

Hydroxyprolylserine Derivatives JBP923 and JBP485 Exhibit the Antihepatitis Activities after Gastrointestinal Absorption in Rats¹

KE-XIN LIU, YUKIO KATO, TAI-ICHI KAKU, TOMOFUMI SANTA, KAZUHIRO IMAI, AKIRA YAGI, TAKASHI ISHIZU, and YUICHI SUGIYAMA

Graduate School of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunkyo-ku, Tokyo (K.L., Y.K., T.S., K.I., Y.S.); Japan Bioproducts Industry Co. Ltd., Tomigaya, Shibuya-ku, Tokyo (T.K.); and Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, Gakuencho, 1, Fukuyama, Hiroshima (A.Y., T.I.), Japan

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ABSTRACT

It has been a desire to develop orally effective therapeutic agents that restore the liver function in chronic injury. Here we demonstrated that *trans*-4-L-hydroxyprolyl-L-serine (JBP923) and *cyclo-trans*-4-L-hydroxyprolyl-L-serine (JBP485), which was previously isolated from hydrolysate of human placenta, exhibit potent antihepatitis activity after their oral administration. The increase in bilirubin concentration and activities of liver cytosolic enzymes in serum caused by α -naphthylisothiocyanate intoxication in rats were significantly countered both after i.v. and oral administration of these dipeptides, whereas glycyrrhizin, which has been used in the treatment of chronic hepatitis, is active only after its i.v. administration. Antihepatitis activity of dipeptides results, at least partially, from their direct

effect on hepatocytes because glutamic-oxaloacetic transaminase and lactate dehydrogenase activities in the medium of hepatotoxin-exposed primary cultured hepatocytes were reduced by these compounds. When comparing the plasma concentration-time profile of JBP923 after its i.v., oral, and portal vein injection, it is suggested that JBP923 is almost completely absorbed from gastrointestinal lumen, and hepatic first-pass removal is minor. JBP923 inhibited the proton-dependent transport of glycylsarcosine in brush-border membrane vesicles, suggesting that peptide transport system(s) may recognize JBP923. Thus, these dipeptides are potent antihepatitis reagents that are still active after oral administration and may be useful for clinical applications.

Several types of drugs have been used to treat chronic hepatitis and cirrhosis. These include prednisone and azathioprine for the treatment of autoimmune chronic hepatitis (Bellary et al., 1995; Czaja, 1999) and interferons for viral hepatitis (Dumoulin et al., 1999; Par et al., 1999; Shiffman et al., 1999). To improve the liver function in chronic hepatitis, glycyrrhizin, one of the main constituents of *Glycyrrhiza glabra* L, which has antiallergic, anti-inflammatory, and antihepatitis activities, is frequently used (Nose et al., 1994, 1996; Wang et al., 1994; Takeda et al., 1996; Arase et al., 1997). However, glycyrrhizin is usually administered i.v. because it is inactive after oral administration. Therefore, for the treatment of chronic liver injuries, orally effective therapeutic agents have to be developed.

cyclo-trans-4-L-Hydroxyprolyl-L-serine (JBP485) (Fig. 1)

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was first isolated from Laennec, a trade name for the hydrolysate of human placenta, as mitogens for a baby hamster kidney cell line, and subsequently it has been enantioselectively synthesized by chemical means (Yagi et al., 1998). Laennec is produced by Japan Bioproducts Industry Co. Ltd. (Tokyo, Japan) by purification of human placental extracts involving dialysis, heat treatment, and hydrolysis. Laennec has been clinically used to treat chronic hepatic injuries for over forty years in Japan. Recently we found that Laennec stimulates liver regeneration and decreases cytosolic enzyme [glutamic-pyruvic transaminase (GPT), alkaline phosphatase (ALP), leucine aminopeptidase (LAP), γ -glutamyltransferase (γ -GTP)] activities in serum in α -naphthylisothiocyanate (ANIT)-intoxicated rats (Liu et al., 1995). We have found that Laennec also contains *trans*-4-L-hydroxyprolyl-L-serine (JBP923). Those findings prompted us to synthesize these dipeptides and investigate their protecting effect on hepatocytes from hepatotoxin treatment.

ABBREVIATIONS: JBP485, *cyclo-trans*-4-L-hydroxyprolyl-L-serine; JBP923, *trans*-4-L-hydroxyprolyl-L-serine; ANIT, α -naphthylisothiocyanate; TFA, trifluoroacetic acid; BIL, bilirubin concentration; GOT, glutamic-oxaloacetic transaminase; GPT, glutamic-pyruvic transaminase; LAP, leucine aminopeptidase; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; DMSO, dimethyl sulfoxide; BBMVs, brush-border membrane vesicles; Gly-Sar, glycylsarcosine; γ -GTP, γ -glutamyltransferase.

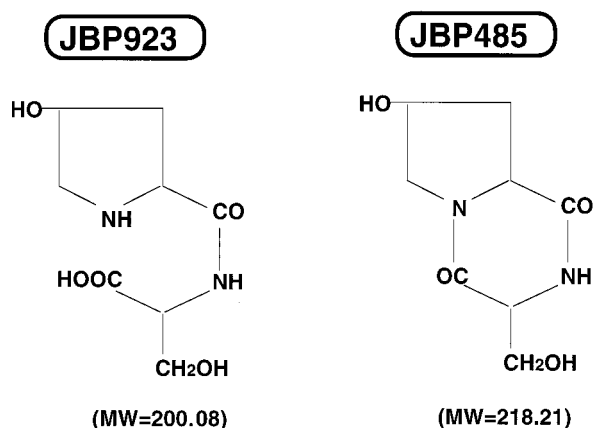


Fig. 1. Chemical structures of JBP923 and JBP485.

Although both JBP923 and JBP485 have simple chemical structures with dipeptide backbone (Fig. 1), here we report their potent antihepatotoxic activity in rats. It is notable that these compounds are active in vivo after oral administration. To support our hypothesis that these dipeptides are orally absorbed and directly interact with hepatocytes to restore their functions, we investigated the gastrointestinal absorption in vivo and antihepatitis activity in primary cultured hepatocytes. Our findings demonstrate that these dipeptides may be applicable as oral drugs for the treatment of liver injuries.

Experimental Procedures

Animals and Materials. Male Wistar rats weighing 250 and 150 g (Nisseizai, Tokyo, Japan) for in vivo and in vitro studies, respectively, were used throughout the experiments. All animals were treated humanely. The studies reported in this article have been carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the National Institutes of Health. JBP923 and JBP485 were synthesized by Watanabe Chemical Industries Company (Hiroshima, Japan). Acetonitrile, tetrahydrofuran, trifluoroacetic acid (TFA), dioxane, and distilled water, all of HPLC grade, were purchased from Wako Pure Chemical Industries (Osaka, Japan). 4-Fluoro-7-nitro-2,1,3-benzoxadiazole was from Tokyo Kasei Co. (Tokyo, Japan).

Antihepatotoxic Activities In Vivo. ANIT (Sigma, St. Louis, MO) dissolved in olive oil was injected i.p. at a dose of 50 mg/kg body wt. For i.v. administration, JBP923 and JBP485 (25 mg/ml), dissolved in saline, or glycyrrhizin injection (Minophagen Pharmaceutical Co., Tokyo, Japan) were administered through the penis vein. For oral administration, JBP923 and JBP485 dissolved in saline or glycyrrhizin tablets (Minophagen Pharmaceutical Co.) dissolved in 5% glucose solution were administered via esophagus with a gastric sonde. Administrations of these drugs were performed at 30 min before and 8, 22, 32, and 46 h after ANIT treatment. All the administrations were performed under ether anesthesia. Serum was collected 48 h after ANIT treatment. For serum collection, rats were anesthetized with ether, and approximately 10 ml of blood was sampled from the aorta abdominalis. Blood was then left on ice for 20 min and centrifuged at 1000g for 5 min to obtain the supernatant as serum. The total bilirubin concentration (BIL) and activity of liver-specific cytosolic enzymes, such as GPT, LAP, ALP, glutamic-oxaloacetic transaminase (GOT) and γ -GTP, in the rat serum were determined using the appropriate assay kits (Wako Pure Chemical Industries).

Determination of Biochemical Marker Leakage from Primary Cultured Rat Hepatocytes. Parenchymal hepatocytes were plated at a density of 1.25×10^5 cells/1.88 cm² and cultured for 24 h

as described previously (Kato et al., 1994). Briefly, isolated hepatocytes suspended in Williams' medium E supplemented with 5% calf serum, 10^{-9} M insulin, and 10^{-9} M dexamethasone were plated onto 24-well plastic dishes coated with type I collagen. The nonattached cells were removed by washing with fresh culture medium at 3 h after plating. CCl₄, first dissolved in dimethyl sulfoxide (DMSO) at 1.0 M, was diluted with fresh medium containing JBP923, JBP485, glycyrrhizin, or 18 β -glycyrrhetic acid (Sigma) to give a final concentration of 5 mM. Control experiments were performed in the presence of only DMSO. At 24 h after plating, the medium was replaced with the buffer containing both CCl₄ and an appropriate drug. The monolayers were further cultured for 24 h, and culture medium was collected and centrifuged at 24,000g for 20 min. GOT and lactate dehydrogenase (LDH) were assayed as described above. One unit was defined as the amount of activity catalyzing formation of 1 μ mol of product/1 min.

Pharmacokinetic Analysis in Normal Rats. Under ether anesthesia, JBP923 (3.13 or 25 mg/kg) dissolved in saline was administered through the penis vein, through the portal vein, or into the stomach with a gastric sonde. This ether anesthesia was sufficient to allow portal vein injection, which was performed over a period of 10 min using an infusion pump. Plasma was collected from the external jugular vein at the indicated times, and the JBP923 concentration in plasma was determined by HPLC as described below. The plasma concentration (C_p)-time profiles of JBP923 after i.v. and oral administration were fitted to the following equations, respectively:

$$C_p = A \exp(-\alpha t) + B \exp(-\beta t) \quad (1)$$

$$C_p = k_a F \exp(-k_a t) (A(1 - \exp(-(\alpha - k_a)t))/(\alpha - k_a) + B(1 - \exp(-(\beta - k_a)t))/(\beta - k_a)) \quad (2)$$

where k_a and F were absorption rate constant and bioavailability, respectively. The plasma clearance (CL_{plasma}) was calculated by:

$$CL_{\text{plasma}} = \text{Dose}/AUC_{i.v.} \quad (3)$$

where AUC_{i.v.} is area under the plasma concentration-time profile after i.v. injection.

$$AUC_{i.v.} = A/\alpha + B/\beta \quad (4)$$

The hepatic availability (F_h) was calculated as:

$$F_h = AUC_{pv}/AUC_{i.v.} \quad (5)$$

where AUC_{pv} was AUC after portal vein injection. The AUC during the 10-min portal vein infusion was calculated by trapezoidal rule. The AUC after the end of infusion was obtained by eq. 4, where the plasma concentration-time profile was fitted also to eq. 1. The input data for all the fitting were weighted as the reciprocal of the square of the observed values, and the algorithm used for the fitting was the damping Gauss-Newton method.

Determination of JBP923 in Plasma by HPLC. To 12.5 μ l of plasma, 500 μ l of methanol was added, and the mixture was centrifuged at 600g for 5 min for deproteinization. The supernatant was collected and dried under reduced pressure using a centrifugal evaporator. For the derivatization of an imino group in JBP923, 20 μ l of 50 mM borate buffer (pH = 8.0) and 30 μ l of 20 mM 4-fluoro-7-nitro-2,1,3-benzoxadiazole dissolved in acetonitrile were added to the dried sample (Fukushima et al., 1995). The reaction mixture was heated at 60°C for 5 min, and 450 μ l of 1% TFA in water was added to the mixture to stop the reaction. Twenty microliters of the resultant solution was subjected to HPLC analysis. The HPLC system consisted of a model L-6320 Intelligent pump (Hitachi, Tokyo, Japan) and a model F-1050 fluorescence spectrophotometer (Hitachi). An ODS-COSMOSIL (4.6 \times 150 mm, i.d.) column (Nacalai Tesque, Tokyo, Japan) was used. The excitation and emission wavelengths were fixed at 470 and 540 nm, respectively. A gradient HPLC system was adopted. Eluent A, water/acetonitrile (95.5:4.5, v/v) containing 4.5%

dioxane, 1% tetrahydrofuran, and 0.05% TFA, and eluent B (acetonitrile) were used. The elution program was as follows: eluent A, 100 to 0% from 0 to 18 min; eluent B, 100 to 0% from 18.1 to 35 min; eluent A, 100% from 35.1 to 45 min. The flow rate was 1 ml/min.

Uptake Study in Brush-Border Membrane Vesicles (BBMVs). BBMVs were prepared by the method of Kessler (Kessler et al., 1978). Briefly, the approximately 50-cm proximal portion of the jejunum was isolated from male rabbits (2.0–2.5 kg; Nisseizai). The mucosa was scraped off and homogenized in a volume of ice-cold buffer A (2 mM Tris/HEPES buffer containing 50 mM D-mannitol, pH = 7.1). The homogenization was carried out with a Waring blender for 2 min at a speed of 18,000 rpm. Solid CaCl₂ was added to the homogenate to give a final concentration of 10 mM, and the mixture was stirred in an ice bath for 15 min. It was then centrifuged at 500g for 15 min, and the supernatant was centrifuged at 1500g for 30 min. The pellet was homogenized with buffer A in a glass/Teflon Potter homogenizer at a speed of 1000 rpm. The mixture then was centrifuged at 750g for 30 min. The pellet was homogenized in a glass/Teflon Potter homogenizer again with the same buffer and the speed mentioned above. The supernatant was centrifuged at 48,000g for 30 min, and then the pellet was suspended with a 23-gauge needle. The centrifugation was performed again with the same speed and time, and then the pellet was suspended with a 27-gauge needle. Finally, protein concentration in the suspension was determined using a Bio-Rad protein assay kit with BSA as a standard; the concentration was 25 mg/ml with transport buffer (10 mM Tris/HEPES buffer with 270 mM D-mannitol, pH = 7.5).

Uptake of [¹⁴C]glycylsarcosine (Gly-Sar) by BBMVs was measured by the rapid filtration method described by Hopfer (Hopfer et al., 1973). The uptake was started by adding 4 μl of BBMVs (100 μg) to 16 μl of transport buffer (20 mM Tris-citrate buffer, pH = 5.5) containing JBP923, JBP485, and [¹⁴C]Gly-Sar at 37°C. The final substrate concentration was 68 and 600 μM [¹⁴C]Gly-Sar (2.96 GBq/mmol) and unlabeled Gly-Sar, respectively. The reaction was stopped at the desired time by adding 1 ml of ice-cold stop buffer (pH = 7.5), which contained 20 mM Tris, 20 mM HEPES, and 300 mM mannitol. Then, 0.9 ml of the diluted sample was applied immediately on a Millipore filter (HAWP, 0.45-μm pore size) and washed rapidly twice with 5 ml of ice-cold stop buffer. The uptake of [¹⁴C]Gly-Sar by BBMVs trapped on the Millipore filter was measured in a liquid scintillation spectrometer. The inhibition constant (K_i) was obtained by fitting the data to the following equation:

$$V_{(+I)}/V_{(-I)} = 1/(1 + I/K_i) \quad (6)$$

where V_(+I) and V_(-I) represent the transport velocity in the presence and absence of inhibitor, respectively; I is the inhibitor concentration. Equation 6 is based on the assumption of competitive inhibition in a case when the Michaelis constant (K_m) is much higher than the substrate concentration. In a preliminary study, we found that the K_m for Gly-Sar uptake was 15.5 mM in rabbit BBMVs, and therefore the substrate concentration chosen for this experiment was 0.668 mM.

Statistical Analysis. Statistical analysis was performed by Student's *t* test to identify significant differences between various treatment groups.

Results

Antihepatotoxic Effect of JBP923 and JBP485 in ANIT-Intoxicated Rats. To examine whether JBP923 and JBP485 promote the repair of injured liver function in ANIT-intoxicated rats, we determined the change in BIL and activities of liver cytosolic enzymes in serum of ANIT-intoxicated rats after administration of JBP923 and JBP485 (Table 1). The increase in BIL and liver cytosolic enzyme activities caused by ANIT intoxication were countered by i.v. and oral administration of JBP923 and JBP485 (Table 1). The reduction in all the marker values were significant at i.v. and oral doses of more than 1.36 and 25 mg/kg, respectively, both for JBP923 and JBP485 (Table 1). When the i.v. and oral doses were increased up to 6.25 and 25 mg/kg, respectively, the bilirubin, LAP, and ALP levels were almost comparable with those values in normal rats (Table 1).

Comparison of the Antihepatotoxic Effect of JBP923 and JBP485 with Glycyrrhizin. Because glycyrrhizin is one of the most frequently administered drugs in chronic liver-injured patients, the antihepatitis activity was compared among JBP923, JBP485, and glycyrrhizin (Fig. 2). Intravenous or oral administration of JBP923 caused the reduction of GPT at almost the same doses (Fig. 2A). The reduction in GPT activity was also observed after i.v. or oral administration of JBP485, although oral administration exhibited weaker antihepatitis activity (Fig. 2B). Minimal reduction was found after oral administration of glycyrrhizin, whereas its i.v. administration decreased GPT level (Fig. 2C).

TABLE 1

Change in BIL and activity of liver cytosolic enzymes in serum in ANIT-intoxicated rats treated with JBP923 and JBP485. Each value represents the mean ± S.E. of three animals.

| Dose | | Bilirubin | GPT | LAP | ALP | γ-GTP | |
|---------|---------------|----------------------|---------------|-----------|---------------|---------------|---------------|
| mg/kg | | mg/dl | | I.U./l | | mU/ml | |
| JBP 923 | i.v. | 0 (normal rat) | 0.49 ± 0.06 | 39 ± 5 | 28 ± 7 | 109 ± 10 | 3.0 ± 0.3 |
| | | 0 (ANIT-treated rat) | 5.8 ± 0.4 | 330 ± 44 | 115 ± 3 | 314 ± 34 | 17.2 ± 1.8 |
| | | 1.36 | 0.74 ± 0.03** | 130 ± 9** | 64 ± 23* | 136 ± 3** | 6.7 ± 0.6** |
| | p.o. | 6.25 | 0.46 ± 0.06** | 99 ± 15** | 29 ± 9** | 120 ± 10** | 3.65 ± 0.06** |
| | | 0 (normal rat) | 0.51 ± 0.07 | 35 ± 6 | 24 ± 6 | 130 ± 8 | 3.4 ± 0.4 |
| | | 0 (ANIT-treated rat) | 6.5 ± 0.5 | 385 ± 56 | 110 ± 16 | 363 ± 55 | 21.5 ± 2.1 |
| 6.25 | 0.79 ± 0.14** | 233 ± 67 | 47 ± 7** | 196 ± 35* | 5.77 ± 0.08** | | |
| 25 | 0.66 ± 0.09** | 68.3 ± 1.7** | 21.9 ± 2.0** | 113 ± 7** | 5.91 ± 0.27** | | |
| JBP 485 | i.v. | 0 (normal rat) | 0.49 ± 0.06 | 39 ± 5 | 28 ± 7 | 109 ± 10 | 3.0 ± 0.3 |
| | | 0 (ANIT-treated rat) | 5.8 ± 0.4 | 330 ± 44 | 115 ± 3 | 314 ± 34 | 17.2 ± 1.8 |
| | | 1.36 | 1.11 ± 0.06** | 238 ± 16* | 54 ± 3** | 253 ± 33* | 7.9 ± 0.5** |
| | p.o. | 6.25 | 0.46 ± 0.07** | 153 ± 3** | 25.5 ± 2.0** | 133 ± 10** | 3.65 ± 0.06** |
| | | 0 (normal rat) | 0.51 ± 0.07 | 35 ± 6 | 24 ± 6 | 130 ± 8 | 3.4 ± 0.4 |
| | | 0 (ANIT-treated rat) | 6.5 ± 0.5 | 385 ± 56 | 110 ± 16 | 363 ± 55 | 21.5 ± 2.1 |
| | 6.25 | 1.2 ± 0.3** | 119 ± 51** | 49 ± 5** | 194 ± 11* | 9.8 ± 0.8** | |
| | 25 | 0.68 ± 0.21** | 62 ± 13** | 28 ± 5** | 104 ± 24** | 4.52 ± 0.11** | |

* *P* < .05, ** *P* < .01, significantly different from ANIT-treated rats.

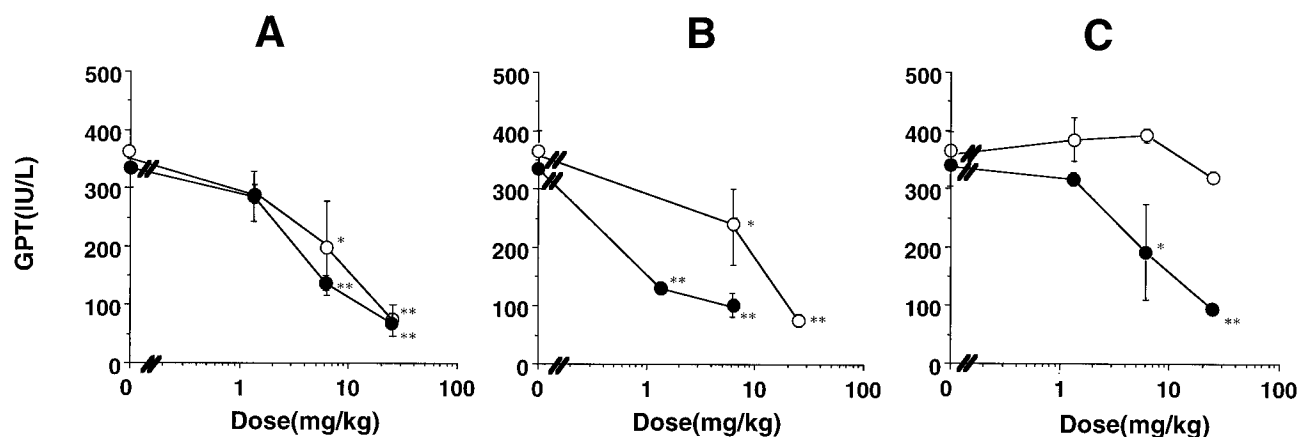


Fig. 2. Comparison of antihepatitis effect on ANIT-intoxicated rats among JBP923 (A), JBP485 (B), and glycyrrhizin (C). JBP923, JBP485, or glycyrrhizin was administered through the penis vein (●) or orally (○) 30 min before and 8, 22, 32, and 46 h after ANIT treatment. Serum was collected at 48 h, and the activity of GPT was determined. The values are expressed as means \pm S.E. of three rats. * $P < .05$; ** $P < .01$, significantly different from saline alone.

Thus, these dipeptides exhibit antihepatotoxic effect after oral administration, but glycyrrhizin does not.

Antihepatotoxic Effect on Primary Cultured Hepatocytes. The decrease in leakage of liver cytosolic enzyme by these compounds was also examined in vitro in primary cultured hepatocytes intoxicated with CCl_4 (Fig. 3). The GOT activity in the medium was decreased by addition of JBP923 and JBP485 in a concentration-dependent manner, the GOT activity at the highest concentration being almost comparable with that of hepatocytes without CCl_4 intoxication (Fig. 3A). Both glycyrrhizin and 18 β -glycyrrhetic acid also decreased the GOT activity, although such effect at concentrations greater than 50 μM was smaller than JBP923 and JBP485 (Fig. 3A). The reduction in LDH activity was found in the presence of any compounds examined (Fig. 3B). Such reduction in the presence of JBP923 or JBP485 was found at a lower concentration than that found in the presence of glycyrrhizin or 18 β -glycyrrhetic acid.

Pharmacokinetics of JBP923 in Normal Rats. To examine the gastrointestinal absorption as well as hepatic first-

pass elimination of JBP923, its plasma concentration-time profiles in rats were determined after i.v., oral, and portal vein administrations (Fig. 4). The plasma concentration of JBP923 was gradually decreased after i.v. administration with a terminal phase half-life of 21 to 24 min (Fig. 4). The gastrointestinal absorption of JBP923 was rapid with a k_a of 0.01 to 0.04 min^{-1} and maximum plasma concentration observed within 30 min (Fig. 4 and Table 2). The AUC after oral administration was almost comparable with that after i.v. administration both at 3.13 and 25 mg/kg (Table 2), suggesting almost complete oral absorption. The AUC after portal vein administration was also comparable with that after i.v. administration at 3.13 mg/kg (Table 2), suggesting that hepatic first-pass elimination is not so remarkable.

Effect of JBP923 and JBP485 on Uptake of [^{14}C]Gly-Sar in Intestinal BBMV. To examine the interaction of these dipeptides with oligopeptide-specific transporters expressed in small intestines, their inhibitory effect on uptake of Gly-Sar, a typical substrate of peptide transporter PEPT1, by rabbit intestinal BBMV was investigated (Fig. 5). The

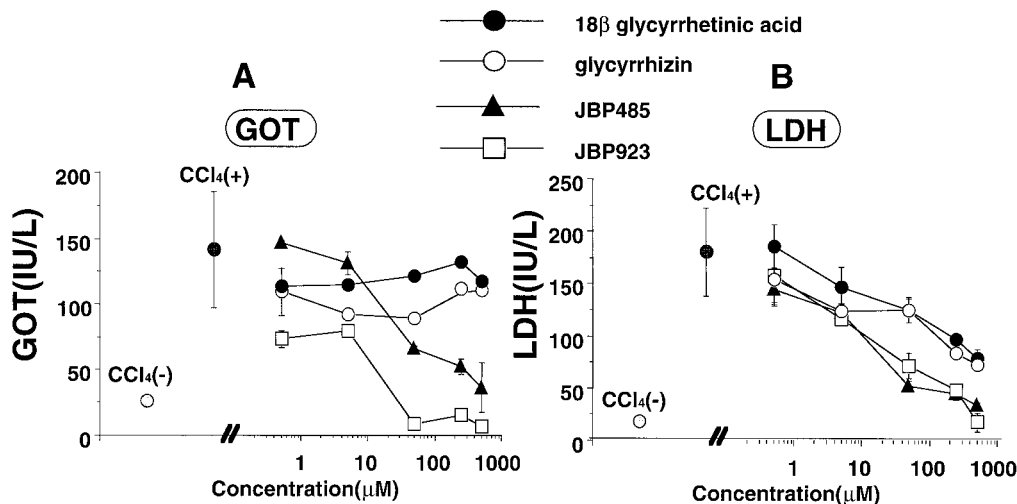


Fig. 3. The recovery of the GOT and LDH leakage by JBP923, JBP485, glycyrrhizin, or 18 β -glycyrrhetic acid in the medium of primary cultured rat hepatocytes exposed to CCl_4 . Parenchymal hepatocytes were cultured for 24 h, then the cultured medium was changed to the fresh medium containing 5 mM CCl_4 (control) or 5 mM CCl_4 with various concentrations of the indicated compounds. Hepatocytes were further cultured for 24 h, and the activities of GOT and LDH in the medium were determined. $\text{CCl}_4(-)$, medium without treatment of CCl_4 ; $\text{CCl}_4(+)$, medium with CCl_4 in 0.45% of DMSO; DMSO, medium containing DMSO alone. The values are expressed as means \pm S.E. of three rats.

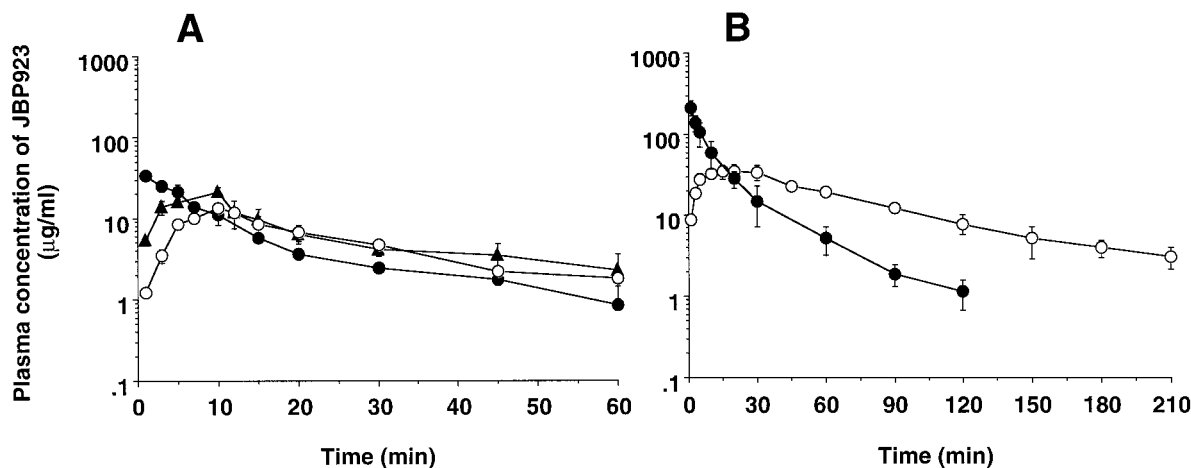


Fig. 4. Plasma concentration-time profiles of JBP923 after i.v., portal vein, and oral administrations in rats. JBP923 at 3.13 (A) or 25 (B) mg/kg was administered through the penis vein (●), portal vein (▲), and orally (○) to rats. Plasma was collected from the external jugular vein at the indicated time, and the JBP923 concentration in plasma was determined by HPLC. Points are expressed as means \pm S.E. of three to five rats.

TABLE 2

Pharmacokinetic parameters of JBP923 in rats

| Route of Administration | Dose | AUC | CL _{plasma} | <i>F</i> or <i>F</i> _h | <i>k</i> _a |
|-------------------------|-------|--|----------------------|-----------------------------------|--------------------------------|
| | mg/kg | $\mu\text{g} \cdot \text{min} / \text{ml}$ | ml/min/kg | | |
| i.v. | 3.13 | 380 \pm 14 | 8.25 \pm 0.31 | | |
| i.v. | 25 | 2730 \pm 168 | 9.25 \pm 0.49 | | |
| p.o. | 3.13 | 422 \pm 49 | | 1.11 \pm 0.13 ^c | 0.040 \pm 0.006 ^c |
| p.o. | 25 | 3068 \pm 348 | | 1.12 \pm 0.12 ^c | 0.014 \pm 0.002 |
| pv | 3.13 | 399 \pm 45 | | 1.05 \pm 0.12 ^b | |

pv, portal vein.

^a Bioavailability (*F*) obtained from eq. 2.

^b Hepatic availability (*F*_h) obtained from eq. 5.

^c Significantly different (*P* < .05).

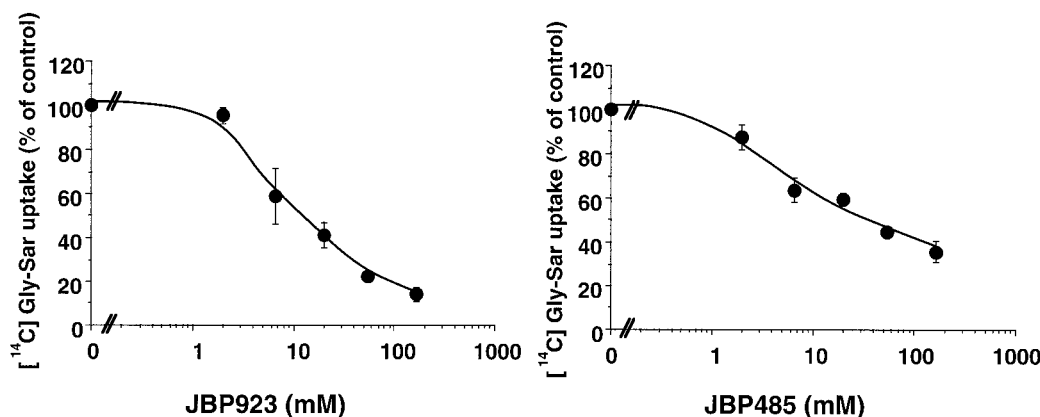


Fig. 5. Inhibitory effects of JBP923 and JBP485 on [¹⁴C]Gly-Sar uptake by rabbit intestine BBMV. Membrane vesicles were preloaded with transport buffer (pH = 7.5). Uptake of [¹⁴C]Gly-Sar (68 μM) with cold Gly-Sar (600 μM) in 20 mM Tris-citrate transport buffer (pH = 5.5) was measured at 37°C for 2 min as a control. After incubating the membrane vesicles containing various concentrations of JBP923 and JBP485 at 37°C for 2 min, the uptake of [¹⁴C]Gly-Sar (68 μM) with cold Gly-Sar (600 μM) was measured. Points are expressed as means \pm S.E. of three independent experiments.

uptake of [¹⁴C]Gly-Sar exhibited proton dependence because the uptake was higher in medium at pH 5.5 than that in medium at pH 7.5 (data not shown). Both JBP923 and JBP485 inhibited the uptake of [¹⁴C]Gly-Sar in a concentration-dependent manner (Fig. 5) with *K*_i values of 13 and 31 mM for JBP923 and JBP485, respectively.

Discussion

In this study, we found two dipeptide compounds, JBP923 and JBP485, that decrease BIL and hepatic cytosolic enzymes activities in serum of ANIT-intoxicated rats (Table 1).

The antihepatitis activity of JBP923 was observed both after its i.v. and oral administration. This was compatible with our finding that gastrointestinal absorption of JBP923 is almost complete (Table 2). JBP923 decreased both GOT and LDH activity in the medium of in vitro primary cultured hepatocytes (Fig. 3). Such direct effect on hepatocytes was found at the concentrations above \sim 50 μM . On the other hand, the plasma JBP923 concentration after its oral administration at 25 mg/kg was higher than 50 μM (10 $\mu\text{g}/\text{ml}$) until at least 2 h (Fig. 4B). This means that effective JBP923 concentration was maintained after oral administration. Thus, it seems to

be that JBP923 showed pharmacological activity after its gastrointestinal absorption and subsequent interaction with hepatocytes, although this finding does not deny the possibility of the existence of its active metabolites.

Although glycyrrhizin also decreased the liver function marker enzyme both in vivo and in vitro (Figs. 2 and 3), its pharmacological effect was minimal after oral administration (Fig. 3C). This is compatible with the fact that glycyrrhizin was usually administered i.v. for the treatment of chronic hepatic injuries. Glycyrrhizin was reported to be hydrolyzed by bacteria in the stomach and large intestinal content, and the first-pass elimination might be the reason for its low bioavailability (Wang et al., 1994; Takeda et al., 1996). Wang et al. (1994) reported that glycyrrhizin was metabolized to 18 β -glycyrrhetic acid. Nose et al. (1994) suggested that 18 β -glycyrrhetic acid was more potent than glycyrrhizin in terms of its antihepatotoxic activity toward CCl₄-treated primary cultured hepatocytes. Also in this study, 18 β -glycyrrhetic acid decreased LDH activity in the medium of cultured hepatocytes (Fig. 3). This active metabolite was also found in plasma of humans after oral administration of 100 mg of glycyrrhizin, although its concentration was at most 0.5 μ g/ml, corresponding to 1.1 μ M, which was less than the effective concentration found in this study and the report by Nose et al. (1996). Thus, oral administration of glycyrrhizin does not exhibit clear antihepatotoxic activity, and it is anticipated that orally active antihepatitis drugs will be developed for the clinical treatment of chronic hepatitis.

JBP923 exerts antihepatitis activity after oral administration (Table 1). JBP923 was found in plasma only 1 min after its oral administration (Fig. 4). This finding as well as its complete gastrointestinal absorption suggests that a certain specific mechanism may contribute to the gastrointestinal transport. It also should be noted that the k_a was significantly lower at 25 mg/kg than at 3.13 mg/kg, suggesting the slower absorption at a higher dose. It has been reported that certain hydrophilic β -lactam antibiotics can be transported by oligopeptide transporters in gastrointestinal tissues. This transport system(s) also accepts other types of pharmaceutical agents such as angiotensin-converting enzyme inhibitors, renin inhibitors, and thrombin inhibitors (Gannapathy et al., 1984; Li and Hidalgo, 1996; Tsuji and Tamai, 1996; Kitagawa et al., 1999; Guo et al., 1999). In this study, both JBP923 and JBP485 inhibited the uptake of Gly-Sar, a typical substrate of oligopeptide transporter, by intestinal BBMVs in a concentration-dependent manner (Fig. 5). This suggests the possibility that these small dipeptides are recognized by the transporter, resulting in its rapid and complete absorption. Further studies are needed to identify the transport system(s).

Here we reported two novel dipeptide compounds that can repair liver function after both i.v. and oral administrations. These compounds can also exert an antihepatitis effect directly on cultured hepatocytes. It should also be noted that glycyrrhizin has been reported to exert many types of biological activity, including antioxidant effect, anti-apoptosis action, and enhancement of nitric oxide production from activated macrophages, although it is still unknown which of

these activities are actually related to its protective effect on liver function (Yi et al., 1996; Liu et al., 1998; Shaikh et al., 1999). Therefore, the mechanism of antihepatitis activity of these two dipeptides should also be clarified to further demonstrate their applicability to clinical stages.

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Send reprint requests to: Professor Yuichi Sugiyama, Ph.D., Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, unkyo-ku, Tokyo 113-0033, Japan. E-mail: sugiyama@seizai.f.u-tokyo.ac.jp