PACIDAMYCINS, A NOVEL SERIES OF ANTIBIOTICS WITH ANTI-*PSEUDOMONAS AERUGINOSA* ACTIVITY

III. MICROBIOLOGIC PROFILE

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Pacidamycins are nucleosidyl-peptide antibiotics which have activity only against *Pseudomonas aeruginosa*. Their MICs for other organisms such as Enterobacteriaceae, *Staphylococcus aureus*, most Streptococci and other *Pseudomonas* species are >100 μg/ml. These compounds had no activity against erythromycin-susceptible Streptococci. The MICs for *Streptococcus pyogenes* with constitutive- and inducible-type of macrolide-lincosamide-streptogramin resistance were 12.5 and 25 μg/ml, respectively. The MICs against *P. aeruginosa* ranged from 8 to 64 μg/ml. The activity of these compounds was 1 to 2-fold less in serum than broth. Time-kill curves were performed using 4 and 8 times the MIC of pacidamycin 1. It was bactericidal against *P. aeruginosa* (3 $\log_{10}$ decrease in 4 to 6 hours). At 24 hours, resistant mutants were found in the cultures. The MICs of piperacillin and gentamicin for these mutants were the same as for the parent strain. The frequency of resistance to these compounds was $<3.5\times10^{-6}$. The resistant mutants were stable after 10 transfers in antibiotic-free medium. The pacidamycins were inactive against *P. aeruginosa* in mouse protection tests. After a single subcutaneous injection of 25 mg/kg of pacidamycin 1, the Cmax was approximately 50 μg/ml and the serum half-life was 0.5 hour.

Pseudomonas aeruginosa infections are a problem to treat in spite of the availability of large numbers of antibiotics because of their intrinsic resistance and their ability to become resistant to new classes of antibacterial agents[1]. Pacidamycins are a novel class of antibiotics which have been isolated from a screen designed to find antibiotics with activity against *P. aeruginosa*. The taxonomy of the producing culture, the fermentation, isolation and chemical structures of these compounds are described in the preceding manuscripts[2,3]. The biological activities of these compounds will be described in part at the 28th Intersci. Conf. on Antimicrob. Agents Chemother.[4].

Materials and Methods

Antibiotics

The pacidamycins were isolated at Abbott Laboratories. The majority of the experiments were performed with pacidamycin 1 (A-68567), the alanine-tryptophan analog. Pacidamycins 2 (alanine-phenylalanine analog), 3 (alanine-*meta*-tyrosine) and 5 (phenylalanine analog) were also tested when sufficient quantities were available

In Vitro Antibacterial Activity

The bacterial strains used in this study were clinical isolates or cultures obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, U.S.A.) which are maintained frozen in our laboratory. MICs were determined by the agar dilution method using brain heart infusion agar and Mueller-Hinton agar[5].

Effect of Serum and pH on the *In Vitro* Potency

The effect of serum and pH on the MICs of pacidamycins 1, 2 and 3 was determined as described previously[6]. The MICs were determined in the broth containing 50% human or mouse serum in a microdilution test using Mueller-Hinton broth as the test medium for *P. aeruginosa* A5007 and *P. aeruginosa* ATCC 27853 and brain heart infusion broth for *Streptococcus pyogenes* 930. The effect of pH on the *in vitro* potency of pacidamycin 1 was determined by measuring the MIC in Mueller-Hinton broth adjusted to pH 6.5, 7.2 and 8.0.

Time-kill Curves

The kinetics of the bactericidal activity of pacidamycin 1 were determined as described previously[6]. Four and eight times the MIC of pacidamycin 1 were added to logarithmic phase cultures of *P. aeruginosa* ATCC 27853 grown in Mueller-Hinton broth. Aliquots of the cultures were removed at 30 minutes, 1, 2, 4, 6 and 24 hours and cultured quantitatively to determine the viable bacterial counts.

Resistance Frequency

The frequency of resistance to pacidamycin 1 was determined as described previously[7]. An 18-hour culture grown from a single colony of *P. aeruginosa* ATCC 27853 was diluted in 10-fold dilutions and plated on Mueller-Hinton agar containing 4 and 8 times the MIC of pacidamycin 1. The stability of resistance in the mutants was determined by serially passing the cultures on antibiotic-free agar and then re-testing the susceptibility of the resistant mutants.

Mouse Protection Test

CF-1 female mice (Sasco, Oregon, Wisconsin) were infected intraperitoneally with 1×10^6 cfu of *P. aeruginosa* A5007 suspended in 5% hog gastric mucin. The mice were treated subcutaneously with 100, 25 and 6.3 mg/kg/day at 1 and 5 hours after infection and the median effective dose (ED_{50}) was determined on the basis of cumulative mortalities on the 6th day after infection.

Pharmacokinetic Studies

The serum and urine concentrations of pacidamycins 1, 2 and 3 were each determined by HPLC after a single dose of 25 mg/kg administered subcutaneously and orally. The chromatograph was comprised of a Waters model 6000A solvent delivery system, Rheodyne model 7125 injector and a Kratos SF 770 UV detector set at 258 nm. Pacidamycins 1, 2 and 3 were quantitated using the external standards method on a Waters 0.39×30 cm μBondapak TM (C-18, 10 μm) column by isocratic elution with CH_3CN - 0.01 M TFA in ratios of 23:77, 22:78 and 20:80, respectively. At a flow rate of 1 ml/minute, the retention times for pacidamycins 1, 2 and 3 were 10, 8 and 5 minutes, respectively. Serum samples were assayed directly, whereas urine samples were subjected to solid phase extraction using 3 ml Bond Elut columns (Analytichem). The solid phase extraction procedure consisted of the following steps: 1) Column conditioning in water, 2) sample application, 3) elution with water, 4) elution with 10% methanol and 5) elution of the pacidamycins in 100% methanol. Recovery by this method was 50 ± 4%.

Results

In Vitro Activity

The MICs of pacidamycins $1\sim3$ and 5 against a wide variety of bacteria are shown in Table 1. They have no activity against Gram-positive bacteria, such as *Staphylococcus aureus* and *S. pyogenes*, except against Streptococci with the inducible (*S. pyogenes* 2548) and constitutive-type (*S. pyogenes* 930) of macrolide resistance. Enterobacteriaceae were not susceptible to 100 μg/ml. The lack of activity against Enterobacteriaceae is probably related to impermeability of the lipopolysaccharide layer to pacidamycins, since *Escherichia coli* SS, a strain which is deficient in its lipopolysaccharide layer and supersensitive to most classes of antibiotics, is sensitive to the pacidamycins. The pacida-

Table 1. *In vitro* activity of pacidamycins 1~3 and 5 against aerobic bacteria.

Organisms	MIC (μg/ml)			
	1	2	3	5
Staphylococcus aureus ATCC 6538P	>100	>100	>100	>100
S. aureus CMX 686B	>100	>100	>100	>100
S. aureus A5177	>100	>100	>100	>100
S. aureus 45	>100	>100	>100	>100
S. aureus 45 RAR2	>100	>100	>100	>100
S. aureus CMX 503A	>100	>100	>100	>100
S. aureus CMX 553	>100	>100	>100	>100
S. epidermidis 3519	>100	>100	>100	>100
Micrococcus luteus ATCC 9341	>100	>100	>100	>100
M. luteus ATCC 4698	>100	>100	>100	>100
Enterococcus faecium ATCC 8043	>100	>100	>100	>100
Streptococcus bovis A5169	>100	>100	>100	>100
S. agalactiae CMX 508	>100	>100	>100	>100
S. pyogenes EES61	>100	>100	>100	>100
S. pyogenes 930 CONST	12.5	50	25	100
S. pyogenes 2548 INDUC	25	>100	100	>100
Escherichia coli Juhl	>100	>100	>100	>100
E. coli SS	1.56	6.25	6.25	6.25
E. coli DC-2	>100	>100	>100	>100
E. coli H560	>100	100	>100	>100
E. coli KNK 437	>100	>100	>100	>100
Enterobacter aerogenes ATCC 13048	>100	>100	>100	>100
Klebsiella pneumoniae ATCC 8045	100	>100	>100	>100
Providencia stuartii CMX 640	>100	>100	>100	>100
Pseudomonas aeruginosa BMH 10	50	50	100	50
P. aeruginosa A5007	50	25	50	12.5
P. aeruginosa K799/WT	>100	>100	>100	50
P. aeruginosa K799/61	1.56	3.12	1.56	0.78
P. aeruginosa BMH 1	25	25	12.5	12.5
P. aeruginosa A5000	64	ND	ND	ND
P. aeruginosa A5005	64	ND	ND	ND
P. cepacia 296I	>100	>100	>100	>100
P. fluorescens CFS 109	>128	ND	ND	ND
P. pseudoalcaligenes CFS 158A	>128	ND	ND	ND
P. maltophilia CMX 661E	>128	ND	ND	ND
Acinetobacter sp. CMX 669	>100	>100	>100	>100
Acinetobacter sp. CMX 662C	>128	ND	ND	ND
Candida albicans ATCC 10231	>128	ND	ND	ND

ND: Not done.

mycins had selective activity against *P. aeruginosa*. Therefore, the compounds were tested against a variety of *P. aeruginosa* using Mueller-Hinton agar as the test medium. The results are shown in Table 2. *Pseudomonas* species other than *P. aeruginosa* were generally resistant to pacidamycins.

Effect of Serum

The MICs of pacidamycins 1, 2 and 3 against *P. aeruginosa* were increased by two to 6-fold by the addition of human or mouse serum to the medium. The MICs were decreased at least 2-fold when the pacidamycins were tested against *S. pyogenes* 930 (Table 3).

Table 2. *In vitro* activity of the pacidamycins 1~3 and 5 against non-fermentative Gram-negative bacilli.

Organisms	MIC (μg/ml)			
	1	2	3	5
Pseudomonas aeruginosa ATCC 27853	8	16	16	8
P. aeruginosa CFS 387C	16	32	16	8
P. aeruginosa CFS 389	16	64	32	16
P. aeruginosa CFS 350F	64	128	64	32
P. aeruginosa CMX 719A	>128	>128	>128	>128
P. aeruginosa A5000	32	64	64	16
P. aeruginosa A5005	32	64	32	16
P. aeruginosa BMH 1	16	32	16	8
P. fluorescens CFS 109	>128	>128	>128	128
P. fluorescens CFS 221	>128	>128	>128	>128
P. pseudoalcaligenes CFS 158A	>128	>128	>128	128
P. pseudoalcaligenes CFS 217E	>128	>128	>128	>128
P. pseudoalcaligenes CFS 290C	>128	>128	>128	>128
P. pseudoalcaligenes CFS 314	64	128	128	32
P. pseudoalcaligenes CMX 663N	>128	>128	>128	>128
P. maltophilia CMX 661E	>128	>128	>128	>128
Alcaligenes sp. CMX 688E	>128	>128	>128	>128
Acinetobacter sp. CMX 662C	>128	>128	>128	>128
Acinetobacter sp. CMX 675F	>128	>128	>128	>128
Acinetobacter sp. CMX 676C	>128	>128	>128	>128

Table 3. Effect of serum on the *in vitro* activity of pacidamycins 1~3.

Organism	Growth medium	MIC (μg/ml)		
		1	2	3
Streptococcus pyogenes 930	BHI broth[a]	16	>128	128
	Human serum	8 (−1)[c]	32 (>−2)	16 (−3)
	Mouse serum	8 (−1)	32 (>−2)	32 (−2)
Pseudomonas aeruginosa A5007	MH broth[b]	32	64	>128
	Human serum	64 (+1)	16 (−2)	64 (>−1)
	Mouse serum	>128 (>+2)	128 (+1)	>128 (0)
P. aeruginosa ATCC 27853	MH broth	16	16	32
	Human serum	32 (+1)	16 (0)	32 (0)
	Mouse serum	>128 (>+3)	64 (+2)	128 (+2)

[a] Brain heart infusion broth.
[b] Mueller-Hinton broth.
[c] Number of 2-fold dilution differences from MIC without serum.

Effect of pH

Pacidamycin 1 was 2-fold more active at pH 6.5 than at pH 7.2. At the basic pH (pH 8.0) pacidamycin 1 was two to 4-fold less active against *P. aeruginosa* and 2-fold more active against *S. pyogenes* than at pH 7.2 (Table 4).

Killing Kinetics

Pacidamycin 1 was bactericidal at 4 and 8 times the MIC (Fig. 1). The bacterial counts were reduced by 3 $\log_{10}$ cfu/ml in 4 hours. However, at 24 hours the cultures had re-grown to the same level as the controls. Bacterial colonies isolated from these cultures were resistant to 4 and 8 times the MIC of pacidamycin 1.

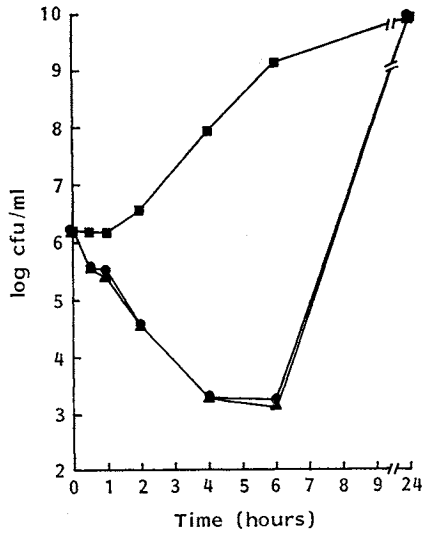
Table 4. Effect of pH on *in vitro* activity of pacidamycin 1.

Organism	Growth pH	MIC (μg/ml)
Streptococcus pyogenes 930	6.5	16 (−1)[a]
	7.2	32
	8.0	16 (−1)
Pseudomonas aeruginosa A5007	6.5	16 (−1)
	7.2	32
	8.0	64 (+1)
P. aeruginosa ATCC 27853	6.5	8 (−1)
	7.2	16
	8.0	64 (+2)

[a] Number of 2-fold dilution differences from MIC at pH 7.2.

Fig. 1. Time-kill curves of pacidamycin 1 against *Pseudomonas aeruginosa* ATCC 27853.

■ Control (no antibiotic), ● 64 μg/ml (4×MIC), ▲ 128 μg/ml (8×MIC).



Resistance Frequency

The frequency of resistance of *P. aeruginosa* ATCC 27853 to pacidamycin 1 at 4 times the MIC was 4.4×10^{-6} and at 8 times the MIC was 3.5×10^{-6}. The MIC of pacidamycin 1 for *P. aeruginosa* ATCC 27853 was 8 μg/ml and the MICs for the resistant mutants were >32 μg/ml. The resistant mutants remained resistant after 10 transfers in antibiotic-free medium. The MICs of piperacillin and gentamicin were the same for the resistant-mutants and the wild-type parent strain suggesting that the mutants had changes in their cell envelope only causing permeation resistance to limited antibiotics such as pacidamycins.

Mouse Protection Test

Pacidamycin 1 was inactive *in vivo* when tested against *P. aeruginosa* A5007 in mice even after administration of 100 mg/kg/day.

Pharmacokinetics in Mice

The compounds were not absorbed after oral administration. After subcutaneous administration of 25 mg/kg, pacidamycins 2 and 3 were not detected in the serum at 30 minutes to 24 hours. The percent urine recoveries were 50.4 for pacidamycin 2 and 32.8 for pacidamycin 3. The peak serum concentration (Cmax) of pacidamycin 1 was 50 μg/ml at 0.5 hour after a single subcutaneous dose of 25 mg/kg. The serum half-life was 0.5 hour and the area under the serum curve was 31.3 μg·hour/ml. The percent urine recovery was 20%. The reason for not finding pacidamycins 2 and 3 in the serum could be a result of a shorter serum half-life for pacidamycins 2 and 3 relative to pacidamycin 1, since the first determination was at 0.5 hour.

Discussion

Antibiotics which have activity against *P. aeruginosa* are not commonly found in nature. This may be related to the ubiquitous occurrence of this bacteria in our environment and its ability to survive in the environment. Therefore, pacidamycins are unusual antibiotics in having selective activity against *P. aeruginosa*. The only other organisms that pacidamycins had activity against were *S. pyogenes* strains carrying macrolide-lincosamide-streptogramin (MLS)-type resistance. We do not

know why pacidamycins have activity against MLS-resistant Streptococci but have no activity against macrolide susceptible Streptococci.

Pacidamycin 1 was initially bactericidal, but resistant colonies were soon selected since the frequency of resistance was quite high. The resistant mutants have not been characterized to determine if they were mutants in the peptide transport system. These mutants, however, were stable in the absence of antibiotic and their susceptibility to piperacillin and gentamicin was the same as that of the parent strain.

Although pacidamycins are peptide antibiotics, they are relatively stable in serum. However, they were inactive *in vivo* in mouse protection tests. Since high serum concentrations were achieved by pacidamycin 1, the lack of *in vivo* activity is probably related to a combination of factors such as selection of resistant bacteria and also decreased activity of pacidamycin 1 in the presence of serum.

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