SYN-1012: A New β-Lactamase Inhibitor of Penem Skeleton

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A new β-lactamase inhibitor, SYN-1012, with a penem skeleton was synthesized and its biological activity compared with clavulanic acid, sulbactam, tazobactam and BRL-42715. The β-lactamase inhibitory activity of SYN-1012 was comparable to BRL-42715. Clavulanate and penam sulphones (sulbactam and tazobactam) were more active against TEM-1 and OXA-1, but were less active against TEM-3 and cephalosporinase (Case) than SYN-1012. In combination with piperacillin, SYN-1012 exhibited comparable or slightly lower synergistic effects than BRL-42715 against all the Gram-positive and Gram-negative isolates tested with only exception of *Pseudomonas aeruginosa*. The separate combinations of SYN-1012 and BRL-42715 with ceftazidime and cefotaxime provided comparable results against Gram-negatives, but not against Gram-positive isolates. Tazobactam was inferior to SYN-1012 in all cases. In comparison to tazobactam, SYN-1012 and BRL-42715 were relatively unstable in human and mouse plasma, and in mouse liver and kidney homogenates. Serum level of SYN-1012 and BRL-42715 after an intravenous administration of 20 mg/kg in rabbit was undetectable after 1 hour.

The most commonly prescribed antimicrobial agents in the United States are the β-lactam antibiotics, and over time many bacterial species have become resistant to these agents[1]. The most common mechanism of bacterial resistance to β-lactam antibiotics is their inactivation by β-lactamases[2]. The strategy to counter β-lactamase activity by the use of β-lactam antibiotics resistant to β-lactamases has not been very successful, because the extensive and indiscriminate usage of such agents has been shown to confer high levels of resistance in bacteria. However, the use of inactivators for β-lactamases has extended the clinical utility of β-lactamase-susceptible penicillins[2,3]. The success of clavulanic acid and the recent introduction of other classes of β-lactamase inhibitors such as sulbactam and tazobactam appear to be of great benefit. Unfortunately, the emergence of bacteria producing new extended spectrum β-lactamases including the plasmid and chromosomally mediated AmpC-types poses further threat to the armamentarium of β-lactam antibiotics[4~9]. Therefore, the need for broad-spectrum β-lactamase inhibitors with suitable physico-chemical and pharmacodynamic profile for oral as well as parenteral use remains a subject of high interest among medicinal chemists.

The extraordinary β-lactamase inhibitory activity of penem skeleton, as seen in the BRL-42715 and its analogs[10~14], prompted us to prepare related penem derivatives for biological evaluation. Some of our earlier work had focused on synthesis of penam sulphones with substitutions similar to that of BRL-42715 series at C-6 position[15,16]. Thereafter, we sought to introduce a substituted conjugated double bond at C-6 position of the penem nucleus in an effort to further define the structural parameters for favorable β-lactamase inhibitory and pharmacokinetic profile. Of several compounds prepared we selected SYN-1012 for extensive studies. Although SYN-1012 has the same methyltriazolyl substitution at C-6 position as present in BRL-42715, but the side chain linking the triazolyl moiety at C-6 is two carbon atoms longer with an additional conjugated double bond in SYN-1012. In the present paper, we report a comparative β-lactamase inhibitory activity, synergistic activity with known β-lactams and pharmacokinetic properties of a new compound, SYN-1012, and two well-known compounds, BRL-42715 and tazobactam.

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Materials and Methods

Synthesis of SYN-1012: Sodium (5*R*)-6-[(*Z*,*E*)-{3-(1-Methyl-1*H*-1,2,3-triazol-4-yl)}-2-propenylidene]-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-en-2-carboxylate

The 6-bromopenem-3-carboxylic acid prepared according to the literature procedure[14] was reacted with sodium bicarbonate and allyl iodide in DMF to give allyl-6-bromopenem-3-carboxylate (yield 26%). The reaction of the allyl-6-bromopenem-3-carboxylate with lithium diphenylamide, β(*E*)-(1-methyl-1,2,3-triazole-4-yl)acrolein[17], and acetic anhydride gave the diastereoisomeric acylated bromohydrins (yield 39%). Reductive elimination of the acylated bromohydrins using powdered zinc in THF afforded the allyl ester of SYN-1012 (yield 32%) and the other geometric isomer (yield 35%). Deprotection of the allyl ester of SYN-1012 using tetrakis(triphenylphosphine)palladium, triphenylphosphine and sodium-2-ethylhexanoate in a mixture of ethyl acetate and dicloromethane (1 : 1) gave SYN-1012 (yield 92%) after purification

Synthesis of BRL-42715

BRL-42715 was synthesized by the literature procedure[15].

Bacterial Strains

Clinical isolates were collected between 1987 ~ 92 from various medical centers in Japan. Identification of each culture was done by conventional methods: Gram-negative bacilli by API 20E (Analytab Products, Plainview, NY.) and NF systems (Remel, Lenexa, Kans.), and staphylococci by Staph Trac (Analytab Products). All isolates were stored frozen in skim milk at −80°C.

Media and Chemicals

All media were prepared in distilled deionized (DI) water. Mueller-Hinton (MH) medium was purchased from Becton Dickinson Microbiology Systems, Cockeysville, MD. All control antimicrobial agents were purchased from Sigma Chemical Co., St. Louis, MO. Clavulanic acid was isolated and purified from the marketed formulation. Tazobactam, sulbactam, SYN-1012 and BRL-42715 were synthesized in our laboratory.

In Vitro Susceptibility Testing

The *in vitro* antibacterial activities were determined by the agar dilution method, as recommended by the National Committee for Clinical Laboratory Standards[18]. Mueller-Hinton II agar (MHA) plates containing the antibiotics at 0.10 to 200 μg/ml (alone or in combination with 5 μg/ml of the β-lactamase inhibitor) were prepared. Inocula were adjusted to a density of 10^7 CFU/ml and approximately 3 μl were applied to the agar surface with a Steers replicator. The test plates were incubated at 37°C for 18 hours. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of antimicrobial agent that completely inhibited visible growth of the organism.

Isolation of β-Lactamases

The overnight culture grown in MHB was diluted 100-fold into the same broth that was prewarmed at 37°C. The diluted culture (100 ml in 500 ml Erlenmeyer flask) was incubated for 4 hours at 37°C and 200 rpm. The cells were cetrifuged at 15,000 *g* for 15 minutes at 37°C, the pellet was washed once with 50 mM phosphate buffer (pH 7.0) and resuspended (10^{10} cells/ml) in the buffer. The cells were disrupted by sonication (3 × 30 seconds pulse at 0°C) and cell debris was removed by centrifugation (15,000 *g*, 15 minutes, 4°C). The supernatant was used as the crude extract containing the β-lactamase.

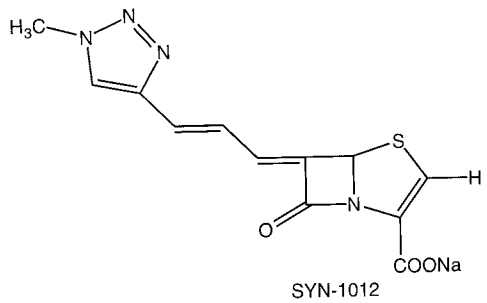
β-Lactamase Inhibitory Activity

The β-lactamase inhibitory activity was performed by UV spectrophotometry using freshly prepared antibiotic solutions in 50 mM phosphate buffer at pH 7. The assay was run at 30°C, and the following wavelengths were used; 235 nm for ampicillin and 260 nm for cephaloridine. At the various concentrations of inhibitors (up to 10 μg/ml), tazobactam, sulbactam, clavulanic acid, SYN1012, and BRL42715 were tested for the inhibitory activity of the cephaloridine hydrolysis rate for TEM-3 (CTX-1, cefotaximase from *K. pneumoniae*), Case (cephalosporinase form *E. cloacae* P99) or the ampicillin hydrolysis rate for TEM-1, PCase Type I, and OXA-1 from different strains of *E. coli*.

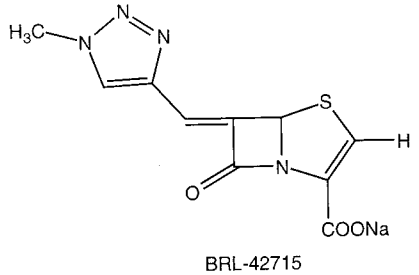
Stability in Plasma, Liver Homogenate, and Kidney Homogenate

Male ddy mice weighing 18 ~ 22 g were purchased from SLC, Shizuoka, Japan. Blood was collected from the cut axillary region of humanely killed mice. Mouse kidney

Fig. 1. Chemical structure of SYN-1012 and BRL-42715.



SYN-1012



BRL-42715

Table 1. Comparative β-lactamase inhibitory activity of SYN-1012 and known inhibitors.

Enzyme type	Enzyme activity (U/ml)	Inhibitor	Inhibition (%) of β-lactamase activity			
			Inhibitor concentration (μg/ml)			
			0.001	0.01	0.1	1.0
TEM-1	1.84	SYN-1012	17.9	36.4	100	100
(*E. coli*)		BRL-42715	14.1	54.9	100	100
		Tazobactam	17.4	40.8	96.2	100
		Sulbactam	17.4	19.0	21.7	65.8
		Clavulanic acid	15.8	20.1	34.8	100
OXA-1	1.82	SYN-1012	12.4	52.7	100	100
(*E. coli*)		BRL-42715	22.0	50.8	98.8	100
		Tazobactam	18.1	24.2	20.9	17.6
		Sulbactam	14.8	19.8	18.1	19.8
		Clavulanic acid	22.5	16.5	13.8	27.3
TEM-3	0.154	SYN-1012	0	4.5	27.3	83.1
(*K. pneumoniae*)		BRL-42715	5.8	1.3	28.0	77.3
		Tazobactam	6.5	12.3	55.5	99.1
		Sulbactam	20.2	15.3	51.9	100
		Clavulanic acid	NT	NT	NT	NT
Case	1.82	SYN-1012	5.9	16.1	37.9	98.4
(*E. cloacae*)		BRL-42715	5.0	10.2	67.9	100
		Tazobactam	10.7	4.1	14.8	6.6
		Sulbactam	4.1	3.3	11.1	3.3
		Clavulanic acid	NT	NT	NT	NT

Table 2-1. Synergistic effect of SYN-1012 and selected β-lactamase inhibitors in combination with β-lactam antibiotics.

Organism (No. of strains)	Antibiotic +inhibitor	Range	MIC (μg/ml)	
			50%	90%
MRSA (15)	Piperacillin	200 ~ >200	200	>200
	+SYN-1012	6.25 ~ 200	25	100
	+BRL-42715	0.78 ~ 100	3.13	100
	+tazobactam	6.25 ~ 200	25	100
	Ceftazidime	25 ~ >200	100	>200
	+SYN-1012	25 ~ >200	50	>200
	+BRL-42715	3.13 ~ >200	25	>200
	+tazobactam	12.5 ~ >200	50	>200
	Cefotaxime	12.5 ~ >200	25	>200
	+SYN-1012	6.25 ~ >200	25	>200
	+BRL-42715	0.78 ~ >200	6.25	>200
	+tazobactam	6.25 ~ >200	50	>200
	SYN-1012	10 ~ >200	200	>200
	BRL-42715	25 ~ >200	50	>200
	Tazobactam	>200	>200	>200
MRSE (7)	Piperacillin	12.5 ~ >200	200	>200
	+SYN-1012	0.39 ~ 200	25	200
	+BRL-42715	0.1 ~ 100	12.5	100
	+tazobactam	1.56 ~ 200	12.5	200
	Ceftazidime	12.5 ~ >200	100	>200
	+SYN-1012	3.13 ~ >200	100	>200
	+BRL-42715	0.1 ~ >200	12.5	>200
	+tazobactam	1.56 ~ 200	12.5	200
	Cefotaxime	3.13 ~ >200	50	>200
	+SYN-1012	3.13 ~ >200	50	>200
	+BRL-42715	0.1 ~ >200	25	>200
	+tazobactam	6.25 ~ >200	50	>200
	SYN-1012	50 ~ >200	>200	>200
	BRL-42715	12.5 ~ >200	200	>200
	Tazobactam	>200	>200	>200

Table 2-2. Synergistic effect of SYN-1012 and selected β-lactamase inhibitors in combination with β-lactam antibiotics.

Organism (No. of strains)	Antibiotic +inhibitor	Range	MIC (μg/ml)	
			50%	90%
E. cloacae (5)	Piperacillin	3.13 ~ >200	100	>200
	+SYN-1012	3.13 ~ 50	6.25	50
	+BRL-42715	1.56 ~ 25	6.25	25
	+tazobactam	1.56 ~ 200	25	200
	Ceftazidime	0.39 ~ >200	50	>200
	+SYN-1012	0.2 ~ 3.13	3.13	50
	+BRL-42715	0.2 ~ 3.13	0.78	3.13
	+tazobactam	0.39 ~ >200	6.25	>200
	Cefotaxime	0.1 ~ >200	100	>200
	+SYN-1012	0.1 ~ 50	3.13	50
	+BRL-42715	0.1 ~ 6.25	0.39	6.25
	+tazobactam	0.1 ~ >200	50	>200
	SYN-1012	>200	>200	>200
	BRL-42715	200 ~ >200	>200	>200
	Tazobactam	>200	>200	>200
E. aerogenes (6)	Piperacillin	25 ~ 100	50	100
	+SYN-1012	6.25 ~ 50	6.25	50
	+BRL-42715	3.13 ~ 25	6.25	25
	+tazobactam	12.5 ~ 50	25	50
	Ceftazidime	12.5 ~ 100	25	100
	+SYN-1012	0.78 ~ 6.25	1.56	6.25
	+BRL-42715	0.39 ~ 0.78	0.78	0.78
	+tazobactam	12.5 ~ 100	25	100
	Cefotaxime	6.25 ~ 50	12.5	50
	+SYN-1012	0.2 ~ 6.25	1.56	6.25
	+BRL-42715	0.1 ~ 6.25	0.1	6.25
	+tazobactam	12.5 ~ 50	25	50
	SYN-1012	>200	>200	>200
	BRL-42715	200 ~ >200	>200	>200
	Tazobactam	>200	>200	>200
S. marcescens (6)	Piperacillin	>200	>200	>200
	+SYN-1012	6.25 ~ 50	6.25	50
	+BRL-42715	1.56 ~ 12.5	3.13	12.5
	+tazobactam	50 ~ >200	100	>200
	Ceftazidime	0.78 ~ 1.56	0.78	1.56
	+SYN-1012	0.78 ~ 1.56	0.78	1.56
	+BRL-42715	0.78 ~ 1.56	0.78	1.56
	+tazobactam	0.78	0.78	0.78
	Cefotaxime	0.78 ~ 1.56	0.78	1.56
	+SYN-1012	0.39 ~ 1.56	0.39	1.56
	+BRL-42715	0.39 ~ 1.56	0.39	1.56
	+tazobactam	0.78 ~ 3.13	0.78	3.13
	SYN-1012	>200	>200	>200
	BRL-42715	>200	>200	>200
	Tazobactam	>200	>200	>200
P. aeruginosa (9)	Piperacillin	6.25 ~ >200	6.25	>200
	+SYN-1012	3.13 ~ 200	6.25	200
	+BRL-42715	6.25 ~ 12.5	6.25	12.5
	+tazobactam	6.25 ~ >200	6.25	>200
	Ceftazidime	1.56 ~ 100	3.13	100
	+SYN-1012	1.56 ~ 50	3.13	50
	+BRL-42715	1.56 ~ 6.25	3.13	6.25
	+tazobactam	1.56 ~ 100	3.13	100
	Cefotaxime	12.5 ~ >200	25	>200
	+SYN-1012	12.5 ~ >200	25	>200
	+BRL-42715	12.5 ~ 50	12.5	50
	+tazobactam	12.5 ~ >200	25	>200
	SYN-1012	>200	>200	>200
	BRL-42715	>200	>200	>200
	Tazobactam	>200	>200	>200

homogenate, liver homogenates, plasma, and human plasma from a healthy volunteer were prepared in 1/15 M phosphate buffer (pH 7.0). Compounds were mixed in at 20 μg/ml concentration and incubated at 37°C. Samples were analyzed for the compounds remaining at 0, 30, 60, 120, and 240 minutes. The sample (200 μl) was mixed with an equal volume of acetonitrile (CH_3CN) and filtered through an ultrafilter (YM10 membrane, Amicon Corp., Beverly, Mass) to remove the unwanted proteins (cutoff, 10 kDa). The filtrate was analyzed by HPLC using TSK-gel, ODS-80Tm column (Tosoh, Tokyo, Japan) and 25 % CH_3CN with 0.01% trifluoroacetic acid as the eluent at a flow rate of 1 ml/minute. The experimental data were fitted to the 1-exponential equation by using the nonlinear regression program, MULTI (YAMAOKA, 1981)[19].

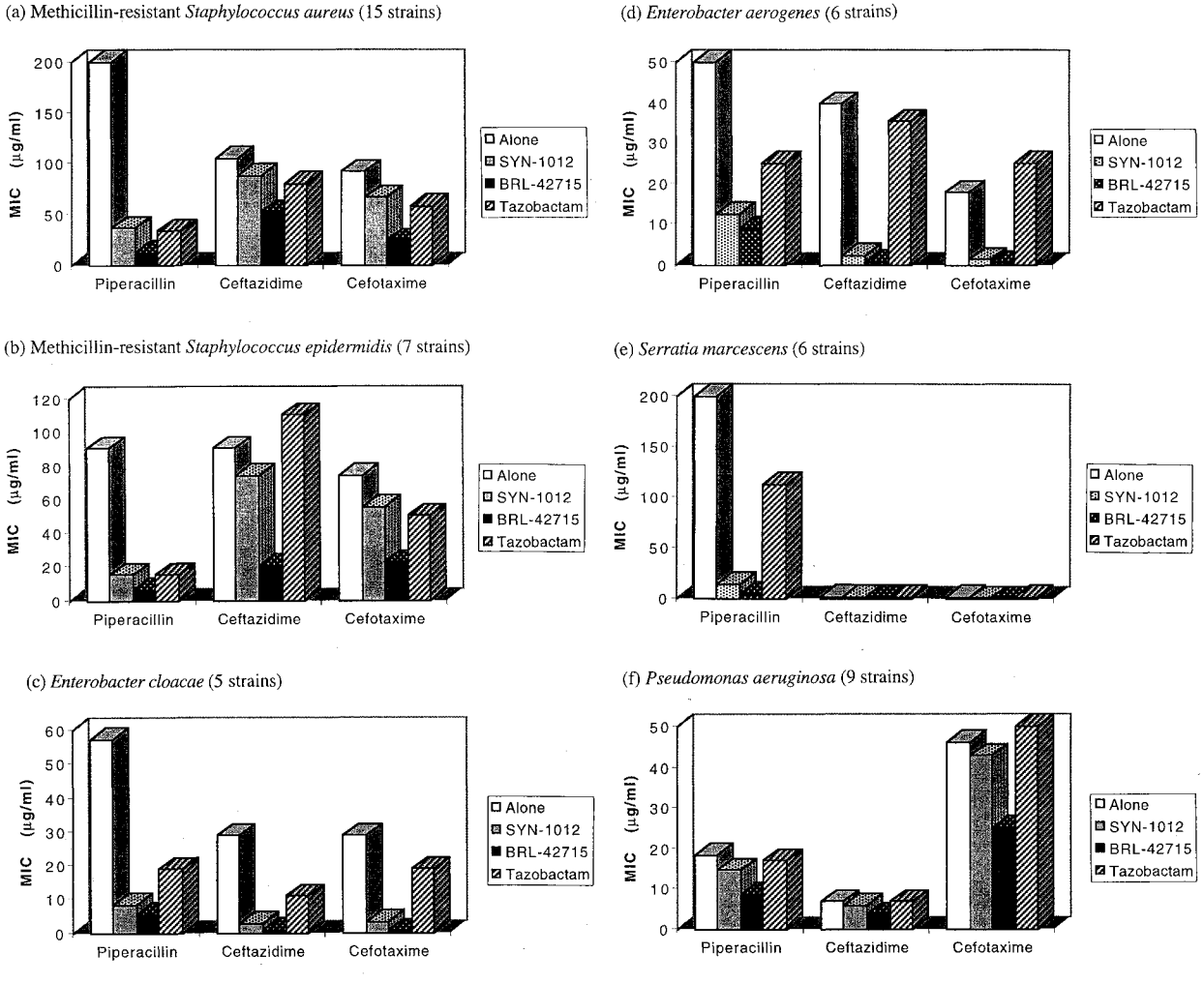
Pharmacokinetic Studies in Rabbit

SYN-1012 and BRL-42715 were dissolved in saline for intravenous (iv) administration. Male NZW rabbits (Nihon Nosan Kogyo K. K., Kanagawa, Japan) weighing 3.88 and 4.01 kg were injected (iv) with the drug (20 mg/kg body weight). Blood samples were taken at 0, 5, 15, 30, 60, 120, 240 minutes post iv administration. The blood sample (200 μl) was analyzed by HPLC method as described above.

Results and Discussion

SYN-1012 (Fig. 1), a new β-lactamase inhibitor with a penem skeleton, was synthesized by a synthetic route similar to that of BRL-42715. The biological activities were compared with well-known inhibitors such as clavulanic acid, sulbactam, tazobactam and BRL-42715. The comparative β-lactamase inhibitory activity of SYN-1012 and other known inhibitors against selected enzymes are given in Table 1. Inhibitory activity against various β-lactamases varied depending upon the nature of the molecule and the enzyme. The order of their activities were as follows: TEM-1 enzyme: sulbactam $\geq$ clavulanic acid > SYN-1012 > tazobactam $\geq$ BRL-42715; OXA-1: clavulanic acid > sulbactam > tazobactam > SYN-1012 > BRL-42715; TEM-3 (cefotaximase): BRL-42715 $\geq$ SYN-1012 > tazobactam > sulbactam; Case (ceph-

Fig. 2. Mean MIC (μg/ml) values of β-lactam antibiotics: alone and in combination with 5 μg/ml of β-lactamase inhibitors.



alosporinase): SYN-1012 > BRL-42715 > sulbactam > tazobactam. It is apparent that the penam sulphones (sulbactam and tazobactam) were superior inhibitors of TEM-1 and OXA-1, but the penems (SYN-1012 and BRL-42715) were better against TEM-3 and cephalosporinase.

The antibacterial activity of inhibitors and three β-lactam antibiotics were determined separately and in combinations by the agar dilution method. MICs of these antibiotics were reduced by several fold when tested in combination with the inhibitor. The synergistic effect varied depending upon the antibiotic and the organism involved (Table 2 and Fig. 2a~f). In combination with piperacillin, SYN-1012 exhibited a comparable or slightly lower synergistic effect than BRL-42715 against all the Gram-positive and Gram-negative isolates tested with the only exception of *Pseudomonas aeruginosa*, against which BRL-42715 was the most active inhibitor. The difference in the activity of SYN-1012 and BRL-42715 against the pseudomonads could have been either due to their different permeability profile through the outer membrane or due to their differencce in β-lactamase inhibitory activity. We could not establish the exact reason for their difference in activity. The combination of SYN-1012 with ceftazidime and cefotaxime provided comparable results to that of BRL-42715 combinations against Gram-negative isolates, but BRL-42715 was the best inhibitor against Gram-positive isolates. Tazobactam was inferior to SYN-1012 in all cases.

In comparison to tazobactam, SYN-1012 and BRL-42715 were relatively unstable in human and mouse plasma, and in mouse liver and kidney homogenates (Fig. 3). SYN-1012 and BRL-42715 were similar in their instability in mouse plasma and mouse liver homogenate (Fig. 3a and 3c), but SYN-1012 was marginally more stable in human plasma and in mouse kidney homogenate (Fig. 3b and 3d). The stability of BRL-42715 analogs in human kidney homogenate has been reported to vary considerably[12]. In general 5-R enatiomers were more active than 5-S enatiomers. Although the 5-R enantiomer of BRL-42715 has been reported to be relatively stable in human kidney homogenate, it degraded rather rapidly in mouse kidney homogenate when tested in our laboratory.

Fig. 3. Stability of SYN-1012, BRL-42715 and tazobactam in mouse plasma (a), human plasma (b), mouse liver homogenate (c), and mouse kidney homogenate (d).

○ SYN-1012, □ BRL-42715, △ tazobactam.

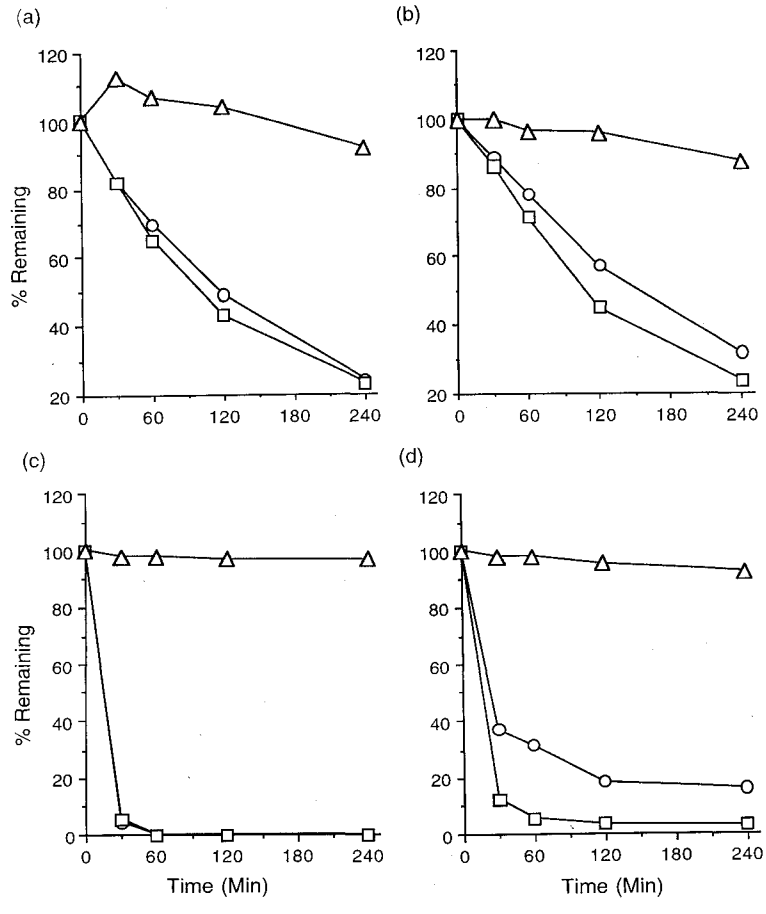
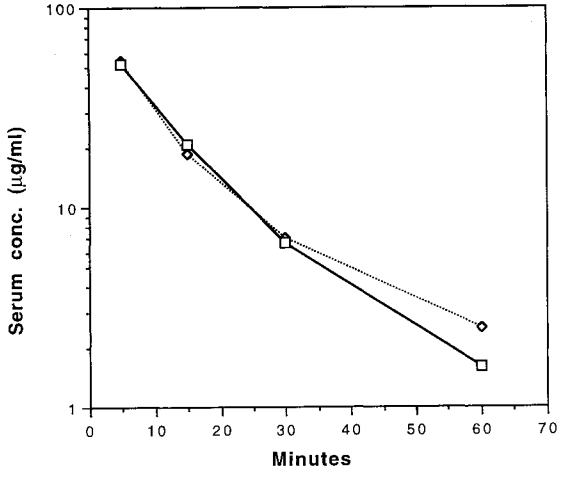


Fig. 4. Serum level of SYN-1012 and BRL-42715 after dosing 20 mg/kg (iv) in rabbit.

□ SYN-1012, ◇ BRL-42715.



Pharmacokinetic studies of SYN-1012 and BRL-42715 were done in male NZW rabbits after intravenous administrations of 20 mg/kg body weight. Serum levels of SYN-1012 and BRL-42715 were found to recede rapidly and were undetectable after 1 hour (Fig. 4).

Although SYN-1012 has very good β-lactamase inhibitory activity its instability in the plasma, kidney and liver homogenates, and fairly short $T_{1/2}$ do not seem to favor further development of this compound.

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