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In vitro blood distribution and plasma protein binding of the iron chelator deferasirox (ICL670) and its iron complex Fe-[ICL670]$_2$, for rat, marmoset, rabbit, mouse, dog and human

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Running title: Plasma protein binding of deferasirox and its iron complex

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Nonstandard abbreviations:

ADME, absorption, distribution, metabolism, elimination; f_p, fraction in plasma of total amount in blood; f_u, unbound fraction in plasma; HDL, high density lipoprotein; HSA, human serum albumin; ICL670, compound code for deferasirox; LDL, low density lipoprotein; PBS, phosphate buffered saline; VLDL, very low density lipoprotein.

Abstract

Deferasirox (Exjade®, ICL670) is an orally active iron chelator. Two molecules of deferasirox can form a complex with ferric iron (Fe-$[ICL670]_2$) which can be excreted, reducing body iron overload. The blood binding parameters across species and the interaction with human serum albumin were analyzed for deferasirox and its iron complex. Both molecules were very highly bound to plasma proteins in all tested species with unbound fractions in plasma in the range of 0.4 – 1.8% and 0.2 – 1.2% for deferasirox and Fe-$[ICL670]_2$, respectively; binding of the iron complex was either similar or higher in all species. The high plasma protein binding was in line with a distribution mainly into the plasma fraction of blood; the fraction in plasma was around 100% for Fe-$[ICL670]_2$ in all species and 65 – 95% for deferasirox depending on the species. Investigations with isolated proteins pointed to serum albumin as the principal binding protein for deferasirox and its iron complex in human plasma. Competition binding experiments indicated that deferasirox at high concentrations displaced markers from the two main drug binding sites of human albumin, whereas Fe-$[ICL670]_2$ displaced only warfarin. In the context of the pharmacokinetic properties of deferasirox and Fe-$[ICL670]_2$ the data indicate the importance of plasma protein binding for their disposition and support a comparison of the pharmacokinetics of deferasirox and its iron complex across species. The low likelihood of clinically relevant drug displacement by deferasirox in plasma is discussed.

Iron accumulation to toxic and eventually lethal levels can result from repeated blood transfusions, e.g. in β-thalassemia major and sickle cell disease, or from excessive dietary iron uptake, since humans are unable to actively eliminate iron from the body. Iron chelators slowly mobilize deposits of accumulated iron, most likely by continuously binding the small amounts of soluble iron in the "labile pool", which are in equilibrium with the insoluble hemosiderins (Crichton and Ward, 2003). Deferasirox (Exjade®, ICL670, Novartis Pharma AG, Basel, Switzerland) is an orally active iron chelator under development (Nick et al., 2003; Nisbet-Brown et al., 2003) and recently approved by some health authorities including the American Food and Drug Administration. Two molecules of deferasirox can form a complex with ferric iron (Fe-$[ICL670]_2$, Figure 1) (Steinhauser et al., 2004); solubilized, chelated iron is then excreted.

For many years Desferal® has been the only iron chelator approved for general use. However, its unfavorable pharmacokinetics (very short plasma half-life and poor oral bioavailability (Porter, 2001)) necessitate special modes of application (daily subcutaneous or intravenous infusions) which are poorly accepted by many patients. This leads to a poor compliance, despite the bad prognosis of untreated iron overload. An orally active iron chelator would be expected to improve the compliance and therefore the clinical responses, will be usable also in areas with less developed infrastructure and facilitate the treatment of other diseases, including non-transfusion dependent thalassemias, anemias and possibly hemochromatosis. The blood binding parameters of a drug can be critical for its pharmacokinetics and are needed for a comparison of pharmacokinetics across species. Here we report the blood distribution and plasma protein binding of deferasirox and its iron complex for species used in preclinical safety investigations and healthy humans, and characterize the interaction of the two molecules with human serum albumin (HSA). The employed concentrations cover the clinically and pre-clinically relevant range.

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Methods

Materials. Deferasirox (4-[3,5-bis-(2-hydroxy-phenyl)-[1,2,4]triazol-1-yl]-benzoic acid, $C_{21}H_{15}N_3O_4$, molecular weight 373.4), Fe-[ICL670]$_2$ (tri-sodium salt, > 95% pure), [^{14}C]deferasirox (0.29 - 2.0 MBq/mg, 98% pure) and [^{14}C]Fe-[ICL670]$_2$ (0.25 - 1.7 MBq/mg, tri-sodium salt, 98% pure), were synthesized at Novartis Pharma AG, Basel (Figure 1). [^{14}C]diazepam (7.2 MBq/mg, 99% pure) and [^{14}C]warfarin (6.68 MBq/mg, 99% pure) were from Amersham Pharmacia Biotech (Little Chalfont, UK). Used stock solutions were in ethanol (deferasirox, warfarin, diazepam) or water (Fe-[ICL670]$_2$). Centrifree and Microcon devices (molecular cut-off of 30 kDa) were from Amicon (Beverly, MA, USA), PBS from Gibco (D-PBS, Paisley, Scotland). HSA (A 1887), α_1-acid glycoprotein (G 9885) and γ-globulins (G 4386) were purchased from Sigma (St. Louis, MO, USA). Human HDL (437641), LDL (437644) and VLDL (437647) were purchased from Calbiochem (San Diego, CA, USA). Solutions of HSA, α_1-acid glycoprotein and γ-globulins were prepared in PBS. The lipoproteins were delivered as solutions in 150 mM NaCl and 0.01% EDTA, pH 7.4. The concentrations of the lipoproteins as given by the manufacturer were adjusted to the used concentrations with PBS. Human blood was taken from healthy male volunteers. Rat blood was from male albino rats (Hanover, Wistar), mouse blood from male p53 wild type mice (B6.129-Trp53$^{tm1Brd+}$ N5), dog blood from male beagles, monkey blood from male and female marmoset monkeys (Callithrix Jacchus) and rabbit blood from male or female rabbits (New Zealand White). Heparin was used as anticoagulant, all blood and plasma specimen were pooled (n ≥3, with the exception of marmoset blood which was from one male and one female animal). Fresh blood was used within 24 h (marmoset blood was slightly older due to the necessity of shipment); plasma was defrosted from storage at –20°C.

Control experiments. Control experiments in PBS at 20 and 80 μg/mL of deferasirox or Fe-[ICL670]$_2$ indicated that ultrafiltration was a suitable method to determine the protein

binding: the permeation through the filtration membrane was ≥ 0.8 for deferasirox and ≥ 0.95 for Fe-$[ICL670]_2$, indicating no major retention. Only total radioactivity was measured in all experiments, therefore some bias due to labeled impurities or degradation products can not be completely excluded. 30 dpm above background (^{14}C) were defined as the LOQ for radioactivity analysis; all reported values were above this limit.

In vitro interconversion between deferasirox and its iron complex was investigated in rat blood and plasma at 37°C (Novartis, non published data). Iron complex formation in plasma spiked with 4 or 80 μg/mL of deferasirox was ≤ 5 or ~ 2%, respectively, after one hour of incubation. Iron complex decay in plasma spiked with 4 or 80 μg/mL of Fe-$[ICL670]_2$ was about 22 or 7%, respectively, after one hour of incubation. Since both forms of deferasirox are very highly bound to plasma proteins the data on binding in plasma reported here do not contain a strong bias due to complex formation or decay. Iron complex formation in blood spiked with 4 or 80 μg/mL of deferasirox was ≤ 5 or ~ 2%, respectively, after one hour of incubation. Iron complex decay in blood spiked with 4 or 80 μg/mL of Fe-$[ICL670]_2$ was about 17 or 5%, respectively, after one hour of incubation. Since the blood distribution of both forms of deferasirox was similar (see Table 1) most of the data on blood distribution reported here do not contain a relevant bias due to complex formation or decay. Only incubations with low concentrations of $[^{14}C]$Fe-$[ICL670]_2$ might slightly underestimate f_p due to some decay of the iron complex.

Blood distribution. Fresh blood was spiked to the desired compound concentrations. First, kinetics of distribution were determined. In all experiments reported here incubations were for 1 h at 37°C, which was sufficient to reach an equilibrium. Sedimentation of cells was by centrifugation (1500 g, 10 min, 37°C). Radioactivity was measured in blood (C_b) before and in plasma (C_p) after centrifugation, hematocrit (H) was determined in triplicate. The fraction in plasma (f_p) was calculated as follows: $f_p(\%) = (C_p/C_b) \times (1-H) \times 100$

Plasma protein binding. Plasma or solutions of plasma proteins in PBS were spiked to the desired compound concentrations. All incubations were for 1 h at 37°C before centrifugation in Centrifree or Microcon (mouse and marmoset plasma and lipoprotein solutions) devices (7-10 min, 2000 g, 37°C, ~ 20% of sample filtered, shorter centrifugation times for protein solutions). Radioactivity was determined in the spiked solution (C_t) and the ultrafiltrate (C_u). The unbound fraction (f_u) was calculated as follows: $f_u(\%) = C_u/C_t \times 100$

IC_{50} values were determined from competition data by nonlinear least-squares fitting to the four-parameter logistic function using Sigma Plot (Systat Software Inc., Richmond, CA, USA).

Results

In the investigated concentration range (4 – 80 µg/mL, 11 – 214 µM for deferasirox and 5.4 – 107 µM for Fe-$[ICL670]_2$, see Table 1 for details) [^{14}C]deferasirox as well as [^{14}C]Fe-$[ICL670]_2$ were mainly located in the plasma fraction of blood. In all investigated species f_p was clearly higher for the iron complex [^{14}C]Fe-$[ICL670]_2$, which was almost quantitatively located in plasma (f_p ~ 100%). Blood distribution of [^{14}C]deferasirox was species dependent (Table 1) with humans showing the highest plasma fraction (95%) and rat the lowest (about 67%). In some species (male and female mouse, male and female rabbit) a trend for a decrease in f_p with increasing compound concentration was visible whereas in others this was not apparent.

Both deferasirox as well as its iron complex Fe-$[ICL670]_2$ were very highly bound to plasma proteins with unbound fractions in the range of 0.4 – 1.8% and 0.2 – 1.2% for deferasirox and Fe-$[ICL670]_2$, respectively (Table 1). The unbound fraction of Fe-$[ICL670]_2$ was similar or slightly smaller as compared to deferasirox for mouse, rabbit, marmoset and human, and clearly smaller for rat and dog. Species differences in plasma protein binding were apparent for deferasirox and Fe-$[ICL670]_2$; from the tested species human showed the highest bound fraction for deferasirox (99.5%) in line with the highest fraction in plasma. For deferasirox

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and Fe-$[ICL670]_2$ a slight trend for increasing f_u with higher compound concentrations was evident in some species but clearly not in marmoset.

Binding to isolated human plasma proteins was measured at physiologically relevant concentrations of the major plasma proteins involved in drug binding (Table 2). Unbound fractions determined in presence of the tested plasma proteins indicated, that both deferasirox as well as its iron complex were predominantly bound to HSA. Both deferasirox and Fe-$[ICL670]_2$ were not significantly bound to hemopexin, a heme-binding plasma glycoprotein (data not shown).

In human plasma displacement of both diazepam and warfarin at high concentrations of deferasirox was found (Table 3). At a concentration of 100 µg/mL (268 µM) deferasirox the unbound fractions of diazepam and warfarin in human plasma were 3.5 and 1.5%, respectively, as compared to 2.3 and 1.2% in absence of deferasirox. In contrast, no increase of the unbound fraction of diazepam and warfarin occurred in human plasma at concentrations of up to 100 µg/mL (134 µM) Fe-$[ICL670]_2$**.** In addition to the displacement experiments in plasma displacement of diazepam and warfarin in a solution of HSA (1 mg/mL, 15 µM, approximately 2.5% of the plasma albumin concentration) was analyzed. Deferasirox displaced diazepam (0.1 µg/mL, 0.35 µM) from HSA with an IC_{50} of 46 µg/mL (123 µM), whereas Fe-$[ICL670]_2$ did not at concentrations of up to 100 µg/mL (134 µM). Warfarin (1 µg/mL, 3.2 µM) was displaced from HSA by deferasirox as well as Fe-$[ICL670]_2$ with IC_{50} values of 84 µg/mL (225 µM) and 41 µg/mL (55 µM), respectively (Table 3).

Discussion

Deferasirox as well as its iron complex were very highly bound to plasma proteins in all investigated species. The very high binding to plasma protein is in line with the predominant distribution of deferasirox and Fe-$[ICL670]_2$ into the plasma fraction of blood. The very high protein binding in combination with a small to moderate (deferasirox, determined for rat and human) or small (Fe-$[ICL670]_2$, determined for rat) volume of distribution (Galanello et al.,

2003) (Novartis unpublished data; Wiegand et al., manuscript in preparation) emphasizes the relevance of the plasma protein binding for the distribution, the systemic exposure and the pharmacokinetics of deferasirox and its iron complex. For iron chelators a reasonably high systemic exposure and lasting presence in plasma should allow for an efficient protection against the harmful effects of circulating non-transferrin-bound plasma iron (Hershko et al., 1998). The reported protein binding and blood distribution data are significant for a comparison of pharmacokinetics of deferasirox and its iron complex across species, for interspecies scaling and allow for a meaningful referencing of pharmacokinetic parameters to either blood or plasma concentrations (Hinderling, 1997).

The overall binding of deferasirox and its iron complex in human plasma is mainly due to binding to albumin. For Fe-$[ICL670]_2$ the unbound fraction in presence of 40 mg/mL HSA, a concentration typically found in human plasma, was virtually identical to the unbound fraction found in plasma, whereas binding to all other tested plasma proteins was in comparison negligible (Table 2). This suggests that protein binding of Fe-$[ICL670]_2$ in human plasma is almost exclusively due to binding to HSA. For deferasirox the unbound fraction in presence of 40 mg/mL HSA was somewhat higher as compared to the unbound fraction in human plasma. Therefore binding to e.g. lipoproteins may contribute to some extent to the overall binding in plasma, but HSA is again clearly the main binder. No strong increase of the unbound fraction at high concentrations (80 µg/mL) of deferasirox or its iron complex indicated a very high binding capacity for both molecules in plasma of all tested species. This is in agreement with albumin being the main binding protein, since albumin is - in absence of severe clinical condition like liver impairment - present at high concentrations.

Deferasirox at high concentrations displaced marker compounds for the two main binding sites for anionic drugs on HSA (Sudlow et al., 1976) in whole human plasma as well as on the isolated HSA. The observed displacement could be due to a direct competition at the binding site and/or an allosteric interaction. Despite the very high plasma protein binding of the iron

complex, neither warfarin (marker for Sudlow's Site I) nor diazepam (marker for Sudlow's site II) were displaced by the complex in plasma, the unbound fraction of diazepam was even slightly reduced. Also, in experiments with isolated HSA the unbound fraction of diazepam decreased in presence of Fe-$[ICL670]_2$ (data not shown). The increased binding of diazepam in presence of Fe-$[ICL670]_2$ points to an allosteric interaction of the complex with HSA, a frequently observed phenomenon (Chen and Hage, 2004). From isolated HSA warfarin was displaced by Fe-$[ICL670]_2$, whereas in plasma no displacement was evident (Table 3), likely due to the higher albumin concentration in the latter experiment. The observed displacement from HSA could be due to a direct competition at the binding site or an allosteric interaction. Recent data from co-crystallization experiments indicate that phenylbutazone and indomethacin can bind simultaneously to Sudlow's Site I (Ghuman et al., 2005). These structural data show that the size and the flexibility of this binding site should not only allow for binding of deferasirox, but also of its relatively bulky iron complex. In addition Ghuman and coworkers show that many drugs bind to albumin also at a secondary or even tertiary binding site. In case of deferasirox and its iron complex binding to several sites could explain the relatively high concentrations of the compounds necessary to displace warfarin, despite their very high binding to albumin.

Patients treated with deferasirox in clinical trials experienced high plasma concentrations of up to 250 µM deferasirox at supra therapeutic doses, concentrations of the iron complex where several fold lower (Galanello et al., 2003) (Nisbet-Brown et al., 2003). Even though the binding capacity of albumin in plasma is very high due to its high concentration of about 40 mg/mL (0.6 mM) in healthy human subjects, some displacement of other HSA bound drugs can occur at such high drug concentrations. Displacement of drugs from binding sites on plasma proteins has been discussed as a mechanism of clinically relevant drug-drug-interaction. However, there is a broad agreement that even though displacement can occur, the effect is usually limited to a change in the total plasma concentration of the displaced drug

with an unchanged or only temporary changed exposure to unbound drug and no need for a dose adjustment (Benet and Hoener, 2002; Rolan, 1994; Sansom and Evans, 1995). A clinical significance of displacement by deferasirox can not be completely excluded but is restricted to the very rare cases of highly protein bound drugs with a narrow therapeutic index that are either given parenterally and have a high extraction ratio or that are given orally and have a very rapid pharmacokinetic-pharmacodynamic equilibration time (Benet and Hoener, 2002; Sansom and Evans, 1995).

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Legend to Figure

Fig. 1. *Structure of ^{14}C labeled Deferasirox (A) and its iron complex (B)*

Deferasirox was labeled with one ^{14}C atom per molecule, however, in each molecule the two given different positions of the label (*) are theoretically possible. Deferasirox binds iron (Fe^{3+}) in a meridional orientation. At physiological pH deferasirox and its iron complex carry one and three negative charges, respectively.

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TABLE 1

In vitro blood distribution and plasma protein binding of [^{14}C]deferasirox and its iron complex [^{14}C]Fe-[ICL670]$_2$ for rat, marmoset, rabbit, mouse, dog and human.

Given numbers are means and standard deviations of triplicate determinations.

Species:	Conc. (µg/mL)	Blood distribution, fraction in plasma (%)		Plasma protein binding, unbound fraction in plasma (%)	
		Deferasirox	Fe-[ICL670]$_2$ [a]	Deferasirox	Fe-[ICL670]$_2$ [a]
Rat, male (H = 0.45 for Deferasirox and 0.48 for Fe-[ICL670]$_2$)	4	63 ± <1	93 ± 2	0.8 ± <0.1	0.3 ± <0.1
	20	68 ± 2	96 ± 1	0.7 ± <0.1	0.3 ± <0.1
	80	69 ± 2	100 ± 2	1.0 ± <0.1	0.3 ± <0.1
Mouse, male (H = 0.46)	4	76 ± 1	97 ± 1	1.5 ± 0.3	1.0 ± 0.1
	20	74 ± 1	100 ± 1	1.1 ± 0.1	0.9 ± <0.1
	80	70 ± 1	99 ± 1	1.2 ± <0.1	1.1 ± 0.1
	160	64 ± 0.2	101 ± 1	1.6 ± 0.1	1.2 ± 0.1
Mouse, female (H = 0.40)	4	85 ± 2	106 ± 1	ND	ND
	20	ND	ND	0.9 ± <0.1	0.9 ± <0.1
	80	79 ± <1	108 ± 2	1.1 ± <0.1	1.0 ± <0.1
Dog, male (H = 0.49)	4	ND	104 ± 1	ND	0.2 ± <0.1
	20	90 ± 1	102 ± 2	0.6 ± <0.1	0.2 ± <0.1
	80	91 ± 3	103 ± 1	0.7 ± 0.1	0.2 ± <0.1
Rabbit, male (H = 0.40)	4	87 ± 2	96 ± 1	0.9 ± <0.1	0.8 ± <0.1
	20	83 ± 3	101 ± 1	0.9 ± <0.1	0.8 ± <0.1
	80	78 ± 1	102 ± 1	1.2 ± <0.1	0.9 ± <0.1
Rabbit, female (H = 0.38)	4	92 ± 2	97 ± 1	0.7 ± <0.1	0.6 ± <0.1
	20	93 ± 1	101 ± 2	0.7 ± <0.1	0.6 ± <0.1
	80	89 ± 1	101 ± 2	0.9 ± <0.1	0.6 ± <0.1
Marmoset, pool of male and female (H = 0.46)	4	73 ± 1	99 ± 1	ND	1.2 ± <0.1
	20	ND	104 ± 4	1.8 ± 0.1	1.0 ± <0.1
	80	77 ± 2	102 ± 1	1.4 ± <0.1	0.9 ± <0.1
Human, male (H = 0.45)	4	95 ± 1	100 ± 2	0.4 ± <0.1	0.3 ± <0.1
	20	95 ± 1	102 ± 1	0.4 ± <0.1	0.3 ± <0.1
	80	94 ± 1	100 ± 2	0.5 ± <0.1	0.3 ± <0.1

H = hematocrit, ND = not determined, [a]: in incubations with low concentrations of [^{14}C]Fe-[ICL670]$_2$ significant decay of the iron complex occurs during incubation, see methods/Control experiments

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TABLE 2

In vitro binding of [^{14}C]deferasirox and its iron complex [^{14}C]Fe-[ICL670]$_2$ to isolated human plasma proteins.

Given numbers are means and standard deviations of triplicate determinations. Used plasma protein concentrations are in the range of concentrations found in plasma of healthy subjects with the exception of 1 mg/mL HSA.

Protein and used concentration	Conc. (µg/mL)	Unbound fraction in protein solution (%)	
		Deferasirox	Fe-[ICL670]$_2$[a]
HSA, 40 mg/mL	4	1.3 ± 0.1	0.4 ± <0.1
	20	1.1 ± <0.1	0.3 ± <0.1
	80	1.3 ± <0.1	0.5 ± <0.1
HSA, 1 mg/mL	4	22 ± 0.3	11 ± 0.4
	20	28 ± 0.2	22 ± 0.3
	80	52 ± 0.9	53 ± 0.2
α_1-acid glycoprotein, 1 mg/mL	20	58 ± 2	91 ± 2
γ globulin, 12 mg/mL	20	67 ± 0.4	70 ± 2
VLDL, 1.3 mg/mL	20	16 ± 0.5	85 ± 2
LDL, 3.6 mg/mL	20	11 ± 0.2	64 ± 1
HDL, 3.9 mg/mL	20	6.8 ± 0.2	27 ± 0.3

[a]: in incubations with low concentrations of [^{14}C]Fe-[ICL670]$_2$ significant decay of the iron complex occurs during incubation, see methods/Control experiments

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TABLE 3

Displacement of [^{14}C]warfarin or [^{14}C]diazepam from human plasma proteins by deferasirox and its iron complex.

Given numbers are means and standard deviations of triplicate determinations for the unbound fractions. For the IC_{50} values 95% confidence intervals of the fit are given in brackets, all R^2 values were > 0.99.

		Unbound fractions of [^{14}C]warfarin or [^{14}C]diazepam in human plasma (%) in absence and presence of deferasirox and Fe-[ICL670]$_2$				
		-	10 µg/mL Deferasirox	100 µg/mL Deferasirox	10 µg/mL Fe-[ICL670]$_2$	100 µg/mL Fe-[ICL670]$_2$
[^{14}C]Warfarin	1 µg/mL	1.16 ± 0.04	1.22 ± 0.02	1.56 ± 0.02	1.15 ± 0.03	1.22 ± 0.03
	5 µg/mL	1.15 ± 0.05	1.19 ± 0.03	1.51 ± 0.01	1.24 ± 0.02	1.21 ± 0.06
[^{14}C]Diazepam	0.1 µg/mL	2.3 ± <0.1	2.4 ± 0.2	3.4 ± 0.1	2.4 ± <0.1	2.0 ± <0.1
	1 µg/mL	2.3 ± <0.1	2.5 ± <0.1	3.6 ± <0.1	2.4 ± <0.1	1.9 ± <0.1

		IC_{50} in HSA solution (1 mg/mL)			
		Deferasirox		Fe-[ICL670]$_2$	
		µM	µg/mL	µM	µg/mL
[^{14}C]Warfarin	1 µg/mL	225	84 (67-106)	55	41 (30-55)
[^{14}C]Diazepam	0.1 µg/mL	123	46 (36-59)	-	> 100

Figure 1