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In Vitro Activities of Free and Liposomal Drugs against *Mycobacterium avium-M. intracellulare* Complex and *M. tuberculosis*

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We compared MICs and MBCs of various free- and liposome-incorporated antimicrobial agents against several patient isolates of *Mycobacterium avium-M. intracellulare* complex and certain American Type Culture Collection strains of *M. avium*, *M. intracellulare*, and *Mycobacterium tuberculosis*. Seven of 19 agents were selected for incorporation into liposomes. The MICs of these agents for 50 and 90% of isolates tested (MIC_{50}s and MIC_{90}s, respectively) ranged from 0.5 to 62 μg/ml. Members of the *M. avium-M. intracellulare* complex were resistant to killing by most of the other agents tested in the free form. However, clofazimine, resorcinomycin A, and PD 117558 showed complete killing of bacteria at concentrations ranging from 8 to 31 μg/ml, represented as MBC_{90}s. Among the liposome-incorporated agents, clofazimine and resorcinomycin A had the highest killing effects (MBC_{90}s, 8 and 16 μg/ml, respectively). Furthermore, both free and liposome-incorporated clofazimine had equivalent growth-inhibitory and killing effects on all American Type Culture Collection strains of *M. avium*, *M. intracellulare*, and *M. tuberculosis* tested. These results show that the antibacterial activities of certain drugs, particularly those of clofazimine and resorcinomycin, were maintained after the drugs were incorporated into liposomes.

Infections caused by members of the *Mycobacterium avium-M. intracellulare* complex (MAC) are the most frequent and fatal complications in patients with AIDS and other immunosuppressed individuals (8, 11, 32, 33). This intracellular pathogen is resistant to many of the standard antituberculous drugs (1, 6). This resistance in many cases is attributed to low permeability of cells to drugs, low levels of intracellular retention inside the cells, or degradation before the drugs reach their tissue targets (6, 9, 10). Another reason for failure to obtain significant activity by certain drugs may be the difficulty in achieving high drug concentrations at sites of the infection. Some drugs used to treat these infections have poor solubilities, stabilities, and absorption properties; others are associated with toxic side effects, poor penetration into macrophages, and low levels of retention or stability in the cells after uptake. Use of liposomes has been shown to overcome these difficulties in the chemotherapy of various diseases (2, 3, 25, 31), in addition to allowing parenteral administration of poorly soluble and toxic drugs. Because of the resistance of these organisms to available drugs, multiple-drug regimens have been used to treat patients infected with MAC; however, the response to multiple-drug regimens is generally poor and is associated with toxicity. We therefore incorporated some of the known agents (cerulenin [23], clofazimine [9, 10, 14], and rifampin [9, 10]) and some new agents (resorcinomycin A [13], PD 117558 [12, 28], PD 117596 [29], and sparfloxacin [20]) into liposomes and studied their antibacterial activities in free and liposomal forms against various MAC strains. A comparison of the drugs' activities in the liposomal form with those of the drugs in the free form allowed us to identify promising candidates that might be useful as effective antimycobacterial agents in liposomal form.

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MATERIALS AND METHODS

Bacterial cell cultures and growth. The MAC isolates used in the present study were obtained from patients with cancer and AIDS who had disseminated MAC infections and who were seen at The University of Texas M. D. Anderson Cancer Center between 1986 and 1989. Seventeen additional isolates obtained from the American Type Culture Collection (ATCC) included strains of *M. avium*, *M. intracellulare*, and *M. tuberculosis*. The MAC 101 (serotype 1) strain, which was obtained from Clark B. Inderlied (Children's Hospital, Los Angeles, Calif.), was also included in the study. The cultures were maintained on slants of Lowenstein-Jensen medium and were subcultured onto Middlebrook agar plates (Remel, Lenexa, Kans.) for 10 to 15 days before use. The cultures from agar plates were then subcultured in Middlebrook 7H9 broth enriched with OADC (Difco, Detroit, Mich.) and were incubated at 37°C on a rotating drum for 3 days.

Antibacterial agents, drugs, lipids, and chemicals. Aclacinomycin was obtained from the National Cancer Institute; compound A-56619 (7, 30) was provided by Abbott Laboratories (North Chicago, Ill.); azalomycin F (16) was from Sankyo Fermentation Research Laboratories, Tokyo, Japan; bafilomycin A_1 (4) was obtained from K. Altendorf at the University of Osnabruck, Osnabruck, Federal Republic of Germany; cerulenin and chlorpromazine were purchased from Sigma Chemical Co. (St. Louis, Mo.); and clofazimine was generously provided by K. Scheibli and H. Schroter at CIBA-GEIGY (Basel, Switzerland). Lanthiopeptin and karnamicin (21, 22) were obtained from Bristol-Myers Research Institute, Tokyo, Japan; compounds PD 117596, PD 117558,

and PD 127391 (for structures, see references 27 to 29) and sparfloxacin (AT-4140) were provided by Parke-Davis Pharmaceutical Research Division (Ann Arbor, Mich.). Pyridotriazines 84-1 and 103-2 (26) were provided by Marvin Reich of American Cyanamid Company (Pearl River, N.Y.), and resorcinomycin A (13) was a gift from Taichiro Komeno of Shinogi Research Laboratories (Osaka, Japan). Rifampin was from Sigma Chemical Co., and roxithromycin was from Hoechst-Roussel Pharmaceuticals (Sommerville, N.J.).

Aclacinomycin and chlorpromazine were dissolved in saline; bafilomycin, cerulenin, and resorcinomycin were dissolved in ethanol; A-56619, PD 117596, PD 117558, PD 127391, pyridotriazine 84-1, rifampin, roxithromycin, and sparfloxacin were dissolved in methanol; karnamicin, lanthiopeptin, and pyridotriazine 103-2 were dissolved in dimethyl sulfoxide (DMSO); azalomycin F was dissolved in 50% DMSO–50% methanol; and clofazimine was dissolved in 10% DMSO–acidified methanol. Drugs were then diluted with saline or water to the required concentrations. Dimyristoyl phosphatidyl choline (DMPC) and dimyristoyl phosphatidyl glycerol (DMPG) were purchased from Avanti Polar Lipids (Birmingham, Ala.); methanol (high-performance liquid chromatography grade) was obtained from Fisher Scientific (Springfield, N.J.). All other chemicals and reagents were of analytical grade.

Preparation and standardization of liposomes. Multilamellar vesicles composed of DMPC and DMPG at a molar ratio of 7:3 were used. These multilamellar liposomes were prepared by the rotary evaporator method as described elsewhere (15, 17–19), and the encapsulation efficiencies of the drugs in various formulations were quantitated by UV spectrophotometry or high-performance liquid chromatography (13, 23, 24).

Determination of MICs and MBCs of drugs against mycobacteria in vitro. The cultures incubated in Middlebrook 7H9 broth (with OADC enrichment) at 37°C for 3 days were diluted in the same broth to yield a concentration of 1 Klett unit per ml by using a Klett-Summerson colorimeter (Klett Manufacturing, Brooklyn, N.Y.). This procedure yielded an actively growing culture containing approximately 10^8 CFU/ml, as confirmed by plate counts on 7H10 agar.

A 20-μl aliquot of this suspension was added to each well of 96-well microtiter plates containing 100 μl of broth and antimicrobial agents. The plates were prepared by adding 100 μl of stock solution (250 to 500 μg/ml) of the antimicrobial agent(s) to the first well and making serial twofold dilutions up to 11 wells with 7H9 broth. Well 12 in all rows, rows A through H, served as a control for bacterial growth; that is, no drug was added to well 12 in any row. The plates were always prepared fresh just before inoculation. After inoculation, the plates were covered, placed in plastic bags, and incubated at 35°C in an ambient atmosphere. The plates were read for no visible growth in the wells, which represented the MIC, on days 5, 10, and 15. The values obtained on day 15 were considered confirmatory, since, in our hands, no strain showing susceptibility on day 10 exhibited resistance on day 15. Deterioration of antimicrobial activity during prolonged incubation was measured by incubating the uninoculated plates for 3 to 6 days and inoculating these and fresh plates with *Escherichia coli* ATCC 27922, *Staphylococcus aureus* ATCC 29213, and *Pseudomonas aeruginosa* ATCC 27853. MICs were recorded on days 1, 4, and 7.

MBCs were determined by subculturing an aliquot from selected wells of microdilution plates onto Middlebrook agar 7H10 plates. After thorough mixing of the wells that showed no visible turbidity, a 0.01-ml aliquot was taken and spread onto 7H10 agar. These plates were then incubated for 7 to 14 days at 35°C or until colonies could be counted with a dissecting microscope. The cultures treated with drug concentrations showing no growth were then taken as the MBCs.

TABLE 1. Activities of free and liposome-encapsulated drugs in vitro against MAC[a]

Antibiotic	MIC (μg/ml)			MBC (μg/ml)	
	Range	50%	90%	50%	90%
Cerulenin	0.2–>125	4	62	>125	>125
Liposome-encapsulated cerulenin	0.4–16	8	16	62	250
Clofazimine	0.2–1.0	0.5	1.0	16	31
Liposome-encapsulated clofazimine	0.1–1.0	0.5	1.0	4	8
Resorcinomycin A	0.5–8	2	8	4	8
Liposome-encapsulated resorcinomycin A	0.5–8	2	8	4	16
Rifampin	0.05–62	31	62	>125	>125
Liposome-encapsulated rifampin	0.05–31	8	16	>125	>125
PD 117596	0.12–31	4	31	>31	>31
Liposome-encapsulated PD 117596	1.0–16	4	16	125	>125
PD 117558	0.25–16	1	2	2	16
Liposome-encapsulated PD 117558	2.0–32	4	32	125	>125
Sparfloxacin	0.10–16	0.5	2	62	125
Liposome-encapsulated sparfloxacin	0.5–16	2	8	125	>125

[a] At least 21 identical isolates tested from a group of 31 isolates.

RESULTS

We studied the antibacterial activities of 19 potential antimicrobial agents against MAC isolates and identified 7 drugs with potentially useful inhibitory activities (MICs for 50% of isolates tested [MIC_{50}s] 0.5 to 31 μg/ml; Table 1) which could be incorporated into liposomes. Liposomes containing these drugs were then prepared and the antibacterial activities of liposome-encapsulated drugs were compared with those of the respective free drugs. The entrapment efficiencies of these drugs in liposomes are given in Table 2. Clofazimine and sparfloxacin showed the highest entrapment efficiencies (up to 100%), whereas the entrapment efficiencies of the other drugs varied from 62 to 86%. The MICs and MBCs of these agents in the free and

TABLE 2. Entrapment efficiencies of antimycobacterial agents in liposomes

Antibiotic	Entrapment efficiency (%)[a]
Cerulenin	85
Clofazimine	95–100
Resorcinomycin A	80
Rifampin	74–86
PD 117596	78
PD 117558	62
Sparfloxacin	75–100

[a] Values represent the percentage of drug in liposomes calculated from the amount added to lipids in a drug-to-lipid ratio of 1:10.

TABLE 3. Antibacterial activities of other agents against MAC strains[a]

Agent	MIC (μg/ml)		MBC (μg/ml)	
	50%	90%	50%	90%
A-56619	7.8	7.8	125	>125
Aclacinomycin	12.5	12.5	250	>250
Azalomycin F	125	125	ND	ND
Bafilomycin A_1	Nil	Nil	ND	ND
Chlorpromazine	15.6	31.2	ND	ND
Karnamicin	125	125	ND	ND
Lanthiopeptin	Nil	Nil	ND	ND
Pyridotriazine 84-1	3.9	7.8	125	>125
Pyridotriazine 103-2	62.5	62.5	ND	ND

[a] The 21 strains used to obtain these data were from the same group of patient isolates used to obtain the data presented in Table 1. Nil, no activity; ND, not done.

liposomal forms for at least 21 patient isolates of MAC are presented in Table 1.

Cerulenin and rifampin showed a wide range of activities against various isolates; the MIC_{50} of cerulenin was found to be 4 μg/ml, and that of rifampin was 32 μg/ml. Liposome encapsulation of both cerulenin and rifampin reduced the MIC_{90} fourfold. Both clofazimine and resorcinomycin A were highly active against all the strains (MIC_{50}s, 0.5 and 2 μg/ml, respectively), with inhibitory activity maintained after liposome encapsulation. Of the quinolones, PD 117558 and sparfloxacin showed excellent activities against all MAC strains, with MIC_{50}s and MIC_{90}s of as low as 0.5 to 2 μg/ml, respectively. These drugs are lipophilic and may be associated with toxic effects, but these toxic effects can be reduced by using them in the liposome-encapsulated form. Therefore, we determined the MICs of the liposome-encapsulated forms of the quinolones PD 117596, PD 117558, and sparfloxacin. Although all of them showed good inhibitory activities, their bactericidal activities were reduced after encapsulation into liposomes. Whether the intracellular antibacterial activity in macrophages is enhanced by using the liposome form remains an important question that must be studied.

The activities of a few other agents tested in free form against various patient isolates are given in Table 3. Most of these agents showed poor activity and were not tested in the liposomal form. Roxithromycin and PD 127391 were tested against the 17 ATCC strains listed in Table 4. Both of them showed good inhibitory activities (MIC_{90}s, 0.48 and 1.95 μg/ml, respectively). Although the bactericidal activities of the agents listed in Table 3 were not significant, roxithromycin and PD 127391 showed high-level killing effects against one strain, *M. avium* ATCC 35713.

We then examined the antibacterial activities of free- and liposome-encapsulated clofazimine against various well-characterized ATCC strains of *M. avium*, *M. intracellulare*, and *M. tuberculosis*. Both free and liposome-encapsulated clofazimine showed similar activities against all the strains (Table 4). An interesting observation was the high level of activity of liposome-encapsulated clofazimine obtained against two strains of *M. avium* and all strains of *M. tuberculosis*, including some drug-resistant strains.

TABLE 4. Antibacterial activities of free and liposome-encapsulated clofazimine against ATCC strains of mycobacteria

Strain	Free clofazimine		Liposome-encapsulated clofazimine	
	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)
M. avium				
ATCC 15769	<0.06	7.8	0.12	3.9
ATCC 35713	<0.12	15.6	0.24	1.95
ATCC 35718	0.48	62.5	0.97	31.2
M. intracellulare				
ATCC 35761	0.97	31.2	1.95	62.5
ATCC 35762	1.95	62.5	0.97	15.6
ATCC 35763	0.48	62.5	0.48	62.5
ATCC 35770	0.48	62.5	0.48	31.2
ATCC 35848	3.9	125	3.9	62.5
M. tuberculosis				
ATCC 27294	<0.06	0.12	0.12	0.48
ATCC 35800	<0.06	0.48	0.12	0.48
ATCC 35801	<0.06	0.48	<0.06	0.48
ATCC 35805	0.12	0.48	0.24	0.98
ATCC 35811	<0.06	0.48	0.12	0.48
ATCC 35812	<0.06	0.24	<0.06	0.24
ATCC 35825[a]	0.24	0.48	0.24	0.48
ATCC 35837[b]	<0.06	0.24	<0.06	0.12
ATCC 35838[c]	<0.24	0.24	<0.24	0.97

[a] Resistant to *para*-aminosalicylic acid, streptomycin, and isoniazid.
[b] Resistant to ethambutol.
[c] Resistant to rifampin.

DISCUSSION

The results presented here indicate that 7 of the 19 drugs used in the present study have good inhibitory activities against MAC, that they can be encapsulated in liposomes, and that their antibacterial activities (MIC) are maintained after encapsulation in liposomes. Although we observed a loss of activity after liposome encapsulation with some other drugs (17), the inhibitory activities of the drugs used in the present study were not reduced except in the case of PD 117558. The bactericidal activities of the quinolones, however, were reduced. These drugs are lipophilic in nature and, like most lipophilic drugs, may be associated with toxic effects. Since liposome incorporation reduces the toxicities of lipophilic drugs, they should be excellent candidates for use in the liposome-encapsulated form. However, it will be important to see whether the intracellular activity is also maintained or is enhanced after liposome encapsulation. Moreover, it is known that quinolones, in general, cannot reach levels in blood necessary to inhibit *M. avium* (5). Although the newer quinolones have been shown to attain higher levels in serum, delivery via liposomes could be useful in this respect, particularly if delivered via the parenteral route. Use of liposomes to deliver these drugs may also enhance intracellular uptake by macrophages, increasing the interaction between the drugs and bacteria in the intracellular compartment and thus producing an enhanced bactericidal effect. The high level of activity observed with roxithromycin against *M. avium* ATCC 35713 (1 to 2 μg/ml) and clofazimine against *M. avium* ATCC 15769 suggests the importance of testing the susceptibilities of mycobacterial strains against various drugs before or during the treatment regimens.

With the recent upsurge of disseminated MAC infections in immunocompromised individuals and of *M. tuberculosis* infections in immunosuppressed as well as healthy individuals, the need for new or more effective agents against these pathogens has greatly increased. Although the activity of clofazimine has been reported earlier (9, 10, 14), our results

confirm the fact that clofazimine is a very potent drug against *M. tuberculosis* and also has considerably good activity against MAC isolates. We also showed that clofazimine can be used in liposomal form without the loss of activity against MAC or *M. tuberculosis*. Also, we identified in the present study other active antimycobacterial agents, such as resorcinomycin A and PD 117558, that may be useful in the treatment of mycobacterial infections in free or liposomal form. The use of liposomes as drug carriers, in general, will help reduce the toxicities of these drugs and allow parenteral administration and targeting of drugs to the macrophages, where mycobacteria reside (2, 3, 31).

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