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Antimicrobial Activities of Clofazimine and B669 Are Mediated by Lysophospholipids

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The susceptibilities of a range of gram-positive and gram-negative microbial pathogens to clofazimine and its analog B669 (0.1 to 32 μg/ml), as well as the effects of these agents on membrane phospholipid metabolism in *Staphylococcus aureus* and *Escherichia coli*, have been investigated in vitro. Gram-positive bacteria were found to be generally susceptible to these agents, whereas gram-negative organisms were uniformly resistant. Exposure of *S. aureus* to both agents (1 to 5 μg/ml), especially B669, caused dose-related enhancement of the activity of phospholipase A_2, according to an increase in the release of ^{3}H-radiolabeled arachidonate and lysophosphatidylethanolamine ([^{3}H]LPE) from bacterial-membrane phospholipids. Treatment of *E. coli* with the riminophenazines also increased the release of [^{3}H]arachidonate and [^{3}H]LPE. Growth of gram-positive but not gram-negative bacteria was inhibited by LPE and lysophosphatidylcholine. Moreover, coincubation with α-tocopherol (vitamin E), a lysophospholipid complex-forming agent, or with lysophospholipase protected gram-positive bacteria against the riminophenazines as well as against lysophospholipids. The results from this study are consistent with a mechanism whereby lysophospholipids mediate the activities of the two drugs.

The antimycobacterial riminophenazine agent clofazimine, which was first described in 1957 (5), is one of the standard drugs for the treatment of leprosy (4, 9, 14, 21) and is now recommended as part of a combination therapy for this disease (22). It is also useful in the antimicrobial-combination chemotherapy of *Mycobacterium avium* infections in AIDS patients (1). Clofazimine also inhibits the growth of tubercle bacilli in vitro and in animal models of experimental infection (7).

Although clofazimine was described in 1957, the biochemical mechanism of its antimicrobial activity has not been established. As a consequence of its highly lipophilic nature and redox potential (−0.18 V at pH 7), it has been proposed that intracellular generation of hydrogen peroxide by redox-cycling mechanisms may contribute to the antimicrobial activity of clofazimine (5). It has also been reported that clofazimine binds to the guanine bases of DNA, thereby blocking the template function of the DNA, leading to inhibition of proliferation (17). The increase in the guanine and cytosine content of microbial DNA relative to that of human DNA may explain the selective inhibitory effects of clofazimine on the proliferation of microbial cells (5).

In the present study, we have investigated the antimicrobial spectrum of clofazimine and its analog B669 (6, 19), as well as the biochemical mechanism of the antimicrobial activities of these agents. B669 was included for comparison on the basis of preliminary screening experiments which indicated that it possessed superior antimicrobial activity, relative to that of clofazimine, for some microbial pathogens. Our data demonstrate that these riminophenazines are broadly active against gram-positive bacteria, whereas gram-negative microorganisms are uniformly resistant. Moreover, antiproliferative lysophospholipids generated in the cell membrane of gram-positive bacteria during exposure to clofazimine or B669 are the primary mediators of the antimicrobial activities of these agents.

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MATERIALS AND METHODS

Antimicrobial agents. The molecular structures of clofazimine [3-(*p*-chloroanilino)-10-(*p*-chlorophenyl)-2,10-dihydro-2-(isopropylimino)-phenazine] and B669 [3-anilino-10-phenyl-2,10-dihydro-2-(cyclohexylimino)-phenazine] are diagrammed in Fig. 1. Both agents were synthesized by J. F. O'Sullivan and dissolved in dimethyl sulfoxide (DMSO) to give stock concentrations of 2 mg/ml. Subsequent dilutions were made in sterile, distilled water, and both agents were used at final concentrations ranging from 0.1 to 32 μg/ml. Appropriate solvent controls were included in the various assays described below.

Chemicals, reagents, and enzymes. Unless otherwise indicated, chemicals, reagents, and enzymes were obtained from the Sigma Chemical Co. (St. Louis, Mo.), and radiochemicals were obtained from Du Pont-NEN Research Products (Boston, Mass.) and Amersham International (Aylesbury, United Kingdom).

Microbial pathogens. The various microbial pathogens and their origins (reference strain or clinical isolate) are listed in Table 1.

MICs and MBCs. The MICs and MBCs of clofazimine and B669 for the various test microbial pathogens were determined by standard bacteriological assays (18), whereas the susceptibilities of *Mycobacterium tuberculosis* H37 were measured with the Bactec TB system (Becton Dickinson Diagnostic Instrument Systems, Towson, Md.). In additional experiments, the susceptibilities to both riminophenazines of the gram-positive facultative anaerobes *Staphylococcus aureus* ATCC 25923, *Streptococcus pyogenes* (clinical isolate), and *Enterococcus faecalis* ATCC 2921 were determined under aerobic (with brain heart infusion

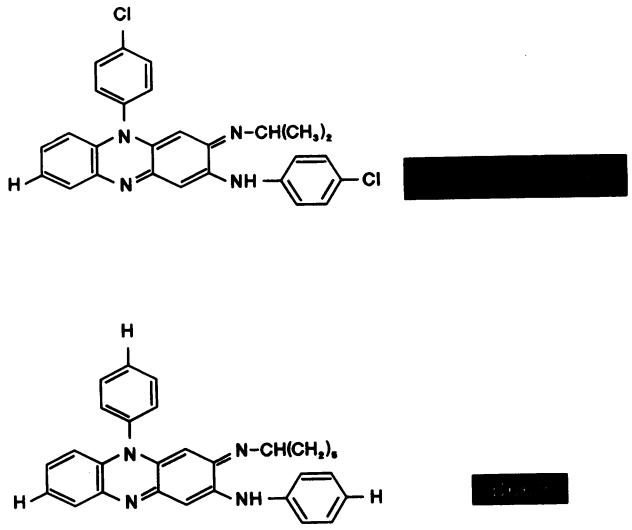

FIG. 1. Molecular structures of clofazimine [3-(*p*-chloroanilino)-10-(*p*-chlorophenyl)-2,10-dihydro-2-(isopropylimino)-phenazine] and B669 [3-anilino-10-phenyl-2,10-dihydro-2-(cyclohexylimino)-phenazine].

broth) and anaerobic (with a prereduced, supplemented brain heart infusion broth) conditions.

Protection experiments with antioxidative agents. The potential of various lipid and water-soluble antioxidant chemicals and enzymes (*dl*-α-tocopherol, *dl*-α-tocopherol acetate, retinol, retinol acetate, butylated hydroxyanisole, ascorbic acid, dithiothreitol, desferrioxamine, catalase, and superoxide dismutase at final concentrations of 25 μg/ml, 25 μg/ml, 5 μg/ml, 50 μg/ml, 10 μg/ml, 100 μg/ml, 100 μg/ml, 100 μg/ml, 500 U/ml and 200 U/ml, respectively) to protect *S. aureus* ATCC 25923 or *Staphylococcus epidermidis* ATCC 2223 against riminophenazine-mediated inhibition of bacterial growth was investigated by a sensitive radioassay based on the uptake of added radiolabeled amino acids by proliferating bacteria. At the predetermined concentrations listed above, none of the test agents per se (i.e., in the absence of the riminophenazines) either inhibited or enhanced the uptake of radiolabeled acids by proliferating bacteria. These assays were performed by inoculating 50 μl of a standardized suspension of the test microbial pathogens into 1 ml of nutrient broth containing 0.25 μCi of radiolabeled amino acids (L-amino acid mixture, L-^{14}C[U]; specific activity, 55 mCi per milliatom of carbon; Du Pont-NEN) and the various protective agents listed above or appropriate solvent controls when necessary. After 15 min of incubation at 37°C, clofazimine or B669 (final concentrations, 0.5 to 8 μg/ml) was added, and the tubes were incubated for 6 h at 37°C, after which the bacteria were centrifuged and washed three times in phosphate-buffered saline. The extent of incorporation of radiolabeled amino acids was measured in a liquid scintillation spectrometer. Bacterial growth is expressed according to the extent of uptake of radiolabeled amino acids in radioactive counts per minute. The Bactec TB system was used to investigate the protective potential of α-tocopherol (25 μg/ml) against riminophenazine-mediated inhibition of the growth of *M. tuberculosis*, with a 48-h incubation period.

In an additional series of experiments, the potential of α-tocopherol (final concentration, 25 μg/ml) to reverse the inhibitory effects of B669 (2 μg/ml) on the growth of *S. aureus* ATCC 25923 were investigated by the radioassay. Briefly, *S. aureus* cells were inoculated into 1 ml of nutrient broth containing the riminophenazines and 0.25 μCi of radiolabeled amino acids, and the mixture was incubated for 18 h at 37°C. Thereafter, α-tocopherol or solvent control was added to the tubes, which were then incubated for a further period of 18 h at 37°C, and reactivation of bacterial growth was measured according to the uptake of radiolabeled amino acids by proliferating bacteria.

Measurement of bacterial PLA_2 activity. The effects of clofazimine and B669 (final concentrations, 1, 2.5, and 5 μg/ml) on phospholipase A_2 (PLA_2) activity in *S. aureus* and *Escherichia coli* were measured according to the extent of release of radiolabeled arachidonate and lysophospholipids by control and riminophenazine-treated bacteria.

Arachidonic acid release. *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were grown for 6 h in nutrient broth at 37°C, washed twice, and resuspended in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered, Ca^{2+}-free Hanks balanced salt solution (pH 7.4) to a concentration of 5×10^{10} cells per ml. The bacteria (1×10^{10} cells per ml) were then coincubated with 5 μCi of radiolabeled arachidonate {[5,6,8,9,11,12,14,15-^{3}H(N)]; specific activity, 79.9 Ci/mmol; Du Pont-NEN} per ml for 30 min at 37°C in Ca^{2+}-free Hanks balanced salt solution to allow incorporation of radiolabeled arachidonate into the C-2 position of the glycerol backbone of membrane phospholipids and then washed and resuspended to 2×10^{10} cells per ml in Hanks balanced salt solution. In a series of preliminary experiments, it was established that coincubation of *S. aureus* or *E. coli* with [^{3}H]arachidonate was accompanied by incorporation of this unsaturated fatty acid into bacterial phospholipid, almost exclusively into phosphatidylethanolamine (PE). This is in agreement with a previous report (10). The bacteria were then preincubated for 5 min at 37°C prior to the addition of the riminophenazines. After the addition of the test agents, the final volume in each tube was 2 ml and contained 2×10^9 bacteria. The tubes were incubated for 120 min (predetermined in preliminary experiments), and the reactions were terminated by the addition of 5 ml of *n*-hexane-isopropanol-concentrated HCl (final concentration, 0.1 M [300:200:4; vol/vol/vol]). Fatty acids were extracted as described previously (20). The upper organic phase was separated, retained, and dried under a stream of nitrogen. The fatty acids were dissolved in 50 μl of hexane-isopropanol (3:2 [vol/vol]) containing 15 μg of unlabeled arachidonate to facilitate visual detection with iodine vapors. Aliquots of 10 μl each were then spotted onto silica gel 60-precoated thin-layer chromatography (TLC) plates (Merck, Darmstadt, Germany). The plates were developed in chloroform-acetone (96:4 [vol/vol]). After exposure to iodine vapors, the arachidonate spots were localized and the silica was removed and assayed for radioactivity.

Lysophospholipid assay. Lysophospholipids in the chloroform-methanol (2:1 [vol/vol]) extracts of control and clofazimine- or B669 (1, 2.5, and 5 μg/ml)-treated *S. aureus* and *E. coli* were measured by a previously described high-performance TLC (HPTLC) method (8). The bacteria were inoculated into 10 ml of nutrient broth containing 50 μCi of radiolabeled palmitate {[9,10-^{3}H(N)]; specific activity, 60 Ci/mmol; Du Pont-NEN} per ml, and the mixtures were incubated for 4 h (predetermined in preliminary experiments) at 37°C to allow incorporation of the radiolabeled saturated fatty acid into the C-1 position of the glycerol backbone of membrane phospholipids. In a series of preliminary experiments, it was established that after coincubation

TABLE 1. MICs and MBCs of clofazimine and B669 for some gram-positive and gram-negative pathogens under aerobic and anaerobic conditions

Organism	Condition	Clofazimine		B669	
		MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)
Gram-positive bacteria					
Staphylococcus aureus[a]	Aerobic	1.0	>32.0	1.0	>32.0
Staphylococcus aureus ATCC 25923	Aerobic	4.0	>32.0	1.0	>32.0
	Anaerobic	1.0	ND[b]	0.5	ND
Staphylococcus saprophyticus ATCC 606	Aerobic	0.5	>32.0	1.0	16.0
Staphylococcus epidermidis ATCC 2223	Aerobic	0.25	>32.0	0.12	16.0
Staphylococcus haemolyticus ATCC 29970	Aerobic	1.0	16.0	0.5	16.0
Streptococcus viridans[a]	Aerobic	>32.0	>32.0	1.0	16.0
Streptococcus viridans NCTC 10712	Aerobic	1.0	2.0	0.5	1.0
Streptococcus pyogenes[a]	Aerobic	2.0	>32.0	1.0	8.0
	Anaerobic	1.0	1.0	0.125	1.0
Streptococcus mutans NCTC 10449	Aerobic	1.0	1.0	0.5	0.5
Enterococcus faecalis ATCC 2921	Aerobic	>32.0	>32.0	2.0	16.0
	Anaerobic	1.0	ND	2.0	ND
Bacillus cereus[a]	Aerobic	0.5	>32.0	0.5	16.0
Listeria monocytogenes[a]	Aerobic	0.25	16.0	0.25	4.0
Listeria monocytogenes NCTC 5214	Aerobic	2.0	32.0	2.0	32.0
Gram-negative bacteria					
Pseudomonas aeruginosa[a]	Aerobic	>32.0	>32.0	>32.0	>32.0
Pseudomonas cepacia NCTC 10734	Aerobic	>32.0	>32.0	>32.0	>32.0
Escherichia coli ATCC 25922	Aerobic	>32.0	>32.0	>32.0	>32.0
Salmonella typhi NCTC 10431	Aerobic	>32.0	>32.0	>32.0	>32.0
Salmonella typhimurium ATCC 14028	Aerobic	>32.0	>32.0	>32.0	>32.0
Klebsiella pneumoniae NCTC 9633	Aerobic	>32.0	>32.0	>32.0	>32.0
Proteus vulgaris[a]	Aerobic	>32.0	>32.0	>32.0	>32.0
Proteus mirabilis ATCC 33583	Aerobic	>32.0	>32.0	>32.0	>32.0
Enterobacter aerogenes[a]	Aerobic	>32.0	>32.0	>32.0	>32.0
Shigella dysenteriae[a]	Aerobic	>32.0	>32.0	>32.0	>32.0
Shigella flexneri[a]	Aerobic	>32.0	>32.0	>32.0	>32.0

[a] Clinical isolate.
[b] ND, not done.

of *S. aureus* for 4 h at 37°C, 85% of the amount of radiolabeled saturated fatty acid incorporated into phospholipids was associated with PE and 15% was associated with phosphatidylcholine (PC). In *E. coli*, almost all of the incorporated [^{3}H]palmitate was associated with PE. In view of the predominant labeling of PE, only lysophosphatidylethanolamine (LPE) was assayed during exposure of the bacteria to the riminophenazines. After labeling, the bacteria were washed and resuspended in Hanks balanced salt solution, and the suspensions were preincubated for 5 min at 37°C, after which the riminophenazines were added. The final reaction volume in each tube was 2 ml and contained 10^9 CFU. After an optimum predetermined incubation time of 5 min at 37°C, the reactions were terminated by the addition of 3 ml of chloroform-methanol (2:1 [vol/vol]). Water (0.3 ml) was added to cause phase separation. After centrifugation, the phospholipid-containing lower phase was removed and evaporated to dryness under a stream of nitrogen. The evaporates were dissolved in 50 μl of chloroform-methanol, and aliquots of 10 μl each containing the appropriate standards (10 μg of PC, lysophosphatidylcholine [LPC], PE, and LPE) were then spotted onto silica gel 60-precoated HPTLC plates (Merck). The plates were developed twice in chloroform–methanol–isopropanol–0.25% KCl–ethyl acetate (3:9:25:6:18 [vol/vol/vol/vol/vol]). After exposure to iodine vapors, the LPE spots were localized and the silica was removed and assayed for radioactivity.

Measurement of the effects of pure LPC and LPE on the growth of a series of gram-positive and gram-negative bacteria. The susceptibilities of the various gram-negative and gram-positive bacterial pathogens to added pure LPC and LPE (0.5 to 20 μg/ml) were investigated by the radioassay. Test bacteria were inoculated into nutrient broth containing 0.25 μCi of radiolabeled amino acids with and without the lysophospholipids, and the mixtures were incubated for 6 h at 37°C, after which bacterial growth was assessed according to the uptake of radiolabeled amino acids. In an additional series of experiments, *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 2223 were exposed to the lysophospholipids (0.5 to 20 μg/ml) in the presence and absence of α-tocopherol (25 μg/ml).

Protection experiments with lysophospholipase. The potential of added, purified lysophospholipase (2-lysophosphatidylcholine acylhydrolase-phospholipase B from *Vibrio* species) to protect *S. aureus* ATCC 25923 against clofazimine- and B669-mediated inhibition of bacterial growth was investigated by the radioassay described above, which is based on the uptake of radiolabeled amino acids by proliferating bacteria. Nutrient broth containing 0.25 μCi of radiolabeled amino acids, with and without 200 mU of lysophospholipase per ml, was inoculated with *S. aureus*, and the tubes were incubated for 15 min at 37°C, after which a sub-MIC concentration (0.5 μg/ml) of clofazimine or B669 or solvent controls was added. The tubes were then incubated at 37°C, and fresh lysophospholipase (200 mU) was added to enzyme-containing tubes after 2 and 4 h, with appropriate volume adjustments made for enzyme-free control tubes. After 6 and 18 h of incubation at 37°C, bacterial growth was

measured as described above according to the extent of uptake of radiolabeled amino acids by proliferating bacteria.

Measurement of bacterial lysophospholipase activities. The lysophospholipase activities of four different species of gram-positive bacteria (*S. aureus* ATCC 25923, *S. epidermidis* ATCC 2223, *S. pyogenes* [clinical isolate], and *E. faecalis* ATCC 2921) and four different species of gram-negative bacteria (*E. coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Pseudomonas aeruginosa* [clinical isolate], and *Klebsiella pneumoniae* [clinical isolate]) were measured by radiometric HPTLC with 0.25 μCi of added radiolabeled LPC ([^{14}C]LPC; L-lyso-3-phosphatidylcholine-1-[1-^{14}C] palmitoyl; specific activity, 56 mCi/mmol; Amersham) and 200 nmol of unlabeled LPC as substrate (8) with intact, viable microorganisms harvested from 4-h cultures in nutrient broth, as well as in the bacterium-free filtrates.

Assay for the activity of purified PLA_2 and lysophospholipase. The effects of the riminophenazines on the activity of purified PLA_2 (PLA_2 from porcine pancreas) were assayed by radiometric TLC (20) with radiolabeled PC (L-3-phosphatidylcholine-1-stearoyl-2-[5,6,8,9,11,12,14,15-^{3}H]arachidonyl; specific activity, 135 Ci/mmol; Amersham) as substrate. The reaction mixtures (1 ml each) contained 20 mM Tris-HCl (pH 8.5), 5 mM $CaCl_2$, 0.625 μCi of radiolabeled PC, 50 nmol of unlabeled carrier PC, 200 nmol of unlabeled LPC, and 5 μg of clofazimine or B669 per ml. Control systems contained the corresponding concentration of DMSO. LPC was included in the assay system to promote the formation of phospholipid micelles (11). Reactions were initiated by the addition of PLA_2 (final concentration, 10 U/ml), and the tubes were incubated for 15 min at 37°C.

The effects of the riminophenazines on the activity of purified lysophospholipase (2-lysophosphatidylcholine acylhydrolase-phospholipase B from *Vibrio* species) were assayed by radiometric TLC (8) with radiolabeled LPC (L-lyso-3-phosphatidylcholine-1-[1-^{14}C]palmitoyl; specific activity, 56 mCi/mmol; Amersham) as substrate. The reaction mixtures (1 ml each) contained 20 mM Tris-HCl (pH 7.0), 0.25 μCi of radiolabeled LPC, 200 nmol of unlabeled LPC, and 5 μg of clofazimine or B669 per ml. Control systems contained the corresponding concentrations of DMSO. Reactions were initiated by the addition of 200 mU (final concentration) of lysophospholipase, and the tubes were incubated for 15 min at 37°C.

Measurement of possible complex formation of α-tocopherol with clofazimine and B669. The UV absorption spectra of mixtures of α-tocopherol (up to 100 μg/ml) and clofazimine or B669 (up to 10 μg/ml) in ethanol relative to identical concentrations of the individual agents were measured as described previously (13) with a Pye Unicam SP 1700 double-beam UV spectrophotometer.

Expression and statistical analysis of results. The results of each series of experiments are presented as the mean values ± standard errors of the mean values (SEMs) for each series of experiments. The numbers of experiments are indicated in the tables and figure legends. Statistical analyses were performed by the Student paired *t* test.

RESULTS

MICs and MBCs. The susceptibilities of a range of gram-positive and gram-negative bacterial pathogens to clofazimine and B669 are shown in Table 1. The gram-positive microbial pathogens were sensitive to one or both riminophenazines, especially B669, whereas the gram-negative pathogens were not inhibited by concentrations of as high as 32 μg/ml. Anaerobiosis increased the susceptibility of the facultative anaerobes *S. aureus* and *S. pyogenes* to the riminophenazines as well as the susceptibility of *E. faecalis* to clofazimine (Table 1).

TABLE 2. Protective effect of α-tocopherol on clofazimine- and B669-mediated inhibition of the growth of *S. aureus*

Treatment	Bacterial growth measured by uptake of ^{14}C-labeled amino acids (cpm)[a]
Control	2,012 ± 240
α-Tocopherol only (25 μg/ml)	1,921 ± 53
Clofazimine (0.5 μg/ml)	97 ± 6
Clofazimine + α-tocopherol	1,411 ± 35
B669 (0.5 μg/ml)	68 ± 5
B669 + α-tocopherol	1,802 ± 241

[a] Data from four separate experiments are presented as the mean values ± SEMs.

Measurement of the protective potential of antioxidative agents. Of the various agents tested, only α-tocopherol protected *S. aureus* and *S. epidermidis* against the antiproliferative effects of both clofazimine and B669. The effects of α-tocopherol (25 μg/ml) on the growth of *S. aureus* coincubated with clofazimine or B669 at 0.5 μg/ml are shown in Table 2. α-Tocopherol also protected *M. tuberculosis* against the inhibitory effects of the riminophenazines (0.25 to 5 μg/ml); these data are shown in Fig. 2.

Retrospective addition of α-tocopherol to *S. aureus*, which had been exposed to B669 (2 μg/ml) during the preceding 18 h, caused reactivation of bacterial growth as measured by the radioassay with an 18-h reincubation period. The level of uptake of radiolabeled amino acids by B669-pretreated (18 h), α-tocopherol-free bacteria was 12.3 ± 9.5 cpm, in comparison with a value of 1,383.3 ± 31.1 cpm in the corresponding α-tocopherol-treated system. The levels of uptake of radiolabeled amino acids by a riminophenazine-free control system without and with α-tocopherol were 1,352 ± 22.4 cpm and 1,637.7 ± 43.5 cpm, respectively. These data demonstrate that α-tocopherol also reverses riminophenazine-mediated inhibition of bacterial growth.

Bacterial PLA_2 activity. The effects of the riminophenazines (1, 2.5, and 5 μg/ml) on the activity of PLA_2 in *S.*

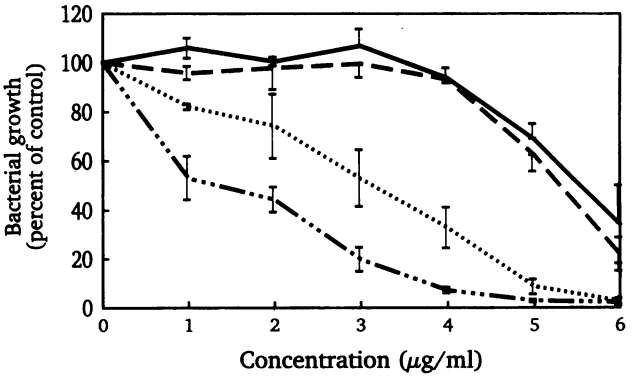

FIG. 2. Effects of clofazimine with (——) and without (——·——) α-tocopherol (25 μg/ml) and of B669 with (— — —) and without (····) α-tocopherol on the growth of *M. tuberculosis*. Data from four experiments are expressed as the mean values ± SEMs. The absolute values for the α-tocopherol- and riminophenazine-free and the α-tocopherol-containing, riminophenazine-free control systems were 394.5 ± 108.2 and 314.7 ± 92.1 cpm, respectively.

TABLE 3. Effects of clofazimine and B669 on the production of [^{3}H]arachidonate and [^{3}H]LPE from *S. aureus* and *E. coli*

Treatment (μg/ml)	Mean % (± SEM)[a] for			
	S. aureus		*E. coli*	
	[^{3}H]arachidonate	[^{3}H]LPE	[^{3}H]arachidonate	[^{3}H]LPE
Clofazimine (1)	173 ± 39	128 ± 9	127 ± 7	141 ± 12
Clofazimine (2.5)	210 ± 60	140 ± 6	161 ± 13	161 ± 14
Clofazimine (5)	247 ± 45	168 ± 9	219 ± 23	164 ± 21
B669 (1)	222 ± 58	157 ± 13	146 ± 8	154 ± 19
B669 (2.5)	257 ± 69	178 ± 29	251 ± 37	147 ± 14
B669 (5)	403 ± 106	243 ± 31	259 ± 46	164 ± 5

[a] Data from four to five experiments are the mean percents ± SEMs of the corresponding riminophenazine-free control systems. For *S. aureus*, the absolute values for these drug-free control systems were 18,935 ± 5,190 and 23,790 ± 7,523 cpm for the release of [^{3}H]arachidonate and [^{3}H]LPE, respectively. The corresponding values for *E. coli* were 20,335 ± 13,130 and 30,800 ± 5,420 cpm, respectively. All values shown are statistically significant ($P < 0.05$ to $P < 0.005$) for increased release of [^{3}H]arachidonate and [^{3}H]LPE in the presence of the riminophenazines.

aureus and *E. coli* were measured according to the release of the two primary hydrolysis products, fatty acid and LPE, from bacterial PE prelabeled in the C-2 and C-1 positions of the glycerol backbone with [^{3}H]arachidonate and [^{3}H]palmitate, respectively. These data for the release of [^{3}H]arachidonate and [^{3}H]LPE are shown in Table 3. The riminophenazines, especially B669, caused dose-related increases in the release of [^{3}H]arachidonate by both *S. aureus* and *E. coli*. Clofazimine and B669 treatment of *S. aureus* and *E. coli* was also associated with dose-related activation of release of LPE; in the case of *S. aureus*, B669 was the more potent of the two riminophenazines. These data demonstrate that exposure to the riminophenazines leads to activation of PLA_2 in both *S. aureus* and *E. coli*.

Effects of lysophospholipids on the growth of gram-positive and gram-negative bacteria. Data for the effects of lysophospholipids on the growth of gram-positive and gram-negative bacteria are shown in Table 4. At concentrations of up to 5 μg of LPC and LPE per ml, none of the gram-negative bacteria was susceptible to either lysophospholipid. However, all of the gram-positive bacteria tested were susceptible to both lysophospholipids at concentrations of 5 and 20 μg/ml. The protective effects of α-tocopherol (25 μg/ml) on LPC- and LPE-mediated inhibition of the growth of *S. aureus* ATCC 25923 are depicted in Fig. 3. α-Tocopherol protected this organism against the antiproliferative effects of the lysophospholipids. Similar results were obtained with *S. epidermidis* ATCC 2223 (results not shown).

Effects of lysophospholipase on clofazimine- and B669-mediated inhibition of the growth of *S. aureus*. Data for the effects of lysophospholipase on riminophenazine-mediated inhibition of the growth of *S. aureus* are shown in Table 5. After 6 h of incubation, only trivial growth was observed in the clofazimine- or B669-containing systems with and without lysophospholipase (data not shown). After extended incubation (18 h), however, lysophospholipase treatment was found to confer partial but significant protection against riminophenazine-mediated inhibition of the growth of *S. aureus*.

Measurement of bacterial lysophospholipase activity. We were unable to detect lysophospholipase activity with intact bacterial cells or in the culture filtrates of any of the gram-positive or gram-negative bacteria tested.

Measurement of the effects of clofazimine and B669 on the activities of purified PLA_2 and lysophospholipase. Neither clofazimine nor B669 at a fixed concentration of 5 μg/ml affected the activities of purified PLA_2 or lysophospholipase. The amounts of arachidonate released from radiolabeled PC in the presence of PLA_2 were 19 ± 1 nmol, 20 ± 1 nmol, and 21 ± 1 nmol for the control system and systems containing 5 μg of clofazimine and B669 per ml, respectively (data from four different experiments). The amounts of LPC hydrolyzed by lysophospholipase were 103 ± 16 nmol, 100 ± 2 nmol,

TABLE 4. Susceptibilities of a range of gram-positive and gram-negative bacteria to LPC and LPE by the radioassay

Microbial pathogen	Percentage of inhibition of bacterial growth with[a]:			
	LPC (5 μg/ml)	LPC (20 μg/ml)	LPE (5 μg/ml)	LPE (20 μg/ml)
Gram-positive bacteria				
Staphylococcus ATCC 25923	42 ± 4	100	83 ± 5	89 ± 4
Staphylococcus epidermidis ATCC 2223	99 ± 35	100	92 ± 12	94 ± 26
Enterococcus faecalis ATCC 29212	82 ± 22	100	6 ± 1	86 ± 4
Streptococcus pyogenes[b]	100 ± 10	100	100	100
Streptococcus viridans[b]	100 ± 10	100	76 ± 11	99 ± 4
Bacillus cereus[b]	100	100	100	100
Gram-negative bacteria				
Escherichia coli ATCC 25922	0	0	0	0
Pseudomonas aeruginosa[b]	19 ± 4	16 ± 3	0	14 ± 7
Salmonella typhimurium ATCC 24028	0	6 ± 12	0	8 ± 3
Klebsiella pneumoniae[b]	0	50 ± 7	0	3 ± 7
Enterobacter agglomerans[b]	0	47 ± 2	0	23 ± 2

[a] Data from four different experiments.
[b] Clinical isolate.

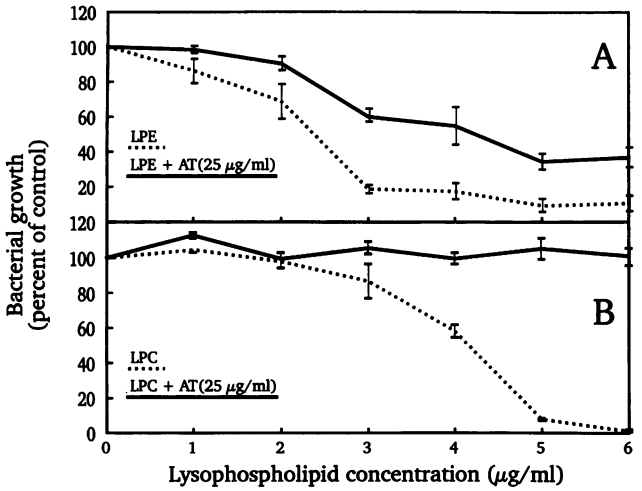

FIG. 3. The effects of LPE (A) with (——) and without (···) α-tocopherol (AT; 25 μg/ml) on the growth of *S. aureus* ATCC 25923 and those of LPC (B) with (——) and without (···) α-tocopherol. Data from four experiments are expressed as the mean percentages ± SEMs of the α-tocopherol- and riminophenazine-free control systems. The absolute values for the α-tocopherol- and riminophenazine-free and the α-tocopherol-containing, riminophenazine-free control systems were 1,174 ± 120 and 1,209 ± 184 cpm, respectively.

and 91 ± 7 nmol for the control system and systems containing 5 μg of clofazimine and B669 per ml, respectively (data from two different experiments).

Spectrophotometric analysis of mixtures of α-tocopherol and clofazimine or B669. The UV spectra of ethanol solutions of clofazimine or B669 before and after the addition of α-tocopherol were unchanged, demonstrating the absence of interactions between the test agents.

DISCUSSION

Although clofazimine is a clinically useful antimycobacterial agent, relatively little is known about its antimicrobial spectrum or mechanism of action. Both aspects have been investigated in the present study. B669, an analog of clofazimine (6), was included for comparison. We have found that gram-positive microorganisms are generally susceptible to both riminophenazines, whereas gram-negative bacteria are uniformly resistant. Of the gram-positive bacteria tested, the reference strain ATCC 2223 and the clinical isolate of *S. epidermidis*, as well as the clinical isolate of *Listeria monocytogenes*, were found to be most susceptible, whereas *E. faecalis* and *Streptococcus viridans* were the least sensitive. With a few exceptions, the gram-positive microorganisms were more susceptible to B669 than to clofazimine. It is noteworthy that the clinical isolate of *S. epidermidis* had been classified as multiresistant by our antibiotic-susceptibility-testing laboratory. The MICs of clofazimine and B669 for the various gram-positive microbial pathogens tested are generally within the range of plasma and tissue concentrations achieved during antimicrobial chemotherapy with clofazimine. Ingestion of 200 or 600 mg of clofazimine daily gives peak serum levels of 0.7 to 1 μg and 3 to 4 μg of the drug per ml, respectively (7, 23). Tissue levels are considerably higher (12).

TABLE 5. Effects of added lysophospholipase on clofazimine- and B669-mediated inhibition of the growth of *S. aureus*

Treatment	Growth of *S. aureus* after 18 h (mean cpm ± SEM)[a]
Control	4,132 ± 85
Lysophospholipase (200 mU)	4,697 ± 81
Clofazimine (0.5 μg/ml)	937 ± 27
Clofazimine (0.5 μg/ml) + lysophospholipase	2,130 ± 88
B669 (0.5 μg/ml)	1,093 ± 29
B669 (0.5 μg/ml) + lysophospholipase	1,732 ± 51

[a] Bacterial growth was measured by the radioassay according to the uptake of radiolabeled amino acids. Data are from three experiments.

As a consequence of the extremely lipophilic properties and redox potential (−0.18 V at pH 7) of clofazimine, it has been proposed that intracellular generation of H_2O_2 by redox-cycling mechanisms may contribute to its antimicrobial activity (5). However, we believe that this is unlikely for the following reasons: (i) the susceptibilities of gram-positive bacteria to clofazimine and B669 are independent of their catalase status (Table 1); (ii) the susceptibilities of *S. aureus*, *S. pyogenes*, and *E. faecalis* to clofazimine and B669 are not eliminated or reduced by anaerobic conditions (quite the opposite, in fact, which suggests that the riminophenazines may be oxidation sensitive); and (iii) coincubation of *S. aureus* or *S. epidermidis* with catalase, superoxide dismutase, or with a range of low-molecular-weight, water-soluble, and lipid-soluble antioxidative agents did not alter the susceptibilities of these bacteria to clofazimine and B669. However, α-tocopherol, originally included as a lipid-soluble antioxidative agent, was a striking exception. When present in a 50-fold excess relative to the riminophenazines, this agent almost completely blocked the inhibitory effects of clofazimine on the proliferative responses of *S. aureus* and *S. epidermidis*. The level of protection afforded by α-tocopherol was somewhat less with B669, probably as a consequence of the greater potency of this agent. It is improbable that the protective effects of α-tocopherol are related to its well-recognized oxidant-scavenging properties, since the other antioxidative compounds and enzymes were ineffective. Alternative mechanisms of α-tocopherol-mediated protection are discussed below.

We have previously observed that clofazimine increases the activity of PLA_2 in human neutrophils (2, 3), mononuclear leukocytes, and a squamous carcinoma cell line in vitro (19a). In the present study, the effects of clofazimine and B669 on the PLA_2 activities of *S. aureus* and *E. coli* were measured according to the extent of release of the primary enzymatic hydrolysis products, unsaturated fatty acid and lysophospholipid (11). To measure release of the fatty acid, the bacteria were labeled with [^{3}H]arachidonate. Although this unsaturated fatty acid is not synthesized by bacteria, exogenous arachidonate can be utilized and is efficiently incorporated into bacterial phospholipid, especially into PE (10). Tritiated palmitate was used to label phospholipids at the C-1 position to enable detection of the lysophospholipid LPE. Treatment of both *S. aureus* and *E. coli* with clofazimine or B669 was associated with increased activity of bacterial PLA_2 according to measurements of the release of [^{3}H]arachidonate (Table 3). Riminophenazine-mediated stimulation of PLA_2 was dose related, with B669 being the more potent of the two antimicrobial agents. The degree of release of LPE was similarly increased in *S. aureus* and *E. coli* treated with clofazimine or B669. Since lysophospholipids are toxic and antiproliferative (11, 15), these agents were identified as being possible mediators of the selective anti-

microbial activities of clofazimine and B669 against gram-positive microorganisms.

The primary involvement of lysophospholipids as mediators of the antimicrobial activities of clofazimine and B669 was suggested by the finding that the superior antimicrobial activity of B669 against *S. aureus* relative to that of clofazimine was correlated with the effects of this agent on release of LPE and was supported by data from other experiments. Firstly, gram-positive microbial pathogens were found to be extremely susceptible to the antiproliferative effects of LPC and LPE, whereas the growth of gram-negative bacteria was unaffected by the lysophospholipids at concentrations of <20 μg/ml. It has previously been reported that LPC is bactericidal for *M. tuberculosis* and *S. aureus* but not for *E. coli* (16). Secondly, α-tocopherol, a lysophospholipid complex-forming and -neutralizing agent (15), protects *M. tuberculosis*, *S. aureus*, and *S. epidermidis* against clofazimine and B669. As mentioned above, these protective effects of α-tocopherol are unrelated to the antioxidative properties of this agent. Tocopherol acetate did not protect gram-positive bacteria against the antiproliferative effects of the riminophenazines and lysophospholipids, indicating a critical requirement for the hydroxyl group on the chromanol nucleus of α-tocopherol. The ability of α-tocopherol to form a complex with and neutralize lysophospholipids is mediated by two types of interaction, namely, formation of a hydrogen bond between the chromanol nucleus hydroxyl group of α-tocopherol and the C-O group of the lysophospholipid and interactions of the acyl chains of the lysophospholipids with the chromanol nucleus methyl groups of α-tocopherol (15). Thirdly, inclusion of lysophospholipase with *S. aureus* during exposure to clofazimine or B669 protected the bacteria against the antiproliferative activity of the riminophenazines (Table 5). Lysophospholipase-mediated protection was delayed and manifested as a slow recovery of bacterial growth. This is probably due to the rapid effects of the riminophenazines on PLA_2 activity, as well as restricted access of the enzyme to the lysophospholipids, leading to a gradual neutralization of these agents. The protective effects of lysophospholipase are the most compelling evidence implicating lysophospholipids as the primary mediators of the antimicrobial activities of clofazimine and B669. The mechanism of riminophenazine resistance operative in all of the species of gram-negative bacteria tested in this study remains unestablished but does not appear to be due to increased lysophospholipase activity.

The activities of purified PLA_2 and lysophospholipase were unaffected by either clofazimine or B669 (5 μg/ml). These observations demonstrate that the riminophenazines do not appear to act directly on either enzyme. In the bacterial-cell membrane, these highly lipophilic riminophenazines may disrupt membrane structure, making the integral phospholipids more susceptible to attack by PLA_2.

In conclusion, the biochemical mechanism of the antimicrobial activities of clofazimine and its analog B669 is related to an increase in the activity of bacterial PLA_2, leading to release and accumulation of lysophospholipids. These agents possess selective antimicrobial activities against gram-positive bacteria.

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