P-glycoprotein (ABCB1) transports the primary active tamoxifen metabolites endoxifen and 4-hydroxytamoxifen, and restricts their brain penetration

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Running title page

Running title: Endoxifen is a P-gp substrate in vitro and in vivo

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Non-standard abbreviations: P-glycoprotein (P-gp); Blood-brain barrier (BBB); ABC: ATP-binding cassette; AUC(0-4h) area under the plasma concentration-time curve; BCRP: breast cancer resistance protein; BSA: bovine serum albumin; LC-MS/MS: liquid chromatography coupled with tandem mass spectrometry; MDCK-II: Madin-Darby Canine Kidney
**Abstract (243/250):**

P-glycoprotein (P-gp, ABCB1) is a highly efficient drug efflux pump expressed in brain, liver, and small intestine, but also in tumor cells, affecting pharmacokinetics and conferring therapy resistance for many anticancer drugs. The aim of this study was to investigate the impact of P-gp on tamoxifen and its primary active metabolites, 4-hydroxytamoxifen, N-desmethyltamoxifen and endoxifen. We used *in vitro* transport assays and *Abcb1a/1b−/−* mice to investigate the impact of P-gp on the oral availability and brain penetration of tamoxifen and its metabolites. Systemic exposure of tamoxifen and its metabolites after oral administration of tamoxifen (50 mg/kg) was not changed in the absence of P-gp. However, brain accumulation of tamoxifen, 4-hydroxytamoxifen and N-desmethyltamoxifen were modestly, but significantly (1.5-2 fold) increased. Endoxifen, however, displayed a 9-fold higher brain penetration at 4 hours after administration. Endoxifen was transported by P-gp *in vitro*. Upon direct oral administration of endoxifen (20 mg/kg), systemic exposure was slightly decreased in *Abcb1a/1b−/−* mice, but brain accumulation of endoxifen was dramatically increased (up to 23-fold at 4 hours after administration). Shortly after high-dose intravenous administration (5 or 20 mg/kg), endoxifen brain accumulation was only 2-fold increased in *Abcb1a/1b−/−* mice, suggesting a partial saturation of P-gp at the blood-brain barrier. Endoxifen, the clinically most relevant metabolite of tamoxifen, is a P-gp substrate *in vitro* and *in vivo*, where P-gp limits its brain penetration. P-gp might thus be relevant for tamoxifen/endoxifen resistance of P-gp-positive breast cancer and of tumors positioned behind a functional blood-brain barrier.
**Introduction**

Discovered in the late 1960’s, tamoxifen remains the most widely used drug for patients with early stage breast cancer and estrogen receptor (ER)-positive tumors (Hoskins, et al., 2009). Active against all stages of hormone-dependent breast cancer, it was only recently fully realized that tamoxifen owes its efficacy mainly to its active metabolites, 4-hydroxytamoxifen and endoxifen (N-desmethyl-4-hydroxytamoxifen). These metabolites exhibit a 100-fold higher binding affinity to the estrogen receptor (ER) and they are more effective in suppressing cell proliferation than tamoxifen (Johnson, et al., 2004; Lim, et al., 2005; Wu, et al., 2009). In humans, the conversion from tamoxifen to endoxifen is predominant, while the conversion via 4-hydroxytamoxifen is much lower. N-desmethyltamoxifen is another important metabolite in these pathways, exhibiting similar activity to tamoxifen (supplemental Figure 1). Therefore, circulating concentrations of endoxifen are considerably higher than those of 4-hydroxytamoxifen, pointing to endoxifen as the clinically most relevant metabolite (Ahmad, et al., 2010a; Ahmad, et al., 2010b; Stearns and Rae, 2008). Tamoxifen and its metabolites can occur in two (geometric) isomeric forms, $Z$ or $E$, and in general the $(Z)$-isomers are pharmacodynamically active (Katzenellenbogen, et al., 1984). $(Z)$-tamoxifen is the isomer employed in the clinic for its antiestrogenic effects, and, upon metabolism in vivo, the $(Z)$-isomers of the metabolites are predominantly formed, whereas the amount of $(E)$-isomers formed through interconversion is negligible (Katzenellenbogen, et al., 1984).

In addition to its ER-mediated effects against breast cancer cells, tamoxifen is reported to be active against brain metastases of ER-positive breast cancer or glioma. These effects are possibly also mediated via inhibition of the protein kinase C pathway (Gupta, et al., 2006; Lien, et al., 1991). Endoxifen exhibits similar effects as tamoxifen on protein kinase C, but its efficacy against brain cancers has not been tested yet (Ali, et al., 2010). Nevertheless, the accessibility of these compounds to the brain tissue (or tumor) might be functionally impaired by the
presence of P-gp (ABCB1, MDR1), an efflux pump which is expressed on the apical membrane of endothelial cells forming the blood-brain barrier, where it efficiently restricts the brain accumulation of a broad range of compounds (reviewed in (Borst and Elferink, 2002)).

Clinically, poor response to tamoxifen treatment can be partially explained by polymorphisms in the gene encoding CYP2D6, the enzyme primarily responsible for the formation of endoxifen, leading to vast inter-individual differences in endoxifen circulating concentrations and response to therapy (Jin, et al., 2005; Schroth, et al., 2010). However, the role of the CYP2D6 genotype alone in predicting tamoxifen-associated outcomes remains controversial. In addition, tamoxifen resistance could perhaps be mediated via P-gp, which has been described to be expressed in the membrane of various breast cancer cells (Faneyte, et al., 2001). Studies conducted in a small number of breast cancer patients indicate that P-gp (over)expression correlates with a poor response to tamoxifen therapy (Keen, et al., 1994; Wang, et al., 1997; Linn, et al., 1995).

However, although tamoxifen can interact with P-gp \textit{in vitro}, it has not been found to be a substrate for transport (Bekaii-Saab, et al., 2004; Callaghan and Higgins, 1995; Mutoh, et al., 2006). Regarding the active metabolites, endoxifen and 4-hydroxytamoxifen, knowledge about their transport properties by P-gp (or by the breast cancer resistance protein, BCRP/ABCG2) is lacking. The aim of this study was to investigate the role of P-gp in serum pharmacokinetics and brain accumulation of tamoxifen and its active metabolites \textit{in vivo}. Further, we focused on the ability of P-gp to transport endoxifen \textit{in vitro} and the impact of P-gp on serum pharmacokinetics and brain penetration of endoxifen after direct administration of endoxifen \textit{in vivo}. We were also interested the factors which can lead to the saturation of P-gp at the blood-brain barrier, like dosage or route of administration.
Materials and methods

Chemicals

Tamoxifen, 4-hydroxytamoxifen and N-desmethyl-4-hydroxytamoxifen (endoxifen) (1:1, E/Z mixture) were purchased from Toronto Research Chemicals (North York, Ontario, Canada). Zosuquidar (Eli Lilly and Company, Indianapolis, IN, USA) was a generous gift from Dr. Olaf van Tellingen. [14C]inulin was from Amersham (Little Chalfont, UK). Isoflurane (Forane) was from Abbott Laboratories (Queenborough, Kent, UK). Bovine serum albumin (BSA) was from Roche (Mannheim, Germany). All other chemicals and reagents were obtained from Sigma-Aldrich (Steinheim, Germany).

Transport assays

Transport assays in polarized canine kidney cell line MDCKII and subclones transduced with human ABCB1 (Evers, et al., 1998) were performed as previously described with minor modifications (Lagas, et al., 2010). Experiments were done in the presence or absence of 5 μmol/L zosuquidar, a specific inhibitor of P-gp (Dantzig, et al., 1996). When zosuquidar was applied, it was present in both compartments during a 2 h preincubation period and during the transport experiment. After preincubation, the experiment was started (t = 0 h) by replacing the medium with fresh Dulbecco’s modified Eagle’s medium (DMEM), containing 10% FCS and 5 μmol/L endoxifen with or without 5 μmol/L zosuquidar. Cells were incubated at 37 °C in 5% CO2. 100 μL aliquots were taken at 4 and 8 h. Transport was calculated as the fraction of drug recovered in the acceptor compartment versus the fraction added in the donor compartment at the beginning of the experiment. Data are represented as mean ±SD (n = 3). Transport ratios (r) were calculated by dividing apically directed translocation by basolaterally directed translocation of endoxifen. Tightness of the monolayers was determined in parallel by
measuring paracellular $[^{14}\text{C}]$inulin leakage ($\sim 4$ kBq/well) in the same cells seeded and cultured the same way. Inulin leakage had to remain below 1% per hour. At the end of the experiment filters with cell layers were washed twice with ice-cold PBS, excised and mixed with 500 μl ice-cold methanol, followed by mixing for 15 min and centrifugation at 5000 rpm (2100 g) for 5 min at 4°C. The protein and endoxifen concentrations were determined in the resulting supernatant.

**Animals**

All mice were housed and handled according to the institutional guidelines complying with Dutch legislation. Animals used for this study were females of >99% FVB genetic background between 8 and 12 weeks of age. Two strains were used for experiments: wild-type and Abcb1a/1b-/- mice (Schinkel, et al., 1997) which lack both functional Abcb1a and Abcb1b genes, that together fulfill the functions of the single human ABCB1 gene. Animals were kept in a temperature-controlled environment with a 12 hour light / 12 hour dark cycle and received a standard diet (AM-II, Hope Farms, Woerden, The Netherlands) and acidified water *ad libitum*.

**Plasma pharmacokinetics and brain accumulation after oral administration of tamoxifen or endoxifen**

Tamoxifen was dissolved in Tween/ethanol (1:1, v/v) (at 20 mg/mL), 4-fold diluted with 0.9% NaCl (to 5 mg/mL) and administered orally at 50 mg/kg (10 mL/kg of mouse). Endoxifen (1:1, E/Z mixture) was dissolved in Tween/ethanol (1:1, v/v) (at 8 mg/mL), 4-fold diluted with 0.9% NaCl (to 2 mg/mL) and administered orally at 20 mg/kg (10 mL/kg). To reduce variation in absorption rates, mice (n = 5 per group) were fasted at least 3 h before tamoxifen or endoxifen was given by gavage into the stomach using a blunt-ended needle. Multiple blood samples
(~50 μL) were collected from the tail vein at 0.25, 0.5, 1 or 2 h in 0.75 mL Eppendorf tubes. At 4h blood was isolated via cardiac puncture under isoflurane anesthesia followed by cervical dislocation. In an independent experiment, 1 h after administration of tamoxifen or endoxifen, blood was isolated via cardiac puncture under isoflurane anesthesia followed by cervical dislocation. Brains were rapidly removed, homogenized on ice in 2 mL of 4 % (w/v) bovine serum albumin and stored at -30 °C until analysis. Serum was obtained by centrifugation of the coagulated blood samples (allowed to clot for a minimum of 1 h at room temperature) at 5000 rpm (2100 g) for 6 min at 4 °C.

**Serum pharmacokinetics and brain accumulation after intravenous administration of endoxifen**

Endoxifen (1:1, E/Z mixture) was dissolved in Tween/ethanol (1:1, v/v) (4 mg/mL or 16 mg/mL), 4-fold diluted with 0.9% NaCl (to 1 mg/mL or 4 mg/mL) and was administered intravenously at 5 mg/kg or 20 mg/kg (5 mL/kg) in the tail vein of the mice. Fifteen minutes after administration blood was collected via cardiac puncture under isoflurane anesthesia followed by cervical dislocation. Brain and blood were processed and stored as described above.

**Drug analysis**

Concentrations of tamoxifen and its metabolites in DMEM, serum and brain homogenate were analyzed by a validated liquid chromatography-tandem mass spectrometry assay as described (Teunissen, et al., 2009). (Z)- and (E)-endoxifen were quantified separately in this assay.

**RNA isolation, cDNA synthesis and RT-PCR**
RNA isolation from mouse small intestine and liver and, subsequent cDNA analysis and RT-PCR were performed as described (van Waterschoot, et al., 2008). Specific primers (Qiagen, Hilden, Germany) were used to detect expression levels of the following mouse genes: Cyp3a11, Cyp3a13, Cyp3a25, Cyp2c38, Cyp2c55, Cyp2c65, Cyp2c66, Abcc2 and Abcg2.

**Pharmacokinetic calculations and statistical analysis**

The AUC was calculated using the trapezoidal rule, without extrapolating to infinity. Brain concentrations of tamoxifen and its metabolites, including endoxifen, were corrected by the amount of drug in the brain vasculature, which corresponds to 1.4% of the serum concentration at the respective time points (Dai, et al., 2003). Relative brain accumulation (brain-to-serum ratio) was calculated by dividing brain concentrations at \( t = 0.25, 1 \) or \( 4 \) h by the serum concentrations at the respective time points. To test the statistical significance, we performed a Student’s t-test and differences were considered significant when \( P < 0.05 \). Data are presented as means ± SD.
Results

Effect of P-gp on serum pharmacokinetics of tamoxifen and its metabolites after oral administration of tamoxifen

As tamoxifen is administered orally to patients, we chose to study its serum pharmacokinetics after oral administration at 50 mg/kg to wild-type and Abcb1a/1b-/ mice. In humans, the conversion to endoxifen is predominant over the conversion to 4-hydroxytamoxifen. Our preliminary studies showed that, in mice, the situation is reversed, with much higher levels of 4-hydroxytamoxifen (Supplemental figure 1, Figure 1 panel A versus B and C). Therefore, we chose a relatively high dosage (relative to the regular prescribed human dosage of 20 mg dd) in order to obtain serum levels of tamoxifen and its most clinically relevant metabolite (endoxifen) in mice similar to steady-state serum concentrations in patients (Furlanut, et al., 2007). As shown in Figure 1 (A, B, C, D) and in Table 1, serum concentrations and AUC(0-4h) for tamoxifen, 4-hydroxytamoxifen, N-desmethyltamoxifen and (Z)-endoxifen were not significantly different between the two mouse strains. These results suggest that absence of P-gp does not significantly affect the oral uptake or elimination of tamoxifen or the formation and elimination of its metabolites.

Effect of P-gp on brain accumulation of tamoxifen and its metabolites after oral administration of tamoxifen

Despite the similar serum levels, brain accumulation of tamoxifen and its metabolites was significantly increased in the absence of P-gp (Figure 2A, B, C, D). Tamoxifen showed only a modest, but significant increase (1.6-fold, \( P < 0.05 \)) in brain concentration in the P-gp knockout mice at 4 hours after oral administration (Figure 2A). When represented as brain-to-serum ratios the difference between the two strains became significant also at 1 hour after administration (Table 1). 4-hydroxytamoxifen and N-desmethyltamoxifen exhibited somewhat
larger effects, with 2- to 2.3-fold \( (P < 0.001) \) higher brain levels (represented as absolute concentrations or brain-to-serum ratios) in \( Abcb1a/1b^{\sim} \) mice at 4 hours after administration (Figure 2B, C, Table 1). The most affected compound was the clinically relevant metabolite, \((Z)\)-endoxifen, which showed increases in brain concentrations in the P-gp knockout mice of 6-fold \( (P < 0.01) \) and 9.4-fold \( (P < 0.001) \) at 1 hour and 4 hours after tamoxifen administration, respectively. Brain-to-serum ratios gave similar results (Table 1).

For some compounds, it has been reported that P-gp and Abcg2 (Bcrp1) can have additive or even seemingly synergistic effects in restricting drug accumulation into the brain (Lagas, et al., 2010). However, pilot experiments including \( Abcg2^{-} \) and \( Abcb1a/1b;\text{A}bcg2^{-} \)-deficient mice failed to indicate any \textit{in vivo} impact of Abcg2 on serum or brain disposition of tamoxifen and its active metabolites (not shown). It thus seems highly unlikely that tamoxifen or its metabolites are substantially affected by BCRP/ABCG2, and we therefore did not pursue this further.

\textit{In vitro} transport of endoxifen

As we observed a pronounced effect of P-gp on \textit{in vivo} brain penetration of endoxifen after oral administration of tamoxifen, we investigated endoxifen as an \textit{in vitro} substrate of ABCB1 by testing its transepithelial transport in polarized monolayers of MDCKII cells stably transduced with ABCB1. Since pure \((Z)\)-endoxifen was not available to us, endoxifen was applied as a 1:1 mixture of two isomers, \((Z)\)- and \((E)\)-endoxifen, which were quantified and represented separately. In parental cells, there was no significant polarized transport of \((Z)\)-endoxifen (Figure 3A, B). In contrast, in the ABCB1-transduced cells there was clear apically directed transport of \((Z)\)-endoxifen (Figure 3C), which was completely inhibited in the presence of the P-gp specific inhibitor, zosuquidar (Figure 3D). \((E)\)-endoxifen showed virtually identical quantitative results (supplemental Figure 2A, B, C, D) indicating that both isomers are equally good P-gp substrates \textit{in vitro}, and also otherwise have similar membrane permeation.
properties. The intracellular concentration of both isomers in the monolayers of the transwell assay at the end of the experiment was significantly reduced in the ABCB1-expressing cells compared to the parental cells, from $1.52 \pm 0.17$ to $0.93 \pm 0.1$ ng/μg protein (1.6-fold, $P < 0.01$) for $(Z)$-endoxifen, and from $1.63 \pm 0.08$ to $0.89 \pm 0.09$ ng/μg protein (1.8-fold, $P < 0.001$) for $(E)$-endoxifen. This indicates that P-gp reduces the intracellular accumulation of endoxifen.

**Effect of P-gp on endoxifen serum pharmacokinetics after oral administration of endoxifen**

Since it has been recognized that the main pharmacodynamically active component of tamoxifen is most likely endoxifen, researchers have considered direct administration of endoxifen, circumventing the complications of interindividually variable conversion of tamoxifen to endoxifen by CYP2D6 (Ahmad, et al., 2010a; Ahmad, et al., 2010b). We therefore administered endoxifen (1:1, $E/Z$ mixture) directly at an oral dose of 20 mg/kg to wild-type and $Abcb1a/1b^{-/-}$ mice. Somewhat unexpectedly, both $(Z)$- and $(E)$-endoxifen serum concentrations and $AUC_{(0-4h)}$ were modestly, but significantly lower (33-37%) in $Abcb1a/1b$ knockout versus wild-type mice (Figure 4A, supplemental Figure 3A, supplemental Table 1). Alternative detoxifying mechanisms whose expression is modestly up-regulated in the small intestine (but not in the liver) of $Abcb1a/1b^{-/-}$ mice as judged by RT-PCR, for instance, the metabolizing enzymes Cyp3a11, Cyp3a25, Cyp2c55 and Cyp2c65, might perhaps explain the decreased oral availability (supplemental Table 2). Also, slightly increased expression of efflux transporters like Abcc2 might have an additional role in limiting oral exposure (supplemental Table 2). It should be noted, though, that very little is known yet about the impact of these or other detoxifying systems on endoxifen disposition.

**Effect of P-gp on endoxifen brain accumulation after oral administration of endoxifen**
In contrast to the somewhat decreased serum exposure of (Z)-endoxifen in Abcb1a/1b knockout mice, brain accumulation of (Z)-endoxifen was highly increased in these mice, both at 1 and 4 hours (P < 0.001) after oral administration of 20 mg/kg of endoxifen (1:1, E/Z mixture) (Figure 4B, C). Fold differences of uncorrected brain concentrations of (Z)-endoxifen between knockout and wild-type mice were higher at the later time point (12.8-fold at 4 hours and 6-fold at 1 hour). When represented as brain-to-serum ratios, the fold differences between the two strains were even higher (23-fold at 4 hours and 6.5-fold at 1 hour) (Figure 4C, supplemental Table 1). For (E)-endoxifen we observed analogous results, although the (E)-endoxifen brain accumulation was overall about 2-fold higher in comparison with (Z)-endoxifen (supplemental Figure 3B, C, supplemental Table 1). The impact of P-gp on limiting brain accumulation of (Z)- or (E)-endoxifen was thus even more pronounced after direct administration of endoxifen compared to administration of tamoxifen.

**Saturation of P-gp-mediated transport after intravenous administration of endoxifen**

To extend our understanding of the P-gp-mediated transport capacity for endoxifen at the blood-brain barrier, we attempted to saturate it using different (high) intravenous dosages, resulting in high initial serum levels of endoxifen. Fifteen minutes after intravenous administration of either 5 mg/kg or 20 mg/kg endoxifen (1:1, E/Z mixture), serum concentrations of either isomer were similar in both strains (Figure 5A, supplemental Figure 4A). At the same time point, the impact of P-gp on brain accumulation of endoxifen was still observed, but much reduced in comparison with oral administration. (Z)-endoxifen brain concentrations in Abcb1a/1b knockout mice were significantly higher (2.8-fold, P < 0.001) in comparison with wild-type mice when endoxifen (1:1, E/Z mixture) was dosed at 5 mg/kg (Figure 5B). However, when 20 mg/kg was employed, the (Z)-endoxifen brain concentrations were not significantly different between the two strains anymore, suggesting a possible near-
saturation of the P-gp-mediated transport in the wild-type mice (Figure 5B). That saturation was not complete was evident from the brain-to-serum ratios, which were still significantly increased (~2-fold, P < 0.05) in Abcb1a/1b knockout versus wild-type mice at both dosages (Figure 5C). Very similar behavior was observed for (E)-endoxifen (supplemental Figure 4B, C). (Z)- and (E)-endoxifen serum concentrations 15 min after intravenous administration were still ~10-fold higher than peak serum concentrations after oral administration (Figure 5A versus Figure 4A, supplemental Figure 3A versus supplemental Figure 4A). These data suggest that when exposed to high (Z)- and (E)-endoxifen serum concentrations, P-gp at the blood-brain barrier can be partially saturated.
Discussion

P-gp has an important role in protecting tissues and P-gp-positive tumor cells from a wide range of compounds. Here we demonstrate its impact on tamoxifen and its active metabolites. Absence of P-gp enhanced the brain accumulation of tamoxifen and its metabolites, but not the serum levels of these compounds. Endoxifen brain accumulation was highly increased in Abcb1a/1b−/− mice, and in vitro we could demonstrate that both endoxifen isomers are P-gp substrates, and that P-gp reduces their cellular accumulation. After direct oral administration of endoxifen (1:1, E/Z mixture), differences in brain penetration of both endoxifen isomers between wild-type and Abcb1a/1b−/− mice were even more pronounced. Finally, P-gp at the blood-brain barrier could be partially saturated after intravenous endoxifen administration when serum concentrations of (Z)- and (E)-endoxifen and thus the relative exposure of the brain were high.

We observed only a small effect of P-gp on tamoxifen brain accumulation after administration of 50 mg/kg tamoxifen, in line with reports that suggest little, if any, P-gp-mediated transport of tamoxifen (Rao, et al., 1994; Callaghan and Higgins, 1995). The brain penetration of the main tamoxifen metabolites, 4-hydroxytamoxifen, N-desmethyltamoxifen and endoxifen is more clearly restricted by P-gp. Our data suggest that 4-hydroxytamoxifen and N-desmethyltamoxifen are relatively weak P-gp substrates in vivo, although Bekaii-Saab et al. could not demonstrate their P-gp-mediated transport in vitro using Caco-2 cells with modest endogenous levels of P-gp (Bekaii-Saab, et al., 2004). This apparent discrepancy between in vitro and in vivo data is probably due to a lower sensitivity of the in vitro assays used in comparison with the blood-brain barrier, a very tight barrier where P-gp activity is very high.

For many years it was thought that 4-hydroxytamoxifen was the main compound responsible for the therapeutic effects of tamoxifen, owing mostly to its 100-fold higher affinity for the estrogen receptor than tamoxifen (Johnson, et al., 2004). With the discovery that endoxifen has a similar activity in its ER interaction as 4-hydroxytamoxifen, and exhibits much
higher serum levels in patients, the view that endoxifen is the clinically most relevant metabolite is becoming more and more accepted (reviewed in (Hoskins, et al., 2009)). Studies addressing its potential as direct therapeutic agent in breast cancer treatment are emerging (Ahmad, et al., 2010a), while endoxifen’s safety, tolerability and adequate systemic oral bioavailability in human subjects was demonstrated in a recent clinical study (Ahmad, et al., 2010b). However, little is known about the transport behavior of endoxifen. Here, we show that (Z)- and (E)-endoxifen are transported substrates of P-gp \textit{in vitro} and \textit{in vivo}. Zosuquidar, commonly employed as a specific inhibitor of P-gp (Dantzig, et al., 1996), could reverse the ABCB1-mediated transport of endoxifen \textit{in vitro}. The substantial role of P-gp in limiting brain penetration of (Z)- and (E)-endoxifen, which is even greater than after administration of tamoxifen, is notable. It is known that only the (Z)-isomers of tamoxifen and 4-hydroxytamoxifen exhibit the desired pharmacodynamic properties (Katzenellenbogen, et al., 1984). Because until recently it was cumbersome to separate the two isomers of endoxifen in large quantities (Fauq, et al., 2010), information regarding the biological activity of the (E)-isomer is lacking. More in depth research comparing the pharmacodynamic properties of the two endoxifen isomers may be of interest.

It is worth noting that in wild-type mice 4 hours after tamoxifen administration, tamoxifen brain concentrations are 60-fold higher than those of endoxifen (Figure 2A versus 2D). If the same situation would hold for the human brain, it could be that tamoxifen, in spite of its 100-fold lower affinity for the ER, still contributes nearly as much as endoxifen to the therapeutic effect for tumor cells positioned behind the BBB.

Absence of P-gp did not have an effect on oral bioavailability of tamoxifen or serum concentrations of metabolites after oral administration. In rats, co-administration with dual P-gp and Cyp3a inhibitors resulted in an increased plasma AUC of tamoxifen, effects possibly mediated via Cyp3a inhibition (Piao, et al., 2008; Shin, et al., 2006). Upon administration of endoxifen itself \textit{in vivo} we could directly observe the impact of P-gp on endoxifen oral
availability. Serum AUC after oral administration of both endoxifen isomers was somewhat decreased in Abcb1a/1b⁻/⁻ mice, an effect which is probably not a direct consequence of the loss of P-gp activity. Possibly up-regulation of alternative detoxifying mechanisms (metabolizing enzymes and efflux transporters) in the small intestine of Abcb1a/1b⁻/⁻ mice might perhaps explain the decreased oral availability. Data regarding endoxifen metabolism or pharmacokinetics in mice are lacking and future studies aiming to elucidate the importance of these alternative mechanisms are required.

The absence of P-gp resulted in a strong increase in brain accumulation of endoxifen, but not in serum AUC levels. A substantial difference between the impact of P-gp on brain penetration (high) and oral AUC (low or absent) is often observed for a diversity of P-gp substrate drugs (Jonker, et al., 1999). This difference might be explained by the different physiological properties and functions of the blood-brain barrier and the small intestinal epithelium. The small intestine facilitates the absorption of heterogeneous food derived-nutrients and it is equipped with a substantial and diverse uptake capacity. In contrast, the blood-brain barrier is a highly selective barrier, protecting the brain from harmful compounds, and thus less prone to protein-mediated uptake (and possibly also to passive diffusion) of a wide range of compounds. Also, the intestinal concentration of oral drugs is usually much higher than plasma concentrations, increasing the likelihood of saturation of P-gp in the intestine. Therefore, the efflux capacity of P-gp in the small intestine can be much more easily overwhelmed by the overall uptake capacity for compounds than in the blood-brain barrier. Finally, P-gp density in the blood-brain barrier might be higher than in the gut. However, these considerations remain hypothetical and further studies will be required to support them.

The impact of P-gp on the accumulation of tamoxifen’s active metabolites, endoxifen, 4-hydroxytamoxifen and even N-desmethyltamoxifen that we observed might have clinical implications for innate or acquired resistance of breast cancers to tamoxifen therapy. It is known that P-gp is expressed in approximately 40% of untreated breast cancers (Leonessa
and Clarke, 2003). A meta-analysis study showed that upon exposure to chemotherapeutic drugs (especially those known to be P-gp substrates), P-gp expression increases in breast cancers and this event is associated with lower response rates (Clarke, et al., 2005). In the current study we demonstrated that P-gp has a high impact on brain accumulation of endoxifen, and a moderate impact on 4-hydroxytamoxifen. Thus, upon tamoxifen treatment, P-gp (over-)expressed in breast cancer cells might also limit the exposure to endoxifen and 4-hydroxytamoxifen, leading to insufficient concentrations of these active metabolites in the tumor cells and poor response to tamoxifen treatment. Accordingly, in the in vitro transport assay we observed significantly lower intracellular concentrations in the cells expressing ABCB1 in comparison with the parental cells. Preliminary clinical evidence supporting this hypothesis is provided by a few clinical studies investigating the effect of P-gp expression in breast tumors on survival after long-term treatment with tamoxifen in a small cohort of patients (Linn, et al., 1995). P-gp-positive cases had a much lower 3-year overall survival than patients with P-gp-negative tumors. Similar results were obtained in another study in which patients whose tumors did not express P-gp after 3-months treatment with tamoxifen had an 2-fold higher response rate than the patients with P-gp positive tumors (Keen, et al., 1994). Since tamoxifen was not considered as a substantially transported substrate for P-gp (Clarke, et al., 2005), it was previously difficult to explain these findings. The insight that endoxifen is a substantially transported substrate for P-gp can provide a straightforward explanation for these results. Unfortunately, in neither study intratumoral concentrations of endoxifen or 4-hydroxytamoxifen were measured. Extensive studies investigating the P-gp profile before and after treatment, correlating treatment response and overall survival with intratumoral levels of endoxifen and 4-hydroxytamoxifen are therefore necessary. Nevertheless, collectively, the findings of our study might provide a plausible explanation for poor response or resistance to tamoxifen treatment in breast cancer patients with P-gp-positive tumors.
Possible benefits of applying efficacious in vivo P-gp inhibitors such as elacridar in order to improve tumor and brain penetration of endoxifen in P-gp-positive breast tumors and in ER-dependent tumors positioned behind a functional blood-brain barrier should now also be considered. It is sometimes considered that the BBB in larger brain metastases of breast cancer is often disrupted (e.g. (Yonemori, et al., 2010)), and that there would therefore be little therapeutic gain of enhancing BBB penetration of anticancer drugs. However, this view disregards the substantial heterogeneity inside (and between) metastases and their vasculature concerning BBB differentiation characteristics and expression of efflux transporters (Regina, et al., 2001; Lockman, et al., 2010), or the fact that the invasive rims of the tumor are likely to be partially protected by the normal BBB in the surrounding brain tissue. The latter will also apply to small micrometastases in the brain that have not yet recruited their own blood vessel formation (Fidler, 2010). In all such cases P-gp inhibition might potentially improve chemotherapy sensitivity.

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Autorship Contributions

Participated in research design: Iusuf, Teunissen, Rosing, Beijnen, Schinkel

Conducted experiments: Iusuf, Teunissen, Wagenaar

Contributed new reagents and analytical tools: Iusuf, Teunissen, Wagenaar, Rosing, Beijnen

Performed data analysis: Iusuf, Teunissen, Schinkel

Wrote or contributed to the writing of the manuscript: Iusuf, Teunissen, Schinkel
References


Footnotes

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Legends for figures

**Figure 1.** Serum concentration versus time curves of (A) tamoxifen, (B) 4-hydroxytamoxifen, (C) N-desmethyltamoxifen and (D) (Z)-endoxifen after oral administration of 50 mg/kg tamoxifen to female wild-type and P-gp knockout mice (Abcb1a/1b^−/−). Points, means; bars, SD (n=5), *, P < 0.05 when compared with wild-type. Note differences in Y-axis scales between panels.

**Figure 2.** Brain concentrations of (A) tamoxifen, (B) 4-hydroxytamoxifen, (C) N-desmethyltamoxifen and (D) (Z)-endoxifen at 1 h and 4 h after oral administration of 50 mg/kg tamoxifen to female wild-type and P-gp knockout mice (Abcb1a/1b^−/−). Columns, means; bars, SD (n=5). *, P < 0.05, **, P < 0.01, ***, P < 0.001 when compared with wild-type. Note differences in Y-axis scales between panels.

**Figure 3.** Transepithelial transport of (Z)-endoxifen was assessed using MDCKII cells, either parental (A and B), or transduced with human ABCB1 (C and D). At t = 0 h, endoxifen (5 μmol/L; 1:1, E/Z mixture) was applied in one compartment (apical or basolateral), and the amount of drug appearing in the opposite compartment at t = 4 and 8 h was plotted as the percentage of the amount of initially applied drug. Zosuquidar (5 μmol/L) was applied to inhibit ABCB1 (D) and/or endogenous canine ABCB1 (B). ○, translocation from basolateral to apical compartment (B-to-A); ▲ translocation from apical to basolateral compartment (A-to-B). Points, mean; bars, SD (n = 3). *, P < 0.05, **, P < 0.01, ***, P < 0.001 when comparing B-to-A versus A-to-B translocation. r = transport ratio calculated as the quotient of B-to-A and A-to-B directed transport at 8 h.
Figure 4. Serum and brain levels of (Z)-endoxifen after oral administration of 20 mg/kg endoxifen (1:1, E/Z mixture) to female wild-type and P-gp knockout mice (Abcb1a/1b−/−). (A) Serum concentration versus time curves, (B) brain concentrations and (C) brain-to-serum ratios of (Z)-endoxifen 1 h and 4 h after administration. Points or columns, means; bars, SD (n = 5). *, P < 0.05, **, P < 0.01, ***, P < 0.001 when compared with wild-type mice.

Figure 5. (A) Serum concentrations, (B) brain concentrations and (C) brain-to-serum ratios of (Z)-endoxifen 15 minutes after intravenous administration of 5 mg/kg or 20 mg/kg endoxifen (1:1, E/Z mixture) to female wild-type and P-gp knockout mice (Abcb1a/1b−/−). Columns, means; bars, SD (n=5). *, P < 0.05, ***, P < 0.001 when compared with wild-type.
Tables

Table 1. Serum AUC_{(0-4)}, serum concentrations and brain-to-serum ratios of tamoxifen and its metabolites in mice at 1 h or 4 h after oral administration of 50 mg/kg tamoxifen. *, P < 0.05, **, P < 0.01 *** P < 0.001 when compared with wild-type. #, note that the t = 1 h data represent an independent experiment from t = 4 h.

<table>
<thead>
<tr>
<th>Tamoxifen</th>
<th>N-desmethyltamoxifen</th>
<th>4-hydroxytamoxifen</th>
<th>(Z)-endoxifen</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Abcb1a/1b^{-/-}</td>
<td>WT</td>
</tr>
<tr>
<td>AUC_{(0-4)}, ng/L.hr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>1319 ± 201</td>
<td>1445 ± 188</td>
<td>347 ± 52</td>
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<tr>
<td>t = 1 h #</td>
<td>Serum (ng/mL)</td>
<td>412 ± 151</td>
<td>289 ± 114</td>
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<tr>
<td>Brain:serum ratio</td>
<td>8.1 ± 1.1</td>
<td>10.5 ± 1.8 *</td>
<td>6.6 ± 3.2</td>
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<td>Fold increase</td>
<td>1</td>
<td>1.3</td>
<td>1</td>
</tr>
<tr>
<td>Serum (ng/mL)</td>
<td>249 ± 119</td>
<td>280 ± 117</td>
<td>116 ± 45</td>
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<tr>
<td>t = 4 h</td>
<td>Brain:serum ratio</td>
<td>23.4 ± 2.3</td>
<td>36.4 ± 7.8 **</td>
</tr>
<tr>
<td>Fold increase</td>
<td>1</td>
<td>1.6</td>
<td>1</td>
</tr>
</tbody>
</table>
Figures
Figure 1.

A) Tamoxifen

B) 4-hydroxytamoxifen

C) N-desmethyltamoxifen

D) (Z)-endoxifen
Figure 2.

(A) Tamoxifen

(B) 4-hydroxytamoxifen

(C) N-desmethyltamoxifen

(D) (Z)-endoxifen

Brain concentration (µg/g) vs. Time (hr)

* p < 0.05

** p < 0.01

*** p < 0.001
Figure 3.

A  Parental  
\[ r = 1.6 \pm 0.7 \]

B  Parental + zosuquidar  
\[ r = 1.6 \pm 0.7 \]

C  ABCB1  
\[ r = 3.3 \pm 0.7 \]

D  ABCB1 + zosuquidar  
\[ r = 1.1 \pm 0.2 \]
Figure 4.

(A) Serum

(Z)-endoxifen serum concentration (ng/mL) vs. Time (hr)

- Wild-type
- Abcb1a/1b^-/-

(B) Brain

(Z)-endoxifen brain concentration (µg/g) vs. Time (hr)

- 1 hr
- 4 hr

(C) Brain-to-serum ratio

(Z)-endoxifen brain:serum ratio vs. Time (hr)

- 1 hr
- 4 hr

* indicates statistical significance.
Figure 5.

A) Serum (Z)-endoxifen serum concentration (ng/mL) for Wild-type and Abcb1a/1b−/− groups at doses of 5 and 20 mg/kg.

B) Brain (Z)-endoxifen brain concentration (µg/g) at doses of 5 and 20 mg/kg, with a significant difference indicated by ***.

C) Brain-to-serum ratio of (Z)-endoxifen brain:serum ratio at doses of 5 and 20 mg/kg, with a significant difference indicated by *.