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In Vitro and In Vivo Activities of Novel 6-Methylidene Penems as β-Lactamase Inhibitors

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Novel penem molecules with heterocycle substitutions at the 6 position via a methylidene linkage were investigated for their activities and efficacy as β-lactamase inhibitors. The concentrations of these molecules that resulted in 50% inhibition of enzyme activity were 0.4 to 3.1 nM for the TEM-1 enzyme, 7.8 to 72 nM for Imi-1, 1.5 to 4.8 nM for AmpC, and 14 to 260 nM for a CcrA metalloenzyme. All the inhibitors were more stable than imipenem against hydrolysis by hog and human dehydropeptidases. Piperacillin was combined with a constant 4-μg/ml concentration of each inhibitor for MIC determinations. The combinations reduced piperacillin MICs by 2- to 32-fold for extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae* strains. The MICs for piperacillin-resistant (MIC of piperacillin, >64 μg/ml) strains of *Enterobacter* spp., *Citrobacter* spp., and *Serratia* spp. were reduced to the level of susceptibility (MIC of piperacillin, ≤16 μg/ml) when the drug was combined with 4, 2, or 1 μg of these penem inhibitors/ml. Protection against acute lethal bacterial infections with class A and C β-lactamase- and ESBL-producing organisms in mice was also demonstrated with piperacillin plus inhibitor. Median effective doses were reduced by approximately two- to eightfold compared to those of piperacillin alone when the drug was combined with the various inhibitors at a 4:1 ratio. Pharmacokinetic analysis after intravenous administration of the various inhibitors showed mean residence times of 0.1 to 0.5 h, clearance rates of 15 to 81 ml/min/kg, and volumes of distribution between 0.4 and 2.5 liters/kg. The novel methylidene penem molecules inhibit both class A and class C enzymes and warrant further investigation for potential as therapeutic agents when used in combination with a β-lactam antibiotic.

The increasing number and variety of β-lactamases produced by bacteria represent a serious threat to the clinical utility of β-lactams. The introduction of β-lactamase inhibitors was thought to have alleviated this problem; however, alterations and mutations within β-lactamases have allowed bacteria to overcome the effects of the β-lactamase inhibitors. The currently marketed inhibitors (tazobactam, clavulanate, and sulbactam) are not active against all β-lactamases. Among the most problematic β-lactamases are the molecular class C enzymes (Bush-Medeiros-Jacoby group 1) (8). Bacterial genera known to produce inducible or stably derepressed AmpC β-lactamases are *Citrobacter*, *Enterobacter*, *Hafnia*, *Pseudomonas*, *Morganella*, *Providencia*, and *Serratia*. Expression of the class C enzymes by these organisms has been shown to confer resistance to the current commercial inhibitor combinations as well as extended-spectrum cephalosporins (7, 24).

Resistance to the known inhibitor combinations has also been exhibited by strains producing multiple enzymes of class A as well as extended-spectrum β-lactamases (ESBLs) (35, 39). The ESBLs are an issue of concern in terms of their incidence and treatment options. This is particularly evident for *Escherichia coli* and *Klebsiella pneumoniae* isolates in many countries (5). Over 100 TEM-derived and 50 SHV-derived enzymes have been identified to date (http://www.lahey.org/studies/temtable.asp). Although the rate of resistance to the extended-spectrum cephalosporins due to these enzymes appears to be stable, it remains clinically significant (17).

The treatment of infections caused by strains producing ESBLs or inducible AmpC β-lactamases in hospitalized patients is a therapeutic challenge. There are currently several different classes of potentially active β-lactamase inhibitors under investigation, including the oxapenems, penicillin derivatives, cyclic acyl phosphonates, and cephalosporin-derived compounds (3, 9, 11, 14, 18, 21, 26).

The medicinal chemistry strategy at Wyeth Research, Pearl River, N.Y., was based on incorporating new heterocycles into penems containing a 6-methylidene moiety. This strategy was successfully carried out using a novel coupling process involving aldol condensation of different aldehydes with the bromopenem followed by reductive elimination (T. Abe, H. Ushirogochi, I. Yamamura, T. Kumagai, T. Mansour, A. Venkatesan, A. Agarwal, P. Petersen, W. Weiss, Y. Yang, and D. Shlaes, Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. F-358, p. 232, 2003). Modeling studies based on the crystal structure of TEM-1, a class A β-lactamase, and P99, a class C β-lactamase, were employed in selecting aldehydes with potentially improved active-site interactions (31). Novel structural features were incorporated based on interactions with active-site residues

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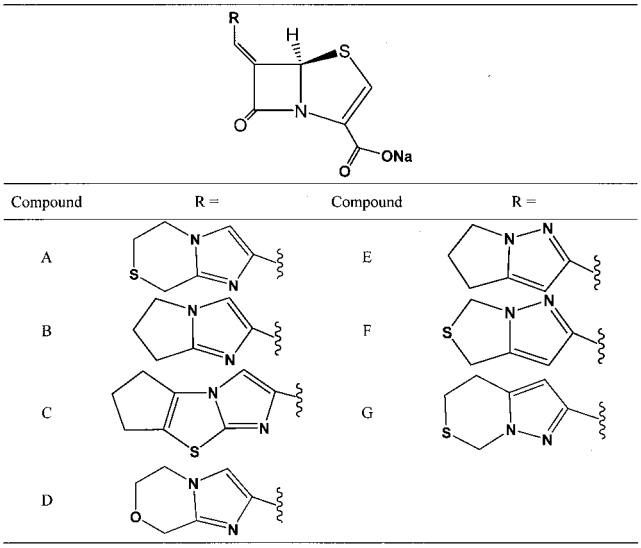

FIG. 1. Chemical structures of substitution-containing 6-methylidene penem molecules.

such as Glu140 (TEM-1) and Gln120 (P99). Mechanism of action of these inhibitors was further elucidated by inhibition studies using X-ray crystallography (30). These approaches resulted in the identification of two distinct chemical series for medicinal chemistry optimization: the bicyclic and the tricyclic series.

MATERIALS AND METHODS

Organisms. All organisms used in this study were taken from the Wyeth Research culture collection and were either recent clinical isolates collected from various medical centers around the United States or standard laboratory strains from the American Type Culture Collection (Rockville, Md.).

Crude β-lactamase extraction and isoelectric focusing. Identification of β-lactamases from clinical isolates was performed as previously described (39). Briefly, the β-lactamases of the clinical isolates were extracted by five cycles of freezing and thawing followed by centrifugation. The β-lactamase activities and pIs were determined by isoelectric focusing electrophoresis on a Multiphor apparatus (LKB-Pharmacia) with prepared polyaerylamide gel plates (pH range, 3.5 to 9.5; Pharmacia). TEM-1, TEM-2, K1, SHV-1, and P99 were used as standards. TEM-10 and TEM-28 were used for comparison. Enzymes were detected after the gel was overlaid with nitrocefin. Several strains yielded multiple β-lactamase bands not fully characterized, and these enzymes are referred to by their pI values only.

Test compounds. Piperacillin and tazobactam powder were obtained from Wyeth Research. The 6-methylidene penem molecules (compounds A, B, C, D, E, F, and G) are novel compounds synthesized as a joint effort between Chemical and Screening Sciences, Wyeth Research, and Wyeth-Lederle Japan (Saitama, Japan) (Fig. 1).

Enzyme inhibition assay. The levels of inhibition of β-lactamase enzymes by penem molecules were determined as previously described (40). Briefly, compounds were diluted in 50 mM phosphate buffer at pH 7.0 to yield concentrations between 100 and 0.1 nM in microtiter plates. An equal volume of diluted enzyme stock was added, and the plates were incubated at 25°C for 10 min. Nitrocefin solution at 50 μg/ml was then dispensed into each well, and the plates were immediately read with the Kinetic program at 495 nm for 5 min on a Spectramax Plus (Molecular Devices, Sunnyvale, Calif.). Maximum rates of metabolism were then compared to those in control wells (enzyme plus nitrocefin), and percentages of enzyme inhibition were calculated for each concentration of inhibitor. The concentrations resulting in 50% inhibition of enzyme activity (IC_{50}s) were calculated using WinNonlin (Pharsight Corp., Mountainview, Calif.).

Preparation of DHP. Dehydropeptidases (DHPs) from fresh hog and mouse kidneys were extracted with butanol and precipitated with ammonium sulfate in 10 mM HEPES buffer (pH 7.2) (10). The enzymes were solubilized from frozen precipitates on each day of testing. A human DHP gene was cloned from a human kidney cDNA gene bank and expressed in human kidney 293 cells (40). DHP was partially purified as described previously (1).

Enzyme stability studies. The levels of hydrolysis of the penem molecules by the DHPs were determined as previously described (40) with 10 mM HEPES buffer (pH 7.2). Two different volumes of enzyme (10 to 50 μl) were used in a total volume of 1,000 μl, and rates were determined as nanomoles of substrate hydrolyzed per microliter of enzyme solution added. Imipenem was included as a reference compound for each set of assays. Relative hydrolysis rates were calculated by normalizing the specific molar hydrolysis rates to those observed with imipenem on the same day.

Antimicrobial susceptibility testing. The in vitro activities of the antibiotics were determined by the broth microdilution method as recommended by the NCCLS (29) by using Mueller-Hinton II broth (BBL, Cockeysville, Md.). Microtiter plates containing serial dilutions of each antimicrobial agent were inoculated with each organism to yield the appropriate density (10^5 CFU/ml) in a 100-μl final volume. The plates were incubated for 18 to 22 h at 35°C in ambient air. The MICs for all isolates were defined as the lowest concentrations of antimicrobial agent that completely inhibited the growth of the organism as detected by the unaided eye.

Time-kill kinetic assay. A time-kill assay was performed by the broth macrodilution method in accordance with the NCCLS guidelines (28). A starting inoculum of approximately 10^6 CFU/ml and a final concentration of the antibiotic equal to four times the MIC were employed for these assays. Flasks containing 50 ml of Mueller-Hinton II broth with the appropriate antimicrobial agent were inoculated with 50 ml of the test organism in logarithmic growth phase. Test flasks were incubated with shaking (150 rpm) in a 35°C water bath. Aliquots were removed for the determination of viable cell counts at 0, 2, 4, 6, and 24 h. Serial dilutions were prepared in sterile 0.85% sodium chloride solution. The diluted samples (0.05 ml) were plated onto Trypticase soy agar plates with a spiral plater (Don Whitley Scientific Ltd.). The plates were incubated at 35°C in ambient air for 18 to 22 h, and the number of colonies was determined using the ProtoCOL plate reader (Don Whitley Scientific Ltd.). Killing curves were constructed by plotting the $\log_{10}$ number of CFU per milliliter versus time over 24 h, and the change in bacterial concentration was determined. Bactericidal activity was defined as a reduction of 99.9% ($\geq 3 \log_{10}$) of the total number of CFU per milliliter in the original inoculum.

Acute lethal infection model. Female mice of strain CD-1 (Charles River Laboratories, Kingston, N.Y.), each weighing 20 ± 2 g, were challenged by intraperitoneal injection of bacterial cells from 5-h broth cultures suspended in either Trypticase soy broth or 5% hog gastric mucin. Five animals were infected at each of five twofold dose levels of the test compound for each organism. Dosing solutions were prepared as piperacillin alone or piperacillin combined with a penem (β-lactamase inhibitor) molecule at a ratio of 4:1 (piperacillin to inhibitor). The bacterial inoculum level for infection was sufficient to result in death of untreated controls within 24 to 48 h. The 7-day survival ratios from three separate tests were pooled for estimation of the median effective dose (ED_{50}) by a computerized program for probit analysis (38). All procedures were carried out using protocols approved by the Wyeth Research Animal Care and Use Committee.

Pharmacokinetics. Pharmacokinetics were investigated using male Wistar rats (200 to 250 g; Charles River Laboratories). Test compounds were administered to groups of three rats each at a dose of 150 mg/kg of body weight at 1 ml/kg by intravenous bolus (i.v.). Blood samples were obtained via a surgically implanted jugular canula at selected time points. Samples were stored at −70°C prior to analysis.

Bioanalytical analysis. Plasma samples were analyzed by liquid chromatography-mass spectrometry methodology by using a Merck Chromolith Speedrod C_{18} column at an ambient air temperature. Sample injection volume was 20 μl at a flow rate of 4 ml/min under gradient conditions consisting of a 95:5 (vol/vol) ratio of 5 mM ammonium acetate in water to acetonitrile and a 95:5 (vol/vol) ratio of 5 mM ammonium acetate in acetonitrile to water. A Micromass Ultima mass spectrophotometer was used in the positive electrospray ionization mode for multiple reaction monitoring. The limit of quantitation was 5 ng/ml.

RESULTS

Inhibition of β-lactamase. The activities of the seven novel methylidene penems and tazobactam against TEM-1, Imi-1 (class A), CcrA (class B), and AmpC (class C) enzymes were compared (Table 1). All of the new inhibitors demonstrated excellent inhibition of the TEM-1 enzyme, with IC_{50}s of 0.4 to

TABLE 1. IC_{50}s of 6-methylidene penem and tazobactam (TZB) molecules for isolated β-lactamases

Inhibitor	IC_{50} (nM) for:			
	TEM-1	Imi-1	CcrA	AmpC
A	1.1	58	260	5.4
B	0.6	20	230	2.2
C	1.4	72	240	2.1
D	0.4	7.8	66	4.8
E	1.2	50	14	1.5
F	1.4	56	110	1.5
G	3.1	ND[a]	ND	3.5
TZB	100	ND	400,000	84,000

[a] ND, not determined.

3.1 nM. Compounds B and D, the most active of the group, were 100- to 200-fold more active than tazobactam against TEM-1. Activity was slightly reduced against Imi-1, a carbapenem-hydrolyzing enzyme (23). IC_{50}s of each of the compounds were 7.8 to 72 nM, with compound D being approximately 2- to 10-fold more active than the other molecules. All of the penem molecules were shown to inhibit the AmpC enzyme, with IC_{50}s of these seven molecules of between 1.5 and 5.4 nM. Tazobactam is a poor inhibitor of the class C AmpC enzyme and was 15,000- to 56,000-fold less active. Inhibition of the CcrA metallo-β-lactamase was observed at concentrations of 14 to 260 nM. Compounds E and D were the most active, with IC_{50}s of 14 and 66 nM, respectively. Tazobactam was a poor inhibitor of the CcrA enzyme.

DHP stability. The relative stability of the penem molecules against renal DHP is summarized in Table 2. Overall, all the inhibitors were more stable against human DHP than against the hog or mouse peptidases. Relative to imipenem, the molecules were 2.5- to 13-fold more stable against the human DHP extract. The relative stabilities of the penems ranged from equivalent to 50- and 7-fold more stable against hog and mouse DHP, respectively. In general, the methylidene penem inhibitors were less stable than tazobactam against each of the DHPs, with the exception of compound D. This compound demonstrated stability equivalent to that of tazobactam against the human and hog enzymes.

In vitro antibacterial activity. When tested alone, neither tazobactam nor any of the penem inhibitors exhibited activity against the isolates used in this study (data not shown). The MICs of piperacillin alone and in combination with 4 μg of each of the inhibitors/ml for ESBL-producing *E. coli* are shown in Table 3. All of the isolates were resistant to piperacillin alone (MIC, >64 μg/ml). The piperacillin MIC at which 50% of the isolates tested were inhibited (MIC_{50}) was reduced to 4 to 16 μg/ml in the presence of a constant 4-μg/ml concentration of each of the inhibitors, including tazobactam, for these *E. coli* isolates. Overall, the addition of the inhibitors restored susceptibility to piperacillin for 65 to 100% of the strains. Compounds C, E, and D displayed the most pronounced enhancement of activity, with 94, 94, and 100% of the ESBL-producing *E. coli* isolates exhibiting susceptibility to piperacillin plus inhibitor, respectively. Each of the inhibitors was less active than tazobactam against the *E. coli* strain expressing the TEM-43 enzyme. This may be due to changes in affinities of the new inhibitors for this enzyme at the Thr182 mutation of TEM-43 (39).

TABLE 2. Stability of 6-methylidene penem molecules against hydrolysis by renal DHP enzymes from various species

Compound	% Hydrolyzed[a] by 50 μM renal DHP from:		
	Human	Hog	Mouse
A	40	74	87
B	25	12	118
C	9.8	25	14
D	7.6	2.0	19
E	32	78	21
F	20	48	40
G	NT	NT	NT
TZB[b]	8.0	<1	<1

[a] Normalized to the level of imipenem hydrolysis, which was set at 100%. NT, not tested.
[b] TZB, tazobactam.

A similar panel of *K. pneumoniae* isolates expressing ESBLs was evaluated with piperacillin plus the inhibitors (Table 4). All isolates were resistant to piperacillin alone (MIC, >64 μg/ml). The addition of 4 μg of each inhibitor/ml reduced the MIC_{50} of piperacillin in all the combinations to 16 to 32 μg/ml. MIC_{90}s of the combinations were two- to fourfold higher. Compounds B, E, and F were the most effective at protecting piperacillin from the ESBLs produced by the *Klebsiella* isolates. Susceptibility to piperacillin at ≤16 μg/ml (in accordance with NCCLS guideline M07-A6, *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, approved standard, 6th ed. [29]) was restored in 83, 83, and 89% of the isolates when the drug was combined with compounds B, E, and F, respectively. The 6-methylidene compounds appeared to be more effective than tazobactam, particularly against strains expressing SHV-13, SHV-4, and SHV-5. Tazobactam, however, was more effective when the TEM-10 enzyme was present alone or expressed with SHV-type enzymes in *K. pneumoniae*. This greater effectiveness of tazobactam was also observed with some of the *E. coli* isolates. Tazobactam and compounds A, C, and G showed similar overall activities, with 44 to 55% of the isolates rendered susceptible to piperacillin.

The activity of piperacillin plus inhibitor against a panel of piperacillin-resistant clinical isolates made up of *Enterobacter aerogenes*, *Enterobacter cloacae*, *Citrobacter* spp., and *Serratia* spp. was evaluated (Table 5). The MIC_{90} of piperacillin alone for all species was >64 μg/ml. The combination of tazobactam with piperacillin demonstrated only a slight increase in activity over piperacillin alone (MIC_{90}s of 64 to >64 μg/ml). When piperacillin was combined with each of the methylidene penem compounds, 100% of the *Enterobacter aerogenes*, *Enterobacter cloacae*, and *Citrobacter* spp. strains were rendered susceptible. MIC_{90}s ranged from 4 to 8 μg/ml for *Enterobacter aerogenes* and 8 to 16 μg/ml for *Enterobacter cloacae* and *Citrobacter* spp. Activity against the *Serratia* isolates was also enhanced with each of the inhibitors. One of the 10 isolates tested was resistant to piperacillin and each inhibitor, but MIC_{90}s of piperacillin were still within the susceptible range (2 to 8 μg/ml).

The effect of inhibitor concentration on the activity of piperacillin against these isolates is shown in Fig. 2. The MICs of piperacillin combined with compounds B, C, and D at concentrations from 4 to 1 μg/ml increased two- to fourfold. Pipera-

TABLE 3. MICs of piperacillin alone and in combination with inhibitors for ESBL-producing *E. coli*

Enzyme(s)	MIC[a] (μg/ml) of piperacillin + inhibitor (4 μg/ml):							
	TZB	A	B	C	D	E	F	G
TEM-4 + TEM-1	>64	64	64	8	8	16	32	16
TEM-1 + SHV-8	>64	64	32	16	16	16	16	16
ACT-1 + TEM-1 + pI 5.6 enzyme	64	4	4	4	4	8	4	4
OXA-1 + TEM-29	64	8	8	4	4	4	16	8
TEM-10	8	64	16	8	16	16	32	16
TEM-10 + SHV-1	8	64	16	8	16	16	32	32
TEM-1 + SHV-7	8	8	8	8	8	8	16	8
TEM-28	8	32	16	8	16	8	16	16
TEM-43	8	64	32	32	32	16	>64	64
TEM-10 + pI 6.3 and 8.1 enzymes	4	16	4	4	4	4	16	8
TEM-10 + pI 6.3 enzyme	4	32	8	4	8	8	16	16
TEM-1 + SHV-7 + CMY-2	4	16	8	8	8	8	8	16
TEM-1 + SHV-5	4	4	4	2	4	4	8	4
TEM-10	2	8	4	4	4	4	8	4
TEM-1 + TEM-10	2	8	2	2	2	4	8	8
TEM-10	2	8	2	2	4	4	8	8
SHV-7	2	4	2	2	2	2	2	2
MIC_{50} (μg/ml)	8	16	8	4	8	8	16	8
MIC_{90} (μg/ml)	64	64	32	8	16	16	32	16
% Susceptible	76	65	82	94	94	100	76	88

[a] The MICs listed correspond to the inhibitory activity of piperacillin. For each ESBL-producing strain, the MIC of piperacillin alone was >64 μg/ml; none of the isolates were susceptible. TZB, tazobactam.

cillin MICs increased eightfold at inhibitor concentrations of 0.5 μg/ml compared to those at a concentration of 4 μg/ml of the same inhibitor. The activity of piperacillin was least affected by decreasing concentrations of compound F. There was only a twofold increase in MICs of piperacillin plus inhibitor over those of piperacillin alone when inhibitor concentrations went from 4 to 0.5 μg/ml.

Time-kill assay. Results of the time-kill assay with piperacillin (8 μg/ml) plus inhibitors (4 μg/ml) representing four times the MIC for a TEM-1-producing *E. coli* strain are presented in Fig. 3. Piperacillin alone and piperacillin plus tazobactam both resulted in a 2 $\log_{10}$ reduction in CFU at 2 h followed by an increase in bacterial counts by 24 h to a level equivalent to that in the control flask. Piperacillin combined

TABLE 4. MICs of piperacillin alone and combined with inhibitors for ESBL-producing *K. pneumoniae*

Enzyme(s)	MIC[a] (μg/ml) of piperacillin + inhibitor (4 μg/ml):							
	TZB	A	B	C	D	E	F	G
pI 5.4, 5.6, 7.6, and 8.7 enzymes	>64	>64	>64	>64	64	64	64	>64
TEM-1 + SHV-5 + SHV-7	>64	>64	64	>64	32	32	32	32
SHV-13	>64	16	8	16	8	8	8	8
TEM-1 + SHV-1 + SHV-5	>64	32	16	32	16	16	16	16
SHV-4	64	4	4	8	2	2	2	2
TEM-1 + SHV-27	32	16	8	16	8	16	16	16
TEM-1 + SHV-5	32	16	16	16	16	16	16	16
TEM-1 + ACT-1	32	16	16	16	16	8	16	16
TEM-26	16	>64	16	64	64	32	16	32
TEM-9 + SHV-1	8	32	16	64	8	8	8	32
TEM-3	8	16	8	16	8	4	8	8
TEM-10 + SHV-1 + SHV	8	>64	16	64	16	32	16	32
TEM-10 + SHV-1	8	>64	32	64	16	32	16	32
CAZ-R, P/T-R strain[b] enzyme	8	64	16	64	16	16	16	32
TEM-10	4	32	4	8	4	8	8	8
TEM-1 + TEM-26 + SHV-1	4	4	4	4	16	16	8	32
SHV-2	4	8	4	8	4	8	8	8
MIC_{50} (μg/ml)	16	32	16	32	16	16	16	16
MIC_{90} (μg/ml)	>64	>64	32	64	32	32	16	32
% Susceptible	50	44	83	50	83	72	89	55

[a] The MICs listed correspond to the inhibitory activity of piperacillin. For each ESBL-producing strain, the MIC of piperacillin alone was >64 μg/ml; none of the isolates were susceptible. TZB, tazobactam.
[b] CAZ-R, ceftazidime resistant; P/T-R, piperacillin-tazobactam resistant.

TABLE 5. MICs of piperacillin alone and combined with constant 4-μg/ml concentrations of inhibitors for various piperacillin-resistant pathogens

Organism (no. of isolates)	Antibiotic[b]	MIC[a] (μg/ml)		
		Range	50%	90%
Enterobacter aerogenes (10)	PIP alone	64–>64	64	>64
	PIP + TZB	32–>64	64	>64
	PIP + A	2–4	4	4
	PIP + B	2–4	4	4
	PIP + C	2–8	4	4
	PIP + D	2–4	4	4
	PIP + E	2–4	2	4
	PIP + F	2–8	2	4
	PIP + G	2–8	4	8
Enterobacter cloacae (20)	PIP alone	>64	>64	>64
	PIP + TZB	2–>64	>64	>64
	PIP + A	2–16	8	16
	PIP + B	2–16	4	8
	PIP + C	2–16	4	16
	PIP + D	2–16	8	16
	PIP + E	2–8	4	8
	PIP + F	2–16	4	8
	PIP + G	4–16	8	16
Citrobacter spp. (10)	PIP alone	64–>64	>64	>64
	PIP + TZB	2–>64	32	>64
	PIP + A	2–16	2	16
	PIP + B	2–16	2	16
	PIP + C	2–16	4	16
	PIP + D	1–16	2	8
	PIP + E	2–8	4	8
	PIP + F	2–8	2	8
	PIP + G	2–16	4	16
Serratia spp. (10)	PIP alone	16–>64	>64	>64
	PIP + TZB	0.5–>64	16	64
	PIP + A	0.5–>64	1	8
	PIP + B	0.5–>64	1	4
	PIP + C	0.5–>64	1	8
	PIP + D	0.25–>64	1	4
	PIP + E	0.25–>64	0.5	2
	PIP + F	0.25–>64	1	2
	PIP + G	1–>64	2	8

[a] The MICs listed correspond to the inhibitory activity of piperacillin.
[b] PIP, piperacillin; TZB, tazobactam.

with compound F achieved a 2 $\log_{10}$ reduction at 2 h which was maintained through 24 h. The remaining compounds D, C, and B achieved a 3 $\log_{10}$ reduction by 6 h followed by a minimal increase by the 24 h time point.

In vivo efficacy. Administered alone, piperacillin had only moderate to poor efficacy (ED_{50}s of 101.8 to 421 mg/kg) against murine infections caused by β-lactamase-producing strains of *E. coli* and *Enterobacter aerogenes* (Table 6). Piperacillin when combined with each of the inhibitors at a 4:1 (piperacillin to inhibitor) ratio and administered intravenously demonstrated a marked reduction in ED_{50}s. The presence of the coadministered inhibitors reduced piperacillin ED_{50}s by 3- to 8-fold for the TEM-1-producing-*E. coli* infection, 1.5- to 3-fold for the TEM-29- and OXA-1-producing-*E. coli* infections, 4- to 7-fold for the CMY-2-producing *E. coli* infection, and 3- to 4-fold for the AmpC-producing *Enterobacter aerogenes* infection. This reduction resulted in ED_{50}s of the piperacillin-methylidene compounds of 19.5 to 31.8 mg/kg compared to ED_{50}s of piperacillin-tazobactam of 46.4 mg/kg for the TEM-1-producing-*E. coli* infection. The ED_{50} of piperacillin for an ESBL-producing strain of *E. coli* was reduced from 127.3 mg/kg to 37.4 to 78.7 mg/kg. The combination of piperacillin and compound F appeared to be the most efficacious combination overall for the infections with class A β-lactamase-producing strains. Similarly, piperacillin ED_{50}s for infections with class C enzyme-producing strains were reduced from 101.8 mg/kg to 14.2 to 22.4 mg/kg for the infection with the CMY-2-producing strain and from 421 mg/kg to 96.4 to 125.3 mg/kg for the infection with the AmpC-producing strain. The piperacillin-tazobactam combination was approximately two-fold less efficacious than the other inhibitor combinations against the *E. coli* CMY-2-producing strain.

Pharmacokinetics. The methylidene penem molecules were administered as single 150-mg/kg i.v. doses to rats, and pharmacokinetic parameters are presented in Table 7. The maximum serum drug concentrations (C_{max}) of compounds B, C, D, and G at 5 min postdosing were comparable, with a range of 345 to 538 μg/ml. Compounds E and F achieved lower C_{max}

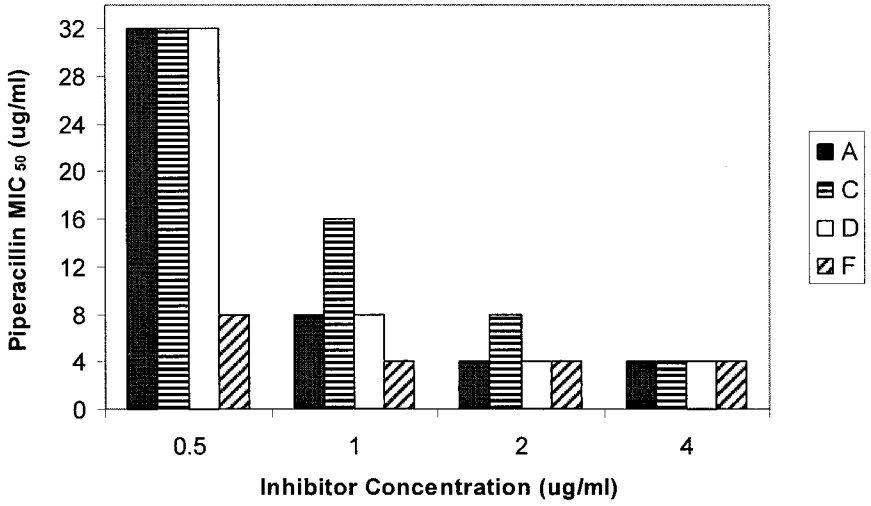

FIG. 2. Effect of inhibitor concentration on the MIC of piperacillin for a combined screen of resistant pathogens: *Enterobacter* spp., *Citrobacter* spp., and *Serratia* spp. (n = 50).

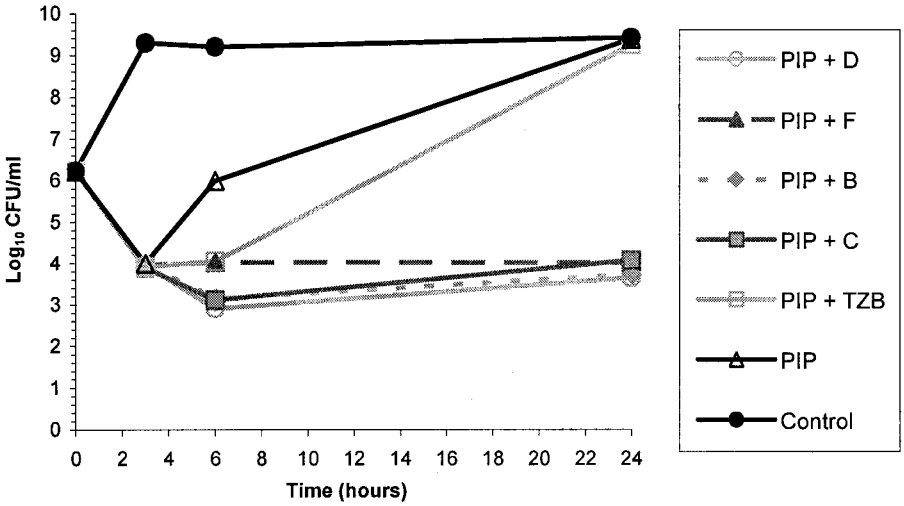

FIG. 3. Time-kill curves for piperacillin (PIP) plus inhibitors (8 plus 4 μg/ml, respectively) against a TEM-1-producing *E. coli* isolate. TZB, tazobactam.

(72 and 152 μg/ml, respectively), which corresponded to the more rapid clearance (approximately 81 ml/min/kg) observed for these two compounds. Compound C was cleared more slowly, at 15.4 ml/min/kg, than any of the others. In terms of overall exposure, compound C also exhibited the greatest area under the concentration curve (AUC), 163 μg · h/ml. This value was two- to fivefold greater than those for the other compounds, which achieved AUCs of 31 to 75 μg · h/ml. The observed volumes of distribution (V_{ss}) of the compounds varied. Compounds F and E exhibited the greatest distribution, with V_{ss} of 2.5 and 1.0 liters/kg, respectively. All other compounds had V_{ss} between 0.32 and 0.45 liters/kg. The mean residence times varied for the compounds, with times ranging from 0.1 h for compounds B and G to 0.2 h for D and E and 0.4 to 0.5 h for C and F.

DISCUSSION

The presence of ESBLs, which is increasing in prevalence, has been observed in most species of *Enterobacteriaceae*. *K. pneumoniae*, causing both nosocomial and community-acquired infections, is the most common pathogen found to produce these ESBLs. However, they have also been found in *E. coli*, *Morganella morganii*, *Serratia* spp., *Shigella* spp., *Enterobacter* spp., *Salmonella* spp., *Proteus* spp., *Citrobacter* spp., and even *Pseudomonas aeruginosa* (4, 36). The spread of these plasmid-mediated enzymes presents a significant clinical problem due to resistance to extended-spectrum cephalosporins as well as current β-lactams and β-lactamase inhibitors (34). The specific increase in the percentages of *E. coli* and *K. pneumoniae* isolates producing ESBLs is causing limitations in treatment options for serious infections (12, 20, 27). Class C β-lactamases, once found to be encoded only on chromosomes, are now found to be encoded on plasmids (2, 37). The potential for transmission of class C enzymes between pathogens exists (13, 32) and could result in increased prevalence of these inhibitor-resistant β-lactamases.

The inhibitors described in this paper represent novel structures compared to the current commercially available penem

TABLE 6. ED_{50}s of combinations of piperacillin and penem molecules for murine acute lethal infections with various pathogens

Compound[b]	ED_{50} (mg/kg) (95% confidence limits)[a] for infection with:			
	TEM-1-producing *E. coli*	TEM-29- and OXA-1-producing *E. coli*	CMY-2-producing *E. coli*	AmpC-producing *Enterobacter aerogenes*
PIP alone	165.8 (120–231)	127.3 (60.7–227)	101.8 (38–430)	421 (191–1,648)
PIP-A	30.4 (22.5–41.4)	44.1 (31.1–66.9)	NT	NT
PIP-B	21.8 (15.9–29.7)	53.1 (33.5–108.4)	14.4 (10.8–19.3)	100.8 (49–463)
PIP-C	19.5 (13.9–26.9)	78.7 (64.3–96.7)	22.4 (15.9–34.2)	102 (50–243)
PIP-D	22.8 (16.3–31.7)	67.6 (39.2–129)	14.2 (8.9–19.4)	96.4 (46.2–185)
PIP-E	24.5 (16.5–38.2)	44.4 (31.2–67.5)	NT	98.1 (68.4–154)
PIP-F	20.0 (15.1–26.5)	37.4 (21.8–65.9)	15.1 (10.3–20.3)	125.3 (71.6–214)
PIP-G	31.8 (23.9–43.1)	37.6 (21.9–66.2)	14.9 (9.6–20.5)	123.2 (62.1–241)
PIP-TZB	46.4 (33.9–67.5)	NT	35.4 (24.1–54.5)	NT
LD_{50}[c] (CFU)	5×10^2	4.7×10^2	5.1×10^3	1.8×10^6

[a] The ED_{50}s listed correspond to the efficacy of piperacillin. NT, not tested.
[b] Piperacillin (PIP)-inhibitor at a 4:1 ratio. TZB, tazobactam.
[c] LD_{50}, 50% lethal dose of organism in each infection.

TABLE 7. Pharmacokinetic parameters of 6-methylidene penem molecules following intravenous administration at 150 mg/kg to male rats

Parameter[a]	Value for compound:						
	A	B	C	D	E	F	G
C_{max} (μg/ml)		345	490	538	72	152	417
$AUC_{0-\infty}$ (μg · h/ml)		75	163	70.1	31	32	50.9
V_{SS} (liters/kg)	NT[b]	0.45	0.39	0.4	1.0	2.5	0.32
CL (ml/min/kg)		52	15.4	35	81	80.7	54
MRT (h)		0.13	0.43	0.2	0.2	0.49	0.1

[a] CL, clearance; MRT, mean residence time.
[b] NT, not tested.

inhibitors. Tazobactam, clavulanic acid, and sulbactam all contain substitutions at the 2 position of their respective thiazole or oxazole rings. These new inhibitors contain bicyclic or tricyclic substituents connected by a methylidene linkage to the 6 position of the β-lactam ring. The unique structure of these compounds imparts potent activity against class A and C β-lactamases, particularly the AmpC enzyme, which is not observed with the other inhibitors (19, 22, 25, 33). The 6-methylidene compounds were equally potent for both the TEM-1 and AmpC enzymes (IC_{50}s of the compounds were between 0.4 and 3.1 nM for TEM-1 and 1.5 and 5.4 nM for AmpC). Comparable data from other studies for tazobactam, clavulanic acid, and sulbactam show them to be 10- to 100-fold less active against the TEM-1 enzyme and 2,000- to 40,000-fold less active against the AmpC and P99 (another class C) enzymes (14, 15, 33).

Activity against isolated β-lactamases has translated to whole-cell bacterial tests also. Each of the inhibitors was partnered with piperacillin. In vitro testing methodology was carried out with a constant 4-μg/ml concentration of each inhibitor in accordance with previously established protocols for piperacillin plus tazobactam (6, 16). Piperacillin plus compounds C, E, D, and G was more active than piperacillin plus tazobactam against ESBL-producing *E. coli*. The addition of these inhibitors rendered approximately 90% of the isolates tested susceptible to piperacillin. The activity of piperacillin was further enhanced over that of piperacillin plus tazobactam when piperacillin was combined with compounds B, E, and F against ESBL-producing *K. pneumoniae*. The methylidene inhibitors also restored the activity of piperacillin against *Enterobacter* spp., *Citrobacter* spp., and *Serratia* spp., all of which are known to produce class C enzymes (8, 17). The MICs of piperacillin plus each of the inhibitors for these strains were in the susceptible range (≤16 μg/ml) for 90% of those isolates tested.

Activity demonstrated in vitro by these combinations was confirmed in murine acute lethal infection models. The efficacy of piperacillin plus the methylidene inhibitors was enhanced compared to that of piperacillin alone against *E. coli* and *Enterobacter aerogenes* organisms producing class A (including ESBL) and class C enzymes. This increase in efficacy was demonstrated by a 1.6- to 8-fold decrease in the overall ED_{50} of piperacillin.

The addition of a bicyclic or tricyclic substitution via a methylidene linkage to the 6 position of the penem molecule confers activity as an inhibitor of class A and C β-lactamase enzymes. The activity is manifested both in vitro and in vivo in the enhancement of the activity and efficacy of piperacillin when administered in combination. The inhibitors all demonstrated equivalent potency in vitro and in vivo. Differences in MICs and ED_{50}s varied by approximately twofold for all the strains tested. Although some compounds were cleared more readily than others, this does not seem to correlate with a significant difference for in vivo efficacy. These new molecules offer an advantage over the current commercial inhibitors and warrant further investigation for development.

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