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Hepatic Clearance of ONO-5046, a Novel Neutrophil Elastase Inhibitor, in Rats and in the Rat Perfused Liver

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Abstract

The hepatic clearance of ONO-5046 (*N*-[2-[4-(2,2-dimethylpropionyloxy)phenylsulphonylamino]benzoyl]aminoacetic acid), a low-molecular-weight neutrophil elastase inhibitor, has been investigated in rats and in the rat perfused liver.

This ester was easily hydrolysed to its inactive metabolite EI-601 (*N*-[2-[(4-hydroxyphenyl)sulphonylamino]benzoyl]aminoacetic acid) in liver homogenate and in erythrocytes suspension in-vitro. On the other hand, it was stable in biological media such as plasma and whole blood, which contain plasma proteins. Scatchard plot analysis of ONO-5046 binding to bovine serum albumin (BSA) in-vitro indicated that the association constant (K) and number of binding sites (n) were $6{\cdot}91 \times 10^4$ (M^{-1}) and 4·33, respectively. Thus, ONO-5046 (100 μM) would bind to plasma proteins to an extent >99% at physiological plasma-protein concentrations. The total plasma clearance of ONO-5046 in rats was constant (approximately 9 mL min^{-1} kg^{-1}) under different steady-state plasma concentrations (5–50 μM) a value equivalent to the hepatic clearance. In the rat perfused liver, the hepatic extraction ratio of ONO-5046 was significantly reduced by adding BSA to the dosing solution. Thus, the relatively low hepatic clearance of ONO-5046, which has an ester linkage in its structure and is naturally susceptible to enzymatic hydrolysis, was found to be because of the extremely high protein-binding of the compound.

Many low-molecular-weight elastase inhibitors have been synthesized, on the basis that a low-molecular-weight inhibitor of neutrophil elastase can make contact with neutrophils whereas close contact is proscribed for endogenous macromolecule anti-proteinases (Campbell et al 1982; Campbell & Campbell 1988; Herbert et al 1992; Fujie et al 1993; Edwards et al 1996; Groutas et al 1997). ONO-5046 (*N*-[2-[4-(2,2-dimethylpropionyloxy)phenylsulphonylamino]benzoyl]aminoacetic acid) is a specific neutrophil elastase inhibitor with a low molecular weight (368·85) and pKa values of 5·3 (carboxyl group) and 8·2 (sulphonylamino group) developed for the treatment of pulmonary emphysema. ONO-5046 suppresses human neutrophil-induced lung haemorrhage in the hamster if administered intra-tracheally, suppresses human neutrophil-induced increase in capillary permeability in the guinea pig if administered intravenously, prevents the ischaemia/re-perfusion-induced liver injury in rats if administered by continuous intravenous infusion, and inhibits neutrophil-induced human pulmonary artery endothelial cell injury, etc. (Kawabata et al 1991; Iwamura et al 1993; Kakimoto et al 1995; Furuno et al 1997).

We have previously analysed the contributions of intestinal and hepatic first-pass metabolism of ONO-5046 after oral administration to rats (Watanabe et al 1997). ONO-5046 was hydrolysed by esterase at a carboxylic ester linkage to an inactive metabolite, EI-601 (*N*-[2-[(4-hydroxyphenyl) sulphonylamino]benzoyl]aminoacetic acid) in the liver and intestine, and the low oral bioavailability of ONO-5046 was found to be mainly because of marked intestinal first-pass metabolism, including metabolism in the intestinal fluid. Unexpectedly, the hepatic first-pass metabolism of ONO-5046 administered into the portal vein at different rates of infusion was relatively small and constant (approximately 20% of the dose), although estera-

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ses have broad substrate specificity and exist in most organs, including the liver, and in biological fluids (Leinweber 1987; Aldridge 1993; Morgan et al 1994). In this study the hepatic clearance of ONO-5046 was further investigated in rats and in the rat perfused liver to clarify the mechanism of clearance of the drug.

Materials and Methods

Materials

ONO-5046 and its metabolite EI-601 were kindly supplied by Ono Pharmaceutical Co (Osaka, Japan). Bovine serum albumin (BSA, fraction V) was obtained from Sigma (St Louis, MO). Other reagents were of the highest grade available.

Stability of ONO-5046 in-vitro

Experiments with animals were performed in accordance with the Guide for Animal Experimentation, Hiroshima University and the Committee of Research Facilities for Laboratory Animal Sciences, Hiroshima University School of Medicine. Male Sprague-Dawley rats, 230–280 g, were anaesthetized with sodium pentobarbital (30 $mg\,kg^{-1}$, intraperitoneal injection) and blood was collected by heart puncture. The liver was excised after infusion of a sufficient amount of cold saline through the portal vein. Part of blood was centrifuged at 1200 *g* for 10 min and the plasma was collected. The erythrocytes precipitated were also collected after removal of the buffy-coat-containing leukocytes. The erythrocytes collected were suspended in cold saline at a volume ratio of approximately 1 : 1, and the suspension was again centrifuged at 1200 *g* for 10 min to remove the buffy coat completely. This washing procedure was repeated twice and the erythrocytes finally obtained were re-suspended in an equal volume of isotonic pH 7·4 phosphate-buffered saline (PBS). Some of the erythrocytes were haemolysed in a 4-fold volume of water and the supernatant of the haemolysate was used after centrifugation at 1200 *g* for 10 min. The excised liver was homogenized in a 99-fold volume of cold PBS by means of a glass/Teflon Potter homogenizer. ONO-5046 (5 mM) was dissolved in water containing 15% propylene glycol and 15% ethanol. The hydrolytic characteristics of ONO-5046 in whole blood, plasma, erythrocyte suspension (50% v/v), erythrocyte suspension containing 3% BSA, haemolysate (20% v/v), haemolysate containing 3% BSA, and 1% liver homogenate were determined by adding ONO-5046 solution (0·1 mL) to each biological sample (4·9 mL; final concentration, 100 μM) at 37°C.

Protein binding of ONO-5046 in-vitro

The binding of ONO-5046 to BSA was determined by use of semi-micro dialysis cells (Sanko Plastic Co, Osaka, Japan) consisting of two 1-mL adjoining chambers separated by semi-permeable cellulose membranes. ONO-5046 (20–600 μM) and BSA (0·1%) were dissolved in PBS and the solutions (1 mL) were placed in opposite chambers of the dialysis cell. Dialysis was performed at 25°C for 20 h with gentle mechanical agitation and samples were taken to determine the concentration of unbound ONO-5046 in the BSA-free chamber and the total (unbound and bound) concentration of ONO-5046 in the chamber containing BSA. Data were analysed by construction of a Scatchard plot.

Hepatic clearance of ONO-5046 in-vivo

Male Sprague-Dawley rats, 250 g, were anaesthetized with sodium pentobarbital (30 $mg\,kg^{-1}$, intraperitoneal injection) and the femoral artery and vein were cannulated with polyethylene tubing (PE-50; Clay Adams, USA). ONO-5046 was administered intravenously at a dose of $C_{ss} \times V_1$, then by constant-rate infusion of $CL_{total} \times C_{ss}$ via the cannula inserted at the femoral vein to effect a steady-state plasma concentration, where C_{ss}, V_1 and CL_{total} denote, respectively, the steady-state plasma concentration (5, 25, or 50 $nmol\,mL^{-1}$), the distribution volume of the central compartment (50 $mL\,kg^{-1}$) and the total plasma clearance of ONO-5046 (8·0 $mL\,min^{-1}\,kg^{-1}$) (Watanabe et al 1997). Blood samples (0·2 mL) were collected from the femoral artery, via the inserted cannula, and from the hepatic vein 30, 40, 50 and 60 min after the initiation of constant-rate infusion. Blood from the hepatic vein was collected through a U-shaped needle (26 gauge), inserted along the hepatic vein, which was connected with polyethylene tubing filled with a heparin solution (200 units mL^{-1}).

Hepatic clearance of ONO-5046 in the rat perfused liver

Isolated rat liver systems were basically prepared according to the method reported by Sugano et al (1978). Briefly, under light ether anaesthesia the bile duct of male Sprague-Dawley rats, approximately 250 g, was cannulated with PE-10 polyethylene tubing and heparin solution (0·6 mL; 200 unit mL^{-1}) was injected into the inferior vena cava. After 30 s the portal vein was rapidly catheterized with an inflow cannula (2·0 mm o.d.), which was connected to the perfusion system, and single perfusion was immediately started at a flow rate of 30 $mL\,min^{-1}$. The perfusate consisted of 115 mM NaCl, 5·9 mM KCl, 1·2 mM $MgCl_26H_2O$, 1·2 mM $NaH_2PO_42H_2O$, 1·2 mM Na_2SO_4, 25 mM $NaHCO_3$

and 10 mM glucose; it was oxygenated with 95% O_2-5% CO_2 to pH 7·4 at 37°C before and during the experiments. The inferior vena cava was catheterized with PE-240 polyethylene tubing as an outflow cannula. The in-flow and out-flow cannulas were tightly ligated in place and the upper hepatic vein was also ligated for isolation of the liver. The isolated liver was perfused at 37°C in a temperature-controlled cabinet and used after a stabilization period of 30 min. ONO-5046 solution (0·25–5·0 μmol in 0·5 mL) was injected as a bolus within 5 s or by constant-rate infusion into the perfusate (perfusate concentration 10–100 nmol mL^{-1}). Total hepatic vein effluent and bile were serially collected at an appropriate time interval. Data were analysed in terms of the hepatic extraction ratio (or recovery) of ONO-5046, recovery of EI-601 in the effluents, and recovery of EI-601 in the bile. The recovery of EI-601 was estimated on a molar basis equivalent to the dose of ONO-5046. In the study to examine the effect of BSA on the hepatic extraction of ONO-5046, drug solution containing different amounts of BSA (1, 2, 5, 10%) was injected, in order, every 10 min. As viability tests of the liver, ONO-5046 solution without BSA was injected twice as the first and sixth runs from the standpoint of the reproducibility of extraction ratio of ONO-5046. Also, the bile flow rate was recorded during the experiment; according to the criteria proposed by Tsao et al (1986) this should be more than 1 μL min^{-1} (g liver)$^{-1}$.

Analysis

Concentrations of ONO-5046 and EI-601 in various biological samples and effluents of the rat perfused liver were analysed by high-performance liquid chromatography equipped with UV detection and a reversed-phase TSK gel ODS-80TM column (Tosoh, Tokyo, Japan) as reported previously (Watanabe et al 1997).

Results

Stability of ONO-5046 in-vitro

The degradation rate constants and half-lives of ONO-5046 at 37°C in various biological media are summarized in Table 1. In erythrocyte suspension, haemolysate and 1% liver homogenate, ONO-5046 was rapidly hydrolysed to EI-601, the hydrolysis following first-order kinetics, whereas it was slowly hydrolysed in whole blood and plasma. The degradation of ONO-5046 in erythrocyte suspension, haemolysate and liver homogenate was significantly inhibited by addition of 3% BSA.

In-vitro binding of ONO-5046 to BSA

Scatchard plot analysis for the binding of ONO-5046 to BSA indicated that ONO-5046 is bound to BSA at one kind of binding site with an association constant (K) of $6{\cdot}91 \times 10^4$ (M^{-1}) and number of binding sites (n) of 4·33 ($r^2 = 0{\cdot}9922$, mean of results from four experiments) (Figure 1). By use of these parameters, the percentage of protein binding of ONO-5046 at physiological protein concentrations (4%, 600 μM) was estimated by use of the equation:

$$\text{Protein binding (\%)} = \{(C_t + K_d + nP) - [(C_t - K_d - nP)^2 + 4K_dC_t]^{1/2}\} \times 100/2C_t \quad (1)$$

where C_t, K_d and P denote the total concentration of ONO-5046 (μM), dissociation constant (14·472 μM) and concentration of BSA (μM), respectively. The estimated percentages of ONO-5046 binding to protein were 99·4 and 99·1% at ONO-5046 concentrations of 100 and 1000 μM, respectively. This extremely high protein binding of ONO-5046 is in good agreement with that in rat plasma previously reported by Watanabe et al (1997).

Table 1. Degradation rate-constant and half-life of ONO-5046 in various biological media at 37°C in the presence or absence of BSA.

Medium	Without BSA		With BSA (3%)	
	Degradation rate-constant (min^{-1})	Half-life (min)	Degradation rate-constant (min^{-1})	Half-life (min)
Whole blood	0·013 ± 0·005	71·7 ± 25·6	ND*	ND
Plasma	0·006 ± 0·001	118·5 ± 14·0	ND	ND
Erythrocyte suspension	0·101 ± 0·021	7·2 ± 1·5	0·009 ± 0·001	80·4 ± 11·1
Haemolysate	0·142 ± 0·007	4·9 ± 0·3	0·014 ± 0·002	50·8 ± 6·9
Liver homogenates	0·038 ± 0·002	18·2 ± 0·8	ND	ND

*Not determined. Erythrocyte suspension: erythrocytes were suspended in an equal volume of pH 7·4 PBS. Haemolysate: erythrocytes were haemolysed in four times their volume of water. The initial concentration of ONO-5046 in each medium was 100 μM. Each value is the mean ± s.e.m. of results from 3 or 4 trials.

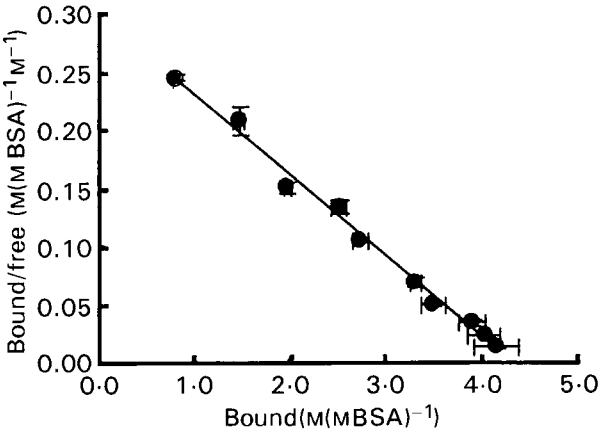

Figure 1. Scatchard plot for binding of ONO-5046 to BSA. Each value is the mean ± s.e.m. of results from four trials.

Hepatic clearance of ONO-5046 in-vivo

Under different steady-state ONO-5046 plasma concentrations, total plasma clearance (CL_{total}) and the hepatic extraction ratio (ER) of ONO-5046 were determined according to the equations $CL_{total} = I_o/C_{ss}$ and $ER = (C_a - C_h)/C_a$, respectively, where I_o, C_{ss}, C_a and C_h denote the constant infusion rate (nmol min^{-1}), the steady-state plasma concentration (nmol mL^{-1}), the arterial plasma concentration (same as C_{ss}), and the hepatic vein plasma concentration of ONO-5046 (nmol mL^{-1}), respectively. Results are summarized in Table 2. In the concentration range 5 to 50 nmol mL^{-1} for C_{ss}, the total plasma clearance and hepatic extraction ratio of ONO-5046 were constant. Supposing that the physiological hepatic plasma flow (Q_{hep}) is 32·7 mL min^{-1} kg^{-1} and the hepatic extraction ratio of ONO-5046 is 26·9% (mean value in this study), the hepatic clearance (CL_{hep}) of ONO-5046 estimated by $CL_{hep} = ER \times Q_{hep}$ was 8·8 mL min^{-1} kg^{-1}. The estimated CL_{hep} was the same as the observed CL_{total} of ONO-5046, indicating that the total plasma clearance of ONO-5046 is essentially accounted for by hepatic clearance.

Hepatic clearance of ONO-5046 in the rat perfused liver

When ONO-5046 alone (without BSA) was injected as a bolus into the perfusate of the rat perfused liver, most of the intact ONO-5046 was recovered in the effluents within 30 s; complete recovery was achieved within 2 min. In contrast, EI-601, the metabolite of ONO-5046, was recovered gradually in the effluents over 25 min. No intact ONO-5046 was detected in the bile, even for high doses (5·0 μmol), and EI-601 alone was excreted over 60 min. Table 3 summarizes the recoveries of ONO-5046 and EI-601 in the effluents and in the bile within 25 and 60 min, respectively, after ONO-5046 bolus injection. The sum of the percentage recoveries of ONO-5046 and EI-601 in the effluents and bile during the experiments depended on the doses administered: 63·2 ± 6·9% at a dose of 0·25 μmol and 98·4 ± 5·2% at a dose of 5·0 μmol. The percentage recoveries of intact ONO-5046 in the effluents increased from 11·4 ± 1·4 to 80·5 ± 5·2% when the dose was increased from 0·25 to 5·0 μmol, and therefore, the extraction ratios of ONO-5046 decreased from 90 to 20% in this dosing range. It was also found that ONO-5046 extracted by the liver was metabolized to EI-601 and approximately 20–25% of the amount extracted was eliminated into the bile, the remainder being effluxed from the liver back into the effluents.

The hepatic extraction ratio of ONO-5046 under constant-infusion at different concentrations also decreased as the concentration of ONO-5046 was increased; the extraction ratio was 99·3 ± 0·6% at a concentration of 10 μM; 99·4 ± 0·01% at 20 μM; 91·8 ± 3·2% at 50 μM; and 75·8 ± 3·9% at 100 μM.

The effects of the concentrations of BSA in the dosing solution on the hepatic extraction of ONO-5046 are summarized in Table 4. The hepatic extraction ratio of ONO-5046 without BSA, injected as the 6th run, was the same as that of the 1st run, indicating that the viability of the liver remained constant. As the concentration of BSA in the dosing solution was increased the hepatic

Table 2. Hepatic clearance of different steady-state plasma concentrations of ONO-5046 in rats.

Parameter	Infusion rate (nmol min^{-1})		
	10	50	100
Arterial plasma concentration (μM)	4.97 ± 0.30	25.83 ± 1.26	52.80 ± 4.5
Hepatic venous plasma concentration (μM)	3.58 ± 0.27	19.38 ± 0.89	38.12 ± 2.98
Total clearance (mL min^{-1} kg^{-1})	8.95 ± 0.56	8.78 ± 0.45	8.65 ± 0.56
Hepatic extraction ratio (%)	28.1 ± 1.3	25.1 ± 1.5	27.5 ± 1.9
Availability (%)	71.9 ± 1.3	74.9 ± 1.5	72.5 ± 1.9

Availability = 100 − hepatic extraction ratio (%). Each value is the mean ± s.e.m. of results from 4 to 6 trials.

Table 3. Recovery of ONO-5046 and EI-601 in effluent and of EI-601 in bile after bolus injection of different doses of ONO-5046 in the rat perfused liver.

Dose of ONO-5046 (μmol)	0·25	0·5	1	5
Recovery of ONO-5046 in effluent (%)	11·4±1·4	32·2±6·3	53·9±2·5	80·5±5·2
Recovery of EI-601 in effluent (%)	26·6±3·1	19·0±6·1	15·2±2·6	13·0±3·0
Recovery of EI-601 in bile (%)	25·2±2·4	15·6±3·2	8·8±1·2	4·9±0·9

Effluent and bile were collected for 25 min and for 60 min, respectively, after injection. Each value is the mean ± s.e.m. of results from 4 or 5 trials.

Table 4. Effect of BSA on the hepatic extraction ratio of ONO-5046 injected at a dose of 0.25 μmol in the rat perfused liver.

	Hepatic extraction ratio (%)
Without BSA	91·5±2·0
With 1% BSA	88·3±3·1
With 2% BSA	83·0±3·5
With 5% BSA	72·9±3·3
With 10% BSA	66·0±2·5
Without BSA	94·2±1·4

Each value is the mean ± s.e.m. of results from 4 trials.

extraction ratio of ONO-5046 was significantly reduced, although the value observed in the presence of BSA was still higher than that observed in-vivo (mean 26·9%) at any BSA concentrations employed.

Discussion

In this study we have examined the hepatic clearance of ONO-5046 both in-vivo and in the rat perfused liver to clarify the mechanism of clearance of ONO-5046. In general, it would be speculated that the total plasma clearance of ester compounds is high, because esterases have broad substrate specificity and exist in most organs and biological fluids (Leinweber 1987; Nakazono et al 1991; Aldridge 1993; Morgan et al 1994). However, the total plasma clearance of ONO-5046, which was equivalent to the hepatic clearance, was relatively low and constant at different steady-state plasma concentrations between 5 and 50 nmol mL^{-1} (Table 2).

In both injection modes, bolus injection and constant-rate infusion of ONO-5046 into the perfusate, the extraction ratio of ONO-5046 was reduced in a concentration (dose)-dependent manner in the rat perfused liver (Table 3). The reduction in the extraction ratio of ONO-5046 with increasing concentration (dose) in the perfusate might be because of the saturation of hepatic intrinsic clearance (CL_{int})—the uptake clearance of ONO-5046 is unlikely to be a rate-determining step because of the high lipophilicity and low molecular weight of ONO-5046. Saturable metabolism of ONO-5046 was also observed in an in-vitro stability study employing 1% liver homogenate; the degradation rate constant was $0{\cdot}038 \pm 0{\cdot}002\ min^{-1}$ at a concentration of 100 μM, $0{\cdot}024 \pm 0{\cdot}002\ min^{-1}$ at 200 μM, and $0{\cdot}016 \pm 0{\cdot}002\ min^{-1}$ at 400 μM.

Thus, CL_{int} could be given by a Michaelis-Menten type equation:

$$CL_{int} = V_{max}/(K_m + C_v \times f) \quad (2)$$

where V_{max}, K_m, C_v and f denote, respectively, the maximum metabolic rate (μmol min^{-1}), the Michaelis constant (μM), the concentration in the effluent, and the unbound fraction of ONO-5046 in the perfusate (f = 1 in this study without BSA). In this analysis, the unbound concentration of ONO-5046 in the liver was assumed to be equal to $C_v \times f$.

Then, the metabolic rate (V) of ONO-5046 under steady-state conditions is described by:

$$V = (C_a - C_v) \times Q = CL_{int} \times C_v \times f = V_{max} \times C_v \times f/(K_m + C_v \times f) \quad (3)$$

or

$$1/V = (K_m/V_{max}) \times (1/C_v \times f) + 1/V_{max} \quad (4)$$

where, C_a and Q denote, respectively, the concentration of ONO-5046 in the perfusate and flow rate of perfusate ($Q = 30\ mL\ min^{-1}$ in this study). When 1/V was plotted against $1/(C_v \times f)$, the regression line fitted the observed values well. Estimated values of V_{max} and K_m were 2·467 μmol min^{-1} and 0·498 μM, respectively.

When extraction ratios for ONO-5046 by the perfused liver were compared for bolus injection and constant-rate infusion, the values for bolus injections of 0·25 μmol in 0·5 mL and 0·5 μmol in 0·5 mL were almost comparable with those after constant infusion at concentrations of 50 nmol mL^{-1} and 100 nmol mL^{-1}, respectively. These results might suggest that ONO-5046 administered as a bolus was diluted approximately

tenfold by the perfusate before extraction by the liver.

BSA in the dosing solution suppressed the extraction ratio of ONO-5046 significantly (Table 4). The decrease in the extraction ratio should be because of the decrease in the unbound fraction of ONO-5046. However, the extraction ratio of ONO-5046 in the presence of 10% BSA was still higher than that observed in-vivo. Although the reason for this is not clear at present, the dilution of BSA itself in the perfusate and the supra-physiological flow rate of perfusate (30 mL min^{-1} per rat liver) might be responsible for the higher extraction ratio in-vitro.

The hepatic uptake of highly protein-bound substances such as warfarin, testosterone and rose bengal exceeds the value evaluated from the unbound plasma fraction, indicating that the unbound fraction and the protein-bound fraction are involved in the uptake (Tsao et al 1986, 1988; Guechot et al 1989). The uptake of protein-bound fractions is now considered to a result of dissociation of the binding in the unstirred layer of the Disse space (Ichikawa et al 1994; Schwab & Goresky 1996). A similar mechanism might be participating in the hepatic uptake of ONO-5046.

In this study, it was found that the total plasma clearance of ONO-5046 in rats is relatively low even though the drug has an ester linkage in its structure, and the total plasma clearance is accounted for by the hepatic clearance. The low clearance or stability, or both, in the central circulation could be because of its extremely high protein binding.

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