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Research Paper

Concentration-dependent plasma protein binding of the novel dipeptidyl peptidase 4 inhibitor BI 1356 due to saturable binding to its target in plasma of mice, rats and humans

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Abstract

Objectives The purpose of this study was to characterise the plasma protein binding of BI 1356.

Methods BI 1356 (proposed trade name ONDERO) is a novel dipeptidyl peptidase 4 (DPP-4) inhibitor, which is under clinical development for the treatment of type 2 diabetes. DPP-4 is expressed in various tissues but soluble DPP-4 is also present in plasma. Therefore, binding to soluble DPP-4 may influence the pharmacokinetics of BI 1356. Plasma protein binding of BI 1356 was determined *in vitro* for wild type mice and rats and the results compared with those for DPP-4 knockout mice and DPP-4 deficient Fischer rats. In addition, protein binding of BI 1356 was examined in plasma from healthy human volunteers and renal excretion of the compound in the DPP-4 knockout mice was compared with that occurring in wild type mice.

Key findings The results showed that BI 1356 exhibited a prominent concentration-dependent plasma protein binding due to a saturable high affinity binding to the DPP-4 target in plasma. Differences in renal excretion of BI 1356 between DPP-4 knockout mice and wild type mice suggested that saturable binding of BI 1356 to DPP-4 in the body also influenced elimination.

Conclusions High affinity, but readily saturable binding of BI 1356 to its target DPP-4 accounted primarily for the concentration-dependent plasma protein binding at therapeutic plasma concentrations of BI 1356.

Keywords BI 1356; dipeptidyl peptidase-4 inhibitor; pharmacokinetics; plasma protein binding; type 2 diabetes

Introduction

BI 1356 (proposed trade name ONDERO) is a novel dipeptidyl peptidase 4 (DPP-4, EC3.4.14.5) inhibitor under clinical development for the treatment of type 2 diabetes.[1] The DPP-4 enzyme cleaves a large number of circulating bioactive peptides and is therefore involved in many physiological processes.[2] One important DPP-4 substrate is glucagon-like peptide 1 (GLP-1) which augments glucose-stimulated insulin secretion.[3] GLP-1 is inactivated after cleavage by DPP-4 and thus, inhibition of DPP-4 leads to an elevation of active GLP-1 levels and a prolonged action of GLP-1. Consequently, therapeutic DPP-4 inhibitors provide a promising strategy for the treatment of type 2 diabetes.[4]

Within plasma, the binding of a drug to proteins may exert a significant effect on its pharmacokinetics.[5] For the vast majority of drugs, the plasma protein binding percentage is virtually constant within each subject, as the most important drug-binding proteins such as albumin and α_1-acid glycoprotein are not saturated at therapeutic plasma levels. However, in cases where the drug-binding proteins can already be saturated at therapeutic doses, this may be a source of nonlinear pharmacokinetics. In particular, when the target protein for a drug is present in the plasma, high affinity but low capacity (saturable) plasma protein binding can occur at therapeutic dose levels. This has been reported, for example, for the angiotensin-converting enzyme (ACE) inhibitors RU44403 and ramipril.[6,7] In addition to prominent

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DPP-4 expression in various tissues, soluble DPP-4 is present in plasma.[2] Characterisation of the plasma protein binding of BI 1356 may provide a useful strategy to help to understand the pharmacokinetics of this compound in humans.

Several animal models have been developed to study the role of DPP-4 in normal physiology and in disease. In DPP-4 knockout mice, the DPP-4 protein is absent from plasma and in DPP-4 deficient Fischer rats only a mutant and enzymatically inactive form of the DPP-4 protein is initially synthesised, preventing processing to the mature DPP-4.[8–10] Very low levels of mature DPP-4 have been identified in lung endothelial cells of DPP-4 deficient rats, but with respect to the majority of tissues and plasma this rat strain can be regarded as a 'protein knockout' model for DPP-4.[11,12] When plasma isolated from these models is studied in parallel with plasma from wild type mice and rats, this offers a means to compare the pharmacokinetics of a compound such as BI 1356 in the presence and absence of DPP-4.

The aim of this study was to characterise the plasma protein binding of BI 1356 in mice, rats and humans. Plasma protein binding of BI 1356 was determined *in vitro* for wild type mice and rats using equilibrium dialysis. The results were then compared with those for DPP-4 knockout mice and DPP-4 deficient Fischer rats.[8,13] Protein binding of BI 1356 was also characterised in the pooled plasma from healthy volunteers. To examine whether the plasma protein binding of BI 1356 observed *in vitro* correlated with urinary excretion *in vivo,* renal excretion of the compound in DPP-4 knockout mice was compared with that occurring in wild type mice.

Materials and Methods

Equilibrium dialysis

A Dianorm Equilibrium Dialyzer (GD-4, Dianorm, Germany) with Teflon Macro 1 cells and Dianorm membranes (10 000 Da MW cutoff) was used. The cells were loaded with 750 μl plasma on one side and 750 μl dialysis buffer on the other side of the membrane. Dialysis was performed at 37°C for 6 h, which had been shown to provide equilibrium conditions.

Reagents

The dialysis buffer consisted of a 137 mM $NaCl^-$, 2.7 mM KCl^- and 10 mM phosphate buffer (PBS) with dextran (from Leuconostoc ssp., Mr ~40 000 Da, Fluka 31389). Dextran was added to the dialysis buffer to minimise the osmotic volume shift between plasma and buffer and the concentration was adjusted according to species differences in colloid osmotic pressure. The dextran concentrations in the dialysis buffer were 32, 34 or 31 g/L for the experiments with mouse, rat and human plasma, respectively.

Animals

Male and female DPP-4 knockout mice were purpose bred under license (see Acknowledgements) at Charles River, Kisslegg (Germany).[8] Mice of the corresponding wild type strain C57BL/6J with the same genetic background were supplied by Charles River (Germany). In addition, plasma from DPP-4 deficient Fischer rats of the strain F344/DuCrlCrlJ (Charles River, Japan) and the corresponding wild type strain (Charles River, Germany) was used. Animal procedures were approved by the local animal ethics committee and complied with the German Animal Protection Act.

Plasma preparation

Animal blood was collected by exsanguination (heart puncture or puncture of the aorta abdominalis under isoflurane anaesthesia). Blood from male and female healthy volunteers was taken by puncture of an antecubital vein. The blood was collected into tubes containing edetic acid as anticoagulant. After centrifugation for 15 min at 4°C, plasma was harvested. Plasma pools (including male and female) were prepared for each species and the plasma was stored frozen at –20°C. On the day of use, the plasma was thawed and the pH was adjusted to 7.4 using hydrochloric acid (1 mol/L).

The blood sampling from volunteers was conducted after informed consent. This was in compliance with the guidelines on good clinical practice and with ethical standards for human experimentation established by the Declaration of Helsinki and in accordance with applicable regulatory requirements.

[^{3}H]BI 1356 and preparation of stock solutions

^{3}H-Radiolabelling of BI 1356 was performed by Tritec, Teufen, Switzerland, and solutions of [^{3}H]BI 1356 in ethanol were used as stock solutions (83 Ci/mmol). The discovery and synthesis of BI 1356 has been described previously.[1] The radiochemical purity was 98.3% after synthesis and 97.3% at the end of the study. Thus, the purity was greater than 97.3% throughout the study. Unlabelled BI 1356 was obtained from Boehringer Ingelheim Pharma GmbH & Co. KG, Department of Chemical Development, Biberach (Germany). The structural formula of BI 1356 is given in Figure 1. Depending on the target concentrations, the stock solution of [^{3}H]BI 1356 was directly added into plasma or further diluted with deionised water before being added. In addition, for target concentrations exceeding 300 nM BI 1356 in the plasma incubations, the specific radioactivity was decreased by mixing samples of [^{3}H]BI 1356 with a solution of nonlabelled BI 1356 in ethanol accordingly. The achieved plasma concentrations of [^{3}H]BI 1356 at equilibrium were in the range 0.172–13 100 (wild type mice), 0.0628–14 300 (DPP-4 knockout mice), 0.139–12 700 (wild type rats), 0.0636–13 800 (DPP-4 deficient rats) and 0.021–3340 nM (humans). In the case of human plasma, concentrations up to 29 900 nM were also tested. However, as saturation of a second class of binding sites was indicated and as these concentrations were far beyond the expected therapeutic exposure levels in humans, these data were not included in the evaluation.

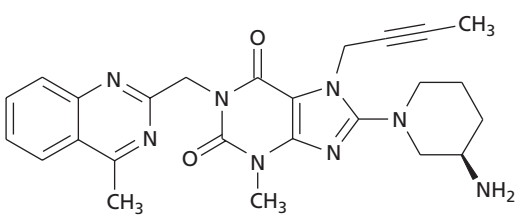

Figure 1 Structural formula of BI 1356.

Quantification of radioactivity

After dialysis, the contents of the dialysis cells were completely transferred into scintillation vials, the weight was captured and 12 ml scintillation fluid (Ultima Gold, Perkin Elmer, US) was added. After a chemiluminescence decay period of approximately 4 h, the vials were counted for 10 min on a liquid scintillation counter (TriCarb 2900 TR, Perkin Elmer, US). The radioactivity was quantified using the tSIE (transformed spectral index of external standard) method for quench correction. Only concentration values based on at least 25 dpm (disintegrations per min) per measured sample were included in the evaluation.

Calculation of radioactivity recovery

Recovery of the radioactivity at the end of the dialysis experiment was calculated as follows:

$$\text{recovery}(\%) = 100 \cdot \left(\frac{C_{don} - C_{acc}}{C_{inc}}\right) \quad (1)$$

where C_{don} and C_{acc} are the radioactivity concentration in the donor and acceptor chamber at equilibrium, respectively, and C_{inc} is the radioactivity concentration in the plasma mixed with stock solution incubation before dialysis, assuming that no volume shift occurs, i.e. the volume of the donor and acceptor chamber at equilibrium are identical (see Discussion). Due to the limited plasma volume available the remaining volume of the plasma mixed with stock solution was not sufficient for measurement of the radioactivity concentration in some of the experiments.

Estimation of BI 1356 binding parameters

The fraction bound (f_B) in percent was calculated as follows:

$$\text{f}_\text{B}(\%) = 100 \cdot \left(\frac{C_{don} - C_{acc}}{C_{don}}\right) \quad (2)$$

where C_{don} and C_{acc} are the radioactivity concentration in the donor and acceptor chamber at equilibrium, respectively. The binding parameters were estimated by fitting the observed data to the following equation (see statistical analysis):

$$C_B = \frac{N_1 \cdot K_1 \cdot C_U}{1 + K_1 \cdot C_U} + (N_2 \cdot K_2) \cdot C_U \quad (3)$$

where C_B and C_U are the molar concentration of bound and unbound [^{3}H]BI 1356 in plasma, N_1 and N_2 are the concentrations of binding sites for the first, saturable and the second nonsaturable binding process, respectively, and K_1 and K_2 are the respective affinity constants. As N_2 and K_2 cannot be determined independently, the product of N_2 K_2 was calculated. C_B was calculated as plasma concentration minus buffer concentration of [^{3}H]BI 1356 related radioactivity at equilibrium. In addition, for correlating f_B with the plasma concentration x in the plasma of wild type mice and rats and humans, the following Hill equation was used:

$$f_B(x) = \min f_B + \frac{(\max f_B - \min f_B)}{1 + 10^{(\log x - \log^{c50}) \bullet H}} \quad (4)$$

where *max* f_B is the maximum extent of protein binding, H is the Hill coefficient, *min* f_B is the minimum extent of protein binding, C50 is the plasma concentration with a half-maximal binding with respect to min f_B. The variable is the plasma concentration x. C50 was calculated from log(C50).

Urinary excretion of [^{14}C]BI 1356 in mice

To study the urinary excretion of BI 1356, three female wild type or DPP-4 deficient mice per dose level were housed together in a metabolic cage separated for wild type and DPP-4 knockout mice. [^{14}C]BI 1356 was synthesised by Boehringer Ingelheim Pharma GmbH & Co. KG. The animals received a single intravenous bolus injection of [^{14}C]BI 1356 dissolved in saline (0.9% NaCl) into a tail vein. The [^{14}C]BI 1356 dose levels were 0.01, 0.1, 0.3, 1, 3 and 10 mg/kg. Pooled urine from each group of three mice was collected up to 72 h after dosing in three time intervals (0–8, 8–24 and 24–72 h). The weight of the urine fraction was captured and the radioactivity concentrations were measured after addition of scintillation fluid (as described above). The urinary excretion was calculated as cumulative amounts of radioactivity (0–72 h) in percent of the administered dose relating to all three mice of one dose group and the same strain.

Statistical analysis

For the statistical analysis weighted nonlinear regression models were fitted. The weights are given by $1/(f_B)^2$ and $1/(C_B)^2$, respectively. Parameter estimates and its standard errors (SE) are reported. To compare the fraction of the bound [^{3}H]BI 1356 in plasma for the different species, $f_B(x)$ was fitted for the three species simultaneously, using the same model with an equation (eqn 4) defined for each species. Moreover, it was assumed that the variances of the errors would depend on the species. More precisely, the variances of the error terms were scaled by $(1 + \sigma_{species})^2$, where σ_{human} was set to 0 and σ_{rat} and σ_{mouse} were estimated from the data. Based on these results differences of the parameters for the different species were estimated. These computations were conducted with SAS (ver 8.2, SAS Institute Inc.), in particular the MODEL procedure was used for the parameter estimation. The corresponding figures were generated with SigmaPlot (ver 10.0, SYSTAT Software Inc., Chicago, Illinois, US). In addition, Scatchard plots were constructed by plotting C_B/C_U against C_B.

Results

Plasma protein binding

The mean ± SD recovery values of radioactivity in the dialysis experiments were 111.5 ± 5.6% (n = 15) for wild type mice, 121.3 ± 18.2% (n = 16) for DPP-4 knockout mice, 100.4 ± 7.5% (n =12) for wild type rats, 113.6 ± 5.0% (n = 16) for DPP-4 deficient rats and 103.6 ± 9.7% (n = 89) for humans. Whereas in rat and human plasma after dialysis only a negligible volume shift was observed (geometric mean of the recovered plasma/buffer volume was 0.98 and 1.06, respectively), the volume shift was more pronounced in plasma of wild type and DPP-4 knockout mice and DPP-4 deficient rats

(geometric mean plasma/buffer ratio = 0.82, 0.85 and 0.80, respectively).

Figure 2 shows the concentration dependency of plasma protein binding by BI 1356. In the wild type mouse and rat, as well as in human plasma, the fraction of [^{3}H]BI 1356 related radioactivity bound decreased from approximately 99% at concentrations below 1 nM to 70–80% at concentrations higher than 100 nM. In contrast, in DPP-4 knockout mice and DPP-4 deficient rats the plasma protein binding was constant at all concentrations investigated (Figure 2). The results of the regression of f_B vs concentration in mouse, rat and human plasma are shown in Table 1. The result of the nonlinear regression C_B vs C_U is shown in Figure 3 for wild type mouse, rat and human plasma. The resulting binding parameters are presented in Table 2. A Scatchard plot of BI 1356 plasma protein binding in all investigated species and strains is shown in Figure 4.

Statistically significant differences were detected for the log(C50) values between human and wild type rat as well as human and wild type mouse plasma ($P < 0.0001$). In addition, a minor difference for log(C50) was detected between wild type mouse and rat plasma ($P = 0.0341$). Besides C50, the mouse showed statistically significant differences in *min* f_B when compared with rat and human plasma ($P < 0.0001$). No statistically significant differences were detected for *max* f_B and the Hill coefficient (H) resulting in P-values between 0.2399 and 0.7497.

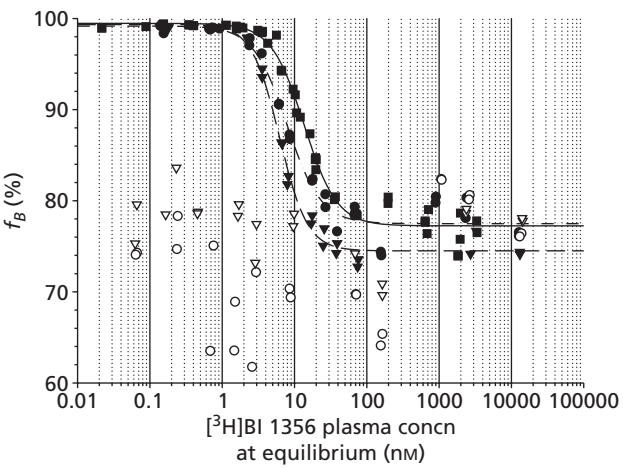

Figure 2 Concentration dependency of the plasma protein binding of BI 1356. Concentration dependency of: human ■, ____; wild type rat ●, –·–·–·–; wild type mouse ▼, – – –; dipeptidyl peptidase 4 deficient rat ○; dipeptidyl peptidase 4 knockout mouse ▽.

Urinary excretion of BI 1356 in DPP-4 knockout and wild type mice

Table 3 shows the amount of radioactivity (expressed as percent of dose) found in urine of female wild type or DPP-4 knockout mice after a single intravenous bolus injection of [^{14}C]BI 1356 (0.01, 0.1, 0.3, 1, 3 and 10 mg/kg). The urinary excretion increased from 2.7% at 0.01 mg/kg [^{14}C]BI 1356 to 24.5% at 10 mg/kg in wild type mice. This contrasted with a virtually constant urinary excretion between 16.8 and 24.7% of the administered dose observed in DPP-4 knockout mice over the identical dose range of [^{14}C]BI 1356.

Discussion

For the first time it has been established that the novel DPP-4 inhibitor BI 1356 exhibited a prominent concentration-dependent plasma protein binding. At concentrations below 1 nM, the fraction of BI 1356 bound to plasma proteins in mouse, rat and human plasma *in vitro* was very high (f_B ~99%). When the concentration of BI 1356 in plasma was increased, its overall protein binding percentage quickly decreased and at concentrations beyond 100 nM, protein binding of approximately 70–80% was observed.

It is worth noting that the radiochemical purity of [^{3}H]BI 1356 (98.3% after synthesis and 97.3% at the end of the experiments) was in the range of the observed maximum protein binding. Thus, impurities and degradation products could have contributed to the buffer concentrations of radioactivity leading to an underestimation of the maximum extent of binding. However, it is unlikely that putative impurities or degradation products would not bind to plasma proteins at all and therefore bias the results only subordinately. In addition, the calculated mean values for the recovery of radioactivity after the dialysis experiments were approximately 100% in the case of human and wild type rat plasma, but exceeded 100% in the case of wild type and DPP-4 knockout mice and DPP-4 deficient rats. Thus, the recovery of radioactivity was complete and nonspecific binding to the dialysis cells or membrane appeared to be of no relevance. However, addition of dextran to the buffer did not fully prevent volume shift across the membrane, as indicated by ratios of the recovered plasma/buffer between 0.8 and 1.06. This is most likely the explanation for the recovery values which exceeded 100%, as recovery was calculated assuming no volume shift. Overall, these technical issues should be considered for the interpretation of the quantitative values for the binding parameters but were regarded as of little relevance with regard to the main purpose of the study, i.e. to demonstrate a concentration-dependent plasma protein binding of BI 1356.

Table 1 Parameters derived from the nonlinear regression of f_B vs the plasma concentration of [^{3}H]BI 1356 BS in three different species

Species	*Max* f_B (%)	*Min* f_B (%)	*H*	log C50 (log(nM))	C50 (nM)
Mouse (wild type)	99.3 ± 0.6	74.5 ± 0.3	2.3 ± 0.2	0.7958 ± 0.02	6.2
Rat (wild type)	99.2 ± 0.8	77.5 ± 0.5	1.9 ± 0.3	0.8991 ± 0.036	7.9
Human (plasma pool)	99.5 ± 0.4	77.3 ± 0.3	1.9 ± 0.2	1.1326 ± 0.018	13.6

Values are mean ± SE. H = Hill coefficient.

Binding parameters for BI 1356 in mouse, rat and human plasma revealed two classes of binding sites. The most prominent class was characterised by a low concentration of binding sites with N_1 of 3.26, 3.84 or 6.35 nM (Table 2), combined with a very high affinity constant K_1 between 1.96 and 3.36 $\times$ 10^{10}/M, respectively. A high affinity, but low capacity binding process therefore appeared to be responsible for the concentration-dependent plasma protein binding by BI 1356, suggesting that a highly specific binding process occurred.

Although the major fraction of DPP-4 expressed in the body is bound to membranes and located in tissues, a soluble form of DPP-4 is also present in plasma.[2,12] In human plasma, protein concentrations of soluble DPP-4 (also known as sCD26) are reported to be in the low nanomolar range, which is in agreement with the number of sites observed for the first class of BI 1356 plasma binding sites in this investigation.[14,15] Furthermore, BI 1356 is a highly potent DPP-4 inhibitor with a very low IC50 (concentration required for 50% inhibition) of approximately 1 nM, demonstrating very high affinity to its target.[16] Specific and high affinity binding of BI 1356 to its target DPP-4 in plasma is therefore a likely explanation for the nonlinear protein binding observed. To test this hypothesis, we analysed the plasma protein binding of BI 1356 in plasma of DPP-4 knockout mice and DPP-4 deficient rats. In both these animal strains, DPP-4 enzymatic activity and protein expression are virtually lacking. The concentration dependency of BI 1356 plasma protein binding was completely abolished in the plasma of both of these animal models and the protein binding percentage (70%) was similar beyond and below the saturation concentration observed in wild type mice and rats (Figure 2). This suggests that only the second class of BI 1356 plasma binding sites remained in these animals. In-vitro experiments with isolated proteins further demonstrated that neither albumin nor α_1-acid glycoprotein could account for the DPP-4 independent BI 1356 binding processes. At approximately 700 nM, only approximately 40% BI 1356 bound to a 45 g/L solution of human albumin or a 1 g/L solution of α_1-acid glycoprotein (data not shown). In addition, at 10 μM BI 1356 indications of saturation of the second class of binding sites were found in human plasma *in vitro*. This was not explored further as this concentration was greatly above the therapeutic plasma levels of BI 1356. The peak plasma levels in humans at the therapeutic dose 5 mg BI 1356 per subject were in the region of 5–10 nM. Therefore, a putative saturation of binding sites at 10 μM is of no relevance for drug therapy. Taken together, our results suggest that high affinity, but readily saturable binding of BI 1356 to its target DPP-4 accounts for the concentration-dependent plasma protein binding.

Small, but consistent species differences in the calculated DPP-4 concentration were suggested by the protein binding data. Assuming an equimolar binding of one molecule BI 1356 per DPP-4 monomer, N_1 essentially reflects the concentration of DPP-4 monomers. Whereas in humans, the estimated DPP-4 monomer concentration was 6.35 $\pm$ 0.43 nM, approximately twofold lower levels were determined for wild type mice (3.36 $\pm$ 0.28 nM) and rats (2.34 $\pm$ 0.35 nM) (Table 2). As the plasma DPP-4 concentration determins the C50 value, this difference is also reflected in the estimated C50 values. The statistical analysis revealed highly significant differences between the log (C50) values of human and rat plasma and also between human and mouse plasma ($P < 0.0001$). A minor difference was detected between mouse and rats ($P = 0.0341$). No statistically significant differences were detected for the maximum

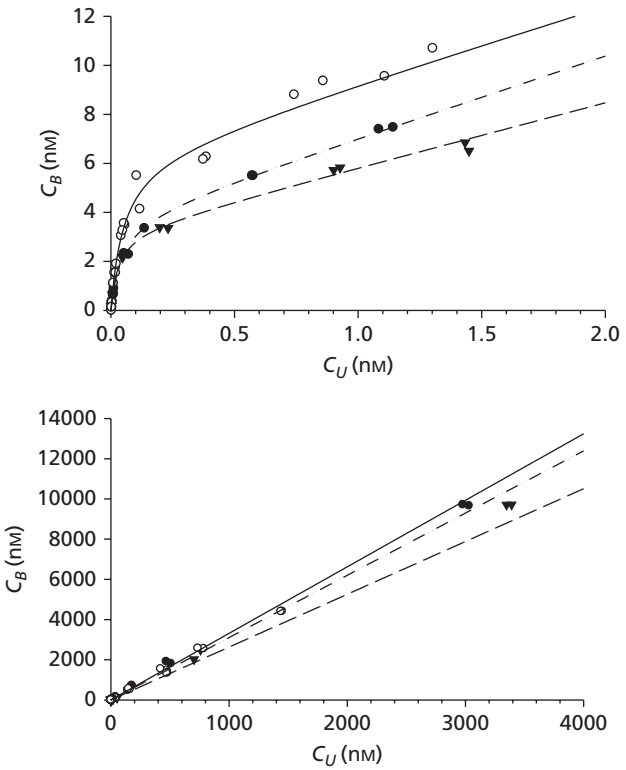

Figure 3 BI 1356 binding to plasma of wild type mice, wild type rats and humans. Binding is saturable and nonsaturable. The results of the nonlinear regression of the bound vs the free concentration are depicted for: human ○, —; wild type rat ●, –··–··–; wild type mouse ▼, - - -.

Table 2 Binding parameters of [^{3}H]BI 1356 in plasma of three different species

Species	K_1 (1/M)	N_1 (nM)	Dipeptidyl peptidase 4 dimer concn (nM)	N_2K_2
Mouse (wild type)	3.36 ± 0.28 (× 10^{10})	3.26 ± 0.19	1.63	2.63 ± 0.07
Rat (wild type)	2.34 ± 0.35 (× 10^{10})	3.84 ± 0.42	1.92	3.31 ± 0.15
Human	1.96 ± 0.18 (× 10^{10})	6.35 ± 0.43	3.18	3.10 ± 0.10

Values are mean ± SE.

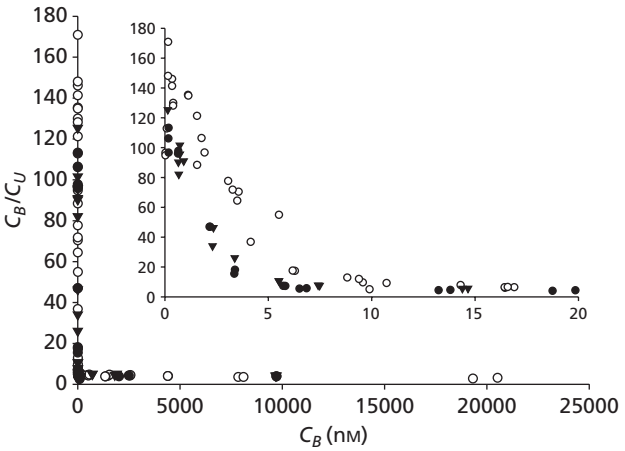

Figure 4 Scatchard plot of binding of [^{3}H]BI 1356 in wild type mouse, wild type rat and human plasma. The insert shows the same plot in different scaling. Human ○; wild type rat ●; wild type mouse ▾.

Table 3 Cumulative urinary excretion of radioactivity after administration of [^{14}C]BI 1356 to wild type or dipeptidyl peptidase 4 knockout mice

Dose (mg/kg)	Radioactivity excreted with urine (% of dose)	
	Dipeptidyl peptidase 4 knockout mice	Wild type mice
0.01	24.2	2.7
0.1	24.7	12.7
0.3	20.3	20.1
1	16.8	16.7
3	19.4	22.8
10	22.8	24.5

The mice were administered a single intravenous bolus injection [^{14}C]BI 1356. The data represent the cumulative urinary excretion from three mice, which were kept together in one metabolic cage, given in percent of the total dose of each group.

protein binding percentage and the Hill coefficient, indicating similar binding properties of BI 1356 to DPP-4 in the investigated species, which was in line with similar binding parameters. In tissue, DPP-4 forms enzymatically active homodimers and proteolytic cleavage of the membrane form results in a soluble form circulating in plasma.[17] In addition, only the DPP-4 dimer form has been reported to be enzymatically active and there is no apparent equilibrium between the monomer and the dimer, suggesting that the dimer formation is very stable.[18] It can be expected that DPP-4 in plasma also forms homodimers. Thus if $N_1/2$ reflects the concentration of DPP-4 dimers, the result can be compared with DPP-4 plasma concentrations determined by ELISA assays. The DPP-4 concentrations in human plasma calculated in this way from our results were well in accordance with the published data.[14,15] However, no reference data for the DPP-4 protein concentrations in mouse and rat plasma were found in the literature and the DPP-4 concentration could not be measured in these investigations. Nevertheless, the similarity between the calculated and reported DPP-4 concentration in human plasma strengthens the plausibility for the DPP-4 plasma concentrations calculated from the protein binding data for the mouse and rat plasma. It should be noted that only frozen plasma was used. This was inevitable due to the duration of the experiments and the need to use plasma pools from each species. Freezing and thawing may change the binding properties of plasma, particularly to DPP-4. However, as DPP-4 enzymatic activity is maintained in previously frozen plasma the plasma protein binding properties of BI 1356 in frozen plasma would be expected to be totally comparable with that of fresh plasma. One additional interesting observation was that the Hill coefficient determined by the regression of f_B vs concentration was approximately 2, suggesting a cooperative effect of BI 1356 binding to DPP-4. As mentioned, DPP-4 in plasma can be expected to form homodimers. In addition, binding of xanthine analogue compounds including BI 1356 induces conformational changes for DPP-4.[1,19] Thus, it cannot be excluded that one-to-one binding of a BI 1356 molecule to a DPP-4 monomer also induced conformational changes into the second monomer leading to an altered, i.e. increased, affinity. The determined affinity constant for binding of BI 1356 to DPP-4 should therefore be regarded as a mixed affinity constant for the first and second binding site in a DPP-4 dimer. The notion of a cooperativity of BI 1356 binding to plasma DPP-4 further points to the presence of dimers of soluble DPP-4 in plasma.

According to the free drug hypothesis, the unbound concentration is the main determinant of many physiological processes like renal glomerular filtration. Hence, plasma protein binding is a key parameter for pharmacokinetics of a compound as it is generally accepted that a high affinity binding in plasma prevents urinary excretion.[5] Concentration-dependent plasma protein binding occurs when the major binding protein in plasma is saturated within the range of therapeutic plasma levels. A special situation is nonlinear plasma protein binding due to binding of the drug to its target in plasma leading to nonlinear pharmacokinetics. This has been observed for the ACE inhibitors ramipril and RU44403.[6,7] As discussed, an analogous profile was observed for BI 1356 plasma protein binding. It is important to consider the role of in-vivo metabolites when extrapolating from in-vitro data. Metabolic clearance of BI 1356 appears to be of only minor relevance. After dosing of radiolabelled BI 1356 to different species, the bulk of radioactivity in plasma appears as the parent compound (approximately 80% of the sample radioactivity in mice, rats and humans, unpublished data). The exposure to most identified metabolites was negligible and only one metabolite with higher abundance was observed which was, however, pharmacologically inactive with respect to DPP-4 inhibition (IC50 > 1 μM). Taken together, a relevant interaction between metabolites and BI 1356 with respect to plasma protein or plasma DPP-4 binding is unlikely. Thus, it was concluded, that the in-vitro data of the parent compound only were sufficient for extrapolation to in-vivo data.

Furthermore, a dose-dependent renal excretion of BI 1356 has been observed in humans: whereas only a negligible amount of the dose was excreted renally at 2.5 mg BI 1356/person, the renal excretion increased to 32.7% at 600 mg/person.[20] To

investigate whether the saturable DPP-4 binding in plasma had any effect on the renal excretion of BI 1356, we compared the urinary excretion of radioactivity in DPP-4 knockout mice with wild type mice after intravenous dosing of [^{14}C]BI 1356. In the in-vivo experiments the ^{14}C-labelled material was preferred to ensure that no exchange of the tritium radiolabel with water occurred, which would impede the data interpretation. On the other hand [^{3}H]BI 1356 was indispensable for the in-vitro plasma protein binding experiments due to the required higher specific radioactivity and for the investigation of very low plasma concentrations. However, there is no reason to believe that the isotopically labelled BI 1356 with either tritium or carbon-14 shows different pharmacokinetic properties to unlabelled BI 1356.

As in plasma, the bulk of the drug related material (approximately 90%) in urine of mice could be assigned to parent compound (data not shown). Thus, in our investigation, renal excretion of radioactivity grossly reflected the renal excretion of BI 1356. In wild type mice, the renally-excreted BI 1356 after different dose levels of [^{14}C]BI 1356 increased dose-dependently between 0.01 and 0.3 mg/kg and remained on a similar level between 0.3 and 10 mg/kg. In contrast, the renal excretion was virtually constant in DPP-4 knockout mice over the entire investigated dose range (Figure 4). This showed that binding of BI 1356 to DPP-4 limited its renal excretion only at doses lower than those needed to reach the saturation of plasma DPP-4. At higher doses, the BI 1356 plasma concentrations remained longer at levels beyond saturation of DPP-4 in plasma and a lower protein binding led to a significant increase in renal excretion. With increasing dose levels, binding of BI 1356 to DPP-4 in plasma and tissue was saturated, and DPP-4 independent processes were preponderant leading to linear, i.e. constant, renal excretion. Thus, the dose-dependent renal excretion of BI 1356 clearly resulted from saturable high affinity binding of the drug to its target. Consequently renal excretion was of low relevance at therapeutically relevant doses. Besides renal excretion, other nonlinear mechanisms were suggested to contribute to the nonlinear plasma pharmacokinetics of BI 1356 observed in volunteers. BI 1356 showed a less than proportional increase of the AUC (area under the curve) value with dose.[20] This can be attributed to saturable binding of BI 1356 to DPP-4 in plasma and also in tissue, as was demonstrated in wild type and DPP-4 deficient rats (Fuchs *et al.* unpublished data).

Conclusions

BI 1356 bound to DPP-4 in plasma with very high affinity. Due to the low concentrations of DPP-4 in plasma, the specific binding sites could be saturated readily. This occurred at therapeutic plasma levels leading to dose-dependent pharmacokinetics and explains the observed nonlinearity in renal excretion and plasma pharmacokinetics in animals and humans. The nonlinear plasma protein binding reflects the binding to its pharmacological target and hence its pharmacodynamic effect, i.e. inhibition of DPP-4 enzymatic activity. Thus, the source of nonlinearity could be well defined, contributing to a better understanding of the pharmacokinetics of BI 1356 in humans.

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Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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