This situation has prompted the development of new anti-infectives for the treatment of pneumococcal infections, particularly those produced by multidrug-resistant pneumococci (19).

Choline is an absolute nutritional requirement for *S. pneumoniae* (42) and is a component of the teichoic and lipoteichoic acids present, respectively, in the cell wall and membrane of this notorious human pathogen (14). Choline serves as an anchor for a well-known family of surface proteins, the choline-binding proteins (CBPs), which are involved in key physiological functions of the bacterium, e.g., remodeling and lysis of the cell wall (26) or adhesion and evasion of complement pathways (2). In fact, elucidation of the three-dimensional structure of the choline-binding domain of several CBPs (12, 13, 17, 18) has led to the use of these proteins as targets for the design of antipneumococcal drugs (11).

The addition of 2% choline chloride to actively growing pneumococci promotes the release of some CBPs from the cell wall (38, 48) and/or the complete inhibition of CBPs with cell wall hydrolase activity (26). Miltefosine (hexadecylphosphocholine) is one of several alkyllyso phospholipid derivatives collectively known as alkylphosphocholines that were originally developed as anticancer agents (6). The biocidal action of miltefosine against *Leishmania* species was demonstrated in the mid 1980s (3, 7). Since then, trials for its clinical evaluation have led to the licensing of miltefosine for the oral treatment of visceral leishmaniasis in India, Colombia, and Germany (37).

Given that miltefosine features a phosphocholine group (Fig. 1), we speculated that this drug might be able to release and/or inhibit pneumococcal CBPs in a manner similar to that of choline itself. We report here that miltefosine shows noticeable bacteriolytic or bactericidal action when added to pneumococcal cultures and that this effect is extended to other pathogenic streptococcal species.

**Miltefosine causes the lysis of pneumococcal cultures.** Pneumococcal strains (Table 1) were grown in C medium (22) supplemented with yeast extract (0.08%) or Todd-Hewitt broth (Difco) containing 0.5% yeast extract (THY) at 37°C with no shaking until the early exponential growth phase. In preliminary experiments, miltefosine (a generous gift from Zentaris GmbH, Frankfurt, Germany) was added to cultures of unencapsulated, antibiotic-susceptible laboratory strain R6 at a final concentration of 100 μM (about 41 μg/ml). Surprisingly, all pneumococcal cultures were lysed within 1 min (data not shown). Several miltefosine concentrations were then tested, and similar lysis was observed at 10 μM, whereas lower concentrations (2.5 and 5 μM) clearly expedited the characteristic autolysis of the cultures in the stationary phase of growth (Fig. 1A). Interestingly, encapsulated, multiply antibiotic-resistant *S. pneumoniae* strains (8249 and Spain23F-1) also underwent rapid lysis upon addition of the drug, although at slightly higher concentrations (Fig. 1B and C). It should be emphasized that South African strain 8249, in addition to being highly penicillin resistant, is tolerant to penicillin and other β-lactam antibiotics yet is lysed by other cell wall inhibitors (cycloserine and vancomycin) and detergents and also undergoes autolysis in the stationary phase of growth (24). As expected, immediate lysis also took place when an encapsulated, antibiotic-susceptible pneumococcal strain (ATCC 6503) was treated with miltefosine (data not shown).

The MIC of miltefosine for pneumococcal strains was calculated according to a standard procedure (Clinical and Laboratory Standards Institute, formerly the National Committee for Clinical Laboratory Standards) (31) in which Mueller-Hinton broth is supplemented with 4% lysed horse blood. This protocol gave rise to MICs of about 60 μM (data not shown). However, it has been consistently found that the hydrophobic moiety of miltefosine binds to serum components, reducing the effective concentration of the drug (16). To minimize this pos-
Bactericidal effects of miltefosine on other pathogenic streptococci. To test whether the lytic effect of miltefosine was restricted to pneumococci, cultures of several pathogenic streptococci were incubated with the drug at concentrations equal to or slightly higher than the MIC. Interestingly, the streptococci were incubated with the drug at concentrations restricted to pneumococci, cultures of several pathogenic bacteria (either pneumococci or closely related streptococcal isolates) produced a LytA-like autolysin. To verify that LytA was involved in the lytic effect, a pneumococcal ΔlytA mutant (strain M32) (Table 1) was incubated in the presence of the drug and a tolerant response was noted (Fig. 2A). Early experiments had shown that the LytA-deficient mutants could be phenotypically “cured” by the addition of exogenous autolysin (44). Thus, curing of the M32 strain with electrophoretically pure LytA amidase (10 µg/ml) (15) prior to the addition of miltefosine was sufficient to restore the characteristic lytic effect of the drug (Fig. 2A). Most interestingly, even in the absence of autolysin, 25 µM miltefosine produced a 2-log drop in viability after only 15 min and a 4-log decrease after 3 h of treatment (Fig. 2A). This indicates that even when a tolerant response is observed, miltefosine is very efficient at killing pneumococcal cells. Although the term tolerance is frequently used as meaning inhibition of growth without cell death, it must be kept in mind that tolerant cells are also killed by the corresponding drugs, but in these cultures the loss of viability occurs at a substantially slower rate than in the case of wild-type cells (43). Experimental evidence showing that the autolysin LytA is responsible for as much as 90% (1 log unit) of the bactericidal action of penicillin against S. pneumoniae has been previously reported (27).

Effects of miltefosine-related compounds on triggering of LytA. The rapid pneumococcal lysis triggered by miltefosine is reminiscent of the bile solubility test used to identify S. pneumo-
moniae in most clinical laboratories (29). It is well known that S. pneumoniae is a major pathogen in various clinical settings, and its resistance to antibiotics has been a significant concern. The drug miltefosine has been shown to have a detergent-like effect, which is critical for its antipneumococcal activity.

**Table 1. Bacterial strains used in the present work and calculated MICs**

| Bacterial species or strain | Relevant characteristic(s) (drug, MIC [µg/ml]) | Miltefosine susceptibility MIC (µM) | Source or reference
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<tr>
<td>S. pneumoniae</td>
<td>Unencapsulated laboratory strain; lytA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>5</td>
<td>ATCC</td>
</tr>
<tr>
<td>R6</td>
<td>Serotype 19A, clinical isolate; lytA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>6.25</td>
<td>ATCC</td>
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<tr>
<td>M32</td>
<td>Serotype 23F; lytA&lt;sup&gt;+&lt;/sup&gt; (penicillin, 1-2; tetracycline, 8; chloramphenicol, &gt;8)</td>
<td>6.25</td>
<td>ATCC</td>
</tr>
<tr>
<td>8249</td>
<td>Serotype 3; lytA&lt;sup&gt;+&lt;/sup&gt;; Preceptrol strain</td>
<td>6.25</td>
<td>ATCC</td>
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<tr>
<td>S. pseudopneumoniae</td>
<td>Type strain; lytA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>ND</td>
<td>1; CCUG</td>
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<tr>
<td>CCUG 49455T</td>
<td>(penicillin, 0.25; tetracycline, 128; erythromycin, &gt;128)</td>
<td>ND</td>
<td>CECT</td>
</tr>
<tr>
<td>Streptococcus sp. strain</td>
<td>LytA&lt;sup&gt;+&lt;/sup&gt; (penicillin, 8; tetracycline, 0.5; erythromycin, 4)</td>
<td>ND</td>
<td>CECT</td>
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<td>Type strain</td>
<td>10</td>
<td>NCTC</td>
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<td>Type strain</td>
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<td>CECT</td>
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<td>Type strain</td>
<td>ND</td>
<td>CECT</td>
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<tr>
<td>E. faecalis</td>
<td>Vancomycin resistant (vanA)</td>
<td>20</td>
<td>F. Baquero, Hospital Ramón y Cajal, Madrid, Spain</td>
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</table>

<sup>a</sup> MICs are the means of three independent determinations; ND, not determined.

<sup>b</sup> ATCC, American Type Culture Collection; CCUG, Culture Collection, University of Göteborg; CECT, Colección Española de Cultivos Tipo; NCTC, National Collection of Type Cultures.

Miltefosine is also active against a variety of protozoa such as Trypanosoma (8), Trichomonas (5), and several amoebas (39, 40, 46). Indeed, in 2005 miltefosine was designated an orphan medicinal product for the treatment of Acanthamoeba keratitis by the European Medicines Agency (http://www.emea.eu.int/pdfs/human/comp/20357405en.pdf). The antifungal activity of miltefosine has also been recently recognized (47). To the best of our knowledge, however, there are no previous reports of the antibacterial effect of miltefosine. Interestingly, the MICs of the drug found here to be effective against pathogenic streptococci (5 to 20 µM, corresponding to 2 to 8 µg/ml) match those described for several pathogenic fungi (47) and, most importantly, are very close to those quoted for several Leishmania species (9). This does not, however, necessarily suggest similar mechanisms of action. Thus, the killing action of miltefosine in Leishmania is induced by a far more complex system than merely detergent-induced lysis. Parasite killing requires drug uptake by the specific transporter LdMT, an aminophospholipid translocase (36), and induction of an apoptosis-like process (35), which would be fairly unlikely if produced solely through a detergent-like effect. The MICs determined here for streptococci are lower than the steady-state miltefosine concentrations (120 µM and >500 nmol/g, respectively) attained.
FIG. 2. Triggering of the autolysin LytA by miltefosine and effect of hydrophobic tail length on pneumococcal lysis. (A) An exponentially growing culture of \textit{S. pneumoniae} strain M32 (ΔlytAΔ32) was incubated in C medium–0.08% yeast extract with (open symbols) or without (solid symbols) the pure LytA enzyme (10 μg/ml) for 1 h at 37°C. The culture was then diluted to an A$_{550}$ of 0.2 and divided into two portions. Miltefosine was added at 25 μM (○, ◦) to one portion, and the other portion was left untreated (□, ○). Incubation was continued at 37°C. Survival of the culture treated only with miltefosine was determined by plating at different incubation times (dotted lines). (B) Growth (and lysis) curves of the pneumococcal R6 strain treated with miltefosine (○), tetradecylphosphocholine (□), or dodecylphosphocholine (△); each drug was added at a final concentration of 25 μM. The growth curve of an untreated R6 culture is indicated by solid circles.

in the serum and lungs of rats after 11 days of daily oral treatment (21). Many pharmacological and pharmacokinetic studies have been done (see reference 41 for a recent review), and oral administration of miltefosine is effective and well tolerated in children (4). Accordingly, this drug could be useful in vivo against streptococcal and, particularly, pneumococcal infections. As a possible shortcoming, it should be considered that these infections develop quite rapidly, sometimes in a few days or even hours, such that orally administrated miltefosine may not achieve sufficiently high concentrations in plasma to kill rapidly multiplying bacteria. It is conceivable, however, that the new miltefosine liposomal formulations (34), nasal instillations complexed with an artificial lung surfactant (45), and/or alternative alkylphospholipid derivatives such as erucylphosphocholine, which can be administered intravenously with no detectable hemolytic effects (10), may prove useful for the in vivo treatment of streptococcal infections in the near future.

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REFERENCES


