Endoxifen, the Active Metabolite of Tamoxifen, Is a Substrate of the Efflux Transporter P-Glycoprotein (Multidrug Resistance 1)

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ABSTRACT:

Tamoxifen is widely prescribed to patients with estrogen receptor-positive breast cancer, and it is a prodrug that requires bioactivation by cytochrome P450 enzymes CYP2D6 and 3A4 to generate the active metabolite, endoxifen. Large interpatient variability in endoxifen plasma levels has been reported, and polymorphisms in CYP2D6 have been implicated as a major determinant of such variability. However, little is known regarding the role of drug transporters such as P-glycoprotein [multidrug resistance 1 (MDR1), ATP-binding cassette B1 (ABCBI)] to endoxifen disposition and response. Therefore, we determined the ability of P-glycoprotein to transport endoxifen in vitro, using a polarized human P-glycoprotein-overexpressing cell line. Markedly higher transport of endoxifen was observed in the basal-to-apical direction, which was abrogated in the presence of the potent and specific P-glycoprotein inhibitor (2R)-anti-5-(3-[4-(10,11-difluoromethanodibenzo-suber-5-yl)piperazin-1-yl]-2-hydroxypropoxy}quinoline trihydrochloride (LY335979). To validate the in vivo relevance of P-glycoprotein to endoxifen disposition, plasma and tissue concentrations were also determined in Mdr1a-deficient mice after oral administration of endoxifen. Plasma endoxifen levels did not significantly differ between wild-type and Mdr1a-deficient mice. However, brain concentrations of endoxifen were nearly 20-fold higher in Mdr1a-deficient mice compared to wild-type mice. Because P-glycoprotein is highly expressed at the blood-brain barrier and in some breast cancer tumors, variation in expression and function of this transporter may alter central nervous system entry and the attained intracellular concentration in such breast cancer cells and therefore may prove to be of relevance to therapeutic outcome.

Introduction

The antiestrogen drug tamoxifen is the most commonly used adjuvant therapy for the treatment of breast cancer in pre- and postmenopausal women (Hoskins et al., 2009). Tamoxifen is considered a prodrug and undergoes extensive metabolism by the cytochrome P450 family of enzymes, namely CYP2D6 and CYP3A4, to form the active metabolite endoxifen (Hoskins et al., 2009). Endoxifen has been shown to bind the estrogen receptor (ER) with near 100-fold greater affinity than tamoxifen, and it is noted to have nearly 6-fold higher plasma levels than 4-hydroxy-tamoxifen, which was originally thought to be the main active metabolite of tamoxifen (Brauch and Jordan, 2009). Plasma endoxifen levels appear to correlate with CYP2D6 enzymatic activity, and interpatient variability of endoxifen levels are thought to be due to genetic polymorphisms and drug-interactions that alter the function of CYP2D6 (Jin et al., 2005). There is now compelling evidence to support an important role of CYP2D6 polymorphisms to disease outcome and recurrence events (Schroth et al., 2009). Therefore, to bypass the role of CYP2D6 and thereby enhance clinical efficacy, the use of oral endoxifen has been suggested as an important next step for the treatment of patients with ER-positive breast cancer (Ahmad et al., 2010). Although the biotransformation of tamoxifen to endoxifen has been well studied and documented, remarkably, there has been no report investigating the involvement of drug transporters to endoxifen disposition. Therefore, a better understanding of the role and relevance of drug transporters to endoxifen disposition will be essential to optimal tamoxifen therapy.

P-Glycoprotein (MDR1, ABCB1) is an ATP-dependent, efflux transporter with broad substrate specificity widely appreciated for its role in mediating cellular resistance to many anticancer agents (Goda et al., 2009). In addition to cancer cells, P-glycoprotein is expressed in epithelial cells of normal tissues involved in drug disposition including the liver, intestine, and kidney while providing a barrier to sites...
such as the brain and testes (Marzolini et al., 2004). P-Glycoprotein expression has been noted to be highly variable in primary breast tumors (detection ranging from 9 to 50% of tumors examined) and is significantly increased after treatment with chemotherapeutic agents; however, its significance to chemotherapy clinical response remains controversial (Koh et al., 1992; Tsukamoto et al., 1997; Rudas et al., 2003; Larkin et al., 2004; Atalay et al., 2006). Several reports determined that P-glycoprotein expression appears to be higher in more aggressive, locally advanced or metastatic tumors and is associated with a shorter overall survival for tamoxifen-treated patients, suggesting its use as a putative prognostic factor (Schneider et al., 1994; Linn et al., 1995; Tsukamoto et al., 1997).

Tamoxifen and 4-hydroxy-tamoxifen are known to bind P-glycoprotein, although they are not believed to be substrates of this transporter (Callaghan and Higgins, 1995; Bekaii-Saab et al., 2004). However, many patients receiving adjuvant tamoxifen therapy are unresponsive due to tamoxifen resistance. The mechanisms resulting in de novo and acquired tamoxifen resistance are likely multifactorial, including modulation of ER expression, cell signaling pathway alterations, changes in growth factor signaling, activity of metabolizing enzymes, and altered cellular accumulation of tamoxifen and its metabolites potentially through the induction of efflux transporters such as P-glycoprotein (Clarke et al., 2001; Musgrove and Sutherland, 2009). Decreased intratumor tamoxifen concentrations were observed in patients with locally recurrent breast cancer with acquired resistance compared to those with de novo resistance, suggesting that efflux transporters may play an important role in a subset of tamoxifen-treated patients (Johnston et al., 1993). Because the active anti-estrogenic compound of tamoxifen is the metabolite endoxifen, we hypothesized that P-glycoprotein may in fact transport endoxifen, thereby altering its disposition. Currently, the importance of active transport processes through P-glycoprotein to endoxifen disposition and response remains unknown. In this study, we show for the first time that endoxifen is a substrate of P-glycoprotein, suggesting the importance of this efflux transporter to tamoxifen therapy.

Materials and Methods

Transport in LLCPK and L-MDR1 Cells. LLCPK and LMDR1 (LLCPK cells overexpressing MDR1) cells were grown with Dulbecco’s modified Eagle’s medium (high glucose) (Lonza Walkersville, Inc., Walkersville, MD) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 2 mM l-glutamine, 50 μU/ml penicillin (Invitrogen), 50 μg/ml streptomycin (Invitrogen), and 1% nonessential amino acids (Invitrogen) and incubated at 37°C in 5% CO2. To maintain P-glycoprotein expression, LMDR1 cells were cultured with 640 nM vincristine (Sigma-Aldrich, St. Louis, MO). Cells were plated at a density of 2 × 10^5 per 0.4-μm cell culture insert (VWR International, Mississauga, ON, Canada) and grown for 6 to 7 days with media changes every 2 to 3 days. Approximately 1 h before the start of the transport experiment, media were removed from each compartment (apical and basal) and washed and replaced with Opti-MEM (Invitrogen). Transport was initiated by removing the Opti-MEM and replacing it with 700 μl of Opti-MEM with or without endoxifen (5 μM) (Toronto Research Chemicals, Toronto, ON, Canada) to the appropriate compartment. The cells were incubated at 37°C in 5% CO2, and 25-μl aliquots were removed hourly from each chamber over 4 h. Drug levels of endoxifen were measured from each aliquot by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Western Blotting. LLCPK and LMDR1 cells were lysed in 5 mM Tris-HCl, containing protease inhibitors (Sigma-Aldrich) and 1% Triton X-100 (WVR International). Protein concentration of whole-cell lysates was determined by Pierce BCA protein assay (Fisher Scientific, Whiby, ON, Canada), and 25 μg was run on a 4–PAGE 12% Bis-Tris gel (Invitrogen). Detection of P-glycoprotein expression was performed by overnight incubation with anti-P-glycoprotein antibody (C219; Covance, Emeryville, CA). To confirm equal loading of lysates, the blot was stripped and incubated with a polyclonal goat anti-actin antibody (C-11; Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Endoxifen Disposition in Mdr1a-Deficient Mice. Male Mdr1a-deficient mice (Crl/Cf-1-Abcb1amds, n = 5), referred to as Mdr1a−/−, 6 to 12 weeks old and age-matched male wild-type (WT) mice (CF-1, n = 6) were obtained from Charles River Laboratories, Inc. (Wilmington, MA). Endoxifen, 5 mg/kg [stock solution of 1 mg/ml dissolved in dimethyl sulfoxide (1%) and (1:1 v/v) PEG-300/sterile water], was administered at 0.5, 1, and 2 h after drug administration. At 4 h, mice were anesthetized using isoflurane, blood was collected by cardiac puncture, and liver and brain tissues were harvested, weighed, and homogenized in 5 mM ammonium acetate (Sigma-Aldrich), pH 4. Endoxifen levels were measured from plasma (30 μl), and tissue homogenates (100 μl) were measured by LC-MS/MS.

Fig. 1. Vectorial transport of endoxifen by P-glycoprotein. A, whole-cell lysates (25 μg) from LLCPK and LMDR1 cells were immunoblotted with an anti-P-glycoprotein antibody. Equal loading was confirmed by immunoblotting for actin. B, LMDR1 cells were grown on cell culture inserts and polarized for 6 to 7 days. Cells were washed and pretreated with Opti-MEM for 1 h before endoxifen was added to either the apical or basal compartment. Aliquots were removed from each chamber immediately after endoxifen treatment and hourly until 4 h. Endoxifen levels were measured in the opposite compartment by LC-MS/MS, and percent transport was determined. C, LLCPK cells were treated as described in B. D, LMDR1 cells were treated as described in B, in the presence of the P-glycoprotein inhibitor, LY335979 (1 μM). ***, p < 0.001.
animal protocols were approved by the University of Western Ontario Animal Care Committee.

**Endoxifen Analysis by LC-MS/MS.** Tissue culture supernatants, plasma, and tissue homogenates were spiked with 15 μl of internal standard (500 ng/ml Tamoxifen-D5; Toronto Research Chemicals). Protein precipitation was performed by addition of 3 volumes of acetonitrile. Samples were mixed, centrifuged, and diluted in 5 mM ammonium acetate, pH 4. Upon injection into the liquid chromatograph (Agilent 1200; Thermo Fisher Scientific, Mississauga, Ontario, Canada), analytes were separated on a reverse-phase column [Hypersil Gold (Thermo Fisher Scientific), 50 × 5 mm, 5 μM particle size] using gradient elution with 5 mM ammonium acetate (pH 4) and acetonitrile (30-80%) with a 7-min run time. Standard curves and quality control (QC) samples were prepared in drug-free plasma. The coefficient of variation (%) for QCs were as follows: low QC 4 ng/ml (10.5%), medium QC 25 ng/ml (8.0%), and high QC 60 ng/ml (7.1%). The lower level of quantitation for endoxifen was 1 ng/ml. The mass spectrometer (Thermo TSQ Vantage; Thermo Fisher Scientific) with heated electrospray ionization source was set in positive mode for detection of endoxifen and tamoxifen-D5 with transitions 374 → 58 m/z and 377 → 72 m/z, respectively.

**FIG. 2.** Endoxifen disposition in Mdr1a-deficient mice. Endoxifen (5 mg/kg) was administered to wild-type (n = 6) and Mdr1a-deficient mice (n = 5), aged 6 to 12 weeks, by oral gavage. A, plasma was collected from blood samples at 0.5, 1, 2, and 4 h postdose. Plasma endoxifen concentrations were measured by LC-MS/MS. B, liver was obtained at 4 h after endoxifen administration, homogenized, and endoxifen tissue concentration was measured. C, liver/plasma ratios at 4 h were determined for wild-type and Mdr1a-def mice. *p < 0.05.

**FIG. 3.** Mdr1a prevents brain entry of endoxifen. Endoxifen (5 mg/kg) was administered to wild-type mice (n = 6) and Mdr1a-deficient mice (n = 5), aged 6 to 12 weeks, by oral gavage. A, four hours after endoxifen administration, mice were anesthetized, brain tissue was obtained, homogenized, and endoxifen concentration was measured by LC-MS/MS. B, brain/plasma ratios at 4 h were determined for wild-type and Mdr1a-def mice. **p < 0.01; ***p < 0.001.

**TABLE 1**

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<thead>
<tr>
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<th>Cellular permeability and efflux ratio of endoxifen (5 μM)</th>
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<tbody>
<tr>
<td></td>
<td>No Inhibitor</td>
</tr>
<tr>
<td></td>
<td>P_{appAB} (S.D.)</td>
</tr>
<tr>
<td>LLCPK</td>
<td>1.83 (0.37)</td>
</tr>
<tr>
<td>LMDR1</td>
<td>1.10 (0.53)</td>
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Statistics. Statistical analysis was done using the unpaired \( t \) test: * \( p < \) 0.05; ** \( p < 0.01 \); and *** \( p < 0.001 \).

Results and Discussion

To test the ability of P-glycoprotein to mediate the transport of endoxifen, we compared LLCPK cells to LLCPK cells overexpressing human P-glycoprotein (LMDR1) (Schinkel et al., 1995; Kim et al., 1998) (Fig. 1A). Vectorial transport of endoxifen was significantly greater in the basal-apical direction (BA/AB; efflux ratio 2.67), when drug was administered to the basal compartment compared to addition of endoxifen to the opposite (apical) compartment in LMDR1 cells (Fig. 1B). Transport of endoxifen in the A-to-B and B-to-A direction was nearly identical in LLCPK cells (efflux ratio 1.07) (Fig. 1). These data suggest the involvement of P-glycoprotein in the differential flux of endoxifen between LMDR1 and LLCPK cells. The efficiency of P-glycoprotein-mediated transport of endoxifen \( (P_{\text{appBA}} = 2.77 \times 10^{-6} \text{ cm/s}) \) (Table 1) in L-MDR1 cells was lower than digoxin (a well established P-glycoprotein substrate) controls \( (P_{\text{appBA}} = 6.74 \times 10^{-6} \text{ cm/s}) \). To confirm the involvement of P-glycoprotein, we added the potent and selective P-glycoprotein inhibitor, LY335979, to this model system and were able to abrogate the B-to-A active transport of endoxifen in LMDR1 cells (efflux ratio 1.25) (Fig. 1D). Together, these in vitro data strongly suggest that endoxifen is a substrate of P-glycoprotein.

To determine the in vivo importance of P-glycoprotein, we assessed plasma and tissue endoxifen levels after oral administration to WT and Mdr1a \( (\text{Mdr1a}^{\text{def}}) \)-deficient male mice. Plasma levels of endoxifen were marginally lower in \( \text{Mdr1a}^{\text{def}} \) mice during the early time points after endoxifen administration; however, they were not statistically different from wild-type mice throughout the 4-h time course (Fig. 2A). At 4 h postadministration, total endoxifen levels in the liver of \( \text{Mdr1a}^{\text{def}} \) mice were slightly lower compared to WT mice (Fig. 2B). Furthermore, the liver/plasma ratio of endoxifen was significantly lower in the absence of P-glycoprotein function (Fig. 2C). Overall, the extent of plasma level or hepatic concentrations were only modestly different in these mice, similar to our previous studies using HIV protease inhibitors (Kim et al., 1998). This result suggests that P-glycoprotein does not likely play a major role in hepatic disposition of endoxifen.

A sensitive in vivo marker to determine P-glycoprotein drug substrates is the extent of their central nervous system entry in P-glycoprotein-deficient male mice. Plasma levels of endoxifen in \( \text{Mdr1a}^{\text{def}} \) mice were markedly lower in \( \text{Mdr1a}^{\text{def}} \) mice compared to wild-type mice (Fig. 3A), exhibiting a near 20-fold greater drug concentration. In addition, the brain/plasma ratio of endoxifen levels was greater than 10-fold higher in the absence of P-glycoprotein (Fig. 3B). Together, these results suggest that endoxifen is a substrate of P-glycoprotein and that P-glycoprotein transport of endoxifen may limit its tissue distribution. We know that P-glycoprotein is highly expressed at the blood-brain barrier and has been shown to provide a protective mechanism to keep many drugs, including chemotherapeutic agents, from entering the central nervous system (Urquhart and Kim, 2009). Tamoxifen therapy has been linked to the onset of depression in some patients, which is thought to be the result of antagonism of the neuroprotective effects of estrogen (Thompson et al., 1999). The entry of endoxifen into the brain may affect regions that are responsible for mood and anxiety symptoms, potentially through ER blockade and decreased serotonin uptake (Thompson et al., 1999). Further studies are required to determine whether there is a link between \textit{MDR1} polymorphisms and the onset of depressive symptoms among tamoxifen-treated patients.

A major complication of many drug therapies, including tamoxifen, is the development of drug resistance. The onset of endocrine resistance likely involves many molecular mechanisms that have been demonstrated in vitro, including most commonly the loss of ER\( \alpha \) expression but also the modulation of cell signaling pathways, growth factor receptors, and cell survival mechanisms (Clarke et al., 2001; Musgrove and Sutherland, 2009). The modulation of HER2 (also known as ERBB2), a member of the epidermal growth factor receptor family, by the ER\( \alpha \)-induced transcription factor PAX2 was recently associated with survival outcomes after tamoxifen treatment (Hurtado et al., 2008). However, as the majority of breast cancers with acquired resistance retain ER\( \alpha \) positivity, other mechanisms including drug efflux transport may play a clinically relevant role. This mechanism seems plausible because the intratumor concentration of tamoxifen was significantly lower in patients with acquired resistance (Johnston et al., 1993). In addition, tamoxifen exposure was shown to induce the expression of efflux transporters in human breast cancer cell lines by activation of the nuclear receptor PXR (Nagaoka et al., 2006). A near 8-fold greater PXR mRNA expression was also observed in malignant breast tissue compared to normal tissue (Meyer zu Schwabedissen et al., 2008). P-Glycoprotein has been detected in malignant mammary tissue, with a correlation for higher expression in more aggressive, locally advanced, or metastatic tumors, and expression is noted to increase after exposure to anticancer agents (Keen et al., 1994; Schneider et al., 1994; Linn et al., 1995). Therefore, one hypothesis may be that exposure to tamoxifen activates PXR, leading to the induction of P-glycoprotein and increased endoxifen efflux from such tumor cells. There appears to be evidence to support this hypothesis because an increase in P-glycoprotein expression was detected in nonresponders after tamoxifen therapy, and, worse, overall survival was observed for tamoxifen-treated patients with higher tumor P-glycoprotein expression (Keen et al., 1994; Linn et al., 1995). Further studies are needed to determine the value of assessing P-glycoprotein expression as a prognostic factor for tamoxifen-treated patients at risk for metastasis or drug resistance.

A potential strategy to alleviate the problems associated with tamoxifen treatment is to administer endoxifen as an oral therapy (Ahmad et al., 2010). Significantly higher systemic exposure of endoxifen was observed in rats treated with oral endoxifen compared to those treated with tamoxifen (Ahmad et al., 2010). The use of endoxifen as an alternative to tamoxifen will likely reduce the extent of interpatient variability in attained endoxifen levels. Because our data show the importance of P-glycoprotein to endoxifen disposition, careful consideration should be given to polymorphisms in this transporter and to P-glycoprotein-associated drug-drug interactions that may alter endoxifen levels, adverse central nervous system side effects, and endoxifen efficacy in some breast tumors.

Authorship Contributions

Participated in research design: Teft and Kim.
Conducted experiments: Teft and Mansell.
Performed data analysis: Teft.
Wrote or contributed to the writing of the manuscript: Teft and Kim.
Other: Kim was the project supervisor.

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