In vitro to in vivo comparison of the substrate characteristics of Sorafenib tosylate towards P-glycoprotein

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Running title page

Running title: Substrate properties of sorafenib towards P-glycoprotein (56/60 characters)

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Words in Discussion: 1044

Abbreviations: ABC, ATP binding cassette; HPLC, high-performance liquid chromatography; i.v., intravenous; KO, knock out; LSC, liquid scintillation counting; P-gp, P-glycoprotein; p.o., per oral; WT, wild-type.
Abstract

Sorafenib (Nexavar™) is a novel oral Raf kinase and vascular endothelial growth factor receptor inhibitor. Most anti-cancer drugs are substrates for ABC efflux pumps especially for P-glycoprotein (P-gp). To evaluate the influence of P-gp on the pharmacokinetics of sorafenib substrate properties for this transporter were investigated. Therefore, permeability of sorafenib across Caco-2 and P-gp overexpressing cells was determined. To determine the in vivo relevance of these in vitro findings, pharmacokinetics of sorafenib in mdr1a/1b−/− and wild-type (WT) mice was studied. Sorafenib is highly permeable and exhibits a slight efflux across Caco-2 cells. In P-gp overexpressing cells a small concentration dependent efflux was observed which was completely blocked by the addition of ivermectin. In mdr1a/1b−/− and WT mice unchanged compound represented by far the majority of radioactivity in plasma. After intravenous and oral administration brain-to-plasma concentration ratios in mdr1a/1b−/− mice were 1.3-fold to 1.5-fold higher compared to WT mice. However, following i.v. or p.o. administration plasma concentrations were similar in both mouse strains. In conclusion, sorafenib is highly permeable and a weak P-gp substrate in vitro. These findings were confirmed by the small factor of 1.3 to 1.5 observed for the brain-to-plasma ratios in mdr1a/1b−/− v.s. WT mice in vivo. Based on these in vitro and in vivo results it is unlikely that P-gp has a major effect on the plasma concentrations of sorafenib in humans. Due to the high permeability and low P-gp mediates transport, sorafenib might be able to cross the blood-brain barrier and target tumors within the brain.
Introduction

Sorafenib tosylate (Nexavar™) is a novel oral Raf kinase and vascular endothelial growth factor receptor (VEGFR) inhibitor that prevents tumor growth by combining two anticancer activities: inhibition of tumor cell proliferation and tumor angiogenesis. Sorafenib inhibits tumor cell proliferation by targeting the Raf/MEK/ERK signaling pathway at the level of Raf kinase, and exerts an antiangiogenic effect by targeting the receptor tyrosine kinases VEGFR-2, VEGFR-3 and platelet-derived growth factor receptor (PDGFR) and their associated signaling cascades (Lyons et al., 2001). Sorafenib has shown marked clinical efficacy and safety in advanced renal cell and hepatocellular carcinoma (Escudier et al., 2007; Kane et al., 2006).

ATP driven efflux pumps such as P-glycoprotein (P-gp, MDR1 or ABCB1), MRP2 (ABCC2) and BCRP (ABCG2) can have a significant effect on chemotherapy. These proteins are localized at apical membranes of epithelial barriers and canicular membranes of hepatocytes facilitating excretion of drugs via liver, intestines and kidneys and limit their distribution to tissues, such as brain and testis (Borst et al., 2002). Furthermore, overexpression of these transport proteins in tumor cells can lead to drug resistances (Gottesmann and Pastan, 1993). At present, one of the best investigated efflux pumps is P-gp, a 170 KDa ATP-driven transport protein (Borst et al., 2002) known to limit distribution of anti-cancer drugs to the brain and cancer cells (Dai et al., 2003, Schinkel et al, 1995). In addition, brain penetration and tumor exposure also depend on the permeability of the drug.

Therefore, the permeability of sorafenib was investigated in vitro using Caco-2 cells. Substrate characteristics of sorafenib towards P-gp were studied in vitro using LLC-PK1 cells overexpressing P-gp (L-MDR1 cells) (Schinkel et al., 1995) in more detail. It has been demonstrated that knock-out mice are a good model to study the influence of transport proteins on the pharmacokinetics of drugs (Schinkel et al, 1995; Schinkel et al., 1997; Lagas et al. 2006). To evaluate the relevance of the in vitro findings for the in vivo situation brain and plasma concentrations of sorafenib in mdr1a/1b−/− and wild-type (WT) mice were determined. The well known P-pg substrate digoxin was used as positive control to validate the in vivo model (Schinkel et al., 1997).
Materials and Methods

Chemicals

$[^{14}\text{C}]$sorafenib was applied as tosylate (99.6% radiochemical purity) and with a specific radioactivity of 4.24 MBq/mg. Tritiated digoxin was purchased from PerkinElmer® Life and Analytical Sciences (Boston, MA, USA) with a specific radioactivity of 11.84 MBq/mg. Ivermectin, Ketoprofen, Metoprolol, Digoxin and Vinblastine were purchased from Sigma Aldrich (Deisenhofen, Germany). Fluvastatin was purchased from Merck KGaA (Darmstadt, Germany).

Cell culture materials were purchased from Gibco (Karlsruhe, Germany) unless otherwise stated. All other chemicals were reagent grade or better.

Culture of Caco-2 cells

Caco-2 cells were purchased from DSMZ (the German Resource Centre for Biological Material Braunschweig, Germany). Cells were seeded at a density of 4 x $10^4$ cells per well on 24 well polycarbonate insert plates, 0.4 $\mu$m pore size (Costar Corning, Inc., Corning, USA) and maintained for 15 days in DMEM medium supplemented with 10% fetal bovine serum albumin, 20 mM glucose, 10 mg/500 mL Streptomycin and 10000 IU/500 mL Penicillin in a humidified incubator at 8% CO$_2$.

The medium was changed twice a week.

Culture of L-MDR1 cells

LLC-PK1 cells overexpressing P-glycoprotein (L-MDR1) cells were kindly provided by Dr. A. Schinkel (Netherlands Cancer Institute, Amsterdam, The Netherlands) (Schinkel et al., 1995). For flux studies cells were seeded at a density of 2 x $10^5$ cells per well on 24 well polycarbonate insert plates, 0.4 $\mu$m pore size, and grown for 4 days in Medium 199 supplement with 2 mM L-Glutamine, 10% fetal bovine serum (FBS), 10 mg/500 mL Streptomycin and 10000 IU/500 mL Penicillin in a humidified incubator at 5% CO$_2$ with a medium change every 2-3 days.

Cellular flux studies

Before running the assay culture medium was replaced by transport buffer consisting of HBSS buffer; pH 7.4, supplemented with 10 mM HEPES and 20 mM glucose (only for Caco-2 cells). The acceptance criteria for acceptable batches of cell monolayers were a TEER value of 600 $\Omega$. Stock solution of $[^{14}\text{C}]$sorafenib tosylate was prepared in DMSO and diluted 100-fold with transport buffer. For inhibition studies ivermectin was added at defined concentrations to the stock solution.
These solutions were applied either to the apical or basal compartment. 1% DMSO was added to the buffer in the trans-compartment. Due to the high non-specific binding of sorafenib to plastic ware the assay was started following a 1 h pre-incubation to ensure equilibrium and saturation of non-specific binding. After 1 h pre-incubation at 37°C an aliquot of the cis-compartment was taken. The solution of the trans-compartment was removed, washed with PBS and fresh transport buffer was added. After 2 h incubation at 37°C samples were taken from both compartments. Furthermore, the trans-compartment was washed with methanol to recover any compound bound to plastics. All samples from the trans-compartment were pooled and analyzed by liquid scintillation. To determine the amount of compound already present in the trans-compartment after the 1 h pre-incubation a parallel experiment was run and terminated after 1 h. The trans-compartment was washed with PBS and rinsed with methanol. These samples were analyzed by liquid scintillation counting. The 2 h trans-values were corrected for the amount of drug permeated during the pre-incubation.

**Measurement of the transepithelial electrical resistance (TEER)**

Transepithelial electrical resistance (TEER) was measured in HBSS buffer before and after the transport studies using a STX 100 TEER electrode (World Precision Instruments, Berlin, Germany). To ensure the integrity of the cell monolayer transepithelial electric resistance (TEER) values were determined before and after the experiment.

**Analysis of reference compounds**

Samples were analyzed by HPLC-MS/MS on an API3000 (PSciex, Darmstadt, Germany) using a Purospher star column. The eluent was 10 mM ammonium acetate buffer, pH 3.0. Compounds were eluted using a gradient from 20% to 90% acetonitrile in 2 min. For the calculation of \( P_{\text{app}} \) values peak heights or peak areas were used. No difference was observed for \( P_{\text{app}} \) values when data was compared with \( P_{\text{app}} \) values calculated based on concentrations (data not shown).

**Calculation of \( P_{\text{app}} \) value and efflux ratio**

The apparent permeability (Papp) was calculated using the following equation:

\[
P_{\text{app}} = \left( \frac{V_r}{P_0} \right) \left( \frac{1}{S} \right) \left( \frac{P_2}{t} \right)
\]

where \( V_r \) is the volume of medium in the receiver chamber, \( P_0 \) is the measured peak height or peak area of the test drug in the donor chamber at \( t = 0 \), \( S \) is the surface area of the monolayer, \( P_2 \) is the measured peak height or peak area of the test drug
in the acceptor chamber after 2 h of incubation, and \( t \) is the incubation time (2 h).

The efflux ratio is defined by the following equation:

\[
\text{Efflux ratio} = \frac{P_{\text{app}(B-A)}}{P_{\text{app}(A-B)}}
\]

where \( P_{\text{app}(B-A)} \) and \( P_{\text{app}(A-B)} \) represent the apparent permeability of test compound from the basolateral to apical and apical to basolateral side of the cellular monolayer, respectively.

Cell data is expressed as arithmetic means and standard deviation. The standard deviation (SD) for the efflux ratio is calculated using linear error propagation.

**In vivo Animal Studies**

Male FVB Tac-(KO)mdr1a-(KO)Mdr1b mice (mdr1a/1b\(^{-/-}\), P-gp double knock-out) and FVB wild-type (mdr1a/1b\(^{+/+}\), WT) mice of approx. 8 weeks were purchased from Taconic Quality Lab. Animals and Services for Research (Germantown, NY, USA). Animals were housed and handled according to institutional guidelines issued by the government of the Federal Republic of Germany (May 25, 1998, BGBI. I S. 1105, 1818I).

Plasma and brain tissue were collected following single intravenous bolus administration (0.5 mg/kg body weight) and single oral administration (0.5 mg/kg and 4 mg/kg body weight) of \([^{14}\text{C}]\text{sorafenib tosylate}\). For intravenous administration \([^{14}\text{C}]\text{sorafenib tosylate}\) (2.13 MBq/kg) was formulated in plasma (1% DMSO + 99% plasma, v/v, 0.137 mg/ml, 5 ml/kg) and injected into the tail vein. For oral administration \([^{14}\text{C}]\text{sorafenib tosylate}\) (2.13 MBq/kg) was dissolved in a NaCl-based formulation (5% ethanol + 25% PEG400 + 5% Tween80 + 65% NaCl, v/v, 0.137 and 1.01 mg/ml, 5 ml/kg) and administrated by gastric gavage. \([^{3}\text{H}]\text{digoxin}\) (0.5 mg/kg) was intravenously administered into the tail vein at a single bolus dose (9.25 MBq/mg) using a formulation according to Leusch (Leusch et al., 2002).

At dedicated time points following oral or intravenous administration, animals were sacrificed under deep Isoflurane\(^{\circledR}\) anaesthesia and blood samples were collected from the carotid artery. Blood cells and plasma were separated by centrifugation and the plasma was stored at \(-20^\circ\)C until radioanalytics. Then, brain tissue was homogenized in 1000 µL demineralized aqua using a potter technique (Wheaton Science International, Millville, NJ, USA). The radioactivity was determined by liquid scintillation counting. In order to determine volatility of the \([^{3}\text{H}]\)-radiolabel of digoxin, two aliquots of plasma and brain samples of mdr1a/1b\(^{-/-}\) and WT mice were freeze-dried, analyzed for total radioactivity and compared to wet plasma and brain
samples. In the present experiments, the $^3$H-radiolabel of digoxin was not volatile as the equivalent concentrations of total radioactivity in wet samples (unchanged compound, radioactive metabolites and tritiated water) were confirmed in freeze-dried samples (removed tritiated water).

**Calculation of brain/plasma ratio of mdr1a/1b$^{-/-}$ vs. WT mice**

To describe the impact of P-gp on drug distribution in brain, the ratio of brain-to-plasma equivalent concentrations was calculated in mdr1a/1b$^{-/-}$ and WT mice. Finally, the ratio of the brain/plasma ratios of mdr1a/1b$^{-/-}$ (KO) and WT (brain/plasma ratio mdr1a/1b$^{-/-}$ / brain/plasma ratio WT) was calculated: Ratio of the brain/plasma ratio KO/WT = (brain/plasma ratio KO) / (brain/plasma ratio WT).

In addition, the area under the curve (AUC) of total radioactivity in plasma and brain of mdr1a/1b$^{-/-}$ (KO) mice was calculated from samples at 0.5 h, 1 h, 2 h and 4 h post dosing and was compared to those of WT mice after intravenous administration.

**Radioanalysis**

Radioactivity of liquid samples was measured by liquid scintillation counting with automatic quench correction by the external standard channel ratio method at 13°C using Ultima Gold® as scintillation cocktail (Packard Instruments BV, Groningen, The Netherlands). Counting time (1-30 min) for radioactivity determination was chosen according to the respective radioactivity concentration of the sample. The equivalent-concentrations were calculated from the radioactivity concentrations (MBq/mL) in the respective samples applying the molecular weight and the specific radioactivity (4.24 MBq/mg) of sorafenib tosylate.

**Metabolic Profiling of Plasma**

Metabolite patterns in plasma samples were determined using high-performance liquid chromatography (HPLC) with off-line radioactivity detection. Sorafenib and its metabolites were quantified using radioactivity in combination with the specific activity of $^{14}$C-sorafenib. Each HPLC run was split every 10 seconds into fractions (83 µL, due to a flow through the column of 0.5 mL/min) and the radioactivity of each fraction was determined by liquid scintillation counting (Wallac 1450 Microbeta™ Plus; Freiburg, Germany). In order to detect potential metabolites plasma samples of WT or mdr1a/1b$^{-/-}$ mice were pooled (50 µL, each) according to the time points investigated (0.5 - 4 h). The combined samples (150 µL plasma) were treated with 600 µL acetonitrile, homogenized using a Vibrofix® VF1 Shaker (IKA® Werke, Staufen, Germany) and proteins were removed by centrifugation (3 min, 13000 rpm).
The recovery ranged from 91 – 114%. Supernatants were concentrated to approx. 120 µL and directly subjected to HPLC with off-line radioactivity detection. Separation of drug and metabolites was carried out on a Prodigy ODS-3 column using phosphate buffer (1 g/L, adjusted with phosphoric acid to pH 2.0) and acetonitrile as eluent. Retention times for Sorafenib, M-2, M-3 and M-4 were 21.3 min, 16.3 min, 17.8 min and 18.8 min respectively. The limit of quantification for sorafenib and its metabolites for metabolite profiling was 20.6 ng/ml.

**Statistical Analysis.**

Unpaired Student’s t test was used to calculate the statistical significance of the concentration dependent decreased of the efflux ratios of sorafenib in L-MDR1 cells and the difference in brain concentrations in WT and P-gp KO mice. All animal data is expressed as geometric means and standard deviation.
Results

Bi-directional flux studies across Caco-2 cells

$[^{14}C]$sorafenib showed $P_{\text{app}}$ values for the apical (A) to basal (B) direction of $16.4 \pm 12.1 \times 10^{-6} \text{ cm/sec}$ and $33.5 \pm 16.3 \times 10^{-6} \text{ cm/sec}$ at 0.1 µM and 1 µM, respectively (Table 1). Sorafenib $P_{\text{app}}$ values were in the range of the $P_{\text{app}}$ values for ketoprofen ($26.5 \pm 6.7 \times 10^{-6} \text{ cm/sec}$), metoprolol ($32.5 \pm 2.4 \times 10^{-6} \text{ cm/sec}$), and fluvastatin ($17.5 \pm 2.0 \times 10^{-6} \text{ cm/sec}$) (Table 1). The low permeable compound atenolol showed a significantly lower permeability of $0.66 \pm 0.048 \times 10^{-6} \text{ cm/sec}$ (Table 1). The known P-gp substrate digoxin showed a low permeability from A to B of $0.37\pm 0.055 \times 10^{-6} \text{ cm/sec}$ and efflux ratio of 59 (Table 1). For sorafenib small efflux ratios of $4.7\pm 3.7$ to $2.5\pm 1.2$ were observed at concentrations of 0.1 and 1 µM, respectively (Table 1).

Bi-directional flux studies across L-MDR1 cells

Active efflux was investigated in more detail by determining bi-directional permeability of $[^{14}C]$sorafenib across P-gp overexpressing L-MDR1 cell monolayers. Efflux ratios of sorafenib were $6.8\pm 2.0$ and $4.3\pm 1.5$ at 0.1 µM and 1 µM, respectively (Table 2). The efflux ratio decreased down to $1.3\pm 0.6$ by the addition of 5 µM ivermectin, a known P-gp inhibitor (Schwab et al. 2003). Digoxin (25 µM) and vinblastine (2 µM) showed efflux ratios of $12\pm 3.5$ and $37\pm 8.8$, respectively.

Brain penetration of digoxin in mdr1a/1b−/− knock out mice

To further evaluate the influence of P-gp on the pharmacokinetics of sorafenib, especially the distribution into the brain, in vivo studies in mdr1a/1b−/− and WT mice were performed. The mdr1a/1b−/− mice model was validated using digoxin, a strong P-gp substrate (Schwab et al., 2003, Schinkel et al., 1997). Four hours after i.v. administration digoxin concentrations in plasma and brain were 1.8-fold and 39-fold higher in KO mice compared to WT mice (Table 3). Not only the distribution into the brain was affected by the knock out of P-gp but also plasma concentrations were elevated. Thus, it is more appropriate to compare the quotient of brain to plasma ratios in knock out and WT mice. Digoxin showed a quotient of 23 for the brain-to-plasma ratios 4 h post administration in KO vs WT mice (Table 3).

Metabolic pattern of sorafenib in mdr1a/1b−/− and WT mice

When using total radioactivity to determine brain and plasma concentrations of sorafenib it is a prerequisite to demonstrate that sorafenib represents the vast majority of circulating radioactivity. Metabolic pattern in mdr1a/1b−/− and WT mice
were investigated up to 4 h following intravenous administration and additionally 1 and 4 h following oral administration of [14C]sorafenib tosylate. In the time interval 0.5 – 4 h following intravenous administration, unchanged compound accounted for 95–99% of the radioactivity present in plasma (Table 4). One hour following oral administration of 0.5 mg/kg and 4 mg/kg [14C]sorafenib tosylate the parent compound represented 91–95% of the radioactivity (Table 4). After 4 h [14C]sorafenib accounted for 82–89% of the radioactivity in plasma. Plasma profiles of WT and mdr1a/1b−/− mice were highly similar at all time points investigated (Table 4). Metabolites M-2 (N-oxide), M-3 (hydroxymethyl derivative) and M-4 (desmethyl derivative) were identified as minor components in plasma. Structures of the metabolites have been reported earlier (Lathia et al., 2006)

Brain penetration and plasma concentrations of sorafenib in mdr1a/1b−/− and WT mice

Whole brain concentrations in mdr1a/1b−/− mice 1 h after intravenous administration of 0.5 mg/kg of [14C]sorafenib tosylate were statistically higher (p<0.05) and showed a tendency towards higher concentrations at the other time points investigated compared to the WT mice (Figure 1). In whole brain the concentrations reached 10-20% and 8–16% of the plasma concentrations in mdr1a/1b−/− and WT mice, respectively. In the brain of WT mice the AUCs of total radioactivity were 96 µg-eq*h/L and 133 µg-eq*h/L in mdr1a/1b−/− mice. The corresponding AUCs of total radioactivity in plasma of WT mice were 1050 µg-eq*h/L and 1038 µg-eq*h/L in mdr1a/1b−/− mice following intravenous administration.

Thus, radioactivity associated with [14C]sorafenib was clearly entering the brain. Differences in the distribution patterns between mdr1a/1b−/− and WT mice are best reflected by comparison of the respective brain to plasma ratios. Brain to plasma ratio in mdr1a/1b−/− was slightly higher (1.3-fold) compared to WT mice (Table 5).

Distribution of radioactivity into the brain was also investigated 1 h and 4 h following oral administration of 0.5 and 4 mg/kg [14C]sorafenib tosylate. Plasma concentrations were not affected by the knock-out of P-gp (Figure 2A). In contrast to the plasma concentrations a slight increase of brain concentrations was observed (Figure 2B). One hour following oral administration for both 0.5 mg/kg and 4 mg/kg brain/plasma ratio was 0.053 in WT mice. In mdr1a/1b−/− mice brain/plasma ratios were slightly higher amounting to 0.068 to 0.078 for the 0.5 mg/kg and 4 mg/kg dose, respectively. 4 hours after oral administration of 0.5 or 4 mg/kg brain/plasma ratios increased in
WT as well as in mdr1a/1b<sup>−/−</sup> mice amounting to 0.075 and 0.11 (WT) and 0.11 to 0.12 (mdr1a/1b<sup>−/−</sup>), respectively. Brain/plasma ratios of mdr1a/1b<sup>−/−</sup> at the respective time points were 1.3-fold and 1.5-fold higher than in WT mice, confirming the ratio of 1.3 observed after intravenous dosing (Table 5). Only 4 h following oral administration of 0.5 mg/kg [14C]sorafenib tosylate no difference of the brain/plasma ratio was observed.
Discussion

In this paper the *in vitro* permeability and substrate characteristics of sorafenib towards P-gp are described. Furthermore, *in vitro* findings were compared with results from *in vivo* studies in mdr1a/1b<sup>-/-</sup> mice. One prerequisite for an effective anti-cancer treatment is a good permeability. According to the FDA guidelines for the Biopharmaceutics Classification System of new chemical entities the P<sub>app</sub> value has to be compared with 20 reference compounds for which the fraction dose absorbed in human is known. Comparison of sorafenib at 1 µM revealed a permeability coefficient that is similar to ketoprofen, metoprolol and fluvastatin, all classified as highly permeable (FDA). Thus, sorafenib was also classified as highly permeable. Sorafenib showed an efflux ratio of > 2 in Caco-2 cells. Compared to other substrates for efflux pumps such as digoxin (Balimane et al., 2004), taxol (Crowe et al., 2002) or vinblastine (Tang et al., 2002), all showing efflux ratios higher than 10, the observed efflux ratio in Caco-2 cells for sorafenib was low.

In P-gp overexpressing L-MDR1 cells an efflux was observed that was completely blocked by the addition of ivermectin, a strong P-gp inhibitor (Schwab et al, 2003), demonstrating that sorafenib is a P-gp substrate. The efflux of sorafenib across L-MDR1 cells was significantly reduced at higher concentrations which might be a hint for a saturable transport (Table 2). With regard to maximal sorafenib plasma concentrations of 6.3 µM (Richly et al., 2006) following oral administration of 400 mg bid (therapeutic dose) the P-gp mediated transport of sorafenib in patients might be saturated. Compared to digoxin, vinblastine (Table 2) and taxol (efflux ratio of 108 (Varma et al., 2005)), for these compounds an *in vivo* effect of P-gp on the pharmacokinetics has been shown (Schinkel et al. 1995, Schinkel et al. 1994, van Asperen et al., 1998), sorafenib showed a lower efflux ratio in L-MDR1 cells. Summarizing the *in vitro* findings, with regard to its efflux ratio sorafenib is classified as weak P-gp substrate. While the observed efflux and high permeability suggest that P-gp would be expected to have only a minor effect on the pharmacokinetics of sorafenib, it is important to confirm these findings in an *in vivo* experiment.

Transgenic mice lacking mdr1a/1b genes and proteins have been generated in order to get a better understanding of the physiological function of P-gp and its influence on the pharmacokinetics of transported drugs (Schinkel et al., 1995). To further investigate the effect of P-gp pharmacokinetics, especially the effect on plasma concentrations and brain penetration of sorafenib, studies in mdr1a/1b knock-out
mice were performed.
The model was validated using [3H]digoxin a well characterized P-gp substrate (Mayer et al., 1996; Kawahara et al., 1999). The observed increase of radioactivity in plasma and brain of KO mice compared to WT mice are in good accordance with earlier publications reporting a 27-fold and 2.9-fold increase of brain and plasma concentrations, respectively (Schinkel et al., 1994; Schinkel et al., 1997). Thus, the used in vivo set-up applying WT and KO mice was considered to be a suitable model to investigate the impact of P-gp on the pharmacokinetics especially the distribution to the brain.

As a next step plasma and brain concentrations of [14C]sorafenib in WT and mdr1a/1b(-/-) mice were determined following oral and intravenous administration at several time points. For these studies radioactively labelled compound was used. To ensure that radioactivity reflects the concentrations of the parent compound a metabolic profile was determined. The majority of radioactivity in plasma was associated with parent compound, indicating that total radioactivity reflects the concentrations of sorafenib adequately. When using whole brain homogenates to determine the distribution of a drug into the brain, the brain vascular space in mice, accounting for approx. 1.5% (Dai et al., 2003; Murakami et al., 2000) of the whole brain volume, should be taken into account. However, this is only relevant for compounds with a low CNS penetration. Brain concentrations for sorafenib in WT and KO mice were higher than 8% relating to the plasma concentrations at all time points, indicating that sorafenib is crossing the blood brain barrier. This is also reflected by the fact that plasma and brain concentration time curves show the same slope and therefore belong to the same compartment.

For both routes of administration plasma concentrations and AUCs (i.v. only) of sorafenib in KO and WT were not different at all time points investigated, indicating that P-gp is unlikely to have a major influence on the absorption and excretion of sorafenib. Brain to plasma ratios of sorafenib in KO mice were 1.3-fold higher than in WT mice. Comparing this small increase after the knock out of P-gp with other substrates such as vinblastine (5.5-fold, Schinkel et al., 1994), imatinib (7-fold, Dai et al., 2003) and digoxin (9.6-fold, Schinkel et al., 1997) the observed increase for sorafenib seems to be of minor relevance. These compounds showed also significantly higher plasma concentrations in KO mice compared to WT mice, which was not observed for sorafenib. Thus, sorafenib is in vivo a weak P-gp substrate.
confirming the *in vitro* findings. Nevertheless of the few time points it seems to be appropriate to translate these observations into an estimation of the effects of P-gp on the pharmacokinetics of sorafenib.

The clinical relevance of the *in vitro* and *in vivo* findings as described in this paper has been demonstrated by other groups. Using cells overexpressing mouse and human P-gp taxol was classified as a strong substrate (Bardelmeijer et al., 2002; Schellens et al., 2000). These findings were confirmed using mdr1a/1b(c-) mice (Lagas et al., 2006) and WT mice co-treated with the P-gp inhibitor cyclosporine A (van Asperen et al., 1998) showing significantly higher plasma and brain concentrations compared to control animals. In clinical studies taxol plasma concentrations with or without co-administration of cyclosporine A were significantly higher in the co-treated group (Meerum Terwogt et al., 1999). Thus, results from mice can reflect the clinical situation in man (Schellens et al., 2000).

Based on these *in vitro* and *in vivo* findings it is unlikely that P-gp is affecting the plasma concentrations of sorafenib in humans and most likely will not limit the entry of sorafenib into the brain or cancer cells. The high permeability of sorafanib tosylate and low *in vitro* P-gp transport rates might be beneficial with respect to treatment of CNS tumors.
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References


Footnotes
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Legends for figures

Figure 1. Equivalent concentrations of radioactivity in plasma (circles) and brain (triangles) after intravenous administration of 0.5 mg/kg $[^{14}\text{C}]$sorafenib tosylate to male mdr1a/1b-/- (open) or WT (black) mice (n = 3 animals per time point). * Statistical different p < 0.05

Figure 2. Plasma (A) and Brain (B) concentrations of $[^{14}\text{C}]$sorafenib after oral administration of 0.5 mg/kg or 4 mg/kg $[^{14}\text{C}]$sorafenib tosylate to mdr1a/1b-/- or WT mice (n=3 animals per time point). 0.5 mg/kg WT (black), 0.5 mg/kg mdr1a/1b-/- (open), 4 mg/kg WT (grey) and 4 mg/kg mdr1a/1b-/- (stripes). * Statistical different p < 0.05
### Table 1. Permeation of [14C]sorafenib, ketoprofen, metoprolol, fluvastatin, digoxin and atenolol across Caco-2 cells after 2 h at 37°C. (n of 3 to 4 wells per direction)

<table>
<thead>
<tr>
<th>Compound / concentration</th>
<th>$P_{app}$ A-B±S.D. [x10^{-6} cm/sec]</th>
<th>$P_{app}$ B-A±S.D. [x10^{-6} cm/sec]</th>
<th>Efflux ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>[14C]sorafenib / 0.1 µM</td>
<td>16.4 ± 12.3</td>
<td>76.6 ± 21.2</td>
<td>4.67 ± 3.73</td>
</tr>
<tr>
<td>[14C]sorafenib / 1 µM</td>
<td>33.5 ± 16.1</td>
<td>83.8 ± 8.60</td>
<td>2.50 ± 1.23</td>
</tr>
<tr>
<td>Ketoprofen / 2 µM</td>
<td>26.4 ± 6.68</td>
<td>18.3 ± 3.98</td>
<td>0.694 ± 0.320</td>
</tr>
<tr>
<td>Metoprolol / 2 µM</td>
<td>32.5 ± 2.44</td>
<td>24.6 ± 3.02</td>
<td>0.756 ± 1.09</td>
</tr>
<tr>
<td>Fluvastatin / 2 µM</td>
<td>17.5 ± 1.95</td>
<td>23.6 ± 2.16</td>
<td>1.35 ± 0.194</td>
</tr>
<tr>
<td>Atenolol / 10 µM</td>
<td>0.662 ± 0.0480</td>
<td>0.860 ± 0.250</td>
<td>1.30 ± 0.389</td>
</tr>
<tr>
<td>Digoxin / 10 µM</td>
<td>0.370 ± 0.0552</td>
<td>22.0 ± 3.53</td>
<td>59.5 ± 13.0</td>
</tr>
</tbody>
</table>
Table 2. Permeation of [14C]sorafenib, digoxin and vinblastine across L-MDR1 cells after 2 h at 37°C with or without the addition of ivermectin (n of 3 to 6 wells per direction). * Statistical different p < 0.05.

<table>
<thead>
<tr>
<th>Compound / concentration</th>
<th>P$_{app}$ A-B±S.D. [x10$^{-6}$ cm/sec]</th>
<th>P$_{app}$ B-A±S.D. [x10$^{-6}$ cm/sec]</th>
<th>Efflux ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>[14C]sorafenib / 0.1 µM</td>
<td>5.82 ± 1.30</td>
<td>39.5 ± 7.62</td>
<td>6.79 ± 2.00</td>
</tr>
<tr>
<td>[14C]sorafenib / 1 µM</td>
<td>12.1 ± 2.90</td>
<td>51.5 ± 13.6</td>
<td>4.26 ± 1.52*</td>
</tr>
<tr>
<td>[14C]sorafenib / 1 µM + ivermectin / 5 µM</td>
<td>17.4 ± 6.03</td>
<td>21.8 ± 6.97</td>
<td>1.25 ± 0.591*</td>
</tr>
<tr>
<td>Digoxin / 25 µM</td>
<td>0.848 ± 0.241</td>
<td>10.2 ± 0.479</td>
<td>12.1 ± 3.48</td>
</tr>
<tr>
<td>Vinblastine / 2 µM</td>
<td>0.355 ± 0.0834</td>
<td>13.2 ± 0.188</td>
<td>37.3 ± 8.78</td>
</tr>
</tbody>
</table>
Table 3. Equivalent concentrations [µg-eq/L] of radioactivity in plasma and brain of WT and mdr1a/1b^{(-/-)} mice (KO) 4 h after intravenous administration of 0.5 mg/kg [³H]digoxin. (n = 3 animals per time point)

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Mean ± S.D. [µg-eq/L]</th>
<th>Ratio KO/WT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>Plasma</td>
<td>210 ± 41.9</td>
<td>379</td>
</tr>
<tr>
<td>Brain</td>
<td>23.2 ± 3.42</td>
<td>904</td>
</tr>
<tr>
<td>Brain/Plasma Ratio</td>
<td>0.111</td>
<td>2.57</td>
</tr>
</tbody>
</table>
**Table 4.** Metabolic pattern in plasma of WT and mdr1a/1b-/- mice following intravenous (i.v.) administration of 0.5 mg/kg or oral (p.o.) administration of 0.5 or 4 mg/kg [14C]sorafenib tosylate (n = 3 animals per time point, pooled plasma (50 µL per animal))

<table>
<thead>
<tr>
<th>Time [h]</th>
<th>i.v. 0.5 mg/kg</th>
<th>% of radioactivity</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Component</td>
<td>M-2</td>
<td>M-3</td>
<td>M-4</td>
<td>Sorafenib</td>
</tr>
<tr>
<td>0.5</td>
<td>WT</td>
<td>--</td>
<td>0.37</td>
<td>0.43</td>
<td>98.1</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>--</td>
<td>1.39</td>
<td>--</td>
<td>98.0</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>--</td>
<td>0.71</td>
<td>1.21</td>
<td>97.3</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>--</td>
<td>1.72</td>
<td>3.23</td>
<td>95.1</td>
</tr>
<tr>
<td>0.5</td>
<td>mdr1a/1b&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>--</td>
<td>0.55</td>
<td>0.14</td>
<td>98.5</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>0.15</td>
<td>0.93</td>
<td>0.48</td>
<td>97.8</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>--</td>
<td>1.52</td>
<td>0.57</td>
<td>93.9</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>--</td>
<td>1.72</td>
<td>2.18</td>
<td>95.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time [h]</th>
<th>p.o. 0.5 mg/kg</th>
<th>% of radioactivity</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Component</td>
<td>M-2</td>
<td>M-3</td>
<td>M-4</td>
<td>Sorafenib</td>
</tr>
<tr>
<td>1</td>
<td>WT</td>
<td>0.93</td>
<td>1.98</td>
<td>0.59</td>
<td>95.1</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>2.35</td>
<td>4.11</td>
<td>4.31</td>
<td>88.8</td>
</tr>
<tr>
<td>1</td>
<td>mdr1a/1b&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>1.32</td>
<td>1.81</td>
<td>0.49</td>
<td>94.4</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>6.21</td>
<td>4.67</td>
<td>4.34</td>
<td>84.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time [h]</th>
<th>p.o. 4 mg/kg</th>
<th>% of radioactivity</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Component</td>
<td>M-2</td>
<td>M-3</td>
<td>M-4</td>
<td>Sorafenib</td>
</tr>
<tr>
<td>1</td>
<td>WT</td>
<td>2.71</td>
<td>2.21</td>
<td>0.88</td>
<td>93.2</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>6.32</td>
<td>3.77</td>
<td>5.29</td>
<td>82.9</td>
</tr>
<tr>
<td>1</td>
<td>mdr1a/1b&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>3.82</td>
<td>2.37</td>
<td>1.21</td>
<td>90.9</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>8.17</td>
<td>3.89</td>
<td>4.67</td>
<td>81.9</td>
</tr>
</tbody>
</table>
Table 5. Ratio of brain/plasma ratios of $[^{14}\text{C}]$sorafenib in mdr1a/1b$^{-/-}$ and WT mice after intravenous or oral administration as its tosylate (n= 3 animals per time point).

<table>
<thead>
<tr>
<th>Dose (related to sorafenib)</th>
<th>Intravenous</th>
<th>Oral</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time [h]</td>
<td>0.5 mg/kg</td>
<td>0.5 mg/kg</td>
</tr>
<tr>
<td>0.5</td>
<td>1.30</td>
<td>--</td>
</tr>
<tr>
<td>1</td>
<td>1.25</td>
<td>1.28</td>
</tr>
<tr>
<td>2</td>
<td>1.50</td>
<td>--</td>
</tr>
<tr>
<td>4</td>
<td>1.27</td>
<td>0.964</td>
</tr>
</tbody>
</table>
Figure 1

Equivalent concentrations [µg-eq/L]

Time [h]

0 1 2 3 4

1 10 100 1000
Figure 2