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SPECIES DIFFERENCES IN HYDROLYSIS OF ISOCARBACYCLIN METHYL ESTER (TEI-9090) BY BLOOD ESTERASES

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Abstract—Species differences in the hydrolysis of isocarbacyclin methyl ester (TEI-9090) in whole blood and in its separated components were studied in rats, dogs and human. Esterase activity in rat whole blood was approximately 100 and 400 times higher than that in dog and human whole blood, respectively, and was attributed to high plasma activity. In contrast, TEI-9090 hydrolysis activities in dog and human blood were due to red blood cells (RBC), whose activity in humans was slightly suppressed by albumin. In dogs, activity in RBC membranes was 10 times greater than in the cytosol, while in human membrane and cytosol activity was virtually the same. The effects of the esterase inhibitor diisopropylfluorophosphate, bis-*p*-nitrophenylphosphate (BNPP), eserine, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and *p*-chloromercuribenzoate showed that the rat plasma and RBC cytosol esterases hydrolysing TEI-9090 were carboxylesterase (CarbE) and arylesterase (ArE), respectively. The esterases in dog plasma and RBC membrane were CarbE, and RBC cytosol esterase was ArE. In humans, the esterase activities in plasma, RBC membrane and cytosol were butyrylcholinesterase, CarbE and ArE, respectively.

Key words: isocarbacyclin; TEI-9090; esterases; red blood cells; plasma; inhibitors

The bioreversible chemical modification of drugs has been regarded as a useful method in overcoming various physicochemical and biopharmaceutical problems [1, 2]. The potency of such drugs depends in general on their conversion efficiency to their active forms. For biologically based drug design, it has become an important issue to consider the conversion site in the body, the corresponding enzymes and their activities. In the case of ester forms, carboxyl ester hydrolases (EC 3.1.1) are particularly worthy of attention. Numerous studies have shown that a variety of xenobiotics are metabolized by these carboxyl ester hydrolases, which exhibit broad substrate specificity and hydrolytic activities which vary among species and individuals [3, 4]. Although a wide distribution of the enzymes in mammalian tissues has been observed, in many cases their physiological functions remain incompletely understood [5].

TEI-9090§ [6], a stable prostaglandin I_2 analogue, was synthesized because it possesses a higher lipophilicity compared to its carboxylic acid form (TEI-7165), resulting in successful entrapment in an oil-in-water emulsion for intravenous administration [7]. Esterases in blood, lung and liver tissues might play an important part in determining pharmaceutical and biological properties of the oil-in-water emulsion containing TEI-9090 dosed intravenously. In our previous paper [8], we demonstrated a possible role for blood esterases in the cleavage of the methyl ester of TEI-9090, triggering release of the drug from the emulsion *in vitro*. In the present study, we have identified the significant species differences in esteratic activities of whole blood and its components, and have attempted to characterize these esterases using selective inhibitors.

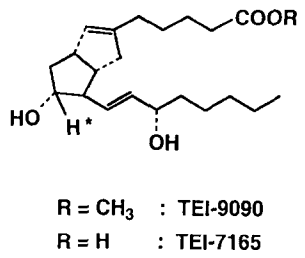

Fig. 1. Chemical structures of [^{3}H]TEI-9090 and [^{3}H]TEI-7165. * indicates the labelled position of ^{3}H.

MATERIALS AND METHODS

Chemicals. 11-β-[^{3}H]TEI-9090 (specific activity

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§ Abbreviations: TEI-9090, isocarbacyclin methyl ester; BC, blood cells; RBC, red blood cells; CarbE, carboxylesterase; ArE, arylesterase; BChE, butyrylcholinesterase; AChE, acetylcholinesterase; DFP, diisopropylfluorophosphate; HSA, human serum albumin; BNPP, bis-*p*-nitrophenylphosphate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); PCMB, *p*-chloromercuribenzoate.

of 0.67 TBq/mmol, > 99% pure) and unlabelled compound were donated by Teijin Co. Ltd, Tokyo. [^{3}H]Isocarbacyclin, TEI-7165, was obtained by hydrolysis of [^{3}H]TEI-9090 as described previously [9]. These chemical structures and labelled positions are shown in Fig. 1. Pig liver CarbE, human serum BChE, human RBC AChE, DFP, eserine and essentially fatty acid free HSA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). BNPP was obtained from Aldrich-Chemie (Steimheim, Germany), and DTNB and PCMB were obtained from Nacalai Tesque Inc. (Kyoto, Japan). All other chemicals were of analytical grade.

Whole blood and its separated components. Fresh whole blood samples were drawn from male Wistar rats (SLC Co., Shizuoka, Japan; 170–180 g), male beagle dogs (Laboratory Research Enterprises, Inc., Tokyo; 12.0–14.5 kg), and from three healthy volunteers aged 25–32 years, who had taken no medication for a week before blood sampling. The blood samples, containing 0.38% (w/v) of sodium citrate, were centrifuged at 4° at 1200 *g* for 10 min to separate the plasma from the BC. The BC were washed five times with equivolumes of isotonic Hank's buffer (Gibco, Grand Island, NY, U.S.A.), and were suspended in adequate volumes of buffer to obtain the same haematocrit values as the blood samples. During the procedure for the preparation of BC described above, the top white layer of the resultant BC, which contained the white BC and platelets, was removed and discarded to prepare RBC suspensions. The RBC were further separated into the membrane suspension and cytosol, according to the method of Dodge *et al.* [10]. In brief, the RBC suspension was diluted in a 10-fold volume of 20 mOsM PBS (pH 7.4) and centrifuged at 20,000 *g* for 30 min at 4° to prepare the cytosol, followed by dilution with an equivolume of 60 mOsM PBS (pH 7.4). The membrane precipitate was then washed three times with a 10-fold volume of Hank's buffer, and suspended in the buffer to make it equivolume with the original blood samples. An RBC suspension containing HSA (40 mg/mL) was prepared to examine the influence of HSA on human RBC esterases.

In vitro *hydrolysis assays.* Standard assay conditions were as follows: Ten microlitres of [^{3}H]TEI-9090 (1.36 μM) in ethanol was added to 1 mL of the enzyme sources, which had been pre-incubated for 10 min at 37°. At different time intervals, the samples were mixed with 4 mL ice-cold ethanol and centrifuged at 1500 *g* for 10 min. After the supernatant was evaporated to dryness under reduced pressure at 40°, the residue was dissolved in 200 μL ethanol. A 50 μL aliquot was injected into an HPLC connected in tandem to a radioactive detector (RI-HPLC), as described previously [7].

The *in vitro* half-lives of [^{3}H]TEI-9090 under different conditions was determined by a least-squares regression analysis of plots of the logarithm of [^{3}H]TEI-9090 concentrations against time. [^{3}H]-TEI-9090 hydrolysis activities in the whole blood and its components (diluted appropriately with Hank's buffer) were assumed to be the initial slopes of the linear regression lines of plots of the generation of TEI-7165 against time. The activities were corrected for dilution and expressed as the rate of [^{3}H]TEI-7165 generation in a unit time per equivalent volume of whole blood. To investigate the effects of the esterase inhibitors on each enzyme source, 10 μL of inhibitor solution (in Hank's buffer) was added to 1 mL of sample for [^{3}H]TEI-9090 hydrolysis assay.

RESULTS

In vitro *half-lives of* [^{3}H]*TEI-9090 in blood samples*

Figure 2 shows the hydrolysis of [^{3}H]TEI-9090 in whole blood, plasma and RBC suspensions obtained from rats, dogs and humans. In the experiments, no metabolite other than TEI-9090 or TEI-7165 was detected on RI-HPLC of all samples (data not shown). In rats, [^{3}H]TEI-9090 was completely hydrolysed within 1 min in whole blood and plasma; however, it was hydrolysed with a half-life of 1.9 min in RBC suspension. The half-life of the drug in dog whole blood was 6.8 min. While only minor hydrolysing activity was observed in dog plasma (a half-life of 140 min), the drug was hydrolysed with a half-life of 2.9 min in RBC suspension. In human whole blood, plasma and RBC, the half-lives of the drug were 17.3 min, 30.6 min and 8.0 min, respectively.

[^{3}H]TEI-9090 was hydrolysed with a half-life of 15.8 min in human RBC suspension containing HSA (Table 1).

Hydrolytic activities in whole blood and its separated components

The generation of TEI-7165 in whole blood and separated components from rats, dogs and humans was measured at different time intervals following addition of 13.6 nM [^{3}H]TEI-9090 (Fig. 3). Hydrolytic activities were calculated from the initial slopes of the linear regression lines in Fig. 3 (Table 2). Activity in rat whole blood was approximately 100 and 400 times higher than that in dog and human blood, respectively. In rats, whole blood esterase activity was nearly equal to that in plasma but in dogs and humans, approximated that in BC. RBC esterase activities were slightly lower than BC activities in all species. Membrane activity was 10 times higher than that in cytosol in dogs. Human RBC membrane and cytosol esterase activity was almost half that of RBC activity. In rats, membrane and cytosol esterase activities were significantly lower than the intact RBC.

Effects of esterase inhibitors on hydrolysis of TEI-9090

The esterase activities possessed by plasma, RBC membrane and cytosol were characterized using selective esterase inhibitors (Fig. 4). For reference, studies on pig liver CarbE, human serum BChE and human RBC AChE were performed.

The [^{3}H]TEI-9090 hydrolysis activity of pig liver CarbE (0.1 μg/mL) was 1.41 pmol/min. CarbE activity was not inhibited by eserine, which is known to inhibit AChE and BChE [11], nor by the arylesterase inhibitors DTNB [11] and PCMB [12]. However, the organophosphorus compound DFP [12, 13] and CarbE inhibitor BNPP [14] completely inhibited activity. Human serum BChE (70 μg/mL)

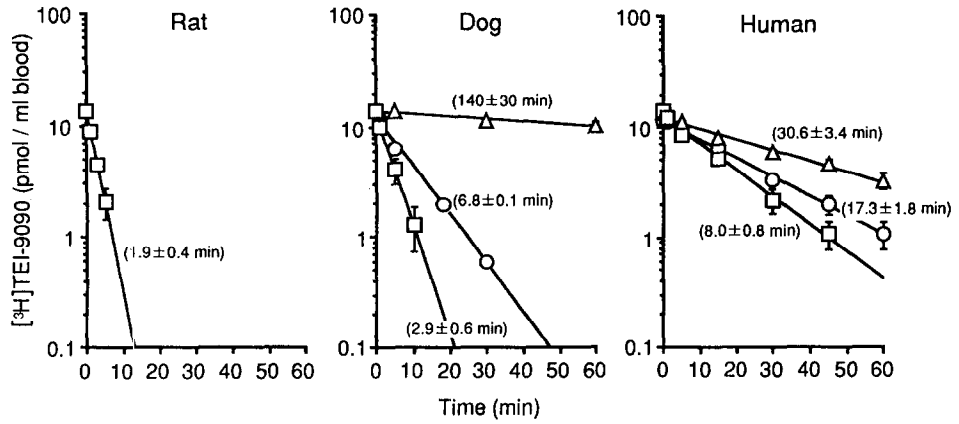

Fig. 2. Plots showing *in vitro* hydrolysis of [^{3}H]TEI-9090 (13.6 nM) in rat, dog and human (○) whole blood, (△) plasma, and (□) RBC suspension at 37°. Parentheses indicate the half-lives. Each point and half-life represents the mean ± SEM of three samples. When the values of SEM were small, they were included within the symbols. In rat whole blood and plasma, [^{3}H]TEI-9090 was completely hydrolysed within 1 min.

Table 1. Half-lives of [^{3}H]TEI-9090 in human whole blood and RBC with and without HSA (mean ± SEM, N = 3)

	Half-life (min)
Whole blood	17.3 ± 1.8
RBC	8.0 ± 0.8
RBC with HSA*	15.8 ± 2.8

* Human RBC suspended in Hank's buffer containing 40 mg/mL of HSA.

hydrolysed [^{3}H]TEI-9090 at 1.04 pmol/min, and was only inhibited by DFP and eserine. Human RBC AChE (4.0 mg/mL) scarcely hydrolysed [^{3}H]TEI-9090 (data not shown).

In dogs, the inhibitor profile of plasma esterase was similar to that of pig liver CarbE, the activity in RBC membrane was inhibited by DFP and BNPP, and that in RBC cytosol inhibited by DTNB and PCMB. In rat plasma and RBC cytosol, the inhibitor profiles were similar to those in dogs, although no clear-cut profile was observed in the experiment on rat RBC membrane.

The hydrolysis of [^{3}H]TEI-9090 in human plasma was not affected by BNPP, DTNB nor PCMB, but was 80% inhibited by eserine and completely inhibited by DFP. In human RBC membrane, the activity was inhibited by DFP, BNPP and PCMB, whereas in cytosol it was strongly inhibited by DTNB and PCMB, as seen in dog RBC cytosol.

DISCUSSION

Rat plasma showed significantly higher TEI-9090 hydrolysis activity than dog and human plasma. These differences were consistent with observations that CarbE activity in rat plasma is higher than that in dog and human plasma [12, 13, 15]. Similar species

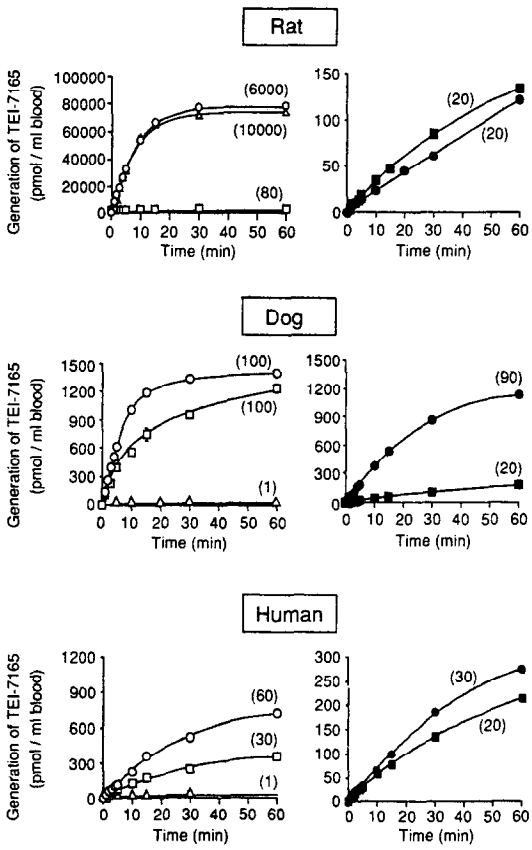

Fig. 3. Time course of generation of TEI-7165 in rat, dog and human (○) whole blood, (△) plasma, (□) RBC suspension, (●) RBC membrane, and (■) cytosol. [^{3}H]-TEI-9090 (13.6 nM) was incubated with the samples, which were diluted appropriately with Hank's buffer, at 37°. The values in parentheses indicate the dilution factors. Each point represents the mean and range of duplicate assays. Small ranges were included within the symbols.

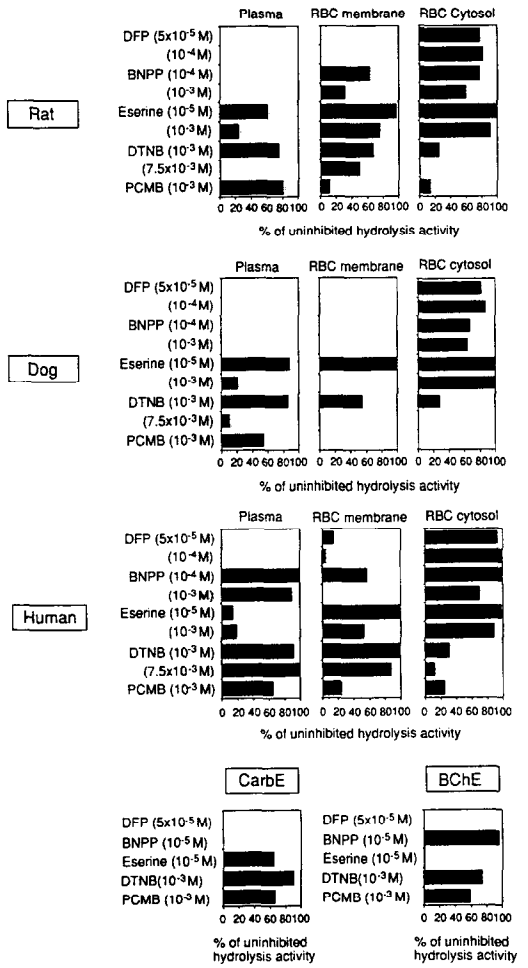

Fig. 4. Effects of various esterase inhibitors on *in vitro* hydrolysis of [^{3}H]TEI-9090 (13.6 nM) in rat, dog and human plasma, RBC membrane and cytosol. Each value represents the mean of triplicate assays. The ranges varied by less than 5% in all cases. The uninhibited hydrolytic rates in rat, dog and human plasma, RBC membrane and cytosol are indicated in Table 2. The uninhibited hydrolysis activities of pig liver CarbE (0.1 μg/mL) and human serum BChE (70 μg/mL) were 1.41 pmol/min and 1.04 pmol/min, respectively.

differences were observed in the hydrolysis of etomidate [16] and vinyl acetate [17]. In human plasma, esterase activity was inhibited by DFP and eserine, a similar pattern to that observed for BChE, known to be the non-specific esterase in human plasma [18] as well as dog plasma [13, 15]. Plasma BChE activity in three male beagles was determined to be 0.17 ± 0.04 μmol/mL/min (the mean ± SEM) by the method of Ellman *et al.* [19] using butyrylthiocholine chloride as substrate, and was completely inhibited by 10^{-5} M of eserine (data not shown). However, TEI-9090 hydrolysis in dog plasma was not affected by eserine but by BNPP as in the case of rat plasma. These observations suggest that TEI-9090 was hydrolysed by CarbE in dog plasma.

The fact that TEI-9090 hydrolysis in BC containing white blood cells and platelets was slightly higher than RBC in all three species suggests that components other than RBC possess esteratic activities. In humans, it has been reported that white blood cells [20, 21] and platelets [22] possess non-specific esterases. Our results seem to indicate that this is also the case for rat and dog BC.

Although the *in vitro* half-life of TEI-9090 in RBC was lower than that in dog and human whole blood (Fig. 2), RBC half-life was elongated by HSA in humans (Table 1). Similar findings with aspirin suggest that albumin molecules protect the drug from RBC esterases in human whole blood [23]. The same observation could be applied to TEI-9090, as the binding rates of TEI-9090 to serum albumin were shown to be higher than 90% in dogs and humans [9]. As demonstrated in Table 2, TEI-9090 hydrolysis activities in dog and human whole blood were higher than those in their RBC suspensions. However, these results do not contradict the results shown in Fig. 2, since albumin concentrations in the whole blood samples were lowered by dilution for the activity measurement (Table 2) in dogs and humans (100 and 60 times, respectively).

The RBC cytosol esterase hydrolysing TEI-9090, which was inhibited by DTNB and PCMB, appears to be an ArE in rats, dogs and humans, as previously seen for aspirin [12], diacetylmorphine [24] and esmolol [25]. A typical RBC membrane esterase, AChE, is known to hydrolyse specific substrates [26], and did not degrade TEI-9090. TEI-9090

Table 2. [^{3}H]TEI-9090 hydrolysis activities (pmol/mL blood/min)* in whole blood and its separated components in rats, dogs and humans

	Rat	Dog	Human
Whole blood	6020	61.5	14.8
Plasma	7820 ± 384	0.12 ± 0.01	0.17 ± 0.05
BC	51.4	77.0	13.9
RBC	39.3 ± 2.4	44.5 ± 3.7	11.3 ± 0.6
RBC membrane	1.82 ± 0.10	32.0 ± 2.9	5.40 ± 0.25
RBC cytosol	2.53 ± 0.02	3.56 ± 0.13	5.04 ± 0.38

* The activities were calculated from the initial slopes of the linear regression lines in Fig. 3, and were expressed as the rate of TEI-7165 generation per equivalent volume of the whole blood sample used (see text for details). Values were expressed as the mean ± SEM of triplicate assays, or the mean of duplicate assays where the range varied by less than 5%.

hydrolysis activities of dog and human RBC membranes were inhibited by DFP and BNPP. Accordingly, it was suggested that CarbE were the RBC membrane esterases in humans and dogs. CarbE binding to the cell membrane has been reported in monocyte [27, 28] and leukocyte [28]. While characteristics of RBC CarbE have also been studied [29–31], its localization in cells is unclear. We have concluded that RBC CarbE is probably located at the membrane, although further studies to confirm this finding are necessary.

We have previously reported that esterase activity is a predominant factor in determining TEI-9090 entrapment by oil-in-water emulsion in serum [8]. In the present study, we have demonstrated that blood cells, as well as plasma, have TEI-9090 hydrolysis activity in rats, dogs and humans. It is therefore conceivable that blood cells play an important role in determining not only the drug-release property of the emulsion containing TEI-9090 but also its biological activity.

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