P-Glycoprotein (ABCB1) Transports the Primary Active Tamoxifen Metabolites Endoxifen and 4-Hydroxytamoxifen and Restricts Their Brain Penetration

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Received December 16, 2010; accepted March 2, 2011

ABSTRACT

P-glycoprotein (P-gp, ABCB1) is a highly efficient drug efflux pump expressed in brain, liver, and small intestine, but also in tumor cells, that affects pharmacokinetics and confers 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- to 2-fold), increased. Endoxifen, however, displayed a 9-fold higher brain penetration at 4 h 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 h after administration). Shortly after high-dose intravenous administration (5 or 20 mg/kg), endoxifen brain accumulation was increased only 2-fold in Abcb1a/1b(−/−) mice compared with wild-type 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 tumors positioned behind a functional blood-brain barrier.

Introduction

Discovered in the late 1960s, 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 ER and 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, whereas the conversion via 4-hydroxytamoxifen is much lower. N-desmethyltamoxifen is another important metabolite in these pathways, exhibiting similar activity to tamoxifen (Supplemental Fig. 1). Therefore, circulating concentrations of endoxifen are considerably higher than those of 4-hydroxytamoxifen, pointing to endoxifen as the clinically most relevant metabolite (Stearns and Rae, 2008; Ahmad et al., 2010a,b). 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 used in the clinic for its antiestrogenic ef-

ABBREVIATIONS: ER, estrogen receptor; P-gp, P-glycoprotein; BBB, blood-brain barrier; ABC, ATP-binding cassette; AUC, area under the plasma concentration-time curve; BCRP, breast cancer resistance protein; MDCKII, Madin-Darby canine kidney II; RT-PCR, reverse transcription-polymerase chain reaction; WT, wild type.
fects, 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 (Lien et al., 1991; Gupta et al., 2006). Endoxifen exhibits similar effects as tamoxifen on protein kinase C, but its efficacy against brain cancers has not been tested (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 that 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 interindividual differences in endoxifen circulating concentrations and response to therapy (Jin et al., 2005; Schröt 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; Linn et al., 1995; Wang et al., 1997).

However, although tamoxifen can interact with P-gp in vitro, it has not been found to be a substrate for transport (Callaghan and Higgins, 1995; Bekaii-Saab et al., 2004; 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 in vivo. Furthermore, we focused on the ability of P-gp to transport endoxifen in vitro and the impact of P-gp on serum pharmacokinetics and brain penetration of endoxifen after direct administration of endoxifen in vivo. We were also interested in which factors can lead to the saturation of P-gp at the blood-brain barrier, such as 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 Inc. (North York, ON, Canada). Zosuquidar (Eli Lilly & Co., Indianapolis, IN) was a generous gift from Toronto Research Chemicals Inc. (North York, ON, Canada). Bovine serum albumin was from Roche Diagnostics (Mannheim, Germany). All other chemicals and reagents were obtained from Sigma-Aldrich (Steinheim, Germany).

Transport Assays. Transport assays were performed in the polarized canine kidney cell line MDCKII and subclones transduced with human ABCB1 (Evers et al., 1998) were performed as described previously with minor modifications (Lagas et al., 2010). Experiments were done in the presence or absence of 5 μM 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. Before preincubation, the experiment was started (t = 0 h) by replacing the medium with fresh Dulbecco’s modified Eagle’s medium, containing 10% fetal calf serum and 5 μM endoxifen with or without 5 μM zosuquidar. Cells were incubated at 37°C in 5% CO2. Aliquots (100 μl) 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 ± S.D. (n = 3). Transport ratios were calculated by dividing apically directed translocation by basolaterally directed translocation of endoxifen. Tightness of the monolayers was determined in parallel by measuring paracellular [14C]inulin leakage (~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 phosphate-buffered saline, excised, and mixed with 500 μl of ice-cold methanol, followed by mixing for 15 min and centrifugation at 5000 rpm (2100g) 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/b+/- 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-h light/12-h 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 (5 ml/kg 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 ratios, 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 4 h 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 (2100g) 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 or 16 mg/ml), 4-fold diluted with 0.9% NaCl (to 1 or 4 mg/ml) and was administered intravenously at 5 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 Dulbecco’s modified Eagle’s medium, serum, and brain homogenate were analyzed by a validated liquid chromatography-tandem mass spectrometry assay as described previously (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 previously (van Water schoot et al., 2008). Specific primers (QiAGEN GmbH, Hilden, Germany) were used to detect expression levels of the following mouse genes: Cyp3a11, Cyp3a13, Cyp3a25, Cyp2e3, 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 corresponded 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 ± S.D.

## Results

**Effect of P-gp on Serum Pharmacokinetics of Tamoxifen and Its Metabolites after Oral Administration of Tamoxifen.** Because tamoxifen is administered orally to patients, we chose to study its serum pharmacokinetics after oral administration at 50 mg/kg to wild-type and $A_{bc}b_{1a/1b}$ knockout mice ($A_{bc}b_{1a/1b}(-/-)$). Points, means; bars, S.D. ($n = 5$). *P < 0.05 compared with wild type. Note differences in y-axis scales.

**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 (Fig. 2). Tamoxifen showed a modest, but significant, increase (1.6-fold, $P < 0.05$) in brain concentration in the P-gp knockout mice at 4 h after oral administration (Fig. 2A). When represented as brain-to-serum ratios the difference between the two strains became significant at 1 h after administration (Table 1). 4-Hydroxytamoxifen and N-desmethyltamoxifen exhibited somewhat larger effects, with 2.6-fold ($P = 0.001$) higher brain levels (represented as absolute concentrations or brain-to-serum ratios) in $A_{bc}b_{1a/1b}(-/-)$ mice at 4 h after administration (Fig. 2B and 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 ($P < 0.001$) and 9.4-fold ($P < 0.001$) at 1 and 4 h after tamoxifen administration, respectively. Brain-to-serum ratios gave similar results (Table 1).

**Table 1**

<table>
<thead>
<tr>
<th>Time</th>
<th>Tamoxifen</th>
<th>N-Desmethyltamoxifen</th>
<th>4-Hydroxytamoxifen</th>
<th>(Z)-Endoxifen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>$A_{bc}b_{1a/1b}(-/-)$</td>
<td>WT</td>
<td>$A_{bc}b_{1a/1b}(-/-)$</td>
</tr>
<tr>
<td>1 h</td>
<td>AUC$_{0-4h}$ [Rg/l·h]</td>
<td>1319 ± 201</td>
<td>1445 ± 188</td>
<td>347 ± 52</td>
</tr>
<tr>
<td></td>
<td>Serum (ng/ml)</td>
<td>412 ± 151</td>
<td>289 ± 114</td>
<td>59.4 ± 26.2</td>
</tr>
<tr>
<td></td>
<td>Brain/serum ratio</td>
<td>8.1 ± 1.1</td>
<td>10.5 ± 1.8*</td>
<td>6.6 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>Fold increase</td>
<td>1.3</td>
<td>1</td>
<td>1.3</td>
</tr>
<tr>
<td>4 h</td>
<td>Serum (ng/ml)</td>
<td>249 ± 119</td>
<td>280 ± 117</td>
<td>116 ± 45</td>
</tr>
<tr>
<td></td>
<td>Brain/serum ratio</td>
<td>23.4 ± 2.3</td>
<td>36.4 ± 7.8**</td>
<td>8.8 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>Fold increase</td>
<td>1.6</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*P < 0.05; **P < 0.01; ***P < 0.001 compared with wild type.
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/Acbg2-deficient mice failed to indicate any in vivo impact of Abcg2 on serum or brain disposition of tamoxifen and its active metabolites (data 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.

**In Vitro Transport of Endoxifen.** Because we observed a pronounced effect of P-gp on in vivo brain penetration of endoxifen after oral administration of tamoxifen, we investigated endoxifen as an in vitro substrate of ABCB1 by testing its transepithelial transport in polarized monolayers of MDCKII cells stably transduced with ABCB1. Because 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 (Fig. 3, A and B). In contrast, in the ABCB1-transduced cells there was clear apically directed transport of (Z)-endoxifen (Fig. 3C), which was completely inhibited in the presence of the P-gp-specific inhibitor zosuquidar (Fig. 3D). (E)-endoxifen showed virtually identical quantitative results (Supplemental Fig. 2), indicating that both isomers are equally good P-gp substrates in vitro and 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 with the parental cells, from 1.52 ± 0.17 to 0.93 ± 0.1 ng/µg protein (1.6-fold, P < 0.01) for (Z)-endoxifen and 1.63 ± 0.08 to 0.89 ± 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 Brain Accumulation after Oral Administration of Endoxifen.** Because 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,b). 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 (Fig. 4A; Supplemental Fig. 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 Cyp2e65, might perhaps explain the decreased oral availability (Supplemental Table 2). In addition, slightly increased expression of efflux transporters such as Abcc2 might have an additional role in limiting oral exposure (Supplemental Table 2). It should be noted, though, that very little is known 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 not available to us, endoxifen was applied as its transepithelial transport in polarized monolayers of MDCKII cells stably transduced with human ABCB1 (C and D). At t = 0 h, endoxifen (5 µM; 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 µM) 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, S.D. (n = 3); *, P < 0.05; **, P < 0.01; ****, P < 0.001 comparing B-to-A and A-to-B translocation. r = transport ratio calculated as the quotient of B-to-A and A-to-B directed transport at 8 h.

**Fig. 2.** Brain concentrations of tamoxifen (A), 4-hydroxytamoxifen (B), N-desmethyltamoxifen (C), and (Z)-endoxifen (D) at 1 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, S.D. (n = 5). *, P < 0.05; **, P < 0.01; ****, P < 0.001 compared with wild type. Note differences in y-axis scales.

**Fig. 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 µM; 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 µM) 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, S.D. (n = 3); *, P < 0.05; **, P < 0.01; ****, P < 0.001 comparing B-to-A and A-to-B translocation. r = transport ratio calculated as the quotient of B-to-A and A-to-B directed transport at 8 h.
ifen was highly increased in these mice, at both 1 and 4 h ($P < 0.001$) after oral administration of 20 mg/kg of endoxifen (1:1, E/Z mixture) (Fig. 4, B and 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 h and 6-fold at 1 h). When represented as brain-to-serum ratios, the fold differences between the two strains were even higher (23-fold at 4 h and 6.5-fold at 1 h) (Fig. 4C; Supplemental Table 1). For (E)-endoxifen we observed analogous results, although the (E)-endoxifen brain accumulation was overall approximately 2-fold higher in comparison with (Z)-endoxifen (Supplemental Fig. 3, B and 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 with 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 or 20 mg/kg of endoxifen (1:1, E/Z mixture), serum concentrations of either isomer were similar in both strains (Fig. 5A; Supplemental Fig. 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 Abcb1a1b 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 (Fig. 5B). However, when 20 mg/kg was used, 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 (Fig. 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 Abcb1a1b knockout versus wild-type mice at both dosages (Fig. 5C). Very similar behavior was observed for (E)-endoxifen (Supplemental Fig. 4, B and C). (Z)- and (E)-endoxifen serum concentrations 15 min after intravenous administration were still ~10-fold higher than peak serum concentrations after oral administration (Fig. 5A versus Fig. 4A; Supplemental Fig. 3A versus Supplemental Fig. 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 me-
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 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. (2004) could not demonstrate their P-gp-mediated transport in vitro using Caco-2 cells with modest endogenous levels of P-gp. This apparent discrepancy between in vitro and in vivo data is probably caused by 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), and 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 in vitro and in vivo. Zosquistadar, commonly used as a specific inhibitor of P-gp (Dantzig et al., 1996), could reverse the ABCB1-mediated transport of endoxifen 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 h after tamoxifen administration tamoxifen brain concentrations are 60-fold higher than those of endoxifen (Fig. 2, A versus D). 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.

The absence of P-gp did not have an effect on oral bioavailability of tamoxifen or serum concentrations of metabolites after oral administration. In rats, coadministration with dual P-gp and Cyp3a inhibitors resulted in an increased plasma AUC of tamoxifen, effects possibly mediated via Cyp3a inhibition (Shin et al., 2006; Piao et al., 2008). Upon administration of endoxifen itself 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 that 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 explain the decreased oral availability. Data regarding endoxifen metabolism or pharmacokinetics in mice are lacking, so 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. In addition, 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-month treatment with tamoxifen had an 2-fold higher response rate than the patients with P-gp-positive tumors (Keen et al., 1994). Because 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.

Benefits of possible applying efficacious in vivo P-gp inhibitors such as elacridar to improve tumor and brain penetration of endoxifen in P-gp-positive breast tumors and 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 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, 2011). In all such cases P-gp inhibition might potentially improve chemotherapy sensitivity.

Acknowledgments
We thank Dr. Birk Poller, Anita van Esch, and Ahmed Elbats for excellent technical assistance and Seng Chuan Tang, Selvi Durmus, and Dr. Guillaume Filion for critical reading of the article.

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

Conducted experiments: Iusuf, Teunissen, and Wagenaar.

Contributed new reagents or analytic tools: Iusuf, Teunissen, Wagenaar, Rosing, and Beijnen.

Performed data analysis: Iusuf, Teunissen, and Schinkel.

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

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

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Supplemental figure 1. Chemical structures of tamoxifen and its main metabolites. In humans the metabolic pathway towards endoxifen is favored, whereas in mice the pathway through 4-hydroxytamoxifen predominates.
Supplemental figure 2. Transepithelial transport of (E)-endoxifen was assessed using MDCKII cells, either nontransduced 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 measured by LC-MS/MS and 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-t-A and A-to-B directed transport at 8 h.
Supplemental figure 3. Serum and brain levels of (E)-endoxifen after oral administration of 20 mg/kg endoxifen (1:1, E/Z mixture) to female wild-type and P-gp knockout mice (Abcb1a/b−/−). (A) Serum concentration versus time curves, (B) brain concentrations and (C) brain-to-serum ratios of (E)-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.
**Supplemental Table 1.** Serum AUC\(_{(0-4)}\), brain concentrations and brain-to-serum ratios of (Z/E)-endoxifen in mice at 1 h or 4 h after oral administration of 20 mg/kg endoxifen (1:1, E/Z mixture). *, \(P < 0.05\), **, \(P < 0.01\), ***, \(P < 0.001\) when compared with wild-type mice. #, note that the t = 1 h data represent an independent experiment from t = 4 h.

<table>
<thead>
<tr>
<th></th>
<th>(Z)-endoxifen</th>
<th>(E)-endoxifen</th>
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<tbody>
<tr>
<td></td>
<td>WT Abcb1a/1b(^{-/-})</td>
<td>WT Abcb1a/1b(^{-/-})</td>
</tr>
<tr>
<td>AUC(_{(0-4)}), ng/L.hr</td>
<td>388 ± 62</td>
<td>243 ± 43*</td>
</tr>
<tr>
<td>Serum (ng/mL)</td>
<td>137 ± 67</td>
<td>130 ± 66</td>
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<tr>
<td>(C_{\text{brain}}) (\mu g/g)</td>
<td>0.08 ± 0.04</td>
<td>0.45 ± 0.26*</td>
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<tr>
<td>(t = 1) h (^{#})</td>
<td>Fold increase</td>
<td>1</td>
</tr>
<tr>
<td>Brain:serum ratio</td>
<td>0.53 ± 0.18</td>
<td>3.42 ± 0.41***</td>
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<tr>
<td></td>
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<tr>
<td>Serum (ng/mL)</td>
<td>65.1 ± 23.3</td>
<td>39.1 ± 21.9</td>
</tr>
<tr>
<td>(C_{\text{brain}}) (\mu g/g)</td>
<td>0.06 ± 0.03</td>
<td>0.72 ± 0.19***</td>
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<td>(t = 4) h</td>
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<td>Brain:serum ratio</td>
<td>0.91 ± 0.35</td>
<td>21 ± 8.7***</td>
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<tr>
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Supplemental table 2. Overview of ΔCt values of the RT-PCR analysis to investigate the expression of several endogenous metabolizing enzymes and efflux transporters in small intestine and liver of female wild-type and *Abcab1a/1b*−/− mice (n = 3, each sample was analyzed in duplicate). Analysis of results was done by comparative Ct method. Quantification of the target cDNAs in all samples was normalized against the endogenous control β-actin (Ct<sub>target</sub> − Ct<sub>β-actin</sub> = ΔCt). Accordingly, the lower the value, the higher the expression level. Data are presented as means ± SD. Each sample was assayed in duplicate. * P < 0.05; * * P < 0.01 when compared with wild-type mice.

<table>
<thead>
<tr>
<th></th>
<th>Small intestine</th>
<th>Liver</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td><em>Abcb1a/1b</em>−/−</td>
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<tr>
<td><strong>Cyp3a11</strong></td>
<td>-0.2 ± 0.4</td>
<td>-1.8 ± 0.2***</td>
</tr>
<tr>
<td><strong>Cyp3a13</strong></td>
<td>10.5 ± 0.5</td>
<td>9.9 ± 0.1</td>
</tr>
<tr>
<td><strong>Cyp3a25</strong></td>
<td>5.5 ± 0.2</td>
<td>4.8 ± 0.4*</td>
</tr>
<tr>
<td><strong>Cyp2c38</strong></td>
<td>9.0 ± 0.7</td>
<td>8.6 ± 3.5</td>
</tr>
<tr>
<td><strong>Cyp2c55</strong></td>
<td>7.8 ± 0.1</td>
<td>6.4 ± 0.2***</td>
</tr>
<tr>
<td><strong>Cyp2c65</strong></td>
<td>3.4 ± 0.2</td>
<td>2.0 ± 0.5*</td>
</tr>
<tr>
<td><strong>Cyp2c66</strong></td>
<td>5.4 ± 0.8</td>
<td>4.4 ± 0.9</td>
</tr>
<tr>
<td><strong>Abcc2 (Mrp2)</strong></td>
<td>0.7 ± 0.8</td>
<td>-0.2 ± 0.3</td>
</tr>
<tr>
<td><strong>Abcg2 (Bcrp1)</strong></td>
<td>3.1 ± 0.6</td>
<td>1.5 ± 0.4*</td>
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</table>
Supplemental figure 4.

Supplemental figure 4 (A) Serum concentrations, (B) brain concentrations and (C) brain-to-serum ratios of (E)-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<sup>−/−</sup>). Columns, means; bars, SD (n = 5). *, P < 0.05, ***, P < 0.001 when compared with wild-type.