STUDIES ON NEW DEHYDROPEPTIDASE INHIBITORS

III. BIOLOGICAL PROPERTIES OF WS1358A1

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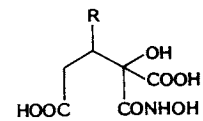
WS1358A1, a novel inhibitor of renal dehydropeptidase (DHP), augmented the urinary recovery of a carbapenem antibiotic imipenem and improved its protective effect in experimental infections when simultaneously administered to mice with the antibiotic.

WS1358A1 was a competitive DHP inhibitor with a *Ki* value of 1.6×10^{-7} M.

In the previous papers[1,2], we reported novel and potent renal dehydropeptidase (DHP-I, EC 3.4.13.11, renal dipeptidase) inhibitors of microbial origin, WS1358A1 and B1. Especially, WS1358A1 has a potent inhibitory activity comparable to that of cilastatin which is a clinically used DHP inhibitor in the antimicrobial combination imipenem/cilastatin[3].

To ascertain the possibility that WS1358A1 can be used as a counterpart in a combination therapy, a preliminary pharmacological evaluation in experimental animals was carried out.

In this paper, we describe the enzyme kinetic study as well as the pharmacological evaluation of WS1358A1.



WS1358A1 $R = CH_3$
WS1358B1 $R = H$

Materials and Methods

Enzyme Assay

Renal DHPs from several species of animal were partially purified by the method described below for porcine renal DHP except that the procedures following the $(NH_4)_2SO_4$ precipitation were omitted.

The effect of WS1358 compounds against the partially purified DHPs was measured by a method similar to that used for porcine DHP[1]. Moreover, to ascertain the specificity of inhibitory activity, the effects of WS1358 compounds against metallo-enzymes other than DHP, such as carboxypeptidase A (CPase A) and leucine aminopeptidase (LAP) were determined. CPase A was assayed by using carbobenzoxyglycyl-L-phenylalanine as substrate. The activity was determined by observing the decline in absorbance at 224 nm. LAP was assayed by using L-leucine-*p*-nitroanilide as substrate. The activity was determined by observing the decline in absorbance at 405 nm. Inhibitory effects on the two enzymes were calculated in the same manner as those on DHP[1].

Purification of Porcine Renal DHP

Purification of DHP from porcine kidney cortex to apparent homogeneity as judged by SDS polyacrylamide gel electrophoresis was carried out using procedures slightly different from those previously reported[4~6].

The cortex was sliced from the intact kidney and homogenized in 25 mM sodium phosphate buffer (pH 8.0) at 4°C in a Polytron homogenizer. The homogenate was adjusted to 20% butanol by the addition of butanol which had been chilled to −20°C. Solubilization of the enzyme was achieved by stirring the

mixture at 4°C overnight. After dialysis for 24 hours against several changes of distilled water, the enzyme activity was concentrated by precipitation between 50~70% $(NH_4)_2SO_4$. This partially purified enzyme was used to search for DHP inhibitors in fermentation broths. Further purification of renal DHP was achieved by means of a HPLC and an affinity column chromatography. HPLC of the $(NH_4)_2SO_4$ fraction was carried out on a TSK SW3000 column using a Waters model 6000 HPLC chromatograph with a Waters model 440 absorbance detector and a Hitach 200 recorder. Affinity column chromatography was performed using a procedure reported by KROPP *et al.*[5]. The DHP inhibitor, cilastatin, was coupled to cyanogen bromide-activated Sepharose 4B following the manufacturer's instruction. The activity of the enzyme at various stages of purification was determined by the method described below.

Enzyme Kinetic Study

The activity of DHP was determined by measuring the rate of enzyme-catalyzed hydrolysis of the unsaturated dipeptide glycyldehydrophenylalanine (GDP) at 37°C. The fall in absorbance of a solution of 5×10^{-5} M of the peptide in 25 mM Tris-HCl buffer (pH 7.7) was measured at 275 nm. Protein concentrations were determined by the method of BRADFORD[7]. Enzyme units are expressed as μmol of substrate hydrolyzed/minute, and specific activity is expressed as μmol of substrate hydrolyzed/minute/mg of protein under the assay conditions described above.

The rates of enzyme-catalyzed hydrolysis of GDP and imipenem in the absence or the presence of an inhibitor were measured in a similar manner. The enzyme concentrations employed in the reactions were 0.1 μg/2.0 ml for GDP and 44 μg/2.0 ml for imipenem.

Determination of Urinary Recovery

For each compound or combination, a group of four mice were given 1 ml water orally and administrated by iv injection. Urine was collected over 0~4 hours period. Antibiotic concentration in urine samples were determined by microbiological assay using *Bacillus subtilis* ATCC 6633 against standard solutions of imipenem prepared in 0.05 M phosphate buffer (pH 7).

Experimental Infection

Female Jcl:ICR mice, 4 weeks of age, were purchased from Shizuoka Laboratory Animal Center, Hamamatsu, Japan. Groups of five mice were intraperitoneally injected with 10^8 *Staphylococcus aureus* 47 suspended in 5% mucin solution. Combined therapies were subcutaneously administrated at 1 hour after the infection. Mortalities were determined after the observation for 5 days.

Materials

CPase A from bovine pancreas (Type II-DEF) and LAP from porcine kidney microsomes (Type IV-S) were purchased from Sigma Chemical Co. Carbobenzoxyglycyl-L-phenylalanine and L-leucine-*p*-nitroanilide were purchased from Peptide Institute, Inc., Osaka, Japan.

Imipenem and cilastatin were isolated from Zienam. GDP[8] was synthesized by our chemists. CNBr-activated Sepharose 4B was purchased from Pharmacia.

Results

Specific Inhibitory Activity of WS1358 Compounds

WS1358A1 and B1 were found to inhibit renal DHPs from several species of animal as shown in Table 1. Especially, WS1358A1 was more potent than cilastatin against all of the DHPs tested. In spite of their potent inhibitory activity against DHP, WS1358A1 and B1 did not inhibit CPase A and LAP at a concentration of 100 μM. Therefore, WS1358 compounds were specific inhibitors against DHP.

Purification of Porcine Renal DHP

It was necessary to purify porcine renal DHP in order to examine the inhibitory mechanism of WS1358A1 against this enzyme. In our procedure of purification, the steps of acetone precipitation, extensive washing with buffer and isoelectric precipitation previously reported have been eliminated to

save labor and time.

A summary of the typical purification is shown in Table 2. From 94 g of porcine kidney cortex, 7.8 g of protein homogenate was obtained. The complete purification from this homogenate yielded 0.6 mg of DHP which was apparently homogeneous and had a MW of about 50,000 as judged by the SDS-polyacrylamide gel electrophoresis (data not shown). The overall purification produced an enzyme approximately 1,800 times more active than the starting homogenate.

Kinetic Study of WS1358A1

Before measuring the kinetic parameters for

Table 1. IC_{50} values of WS1358A1, B1 and cilastatin against various peptidases.

Peptidase	IC_{50} (μM)		
	A1	B1	Cilastatin
Renal DHP:			
Porcine	0.003	0.60	0.13
Rabbit	0.04	2.7	0.075
Mouse	0.01	1.6	0.25
Rat	0.008	1.1	0.20
CPase A	$>10^2$	$>10^2$	$>10^2$
LAP	$>10^2$	$>10^2$	$>10^2$

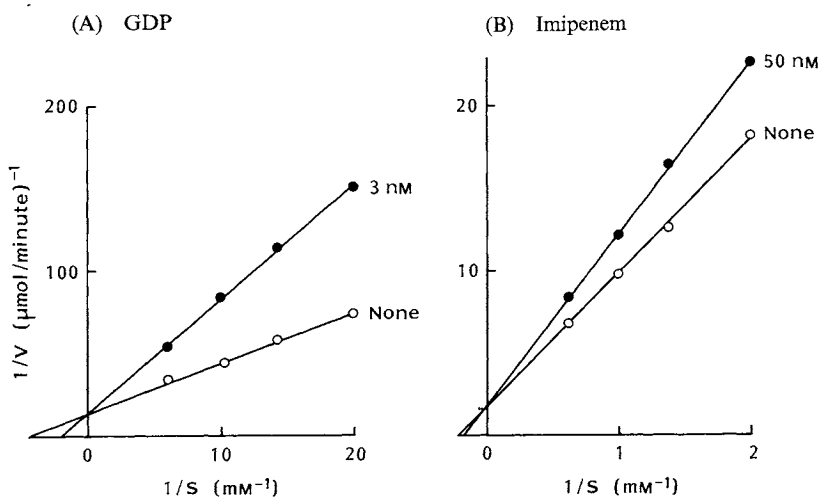
Enzymes were assayed using synthetic peptides as substrate. Enzyme activity was determined by measuring the decline of absorbance in the presence or the absence of an inhibitor. Inhibition percent was calculated as described in the text.

Table 2. Purification of porcine renal dehydropeptidase.

Fraction	Total protein (mg)	Total activity[a] (U)	Activity recovered (%)	Specific activity (U/mg)	Purification (fold)
Homogenate	7,800	78	100	0.010	1
Solubilized	2,688	97	124	0.036	3.6
50~70% $(NH_4)_2SO_4$	128	53.4	69	0.42	42
HPLC	12.2	22.6	29	1.8	180
Affinity chromatography	0.6	11.0	14	18.3	1,830

[a] All fractions were assayed using 50 μM GDP as substrate in 25 mM Tris-HCl buffer (pH 7.7).

Fig. 1. Lineweaver-Burk plot of porcine renal dehydropeptidase-catalyzed hydrolysis in the presence or absence of WS1358A1.



The DHP-catalyzed hydrolysis rates were measured at 37°C in 25 mM Tris-HCl buffer (pH 7.7). The enzyme was mixed with an inhibitor and pre-warmed at 37°C for 1 minute before adding the substrate. Velocity is expressed as μmol/minute. Each point shows the mean of three to four determinations.

Table 3. Inhibition constants of DHP inhibitors.

Inhibitor	*Ki* (μM)	
	GDP	Imipenem
WS1358A1	0.002	0.16
Cilastatin	0.09	0.30

Experimental conditions were the same as in Fig. 1. The concentration tested for *Ki* determination of cilastatin was 0.26 μM.

Table 4. Urinary recovery of imipenem administrated to mice at a dose of 1 mg/kg alone or together with DHP inhibitor.

Inhibitor	(mg/kg)	Urinary recovery (%)
None		44.1±0.6
WS1358A1	0.5	66.1±3.0
	1.0	63.8±7.3
	2.0	65.4±9.5
Cilastatin	0.5	68.9±6.4
	1.0	70.2±6.0
	2.0	63.1±5.6

The quantity of antibiotic excreted in urine within 4 hours after the administration was measured by means of a bioassay.

Table 5. Protective effect of imipenem with or without DHP inhibitor on experimental infection[a].

Drug[b]	Dose (mg/kg)	Survived/treated[c]
Saline		0/5
Imipenem	0.4	0/5
	2.0	0/5
	10.0	3/5
Imipenem + WS1358A1[d]	0.4	2/5
	2.0	5/5
	10.0	5/5
Imipenem + cilastatin[d]	0.4	2/5
	2.0	4/5
	10.0	5/5

[a] ICR mice were intraperitoneally infected with 4×10^8 *Staphylococcus aureus* 47.
[b] Drugs were subcutaneously administrated at 1 hour after the challenge.
[c] Mortalities were determined at day 5 after the infection.
[d] Co-administration at a combination ratio of 1:1 (w/w).

enzyme-catalyzed hydrolysis, a linearity between the purified enzyme concentration and enzyme activity against GDP was established over the enzyme concentration range of 0.05 through 10 μg/ml. The kinetic parameters were then determined for GDP and imipenem in the absence or the presence of inhibitor, using the standard graphical technique of Lineweaver-Burk. Typical reciprocal plots for GDP and imipenem are shown in Fig. 1. The following kinetic parameters of the purified enzyme could be calculated for the two substrates GDP ($Km = 0.3$ mM, Vmax = 800 μmol/minute/mg) and imipenem ($Km = 7.7$ mM, Vmax = 23 μmol/minute/mg).

WS1358A1 as well as cilastatin showed a competitive inhibition and the inhibition constants for WS1358A1 using GDP and imipenem as substrates are *Ki*, 2×10^{-9}, 1.6×10^{-7} M, respectively. The inhibition constants for WS1358A1 compared with those of cilastatin are listed in Table 3.

Effect of WS1358A1 on Urinary Recovery of Imipenem

As shown in Table 4, the mean urinary recovery percents of imipenem intraveneously administrated to mice at a dose of 1 mg/kg without DHP inhibitor were 44.1%. However, when WS1358A1 was co-administrated at a dose of 1 mg/kg with imipenem (1:1), the urine recovery percents increased to 63.8%. The urinary recovery of imipenem was also significantly augmented at the combination ratios of WS1358A1 versus the antibiotic within a range of 0.5:1 to 2:1 by weight. A similar effect was observed when cilastatin was used as inhibitor in place of WS1358A1. Therefore, the effect of WS1358A1 in mice on urinary recovery of imipenem resembles that of cilastatin when co-administrated with imipenem to human volunteers[9].

Effect of WS1358A1 on Experimental Infection

Although carbapenem antibiotics show strong antimicrobial activity *in vitro*[10], their ability to protect mice from experimental infection[11] is somewhat weak because of their susceptibility to inactivation by

renal DHP. As shown in Table 5, imipenem showed no protective activity when it was administrated alone at the doses of 0.4~2.0 mg/kg. In this case, no mice survived at day 5 after challenge. But, it showed an improved protective activity when WS1358A1 was co-administrated with the antibiotic at the combination ratio of 1 : 1. Two and five of five mice were cured at the doses of 0.4 and 2.0 mg/kg, respectively. The similar result was obtained when WS1358A1 was replaced by cilastatin.

Accordingly the improved protective effect of DHP inhibitors in experimental infection related to the augmentation of urinary recovery by the inhibitors.

Discussion

The co-administration of WS1358A1 with a carbapenem antibiotic imipenem increased the urinary recovery of the antibiotic and improved the protective effect on experimental infections. The efficacy of WS1358A1 is comparable to that of cilastatin[3,9]. We can attribute the *in vivo* effects of WS1358A1 to the potent inhibitory activity of the compound on DHP by which carbapenem antibiotic is inactivated.

In conclusion, WS1358A1 should be considered in combination therapy with carbapenem antibiotics like imipenem/cilastatin.

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