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Lack of In Vivo and In Vitro Bactericidal Activity of *N*-Formimidoyl Thienamycin Against Enterococci

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The minimal bactericidal concentrations of *N*-formimidoyl thienamycin (*N*-f-thienamycin) against 21 strains of enterococci isolated from patients with infective endocarditis were determined by macro- and microdilution methods. By a macrodilution technique with the minimal bactericidal concentration defined as ≥99.9% killing of an initial inoculum, all 21 strains of enterococci were found to have minimal bactericidal concentration/minimal inhibitory concentration ratios of ≥32. The mean minimal inhibitory concentration was 1.5 μg/ml (range, 0.5 to 4 μg/ml), and the minimal bactericidal concentration was ≥128 μg/ml. The disparity between the results of our study and those published elsewhere, which reported that *N*-f-thienamycin is bactericidal in vitro against enterococci, may represent the relative insensitivity of the microdilution method in determining ≥99.9% killing. The lack of in vitro bactericidal activity of *N*-f-thienamycin against enterococci was confirmed in vivo in the rabbit model of experimental endocarditis. *N*-f-Thienamycin was no more effective than penicillin alone in the treatment of experimental enterococcal endocarditis and was less effective than the combination of penicillin and gentamicin. The results indicate that *N*-f-thienamycin should not be used alone in the treatment of enterococcal endocarditis.

N-Formimidoyl thienamycin (*N*-f-thienamycin), a derivative of thienamycin, is a new beta-lactam antibiotic which is reported to be inhibitory (3, 5, 6, 8, 12, 14) and bactericidal (9) in vitro against enterococci. Since the successful treatment of enterococcal endocarditis necessitates the use of penicillin, ampicillin, or vancomycin in combination with an aminoglycoside, and since the aminoglycosides are associated with nephro- and ototoxicity, *N*-f-thienamycin might be useful as single-drug therapy for patients with endocarditis if it were bactericidal against enterococci in vitro and in vivo. The purpose of this study was to determine the bactericidal effect of *N*-f-thienamycin against enterococci by macro- and micro-broth dilution techniques and its efficacy in the treatment of experimental enterococcal endocarditis in animals.

MATERIALS AND METHODS

Organisms. A total of 21 strains of enterococci isolated from patients with infective endocarditis were studied. Eighteen were obtained from patients treated at Mayo Clinic-affiliated hospitals, and three were provided by B. V. Reller, University of Colorado Medical Center, Denver. The enterococci were stored in defibrinated sheep blood at −70°C. Before testing, each strain was thawed and subcultured on Trypticase soy agar containing 5% sheep blood. Seventeen strains were relatively susceptible to streptomycin with a minimal inhibitory concentration (MIC) less than 2,000 μg/ml; four were streptomycin resistant (MIC > 2,000 μg/ml).

Antibiotics. *N*-f-Thienamycin was supplied by the Merck Institute for Therapeutic Research, Rahway, N.J. Procaine penicillin was supplied by Wyeth Laboratories, Philadelphia, Pa.; gentamicin was supplied by Schering Corp., Kenilworth, N.J. Antibiotic solutions for in vitro susceptibility studies and for administration to laboratory animals were prepared daily, filter sterilized, and adjusted to pH 7.2 with 1.0 mM sodium phosphate. Dilution of the antibiotic solution for in vitro susceptibility testing was performed in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.).

Susceptibility testing. (i) Macrodilution method. Inocula were prepared from 6-h broth cultures to yield 10^5 to 10^6 colony-forming units (CFU) per ml in serial twofold dilutions of antibiotic in broth. Subcultures were made for quantitation and confirmation of purity of the inoculum. Tubes containing the inoculum in serially diluted penicillin or *N*-f-thienamycin were incubated for 18 h at 35°C in air. The MIC was the lowest concentration of broth without visible growth. The minimal bactericidal concentration (MBC) was determined by subculture of 100 μl of broth from the first tube exhibiting growth and from all tubes without visible growth and was defined as the lowest concen-

tration of antibiotic that killed ≥99.9% of the original inoculum.

(ii) Microdilution method. For the microdilution method the preparation of the inoculum and antibiotic was identical to that for the macrodilution method. Volumes (50 μl) of the diluted antibiotic were distributed with an eight-channel pipette (Sensititre, Seward Laboratory, London, England) into the wells of microtitration plates (Dynatech, Alexandria, Va). A 50-μl volume of inoculum was added to each microtitration well. Each isolate was tested at inocula of 10^4 to 10^5 CFU/ml and 10^5 to 10^6 CFU/ml. No more than two separate isolates were tested per tray. The MIC was defined as the lowest concentration of *N*-f-thienamycin that resulted in no visible turbidity or sediment after 18 h of incubation at 35°C in air.

The wells in trays inoculated with 10^5 CFU/ml were subcultured after mixing on a microshaker, by methods described by Barry and Lasner (1), and the MBC was determined by the following methods: (i) 10 μl was streaked over the surface of a Mueller-Hinton agar plate; (ii) 1 μl was spot inoculated or streaked on the surface of a Mueller-Hinton plate.

All subcultures were incubated at 35°C in air with 5% CO_2 for 72 h. The MBC was defined as the lowest concentration of antibiotic that killed ≥99% or ≥99.9% of the original inoculum (see below and Table 2).

Animal studies. (i) Rabbit model. Experimental aortic valve endocarditis was established in 57 New Zealand white rabbits (weight, ≥2 kg) by a modification of the method described by Garrison and Freedman (4). A total of 78 rabbits were used during the study. Of these 57 were treated according to protocol. The remaining 21 animals (14 control and 7 treated with antimicrobial agents) died less than 3 days after the time of infection and could not be analyzed. Control animals were included throughout the course of the study. Animals were anesthetized with 60 mg of pentobarbital injected through a peripheral ear vein. An incision was made in the neck, and the right carotid artery was exposed. The artery was ligated distally, and a sterile polyethylene catheter (radio-opaque Intramedic, PE 90; Clay-Adams, Parsipanny, N.J.) was inserted into the artery through a small incision and advanced proximally across the aortic valve into the left ventricle. A pressure-sensitive monitoring device was attached to the distal end of the catheter to ensure that the catheter tip crossed the aortic valve and entered the left ventricle. The catheter was left in place in the left ventricle for approximately 5 min and then withdrawn to a position just above the aortic valve. The distal end of the catheter was heat-sealed and tied to the carotid artery, and the wound was closed over the catheter with surgical clips. The catheter was left in place for 24 h before injection of bacteria.

(ii) Isolates. Two strains were selected from among the 18 strains of enterococci isolated from patients with endocarditis seen at the Mayo Clinic. The MIC and MBC of penicillin and *N*-f-thienamycin for the two strains of *Streptococcus faecalis* used were 1 μg/ml and >128 μg/ml, respectively. Of the 57 animals included in the study, 30 were infected with one strain and 27 were infected with the other. Enterococci were inoculated into Mueller-Hinton broth and incubated overnight. A fresh culture of enterococci was prepared daily for animal inoculation. One milliliter of broth, containing 10^7 to 10^8 CFU of enterococci per ml, was injected into a peripheral ear vein 24 h after insertion of the polyethylene catheter. This technique resulted in aortic valve endocarditis in 98.4% of animals studied. The presence of endocarditis was confirmed by blood culture yielding enterococci before the animals were sacrificed.

(iii) Treatment groups. Antimicrobial therapy was initiated 24 h after intravenous injection of enterococci and was administered for 3 days. After day 3 of treatment and at least 12 h after the administration of the last dose of antimicrobial agent(s), animals were sacrificed by intravenous injection of sodium pentobarbital. The chest was opened, the heart was excised and opened, and the aortic valve vegetations were removed aseptically. The vegetations were weighed and homogenized with a sterile mortar and pestle. The number of CFU of enterococci per gram of vegetation was quantitated by using a pour plate method with agar containing bile and esculin as indicators. The results were expressed as the $\log_{10}$ CFU of enterococci per gram of valve vegetation.

Animals were divided into treatment groups as follows: (i) control—seven animals received no antimicrobial therapy; (ii) penicillin alone—20 animals received procaine penicillin at 1.2×10^6 U intramuscularly (i.m.) three times daily; (iii) *N*-f-thienamycin alone—20 animals received *N*-f-thienamycin at 20 mg/kg i.m. three times daily; (iv) penicillin and gentamicin—10 animals received procaine penicillin at 1.2×10^6 U i.m. plus gentamicin at 2 mg/kg i.m., administered three times daily.

(iii) Antibiotic assay. On day 2 of therapy, after the administration of the fourth dose of antibiotic, blood samples were obtained from a peripheral ear vein for measurement of serum concentration. Serum concentrations of penicillin were measured by bioassay (13). Serum concentrations of gentamicin were measured by high-pressure liquid chromatography, and those of *N*-f-thienamycin were measured by a method described previously (7).

(iv) Dose-response curves for *N*-f-thienamycin. Serum samples for assay were obtained by separate venipuncture at 5, 15, 30, 45, and 60 min and at 2, 3, and 4 h after i.m. injection of *N*-f-thienamycin at 20 mg/kg. No heparin was administered to any animals.

(v) SBT. Blood samples for determination of serum bactericidal titer (SBT) were obtained from a peripheral ear vein at 15 min after administration of *N*-f-thienamycin and 1 h after administration of procaine penicillin or procaine penicillin plus gentamicin. Serum specimens were stored at −70°C. The SBT was measured by methods described elsewhere (13).

Analysis of results. Differences in mean $\log_{10}$ CFU of enterococci per gram of vegetation were analyzed statistically by using the Kruskal-Wallis test and individual rank sum tests for differences between pairs (2, 11).

RESULTS

In vitro susceptibility of enterococci to aqueous penicillin G. The mean penicillin G MIC for the 21 strains of enterococci was 1.38 ± 0.62 μg/ml; MBC was >128 μg/ml for all 21 strains.

TABLE 1. Susceptibility of 21 strains of enterococci to *N*-f-thienamycin determined by the macrodilution method

Isolate (no. tested)	MIC (μg/ml)		MBC (μg/ml)
	Mean	Range	
Streptococcus faecalis			
Streptomycin susceptible (13)	1.5	0.5–2.0	>128
Streptomycin resistant (4)	1	1	>128
Streptococcus faecium (3)	1.8	0.5–4	>128
Streptococcus durans (1)	4		128

In vitro susceptibility of enterococci to *N*-f-thienamycin. The MICs and MBCs determined by the macrodilution technique for 21 strains of enterococci are shown in Table 1. All of the 21 strains had MBCs of ≥128 μg/ml.

The effects of the method of subculture and volume subcultured on the bactericidal effect of *N*-f-thienamycin against enterococci are shown in Table 2. When the MBC was defined as ≥99.9% killing of the initial inoculum as determined by the macrodilution technique with subculture of 100 μl, all strains of enterococci had MBC/MIC ratios of ≥32. When the MBC was defined by 99.9% killing as determined by the microdilution technique with subculture of 10 μl, 20 of 21 (95%) strains tested at an inoculum size of 1.8×10^6 CFU per ml and 19 of 21 (90%) strains tested at an inoculum size of 1.8×10^5 CFU per ml had MBC/MIC ratios of ≥32. The maximum percent killing that could be determined by our microdilution method with a subculture volume of 1 μl was 99% rather than 99.9%. As can be seen in Table 2, both the number of colonies used to define ≥99.9% killing and the manner of deposition (streaking versus spotting) of the subculture on the agar surface affected the MBC/MIC ratios of the strains tested.

In vivo activity of *N*-f-thienamycin in animals. The peak (mean, 17.6 μg/ml) serum concentration of *N*-f-thienamycin in seven rabbits occurred 15 min after administration of 20 mg/kg i.m. Four hours later, the mean serum concentration was <1 μg/ml. In animals treated with 2 mg of gentamicin per kg i.m., the mean peak 1-h serum concentration was 8.8 μg of gentamicin per ml. The mean concentration of procaine penicillin 1 h after administration of the dose was 17.6 μg/ml.

The results of treatment of experimental enterococcal endocarditis for 3 days with *N*-f-thienamycin, procaine penicillin, and procaine penicillin plus gentamicin are shown in Table 3. The efficacy of *N*-f-thienamycin was very similar to that of procaine penicillin alone and was less than that of procaine penicillin plus gentamicin ($P < 0.001$).

The peak SBT was ≤1:4 in animals treated with procaine penicillin, ≤1:2 in those treated with *N*-f-thienamycin, and ≥1:8 in those treated with penicillin plus gentamicin (Table 3).

DISCUSSION

Our results agree with those of Eliopoulos and Moellering (3), who found that *N*-f-thienamycin was not bactericidal in vitro against enterococci, and in disagreement with those reported by Livingston et al. (9), who reported that the MBC of *N*-f-thienamycin against enterococci was equal to or no more than twofold greater than the MIC. This disparity may represent the relative insensitivity of the microdilution method in detecting ≥99.9% killing.

With an initial inoculum of 4.5×10^4 to 1×10^5 CFU/ml, subculture volume of 2 μl, and an endpoint of ≤2 CFU, it is only possible to

TABLE 2. Effect of method and volume of subculture on bactericidal effect of *N*-f-thienamycin against 21 strains of enterococci

Inoculum (mean CFU/ml)	Mean no. of surviving colonies for MBC endpoint	Subculture		Number of strains with MBC/MIC ratio of:							
		Vol (μl)	Method of deposition	1	2	4	8	16	32	64	≥128
1.4×10^5	14[a]	100	Streaked						2	7	12
1.8×10^6	18[a]	10	Streaked			1			3	1	16
1.8×10^5	1.8[a]	10	Streaked				2		1	2	16
1.8×10^5	2[b]	1	Streaked	1	4		2		1	2	11
1.8×10^5	2[b]	1	Spotted	7	4			1	2	2	5
1.8×10^5	0[c]	1	Streaked	1	1	1					18
1.8×10^5	0[c]	1	Spotted	6	1	1			1	2	10

[a] Shown as mean number of colonies used on subculture plate to define ≥99.9% killing of initial inoculum.
[b] Shown as mean number of colonies used on subculture plate to define ≥99% killing of initial inoculum.
[c] Percent of kill was indeterminate but at least 99% of initial inoculum.

TABLE 3. Results of antimicrobial therapy of experimental enterococcal endocarditis in rabbits

Antimicrobial therapy	Mean $\log_{10}$ CFU/g of vegetation (±SD)	SBT	
		Mean	Range
Control	9.7 ± 0.67		
Procaine penicillin (1.2×10^6 U i.m. TID[a])	4.2 ± 0.57[b]	1:1.2	0–1:4
N-f-Thienamycin (20 mg/kg i.m. TID)	4.4 ± 0.71[b]	1:1	0–1:2
Procaine penicillin (1.2×10^6 U i.m. TID) plus gentamicin (2 mg/kg i.m. TID)	1.4 ± 1.36[c]	1:32	1:8–1:128

[a] TID, Three times daily.
[b] $P < 0.001$ versus control animals.
[c] $P < 0.001$ versus either procaine penicillin or N-f-thienamycin treatment regimens alone.

determine ≥98 to 99% killing, rather than ≥99.8% as reported by Livingston et al. (9). By subculture of only 1 to 2 μl, therefore, the MBCs approximated the MICs of N-f-thienamycin in 14 to 52% of instances. Lower MBC/MIC ratios were observed when the subcultures were spot inoculated rather than streaked, presumably because of greater residual concentrations of antimicrobial agent at or near the spot (1).

As reported by Pearson et al. (10), the initial inoculum size and volume of samples subcultured are covariant parameters affecting the number of colonies in the samples subcultured and ultimately the definition of killing. Initial inocula of less than 3×10^5 CFU/ml are associated with rejection values (upper limit of CFU representing ≤0.1% survival or assuming ±5% pipetting or sampling errors) of reduced sensitivity and specificity (10).

The low SBT observed in animals treated with N-f-thienamycin is probably the result of defective killing of enterococci by this antibiotic, but may in part reflect the relative instability of N-f-thienamycin in frozen serum specimens. The serum specimens from animals were frozen quickly to −70°C and thawed rapidly for SBT determination. The instability of N-f-thienamycin is minimized in serum that is frozen and thawed rapidly, and the influence on the results of SBTs in our study was probably negligible (H. Kropp, personal communication).

The lack of in vitro bactericidal activity of N-f-thienamycin against enterococci was confirmed in vivo in the rabbit model of experimental endocarditis. The clinical relevance of 99 versus 99.9% killing in bactericidal tests has been a matter of debate. The results of our studies with experimental endocarditis show that antimicrobial agents that kill ≥99% of the inoculum are less effective than those which result in ≥99.9% killing. N-f-Thienamycin was no more effective than penicillin alone in the treatment of experimental enterococcal endocarditis and was less effective than the combination of penicillin and gentamicin. Therapeutic trials with N-f-thienamycin used alone in the treatment of human enterococcal endocarditis do not appear justified. In vitro synergy between N-f-thienamycin and gentamicin is similar to that observed with penicillin and gentamicin (3), but results of studies of experimental enterococcal endocarditis with N-f-thienamycin plus gentamicin have not been published.

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