Arch. Pharm. Res. Vol.21, No.5, pp. 527-530, 1998

Comparative Activities of Novel β-Lactamase Inhibitors, 6-Exomethylene Penamsulfones (CH1240, CH2140) in Experimental Mouse Infection Model

Kye-Whan Park, Chul-Bu Yim and Ki-Ho Kim*

College of Pharmacy, Chung Ang University, Seoul 156-756, Korea

(Received April 30, 1998)

The antibacterial activity of novel β-lactamase inhibitors, 6-exomethylene penamsulfones (CH 1240, CH2140), has been compared *in vivo* with that of sulbactam and clavulanic acid against β-lactamase producing strains. *In vivo* microbiological assessment was used as experimental mouse infection model by gram negative strains. Against *Pseudomonas aeruginosa* F0013, cefoperazone/CH1240 was slightly less active than sulbactam. Ampicillin/CH 1240 was more active than sulbactam against *Citrobacter diversus* species. That of ampicillin/CH 2140 was less effective than sulbactam against *Escheriachia coli* 3457. Especially against *Citrobacter diversus* 2046E, amoxicillin/CH 2140 was the most potent and amoxicillin/CH1240 was slightly more active than clavulanic acid. Consequently the difference in efficacy between the drug combinations appears to be related to the degree of protection afforded the animals by the β-lactamase inhibitors. CH1240 and CH2140 are promising new agents and should undergo further investigations.

Key words : *In vivo*, Novel β-lactamase inhibitors, 6-Exomethylene penamsulfones, Antibacterial activity

INTRODUCTION

Since the first cures were achieved by penicillin in 1941, an expanding family of β-lactam antibiotics has been developed to combat bacteria (Sutherland, 1993). As a result of gene transfer and recombination, an increasing number of bacterial species produce β-lactamase, an enzyme that rapidly transform β-lactam into inactive metabolites by hydrolyzing the β-lactam bond. Although several mechanisms of bacterial resistance to β-lactam antibiotics have been described, enzymatic hydrolysis of the β-lactam ring was the best studied (Nikaido *et al.*, 1993; Smith *et al.*, 1969). Bacterial production of β-lactamase is ubiquitous in the Enterobactericeae and Pseudomonaceae (Nandivado *et al.*, 1990). The continuing problem of β-lactamase-mediated resistance has encouraged the researcher to search for β-lactamase inhibitors (Maddux, 1991). They can, in theory, be used in combination with β-lactam antibiotics in order to extend antibiotics spectrum of established compounds that are inactivated by the strains of bacteria which are resistant by virtue of organisms ability to produce β-lactamases (Donowitz *et al.*, 1988; Reguera *et al.*, 1991). Clavulanic acid and sulbactam were the first broad spectrum β-lactamase inhibitors with demonstrated activity *in vitro* and *in vivo* (Fassand *et al.*, 1989; Livermore, 1993). As there are so many such strains, the improvement of inhibitors is challenging in this area (Payne *et al.*, 1991).

In our former approach, novel β-lactamase inhibitory compounds, 6-exomethylene penamsulfones (CH1240, CH2140) were synthesized and the combination activities of the compounds were compared *in vitro* enzyme assay and antimicrobial inhibitory activities (Park *et al.*, 1997).

CH1240 (Disodium 6(Z)-6-[1-[1-(2-Nicotinate-2yl) thioethyl]1,2,3-triazol-4-yl]methylene)penicillanic acid 1,1-dioxide), CH2140 (Sodium 6(Z)-6-[1-[1[4-(benzoxazoly-2-yl)thiobutyl]-1,2,3-tria-zol-4-yl]methylene] Penicillanate-1,1-dioxide) are several of 6-exomethylene penam sodiums and they were synthesized from oligo intermediates including p-methoxybenzyl- ester of 6-exomethylenepenams (Fig. 1) (English *et al.*, 1986). Two samples were selected through enzyme assay and antimicrobial susceptibility test (Park *et al.*, 1997). In this approach, the antibacterial activities of the compounds were compared with that of the β-lactamase inhibitors against β-lactamase producing strains in ex-

Correspondence to: Ki-Ho Kim, College of Pharmacy, Chung Ang University, 221 Heuksuk-Dong, Dongjak-Ku, Seoul 156-756, Korea

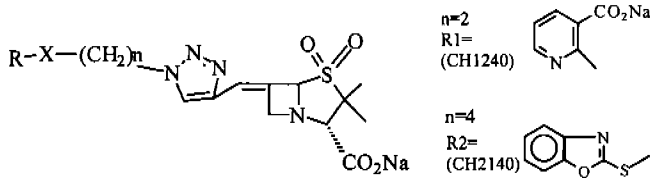

Fig. 1. Structure of heterocyclyl exomethylenepenam derivatives.

perimental mouse infection model. The purpose of this study was to compare the relative efficacy of sulbactam, clavulanate and novel β-lactamase inhibitors, 6-exomethylene penam sulfones to β-lactams (ampicillin trihydrates, amoxicillin-Na, cefoperazone) in experimental mouse infection model (Basker *et al.*, 1979; Uri *et al.*, 1976).

MATERIALS AND METHODS

Antimicrobials

Antimicrobials were obtained as follows: Ampicillin trihydrates (m.w. 463.4), cefoperazone (m.w. 667.6) and amoxicillin sodium (m.w. 387.4) were purchased from Sigma Co. (USA). Sulbactam was supplied from Pfizer Co. (Korea). Clavulanate-K was obtained from Smithkline-Beecham Co. Samples (CH1240: m.w. 536.3, CH2140: m.w. 539.7) were synthesized by author at Medicinal Chemistry Laboratory in College of Pharmacy, Chung Ang University. All of the compounds were stored at 4°C under desiccation and all of them were diluted to final concentration with PBS (pH 7.4). Also β-lactamase inhibitors were dissolved and diluted in saline (NCCLS, 1990).

Bacterial strains

The following strains were tested: *Pseudomonas aeruginosa* F0013 (unknown β-lactamase producing strain), *Citrobacter diversus* 2046E (CHR IND+VE), *Escherichia coli* ML4901/Rms 213 (Va), *Escherichia coli* 3457 (TEM-9) and they are β-lactamase producing strains. The four strains that showed good activities in MIC test and enzyme assay *in vitro* by 6-exopenam sulfones compounds including CH1240 and CH2140 were selected (Park *et al.*, 1997). They were supplied from LG Chemicals in Korea.

Cultures

The cultures used were mainly β-lactamase producing strains. By using 0.5 Mcfarland standard solution, cultures turbidity was adjusted at 10^8 CFU. This culture was diluted as inoculum strains by NCCLS (National Committee for Clinical Laboratory Standard) method (NCCLS, 1990). They were subcultured over two times before using in animal experiments (Comber *et al.*, 1979).

Animals

ICR strain male albino mice (body weight: 18~30 g) were used throughout (supplied from Chung Ang Animal or Sam Yuk Experimental Animal).

Quantitation of lethal inocula in mice (Virulence Titration)

Overnight broth cultures of the test organisms were serially diluted with fresh broth and 0.1 ml aliquots of each dilution were plated on Mueller-Hinton agar. Mice were injected by the intraperitoneally route with 0.5 ml of a suspension in hog gastric mucin (Difco. Co.). Survivors were counted daily over four days (at least 4~7 days) for viable count (Comber *et al.*, 1979). The inoculum required to kill 50% of the test animals (LD_{50}) was calculated by the method of Litchfield and Wilcoxon (Basker *et al.*, 1979).

Protection by β-lactam antibiotics-β-lactamase inhibitor combinations

Acute systemic infections in mice were injected by the intraperitoneal route with 0.5 ml of a suspension of an overnight cultured broth of the test organism in hog gastric mucin (Difco Co.) standardized to give an infective inoculum of 10~100 median lethal doses (Uri *et al.*, 1976). The median lethal doses were obtained each group as 10 mice at each dose level. Each mice was treated SC starting 0.5 hr after bacterial challenge and subsequently from 2, 4, 6, 8 and 24 hrs. (Comber *et al.*, 1979). The dosage regimens consisted of 4 over different concentrations of antibiotics in two-fold dilution series administered to mice per dosage. Each group was organized as 6 mice. Percent survival was recorded after at least four days observation period. After three or four experiments were completed, data on survival were average and PD_{50} (mg/kg per dose) was calculated. Each experiment was prepared as the ratio of β-lactam and β-lactamase inhibitor as 1:1 cefoperazone/sulbactam), 2:1 (ampicillin/sulbactam), 4:1 (amoxicillin/clavulanic acid) combination (Thomson, 1995). The numbers of animals surviving 4 days after infection were recorded. The method of *Litchfield* and *Wilcoxon* was used to calculate the 95% confidence limit (Basker *et al.*, 1979).

RESULTS

Lethality of test strains

The LD_{50}s for *Citro. diversus* 2046E, *E. coli* 3457E, *E. coli* ML4901/Kms 213 and *Ps. aeruginosa* F0013 were 4×10^7, 1×10^8, 9×10^6 and 6×10^6 cfu/mouse,

Table I. Virulence titration (lethal 50% determination)

Infecting organisms	LD_{50} cells/ mouse	95% confidence limits	
		Lower C. L.	Upper C. L.
Citrobacter diversus 2046E	4×10^7	8×10^6	9×10^7
Escherichia coli 3457E	1×10^8	1×10^7	9×10^9
Escherichia coli ML4901/Rms	9×10^6	2×10^6	6×10^7
Pseudomonas aeruginosa F0013	6×10^6	1×10^6	2×10^7

Values are means of four experiments.

respectively. The inocula used in the subsequent experiments varied from 10 to 100 times (Table I). Later in PD_{50} experiment, *E. coli* ML4901/Kms 213 excluded from test strains.

Protection studies

In experimental *Ps. aeruginosa* infections, sulbactam combined with non-protective doses of cefoperazone. It provided protection. However, CH1240 didn't provide protection (Table II). The relative activity ratios (PD_{50}) were 1 (cefoperazone): 1.10 (cefoperazone/sulbactam): 8.28 (cefoperazone/CH1240). Sulbactam was more effective than CH1240 against *P. aeruginosa*. In experimental *E. coli* 3457E infections, sulbactam combined with non-protective doses of ampicillin provided protection. The PD_{50} of CH2140 was more less than sulbactam (Table III).

In the case of experimental *C. diversus* infections, sulbactam was combined with ampicillin. That protection relative per cent was high score. It was regarded that combined regimen was hardly protection against this strains. But CH1240 combined with ampicillin provided protection against *E. coli* 3457E. Then PD_{50} of ampicillin/CH1240 was 59.14% against ampicillin alone (Table IV).

When amoxicillin was combined with clavulanic acid, CH1240 and CH2140 as two-fold diluted doses. Both CH1240 and CH2140 were more effective than clavulanic acid. The effect of CH1240 had 97.07% as PD_{50} against clavulanic acid combined. That dose was 47.68% against amoxicillin alone. On the other hand, the combination of CH2140/amoxicillin was superior to clavulanate/amoxicillin (relative per cent= 47.68%). That dose observed was only 19.70% against PD_{50} of amoxicillin alone. To confirm the most effect, this experiment was continued until four times (Table V).

Table II. Chemotherapeutic activity of cefoperazone/sulbactam (1 : 1) against experimental infection with *P. aeruginosa* F 0013 in mice

Antibiotics	PD_{50} (mg/kg)	95% confidence limits	
		Low C. L.	Upper C. L.
Cefoperazone	256.90	91.552	720.90
Cefoperazone/sulbactam	212.68	64.514	701.13
Cefoperazone/CH1240	283.44	98.801	813.11

Values are means of three experiments.

Table III. Chemotherapeutic activity of ampicillin/ sulbactam (2 : 1) against experimental infection with *E. coli* 3457E in mice

Antibiotics	PD_{50} (mg/kg)	95% confidence limits	
		Low C. L.	Upper C. L.
Ampicillin	49.502	19.485	125.77
Ampicillin/sulbactam (2 : 1)	25.206	5.7320	113.50
Ampicillin/CH2140 (2 : 1)	92.550	29.992	285.60

Values are means of three experiments.

Table IV. Chemotherapeutic activity of ampicillin/sulbactam (2 : 1) against experimental infection with *C. diversus* 2046E in mice

Antibiotics	PD_{50} (mg/kg)	95% confidence limits	
		Low C. L.	Upper C. L.
Ampicillin	82.812	43.456	157.81
Ampicillin/sulbactam (2 : 1)	283.44	77.964	1030.4
Ampicillin/CH1240 (2 : 1)	48.979	10.528	227.86

Values are means of three experiments.

Table V. Chemotherapeutic activity of amoxicillin/ clavulanic acid (4 : 1) against experimental infection with *C. diversus* 2046E in mice

Antibiotics	Infecting organism	95% Confidence limits	
	PD_{50} (mg/kg)	Low C. L.	Upper C. L.
Amoxicillin	251.98	94.290	673.41
Amoxicillin/clavulanic acid (4 : 1)	104.09	23.391	463.12
Amoxicillin/CH1240 (4 : 1)	101.01	39.756	256.62
Amoxicillin/CH2140 (4 : 1)	49.629	28.491	86.448

Values are means of three experiments

DISCUSSION

Among experimental results a outstanding point is when combined with amoxicillin, clavulanic acid has been successfully used in the treatment of infections caused by β-lactamase producing organisms. Furthemore, sulbactam and tazobactam as well as clavulanic acid have been used as good β-lactamase inhibitors. However, there have been the resistances of combination therapy (Fassand, 1989; Livermore, 1993).

CH1240 and CH2140, 6-exomethylene penamsul-

fones, were potentially useful β-lactamase inhibitor as shown by the results of several screening experiments *in vitro* and *in vivo* (Park *et al.*, 1997). Especially, when combined with amoxicillin, CH2140 synergistically inhibited β-lactamase producing strains of *C. diversus* and CH1240 was somewhat more effective than clavulanic acid. The *in vitro* synergy of combination of the β-lactam and β-lactamase inhibitor against the four β-lactamase producing organisms used in this experiment were superior to sulbactam, clavulanic acid (Park *et al.*, 1997).

As a result, the superiority of amoxicillin-CH2140/CH1240 combinations against *C. diversus* appear to relate to the degree of protection provided by amoxicillin alone compared to that of clavulanate. These compunds would be potentially useful agents and the activity of combination against β-lactamase producing organisms should undergo further testing.

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