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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 alkyllysophospholipid 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 Spain^{23F}-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 6303) 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-

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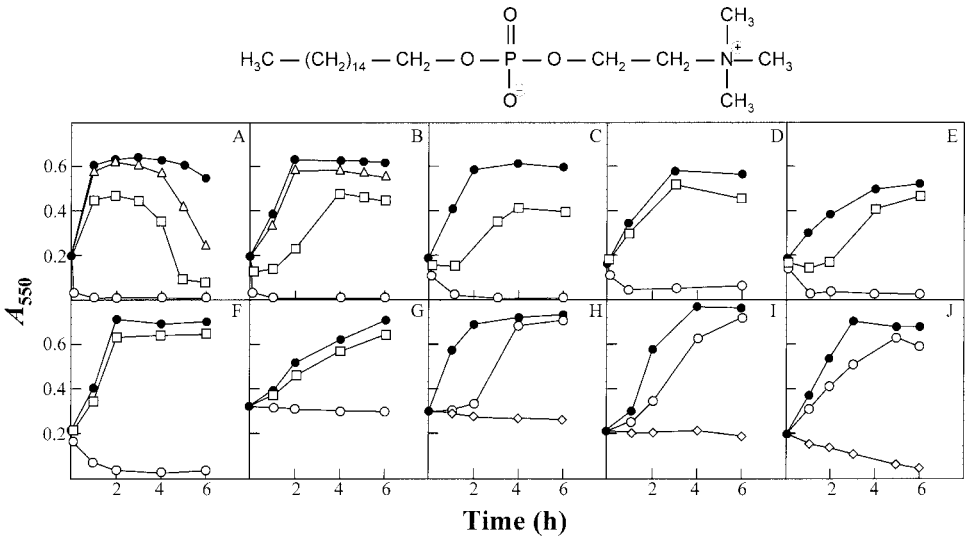

FIG. 1. Effect of miltefosine on cultures of several streptococcal species. Exponentially growing cultures were incubated in THY broth to an A_{550} of about 0.2. Miltefosine was then added to a portion of the culture, and incubation was continued without shaking at 37°C. Panels: A, *S. pneumoniae* R6; B, *S. pneumoniae* Spain^{23F}-1; C, *S. pneumoniae* 8249; D, *S. pseudopneumoniae* type strain; E, *Streptococcus* sp. strain 789/96; F, *Streptococcus* sp. strain 11923/96; G, *S. pyogenes* type strain; H, *S. agalactiae* type strain; I, *S. mutans* type strain; J, *S. sanguinis* type strain. Solid circles represent untreated control cultures. Miltefosine was added at the following concentrations: ◇, 25 μM; ○, 10 μM; □, 5 μM; △, 2.5 μM. The chemical structure of miltefosine is shown at the top.

sibility, the same test was performed with THY broth. Under these conditions, MICs ranged from 5 to 6.25 μM for *S. pneumoniae* strains and from 10 to 20 μM for other streptococci (Table 1). On the other hand, miltefosine showed no effect on the growth of two gram-negative species tested, i.e., *Escherichia coli* and *Pseudomonas aeruginosa* (unpublished observations), suggesting that the outer membrane prevents access of the drug to the cytoplasmic membrane.

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 *Streptococcus pseudopneumoniae* type strain and two streptococci of the mitis group (strains 782/96 and 11923/96) were also lysed rapidly by 10 μM miltefosine (Fig. 1D, E, and F). Remarkably, all of these strains synthesize a partly active LytA-like autolysin, albeit one different from the LytA enzyme characteristic of pneumococci (25, 32). With the possible exception of the *Streptococcus sanguinis* type strain, in which lysis occurred to some extent following the addition of 25 μM miltefosine (Fig. 1J), none of the remaining streptococci, namely, the *Streptococcus pyogenes* type strain, the *Streptococcus agalactiae* type strain, or the *Streptococcus mutans* type strain, showed clear autolytic behavior after exposure to the drug. Moreover, while *S. pyogenes* exhibited a tolerant response when treated with 10 μM miltefosine, *S. agalactiae* and *S. mutans* only stopped growing when at least 25 μM miltefosine was added to the cultures (Fig. 1G, H, and I). The other streptococcal species tested (including a vancomycin-resistant *Enterococcus faecalis* strain) (Table 1) exhibited similar tolerance to miltefosine (unpublished observations). Interestingly, a rapid loss of viability was observed (between 1.5 and 3 log units in 3 h, depending on the species) in experiments performed with streptococcal species that are not lysed by exposure to miltefosine (unpublished data). This behavior resembles the response shown by the autolytically defective strain *S. pneumoniae* M32 (see below).

Miltefosine promotes the uncontrolled action of LytA autolysin. As illustrated above, rapid and complete lysis of bacterial cultures in response to miltefosine only occurred when the 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 Δ*lytA* 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. pneu-*

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)[a]	Source or reference[b]
S. pneumoniae			
R6	Unencapsulated laboratory strain; *lytA*$^+$	5	33
M32	Unencapsulated laboratory strain; Δ*lytA32*	5	28
8249	Serotype 19A, clinical isolate; multiresistant; *lytA*$^+$ (penicillin, 6)	6.25	24
Spain^{23F}-1	Serotype 23F; *lytA*$^+$ (penicillin, 1-2; tetracycline, 8; chloramphenicol, >8)	6.25	30
ATCC 6303	Serotype 3; *lytA*$^+$; Preceptrol strain	6.25	ATCC
S. pseudopneumoniae CCUG 49455^T	Type strain; *lytA*$^+$	ND	1; CCUG
Streptococcus sp. strain 782/96	*lytA*$^+$ (penicillin, 0.25; tetracycline, 128; erythromycin, >128)	ND	32
Streptococcus sp. strain 11923/96	*lytA*$^+$ (penicillin, 8; tetracycline, 0.5; erythromycin, 4)	ND	32
S. mitis NCTC 12161^T	Type strain	10	NCTC
S. oralis NCTC 11427^T	Type strain	10	NCTC
S. pyogenes CECT 985^T	Type strain	10	CECT
S. agalactiae CECT 183^T	Type strain	20	CECT
S. mutans CECT 479^T	Type strain	20	CECT
S. sanguinis CECT 480^T	Type strain	ND	CECT
S. uberis CECT 994^T	Type strain	ND	CECT
E. faecalis	Vancomycin resistant (*vanA*)	20	F. Baquero, Hospital Ramón y Cajal, Madrid, Spain

[a] MICs are the means of three independent determinations; ND, not determined.
[b] ATCC, American Type Culture Collection; CCUG, Culture Collection, University of Göteborg; CECT, Colección Española de Cultivos Tipo; NCTC, National Collection of Type Cultures.

moniae in most clinical laboratories (29). It is well known that bile (i.e., deoxycholate) causes the release of lipoteichoic acid, the natural inhibitor of LytA autolysin (20), from the pneumococcal membrane, allowing this enzyme to rapidly degrade cell wall peptidoglycan. In fact, miltefosine and its related compounds have a detergent effect and dodecylphosphocholine has been used to solubilize membrane proteins (23). Collectively, these results suggest that the primary effect of miltefosine on *S. pneumoniae* and other streptococci may involve its action as a detergent. To establish the influence of the length of the alkyl tail on bactericidal activity, two additional shortened alkylphosphocholines were tested, i.e., dodecylphosphocholine and tetradecylphosphocholine (both purchased from Anatrace, Inc., Maumee, OH). As shown in Fig. 2B, the length of the alkyl chain was found to be directly proportional to the lysis-promoting capacity of pneumococcal strain R6. In fact, dodecylphosphocholine was practically devoid of any lytic activity even when applied at a concentration of 25 μM. These observations are consistent with the critical micellar concentration reported for these compounds (Anatrace, Inc., catalog, 2006 ed. [http://www.anatrace.com/Literature/Catalog%20Sept%202006.pdf]).

In addition to its antitumor and antileishmanial activities, 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

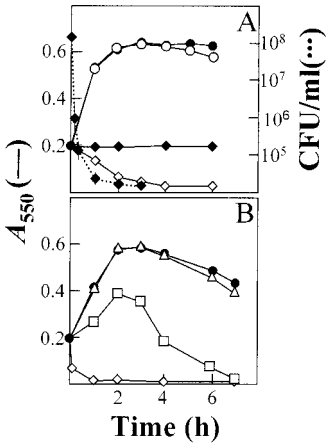

FIG. 2. Triggering of the autolysin LytA by miltefosine and effect of hydrophobic tail length on pneumococcal lysis. (A) An exponentially growing culture of *S. pneumoniae* strain M32 (Δ*lytA32*) was incubated in C medium–0.08% yeast extract with (open symbols) or without (solid symbols) the pure LytA 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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