Digoxin Is Not a Substrate for Organic Anion-Transporting Polypeptide Transporters OATP1A2, OATP1B1, OATP1B3, and OATP2B1 but Is a Substrate for a Sodium-Dependent Transporter Expressed in HEK293 Cells

Mitchell E. Taub, Kirsten Mease, Rucha S. Sane, Cory A. Watson, Liangfu Chen, Harma Ellens, Brad Hirakawa, Eric L. Reyner, Marton Jani, and Caroline A. Lee

Drug Metabolism and Pharmacokinetics, Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, Connecticut (M.E.T., K.M., R.S.S.); Preclinical Drug Metabolism and Pharmacokinetics, GlaxoSmithKline, King of Prussia, Pennsylvania (C.A.W., L.C., H.E.); Solvo Biotechnology, Budaors, Hungary (M.J.); and Drug Safety Research and Development (B.H.) and Pharmacokinetics, Dynamics, and Metabolism (E.L.R., C.A.L.), Pfizer Worldwide Research and Development–La Jolla Laboratories, San Diego, California

Received May 19, 2011; accepted August 16, 2011

ABSTRACT:

Digoxin, an orally administered cardiac glycoside cardiovascular drug, has a narrow therapeutic window. Circulating digoxin levels (maximal concentration of ~1.5 ng/ml) require careful monitoring, and the potential for drug-drug interactions (DDI) is a concern. Increases in digoxin plasma exposure caused by inhibition of P-glycoprotein (P-gp) have been reported. Digoxin has also been described as a substrate of various organic anion-transporting polypeptide (OATP) transporters, posing a risk that inhibition of OATPs may result in a clinically relevant DDI similar to what has been observed for P-gp. Although studies in rats have shown that Oatps contribute to the disposition of digoxin, the role of OATPs in the disposition of digoxin in humans has not been clearly defined. Using two methods, Boehringer Ingelheim, GlaxoSmithKline, Pfizer, and Solvo observed that digoxin is not a substrate of OATP1A2, OATP1B1, OATP1B3, and OATP2B1. However, digoxin inhibited the uptake of probe substrates of OATP1B1 (IC50 of 47 μM), OATP1B3 (IC50 > 8.1 μM), and OATP2B1 (IC50 > 300 μM), but not OATP1A2 in transfected cell lines. It is interesting to note that digoxin is a substrate of a sodium-dependent transporter endogenously expressed in HEK293 cells because uptake of digoxin was significantly greater in cells incubated with sodium-fortified media compared with incubations conducted in media in which sodium was absent. Thus, although digoxin is not a substrate for the human OATP transporters evaluated in this study, in addition to P-gp-mediated efflux, its uptake and pharmacokinetic disposition may be partially facilitated by a sodium-dependent transporter.

Introduction

In the Western world, cardiovascular disease is the most prevalent cause of morbidity and mortality. Over the past decade, the incidences of chronic heart failure have continued to increase, and approximately 5 million people in the United States are now affected by this disease (Klein et al., 2003; Liao et al., 2008). Digoxin, a cardiac glycoside drug, is commonly used for treatment of chronic heart failure and atrial fibrillation (Hunt et al., 2005) and is frequently prescribed to patients who are also taking diuretics, angiotensin-converting enzyme blockers (Hunt et al., 2005). Digoxin has a narrow therapeutic window, and as such even slight changes in plasma exposure have been associated with adverse reactions such as digitalis toxicity, resulting in recommendations for close monitoring of digoxin serum levels (Tuncok et al., 1997).

In humans, digoxin is largely eliminated unchanged with comparable renal and nonrenal clearance in healthy subjects after intravenous digoxin administration (Lacarelle et al., 1991; Ding et al., 2004). However, digoxin, mainly the Lanoxin formulation, can undergo metabolism in a small minority of patients by enteric bacteria (Lindenbaum et al., 1981), in which a double bond in the digoxin lactone ring is reduced. Because gut flora activity can vary in patients, variable levels of digoxin can be absorbed (Saha et al., 1983). Drug-drug interactions (DDIs) resulting in alterations in digoxin plasma exposure have been largely attributed to changes in P-glycoprotein (P-gp) efflux activity in the gut, liver, and kidney (Cavet et al., 1996; Ernest et al., 1997).

All authors contributed equally to this project.

Article, publication date, and citation information can be found at http://dmd.aspetjournals.org. doi:10.1124/dmd.111.040816.

ABBREVIATIONS: DDIs, drug interactions; OATP, organic anion-transporting polypeptide; P-gp, P-glycoprotein; BI, Boehringer Ingelheim; GSK, GlaxoSmithKline; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; HBSS, Hanks’ balanced salt solution; DPBS, Dulbecco’s phosphate-buffered saline; WT, wild type; HEK, human embryonic kidney; CHO, Chinese hamster ovary; MDCK, Madin-Darby canine kidney; MRP, multidrug resistance-associated protein; ASBT, apical sodium-dependent bile acid transporter; SMVT, sodium-dependent multivitamin transporter; SCHH, sandwich-cultured human hepatocytes; O斯塔, organic solute transporter-α; O斯塔β, organic solute transporter-β; LY335979, (R)-4-((1αR,6R,10bS)-1,2-difluoro-1,1a,6,10b-tetrahydrodibenzo[a,e]cyclopropa[c]cycloheptan-6-yl)-α-((5-quinoloxoxy)methyl)-1-piperazineethanol, triflhydrochloride.
Because potential DDIs involving digoxin are clearly undesirable, many biopharmaceutical laboratories have successfully developed assays using digoxin as a probe substrate to evaluate P-gp activity in various multidrug resistance gene-expressing cell lines (Tanigawara et al., 1992; Takara et al., 2002). Several comedications that are commonly prescribed in the cardiovascular field are inhibitors of P-gp, such as quinidine, cyclosporine, and amiodarone (Gillis and Kates, 1984; Robinson et al., 1989; De Lanney et al., 1992; Weiss et al., 2003). These drugs can elevate circulating levels of digoxin via inhibition of P-gp and as such require dose adjustments (Lesko, 1989). Thus, assays used to predict the potential for digoxin to cause a DDI by inhibition of P-gp are considered routine in most drug development programs. Regulatory agencies typically request such in vitro data before final approval of a new drug, and in cases in which a clinical DDI is predicted, a corresponding clinical DDI study using digoxin as a substrate may also be requested.

Recently, digoxin has been reported to be a substrate for organic anion transporting polypeptide (OATP) transporters. In rats, digoxin was reported to be a substrate for Oatp2 and Oatp4 (Cattori et al., 2000; Funakoshi et al., 2005). Digoxin has also been reported to be a substrate for human liver OATP1B3 (Kullak-Ublick et al., 2001) and kidney OATP4C1 (Mikkaichi et al., 2004; Chu et al., 2007) on the basis of studies conducted using recombinant expression systems. Thus, if DDIs involving digoxin administration could also occur upon coadministration with drugs that inhibit OATPs, this may be an additional cause for concern. For example, a clinically relevant increase in exposure of digoxin (area under the curve increase of 212% compared with control) was reported when coadministered with cyclosporine (Dorian et al., 1988) was proposed to be due to inhibition of Oatp2. However, because cyclosporine is also an OATP1B3 inhibitor (Smith et al., 2005), it is important to clarify if inhibition of OATP1B3 contributes to the increase in digoxin exposure, in addition to inhibition of P-gp. Likewise, it has been shown in rats that amiodarone can limit the uptake of digoxin into hepatocytes by inhibition of Oatp2, causing an increase in the plasma exposure of digoxin (Funakoshi et al., 2005). The possibility that an amiodarone-digoxin DDI may also occur in humans (i.e., one that may involve P-gp and OATPs) is an issue that requires further study. Thus, the objective of this investigation is to compare and contrast data generated by multiple laboratories, in order to determine for the individual positive control substrates and transporters. At BI, before conducting uptake experiments in transiently transfected HEK293 cells, the corresponding untransfected HEK293 cells were obtained from Professor Dietrich Keppler (DKFZ, Heidelberg, Germany). Cells were maintained in minimum essential medium with Earle salts and penicillin/streptomycin (1%) (Invitrogen) and were supplemented with 10% FBS and 400 μg/ml geneticin for transfected cell lines. Cells were seeded onto 24-well plates at a density of 2 × 104 cells/well and incubated at 37°C, 5% CO2, and 95% humidity. At 48 h after plating, uptake experiments were conducted.

At Pfizer, HEK293 cells stably transfected with OATP1B1, OATP1B3, and OATP2B1 and the corresponding untransfected HEK293 cells were obtained from Professor Dietrich Keppler (DKFZ, Heidelberg, Germany). Cells were maintained in minimum essential medium with Earle salts and penicillin/streptomycin (1%) (Invitrogen) and were supplemented with 10% FBS and 400 μg/ml geneticin for transfected cell lines. Cells were seeded onto 24-well plates at a density of 2 × 104 cells/well and incubated at 37°C, 5% CO2, and 95% humidity. Daily changes of medium were performed and on the second day after seeding using medium containing 10 mM sodium butyrate. At 72 h after plating, uptake experiments were conducted.

Materials and Methods

Chemicals and Reagents. Unless otherwise noted, reagents were obtained from the following commercial suppliers: HEK293, HEK293-MSR11, and CHO-K1 cells were obtained from American Type Culture Collection (Manassas, VA); calcium chloride, choline chloride, sodium butyrate, genetin, glucose, digoxin, HEPES, ouabain, magnesium sulfate solution, poly-L-lysine, poly-L-ornithine, potassium bicarbonate, potassium phosphate monobasic solution, rifamycin SV, sodium butyrate. At Pfizer, HEK293 cells stably transfected with OATP1B1, OATP1B3, and OATP2B1 and the corresponding untransfected HEK293 cells were obtained from Professor Dietrich Keppler (DKFZ, Heidelberg, Germany). Cells were maintained in minimum essential medium with Earle salts and penicillin/streptomycin (1%) (Invitrogen) and were supplemented with 10% FBS and 400 μg/ml geneticin for transfected cell lines. Cells were seeded onto 24-well plates at a density of 2 × 104 cells/well and incubated at 37°C, 5% CO2, and 95% humidity. Daily changes of medium were performed and on the second day after seeding using medium containing 10 mM sodium butyrate. At 72 h after plating, uptake experiments were conducted.

At Solvo, CHO cells stably transfected with OATP1B1 and OATP1B3 as well as wild type were obtained from Bruno Stieger (University of Zurich, Zurich, Switzerland) and were cultured using DMEM (45%), Ham’s F12 medium (45%), FBS (10%), nonessential amino acid solution (1X), L-glutamine (2 mM), L-proline (50 μg/ml), and a penicillin-streptomycin mixture (100 μg/ml). Wild-type- and OATP2B1-transfected MDCKII cells were obtained from Heyo Kroemer (University of Greifswald, Greifswald, Germany) and cultured in medium containing DMEM, FBS (10% final concentration), nonessential amino acid solution (1X), L-glutamine (2 mM), and a penicillin/streptomycin mixture (100 μg/ml). Cells (100,000/well) were seeded onto 96-well cell culture plates in media supplemented with 5 mM sodium butyrate and incubated at 37°C, 5% CO2, and 95% humidity. At 24 h after plating, uptake experiments were conducted.

Digoxin Uptake in OATP-Expressing HEK, CHO, and MDCK Cells. Digoxin uptake experiments were performed under initial rate conditions as determined for the individual positive control substrates and transporters. At BI, before conducting uptake experiments in transiently transfected HEK293 or CHO cells, culture medium was aspirated and cells were incubated with 400 μl of OptiMEM containing 1 μM [3H]digoxin, in triplicate. OATP probe substrates 1 μM [3H]cholecystokinin-8 (10-min incubation) or 0.5 μM [3H]estrone-3-sulfate (3-min incubation), for OATP1B1 and OATP2B1, respectively, with and without rifamycin SV (10 μM), were also added to triplicate sets of wells as positive controls. Digoxin-treated cells were incubated at 37°C for 30 min. At GS, transduced HEK cells were preincubated at 37°C for 15 to 30 min in 1 ml DPBS with and without inhibitors rifamycin SV (OATP1B1 and
OATP1B3), ketoconazole (OATP1A2), or montelukast (OATP2B1) (Cayman Chemical, Ann Arbor, MI) at 10 μM. After the removal of preincubation solutions, 400 μl of DPBS containing [3H]digoxin (GE Healthcare) and/or cold digoxin, at final concentrations of 0.01, 0.05, 0.25, 0.5, 5, and 50 μM with and without the addition of inhibitors (10 μM), were added to each well. OATP probe substrates [3H]estradiol-17β-glucuronide or [3H]estrone-3-sulfate, for OATP1B1 and OATP1B3, respectively, with and without appropriate inhibitors, were also added to triplicate sets of wells as positive controls. Cells were incubated at 37°C for 3 min (OATP1B1), 10 min (OATP1B3), or 30 sec (OATP2B1 and OATP1A2). At Pfizer, stably transfected HEK cells were first washed (3 × 1 ml) with prewarmed HBSS (pH 7.4) and then were incubated with 250 μl of HBSS containing digoxin at concentrations of 1 or 10 μM. Cells were incubated for 4 min at 37°C for all experiments. To account for passive uptake and nonspecific binding, the uptake study was performed in parallel using untransfected HEK293 cells. At Solvo, stably transfected CHO or MDCK cells were first washed twice with prewarmed Krebs-Henseleit buffer and were then incubated with 100 μl of Krebs-Henseleit buffer containing 0.02 μM [3H]digoxin and cold digoxin with the final concentrations at 1.2 or 11 μM. OATP probe substrates 0.1 μM [3H]estrone-3-sulfate, 10 μM Fluo-3 (Sigma-Aldrich), or 1 μM [3H]estrone-3-sulfate were incubated in OATP1B1, OATP1B3, and OATP2B1 cells, respectively, with and without the positive control inhibitors cerivastatin (OATP1B1, 100 μM) and fluvastatin [OATP2B1 (10 μM) and OATP1B3 (30 μM)]. The treated cells were incubated at 37°C for 15 min. Table 1 summarizes the methodologies used at the various companies.

**Inhibition of OATP-Mediated Transport by Digoxin.** The inhibition experiments were performed under probe substrate initial rate conditions as described above. At BI, cell culture medium was aspirated and the transiently transfected HEK293 cells were then incubated with 100 μl of Krebs-Henseleit buffer containing 0.02 μM [3H]digoxin and cold digoxin with the final concentrations at 1.2 or 11 μM. OATP probe substrates [3H]estradiol-17β-glucuronide or [3H]estrone-3-sulfate, for OATP1B1 and OATP1B3, respectively, with and without appropriate inhibitors, were also added to triplicate sets of wells as positive controls. Cells were incubated at 37°C for 3 min (OATP1B1), 10 min (OATP1B3), or 30 sec (OATP2B1 and OATP1A2). At Pfizer, stably transfected HEK cells were first washed (3 × 1 ml) with prewarmed HBSS (pH 7.4) and then were incubated with 250 μl of HBSS containing digoxin at concentrations of 1 or 10 μM. Cells were incubated for 4 min at 37°C for all experiments. To account for passive uptake and nonspecific binding, the uptake study was performed in parallel using untransfected HEK293 cells. At Solvo, stably transfected CHO or MDCK cells were first washed twice with prewarmed Krebs-Henseleit buffer and were then incubated with 100 μl of Krebs-Henseleit buffer containing 0.02 μM [3H]digoxin and cold digoxin with the final concentrations at 1.2 or 11 μM. OATP probe substrates 0.1 μM [3H]estrone-3-sulfate, 10 μM Fluo-3 (Sigma-Aldrich), or 1 μM [3H]estrone-3-sulfate were incubated in OATP1B1, OATP1B3, and OATP2B1 cells, respectively, with and without the positive control inhibitors cerivastatin (OATP1B1, 100 μM) and fluvastatin [OATP2B1 (10 μM) and OATP1B3 (30 μM)]. The treated cells were incubated at 37°C for 15 min. Table 1 summarizes the methodologies used at the various companies.

**TABLE 1**

<table>
<thead>
<tr>
<th>Expression System</th>
<th>OATPs Investigated</th>
<th>Digoxin Uptake Experiments</th>
<th>Positive Controls/Inhibitors/Negative Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td>Uptake Time</td>
<td>Temperature</td>
</tr>
<tr>
<td><strong>GSK</strong></td>
<td><strong>HEK293 cells transduced with recombinant BacMam virus</strong></td>
<td>OATP1B1</td>
<td>0.01, 0.05, 0.25, 0.5, 5, and 50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>BI</strong></td>
<td><strong>HEK293 and CHO cells transiently transfected with transporter containing plasmid DNA</strong></td>
<td>OATP1B1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pfizer</strong></td>
<td><strong>Stably transfected HEK293 cells</strong></td>
<td>OATP1B1</td>
<td>1 &amp; 10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Solvo</strong></td>
<td><strong>CHO cells transiently transfected with control DNA</strong></td>
<td>OATP1B1</td>
<td>1.2 &amp; 11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

E3S, estrone 3-sulfate; E17BG, estradiol-17β-glucuronide; CCK-8, cholecystokinin-8.
mix scintillation cocktail. OATP1B3-expressing CHO cells were lysed using a solution of 5% SDS and 1 mM CaCl₂. The Fluo-3 content of the lysate was analyzed with a BMG Labtech GmbH Fluostar fluorimeter (Offenburg, Germany) with 485- and 520-nm filters for excitation and emission, respectively.

**Determination of Gene Expression in HEK Cells Using Gene Array.**
Cells were homogenized in Qiazol (QIAGEN, Valencia, CA) with a bead-mill homogenizer. RNA was extracted using the Universal Tissue Protocol (QIAGEN) on a Qiangen M48 Biorobot. RNA quality was assessed with an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA (2 μg) underwent first- and second-strand cDNA synthesis and then in vitro reverse transcription (cRNA synthesis) following protocols within the GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). Hybridization of the fragmented cRNA on the GeneChip Human Genome U133 Plus 2.0 Array and subsequent washing, staining, and scanning steps were also performed per the manufacturer’s protocol. Preliminary image analysis was done with Expression Console software (Affymetrix). GeneSpring software (Agilent Technologies) was used for quality control assessment, data normalization, filtering, and statistical analysis of the data. Multivariate procedures including principal-component analysis and hierarchical clustering were performed to assess overall data quality. The RNA preprocessing algorithm was applied to probe-level data to normalize against the mean of all samples. A data set derived from the MAS5 (Affymetrix Microarray Suite version 5.0; Affymetrix) algorithm was also generated to obtain absent/present calls for individual gene expression values where desired.

**Results**

**Evaluation of Digoxin as a Substrate of OATP1A2, OATP1B1, OATP1B3, and OATP2B1.** The methodologies used at the various companies to evaluate digoxin as an OATP substrate are summarized in Table 1. Digoxin was examined as a potential substrate of OATP1A2 (GSK), OATP1B1 (GSK, Solvo, and Pfizer), and OATP1B3 and OATP2B1 (all companies). Functional expression of the various OATPs was first determined using probe substrates and inhibitors for each OATP isoform before conducting experiments with digoxin (data not shown). BI, Pfizer, and Solvo compared uptake in OATP transfected cells with uptake in vector control or wild-type cells, whereas GSK determined uptake in cells transduced with OATP-BacMam virus in the absence and presence of known OATP inhibitors. Either stably transfected or transiently transfected HEK293, CHO, or MDCK cells were used in these experiments. Digoxin did not exhibit active uptake into cells expressing OATP1B1 (Fig. 1A), OATP1B3 (Fig. 1B), or OATP2B1 (Fig. 1C) at incubation concentrations ranging from 1 to 11 μM. The amount of digoxin in the transfected cells at the end of each experiment compared with the amount of digoxin in the vector control-treated or wild-type cells was near unity. Furthermore, the uptake of digoxin over a range of concentrations (0.01, 0.05, 0.25, 0.5, 5, and 50 μM) in OATP BacMam transduced cells did not change in the presence of inhibitors of OATP1A2 (ketocazole 10 μM; Fig. 2A), OATP1B1 (rifamycin SV, 10 μM; Fig. 2B), OATP1B3 (rifamycin SV, 10 μM; Fig. 2C), and OATP2B1 (montelukast, 10 μM; Fig. 2D), demonstrating that digoxin is not a substrate of these transporters.

**Evaluation of Digoxin as an Inhibitor of OATP1A2, OATP1B1, OATP1B3, and OATP2B1.** Digoxin was evaluated as a potential inhibitor of OATP1A2 (GSK), OATP1B1 (GSK, Solvo, and Pfizer), OATP1B3 (BI, GSK, and Solvo), and OATP2B1 (BI, GSK, and Solvo). The probe substrates used to evaluate digoxin inhibition potential were [3H]estrone-3-sulfate for OATP1A2 (GSK), [3H]estradiol-17β-d-glucuronide (GSK) and [3H]estrone-3-sulfate (BI and GSK) and Fluo-3 (Solvo) for OATP1B3, and [3H]cholecystokinin-8 (BI and GSK) and Fluo-3 (Solvo) for OATP2B1. The inhibitory potency of digoxin was evaluated by BI and GSK in HEK293 cells expressing the various OATPs. IC₅₀ values were determined to be 47 μM for OATP1B1, >8.1 μM for OATP1B3, and >300 μM for OATP2B1; inhibition was minimal for OATP1A2 (Table 2). Because high concentrations of digoxin showed no effect, or only a marginal effect, on OATP2B1 and OATP1A2 probe substrate uptake, digoxin even at the
The uptake of digoxin and ouabain, structurally related cardiac glycosides, was evaluated in wild-type CHO cells and HEK293 cells at 4 and 37°C. After a 30-min uptake experiment, digoxin showed greater uptake into CHO and HEK293 cells when compared with ouabain (Fig. 3). In addition, digoxin exhibited greater temperature-dependent uptake in the HEK293 cell line compared with CHO cells (data not shown).

To further elucidate whether an endogenous uptake transporter for digoxin is present in the HEK293 cells, BI, GSK, and Pfizer evaluated the uptake of digoxin in the absence and presence of prototypical OATP inhibitors. Cells were incubated with [3H]digoxin at concentrations ranging from 0.01 to 50 μM at 37°C using site-optimized incubation times as described under Materials and Methods in the presence or absence of the OATP inhibitors ketoconazole (10 μM, OATP1A2), rifamycin SV (10 μM, OATP1B1 and OATP1B3), or montelukast (10 μM, OATP2B1). A positive control probe substrate [0.2 μM estradiol-17β-glucuronide (EBG) or estrone-3-sulfate (E3S)] was included in each set of experiments. Each column represents the mean uptake of [3H]digoxin ± S.D. in n = 3 wells.

Transporter-Mediated Uptake of Digoxin in HEK293 and CHO Cells. The uptake of digoxin and ouabain, structurally related cardiac glycosides, was evaluated in wild-type CHO cells and HEK293 cells at 4 and 37°C. After a 30-min uptake experiment, digoxin showed greater uptake into CHO and HEK293 cells when compared with ouabain (Fig. 3). In addition, digoxin exhibited greater temperature-dependent uptake in the HEK293 cell line compared with CHO cells (data not shown).

To further elucidate whether an endogenous uptake transporter for digoxin is present in the HEK293 cells, BI, GSK, and Pfizer evaluated the uptake of digoxin in the absence and presence of ouabain as a competitive inhibitor (Fig. 4). Ouabain and digoxin have been reported to be substrates for OATP4C1 (Mikkaichi et al., 2004), with Km values of 0.4 and 8 μM, respectively. All three laboratories demonstrated that the uptake of digoxin (1 μM) at either 3 min (Fig. 4A) or 30 min (Fig. 4B) was not altered in the presence of 10 μM ouabain; however, digoxin uptake was modestly inhibited (~50%) at 30 min in the presence of 100 μM ouabain (Fig. 4B).

Ouabain at concentrations up to 100 μM was not cytotoxic as demonstrated by the CellTiter-Glo Luminescent cell viability assay.

TABLE 2

<table>
<thead>
<tr>
<th>Company</th>
<th>OATP1A2</th>
<th>OATP1B1</th>
<th>OATP1B3</th>
<th>OATP2B1</th>
</tr>
</thead>
<tbody>
<tr>
<td>BI</td>
<td>N.D.</td>
<td>N.D.</td>
<td>54</td>
<td>~300</td>
</tr>
<tr>
<td>GSK</td>
<td>33%  a</td>
<td>47</td>
<td>69</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Solvo</td>
<td>N.D.</td>
<td>52%  a</td>
<td>8.1</td>
<td>48%  a</td>
</tr>
</tbody>
</table>

N.D., not determined; E3S, [3H]estrone-3-sulfate; E17βG, [3H]estradiol-17β-glucuronide; CCK-8, [3H]cholecystokinin-8.

a Inhibition was observed at 300 μM digoxin, but the IC50 value could not be determined.
say. In the presence of 3 μM rifamycin SV, the uptake of digoxin varied from no change (BI and GSK) to modest inhibition (50% observed by Pfizer) after 3- and 30-min incubations. However, BI and GSK showed higher than expected uptake of digoxin at 3 and 30 min when 100 μM rifamycin SV was added.

Because radiolabeled digoxin demonstrated temperature-dependent uptake into HEK293 cells, digoxin uptake was partially inhibited by ouabain (a structurally related molecule; Fig. 4), and mRNA for the sodium-dependent multivitamin transporter (SMVT; gene SLC5A6) is highly expressed in HEK293 cells (Table 2), we then sought to determine whether a sodium-dependent transporter expressed in HEK293 cells facilitates active uptake of digoxin. Studies were conducted in the presence and absence of sodium in the incubation media using osmotically balanced buffers. After a 30-min incubation in media osmotically balanced with potassium and choline instead of sodium, uptake of digoxin (0.1 μM) into HEK293 cells decreased by ~43% (Fig. 5A) compared with incubations conducted using sodium-deficient media. The addition of P-gp inhibitor LY335979 (3 μM) in incubations with OptiMEM culture medium resulted in a ~30% increase in uptake of digoxin compared with incubation without LY335979. Similar results were seen in a time course study (Fig. 5B) in which the uptake of digoxin into untransfected HEK293 cells increased ~2-fold when incubated in the presence of sodium compared with incubation without sodium (30-min time point). Incubation with LY335979 to inhibit P-gp resulted in a sharp increase in digoxin uptake at 3 and 10 min compared with cells incubated without

![Figure 3](https://example.com/fig3.png)

**Fig. 3.** Uptake of digoxin or ouabain in untransfected HEK293 or CHO-K1 cells. Untransfected cells were incubated with [3H]digoxin or [3H]ouabain at a concentration of 0.1 μM at 37°C for 30 min. Each column represents the mean uptake of [3H]digoxin ± S.D. in n = 3 wells.

![Figure 4](https://example.com/fig4.png)

**Fig. 4.** Uptake of digoxin uptake in HEK293 cells in the presence and absence of ouabain or rifamycin SV. Untransfected HEK293 cells were incubated with [3H]digoxin or cold-digoxin (1 μM) alone and in the presence of ouabain (10 and 100 μM) or rifamycin SV (RIF-SV; 3 and 100 μM) at 37°C for (A) 3 min and (B) 30 min. Experiments were conducted at BI (□), GSK (■), and Pfizer (●). Each column represents the uptake of [3H]digoxin in the presence of ouabain or rifamycin SV expressed as a percentage of [3H]digoxin uptake in control (untreated) cells (n = 3).

![Figure 5](https://example.com/fig5.png)

**Fig. 5.** Uptake of digoxin in HEK293 cells in the presence or absence of sodium and/or LY335979. A, untransfected HEK293 cells were incubated with [3H]digoxin (0.1 μM) for 30 min at 37°C in sodium-fortified media, sodium-deficient media, or OptiMEM with or without LY335979 (3 μM). Each column represents the mean ± S.D. of n = 3 wells. B, untransfected HEK293 cells were incubated with [3H]digoxin (1 μM) for 30 min at 37°C in sodium-fortified or sodium-deficient media with or without LY335979 (3 μM). Each point represents the mean ± S.D. of n = 3 wells.

Because radiolabeled digoxin demonstrated temperature-dependent uptake into HEK293 cells, digoxin uptake was partially inhibited by ouabain (a structurally related molecule; Fig. 4), and mRNA for the sodium-dependent multivitamin transporter (SMVT; gene SLC5A6) is highly expressed in HEK293 cells (Table 2), we then sought to determine whether a sodium-dependent transporter expressed in HEK293 cells facilitates active uptake of digoxin. Studies were conducted in the presence and absence of sodium in the incubation media using osmotically balanced buffers. After a 30-min incubation in media osmotically balanced with potassium and choline instead of sodium, uptake of digoxin (0.1 μM) into HEK293 cells decreased by ~43% (Fig. 5A) compared with incubations conducted using sodium-containing media. Moreover, OptiMEM culture medium, which is normally used for incubations with HEK293 cells, demonstrated a ~2-fold increase in the uptake of digoxin compared with sodium-deficient media. The addition of P-gp inhibitor LY335979 (3 μM) in incubations with OptiMEM culture medium resulted in a ~30% increase in uptake of digoxin compared with incubation without LY335979. Similar results were seen in a time course study (Fig. 5B) in which the uptake of digoxin into untransfected HEK293 cells increased ~2-fold when incubated in the presence of sodium compared with incubation without sodium (30-min time point). Incubation with LY335979 to inhibit P-gp resulted in a sharp increase in digoxin uptake at 3 and 10 min compared with cells incubated without
LY335979. At 30 min, the uptake of digoxin was ~3.5-fold higher in cells incubated with sodium and with LY335979 compared with cells incubated without sodium and without LY335979.

**Expression of Endogenous Transporter Genes (mRNA) in HEK293 Cells.** Because digoxin and ouabain have been reported as substrates of OATP4C1 (Mikkaichi et al., 2004; Chu et al., 2007), analysis of the HEK293 cell line using a gene chip was conducted at Pfizer to determine if genes for any known human uptake and efflux membrane transporters are endogenously expressed that may contribute to the active transport of digoxin or ouabain in these cells (Table 3). Among the known transporters identified in this analysis, the monocarboxylic acid transporter-1 gene was the membrane transporter gene demonstrating the highest expression levels with a value of 4028 MAS5 units, which is approximately one-sixth of the level of the most abundant gene (ribosomal protein L13 of ~30,000). There are several multidrug resistance-associated protein (MRP) genes expressed in the HEK293 cell line, including MRP1, MRP4, MRP5, and MRP6. The kidney-specific multidrug and toxin extrusion transporter and P-gp genes were also identified and represented expression levels that were approximately 20% of the monocarboxylic acid transporter-1 levels. The level of expression of the gene for OATP4C1, a kidney-specific transporter, in HEK293 cells was low, and the liver-specific OATP transporters (OATP1B1 and OATP1B3) were absent, a result that is consistent with what has been reported in the literature (Ahlin et al., 2009). It is also notable that mRNA for SLC5A6, a sodium-dependent transporter that has been reported to demonstrate broad substrate selectivity (de Carvalho and Quick, 2011), is highly expressed in HEK293 cells, at 1634 MAS5 units. In contrast, the apical sodium-dependent bile acid transporter (ASBT; gene SLC7A2) is a sodium-dependent bile acid transporter localized to the apical surface of the terminal ileal enterocytes; however, consistent with the relatively low level of mRNA expression determined from gene chip analysis (56 MAS5 units, ASBT is not endogenously expressed in HEK293 cells (Annaba et al., 2008). Likewise, but not surprisingly, mRNA for the sodium taurocholate transporting polypeptide (gene SLC10A1), a sodium-dependent bile acid transporter expressed on the sinusoidal membrane of hepatocytes, was also essentially absent in HEK293 cells (22 MAS5 units; Table 3).

### Discussion

From a drug development and regulatory perspective, there is keen interest in understanding and elucidating the mechanisms involved in DDIs that can result in an increase in the plasma exposure of digoxin. Most clinical investigations conducted to date have focused on P-gp-mediated drug interactions [e.g., DDI studies involving digoxin and amiodarone (Robinson et al., 1989) and quinidine (Rameis, 1985)]. However, two publications suggest that, in addition to P-gp, digoxin is also a substrate for OATP1B3 (Kullak-Ublick et al., 2001) and OATP4C1 (Mikkaichi et al., 2004; Chu et al., 2007). The consequences of a coadministered medication inhibiting the uptake of digoxin into the liver or kidney by inhibiting OATP1B3 or OATP4C1, respectively, could also theoretically result in elevated digoxin exposures that may be indistinguishable from an effect caused by inhibition of P-gp. It has been suggested that certain drugs that are recognized inhibitors and/or substrates of P-gp are likely to inhibit OATPs. For example, Cvetkovic et al. (1999) showed that several HIV protease inhibitors that are also P-gp inhibitors modulate fexofenadine OATP-mediated transport activity, and many of these HIV protease inhibitors more potently inhibited the OATPs compared with P-gp. The potential overlap between interactions of drugs with P-gp and OATP transporters warrants further investigation of whether digoxin is a substrate for OATP transporters.

The original study citing digoxin as a substrate for OATP1B3 was conducted using *Xenopus laevis* oocytes expressing this transporter (Kullak-Ublick et al., 2001). In addition, digoxin was found to be a substrate for OATP4C1, an OATP transporter expressed in the human kidney (Mikkaichi et al., 2004; Chu et al., 2007). To enhance understanding of the role of other OATPs in addition to OATP1B3 and OATP4C1, we conducted a multisite (BI, GSK, Pfizer, and Solvo) investigation using various commonly used human OATP expression systems. To define the contribution of OATP transporters in digoxin uptake into the intestine, liver, and kidney, we investigated digoxin uptake using HEK293 cells transfected with OATP1A2, OATP1B1,
OATP1B3, and OATP2B1. It was surprising to note that in results that were consistent across all laboratories, we found that digoxin is not a substrate for OATP1A2, OATP1B1, OATP1B3, or OATP2B1 (Figs. 1 and 2). Although digoxin does not appear to be a substrate for these intestinal and hepatic OATP uptake transporters, it may be a substrate for OATP4C1, which is expressed in the human kidney, because two laboratories have reported this finding (Mikkaichi et al., 2004; Chu et al., 2007). Although we attempted to express OATP4C1 in HEK293, CHO, and MDCK WT cell lines, functional expression could not be achieved using standard transient transfection techniques that were successful for the other OATPs studied, including but not limited to the techniques described under Materials and Methods (data not shown). By alternative means, the involvement of OATP4C1 as a digoxin uptake transporter in the HEK293 cell line could not be confirmed because 10 µM ouabain did not appreciably impair the uptake of digoxin despite having greater affinity for this transporter compared with digoxin (Mikkaichi et al., 2004). It is worth noting that Mikkaichi et al. (2004) and Yamaguchi et al. (2010) have proposed that substrates for OATP4C1 may not competitively inhibit this transporter because of the presence of multiple binding sites on OATP4C1. For example, Mikkaichi et al. (2004) showed that digoxin and [125I]3,5,3'-triiodo-L-thyronine, substrates of OATP4C1, are not capable of inhibiting OATP4C1-mediated uptake when coincubated. Likewise, Yamaguchi et al. (2010) showed that estrone 3-sulfate is efficiently transported by OATP4C1 with a K_m of 27 µM and uptake is inhibited by [125I]3,5,3'-triiodo-L-thyronine, chenodeoxycholic acid, bromosulphophthalein, and cyclosporine. However, digoxin and ouabain could not alter estrone 3-sulfate transport via OATP4C1. Also, literature evidence suggests that the transport function of OATP4C1 is complex, involving multiple binding sites that may further confound predictive interactions with digoxin.

In addition, adding rifamycin SV did not impair digoxin uptake in transfected HEK293 cells, which is consistent with our finding that digoxin is not a substrate for OATP1A2, OATP1B1, OATP1B3, and OATP2B1. It is interesting to note that in untransfected HEK293 cells, rifamycin SV slightly stimulated the uptake of digoxin (Fig. 4), an unusual observation made independently by BI and GSK. A possible explanation for the enhanced uptake of digoxin may be attributed to inhibition of P-gp-mediated efflux because rifamycin SV has been shown to inhibit P-gp activity (Xifanxan; Salix Pharmaceuticals, Raleigh, NC) and P-gp is expressed endogenously in HEK293 cells (Table 2). Likewise, as shown in Fig. 5, the addition of LY335979 resulted in a ~2-fold increase in the uptake of digoxin in untransfected HEK293 cells after a 30-min incubation, further demonstrating that, in addition to passive and active uptake processes, the P-gp-mediated efflux of digoxin contributes significantly toward its equilibrated accumulation in this human kidney cell line.

Although it has been shown that P-gp plays a role in the oral absorption and renal elimination of digoxin because of its expression in intestinal and renal epithelia, uptake of digoxin into these organs is thought to occur either by passive diffusion or possibly active uptake (Chu et al., 2007). For the first time, we have demonstrated that digoxin is a substrate of a sodium-dependent transporter expressed in WT-HEK293 cells (Fig. 5). Most commercially available or commonly used tissue culture media contain sodium, unless specifically osmotically balanced using other salts. Thus, on the basis of our observations, in previously reported in vitro studies conducted to evaluate digoxin uptake into transfected or untransfected mammalian cells, a sodium-dependent uptake transporter may at least partially contribute to the uptake of digoxin.

The data represented in Fig. 5B depict the kinetic profile of concurrent pathways leading to the intracellular accumulation of digoxin when incubated with HEK293 cells [i.e., passive diffusion, active uptake (sodium-dependent transporter), and active efflux (P-gp)]. It is intriguing that, in the absence of sodium but in the presence of LY335979, intracellular levels of digoxin increased only at 30 min compared with the parallel experiment in the presence of sodium and LY335979, whereas intracellular levels of digoxin at 1, 3, and 10 min were nearly identical under both conditions. We speculate that this effect may be due to differences in the intracellular distribution of digoxin following different pathways of its uptake into HEK293 cells. For example, active uptake of digoxin (i.e., via the sodium-dependent transporter) may result in an intracellular distribution of digoxin that would hinder its immediate access to membrane-bound P-gp. However, digoxin entering the cells by passive diffusion (i.e., in the absence of sodium) may facilitate more favorable access to P-gp because P-gp is expressed on the cell membrane. We further speculate that, at 30 min, a steady state is reached, and digoxin entering cells via the sodium-dependent transporter is distributed into all intracellular compartments, thus resulting in the expected increase in intracellular levels in the presence of sodium and LY335979.

Because it is known that the ASBT (SLC10A2) is expressed on the apical surface of terminal ileal enterocytes, cholangiocytes, and renal proximal tubular cells (Annaba et al., 2008), it was considered a possibility that ASBT may be responsible for the sodium-dependent uptake of digoxin into HEK293 cells. However, ASBT is not expressed in HEK293 cells (Annaba et al., 2008), so this was ruled out as a potential explanation. Another transporter, SMVT (SLC5A6), has recently been described as a sodium-dependent SLC family transporter that demonstrates “surprising substrate versatility” (de Carvalho and Quick, 2011). Although it remains to be determined if SMVT protein is expressed in HEK293 cells, and if digoxin is indeed a substrate of SMVT, we show via gene chip analysis a reasonably high level of expression of SMVT mRNA expression in cultured HEK293 cells. Thus, it remains a possibility that digoxin is a substrate of SMVT, and perhaps ASBT as well; experiments to further elucidate this relationship will be the focus of future studies.

It was recently shown that digoxin uptake in sandwich-cultured human hepatocytes (SCHH) was not inhibited by known inhibitors of OATP1B1, OATP1B3, OATP2B1, organic anion transporter-2, organic cation transporter-1, and monocarboxylate acid transporter-8 (Kimoto et al., 2010). However, a 50% reduction in uptake in SCHH was observed when digoxin was incubated with certain flavonoids (Acharya et al., 2008; Kimoto et al., 2010). The medium used in these SCHH experiments contained sodium; thus, it is possible that a sodium-dependent transporter expressed in SCHH was partially responsible for facilitating active uptake of digoxin. It remains to be determined which uptake transporter, or family of transporters, facilitates the uptake of digoxin into cells such as gastrointestinal enterocytes, hepatocytes, and renal cells. In addition, the relative contribution of active and passive transport toward total digoxin uptake into cells is still unknown (Acharya et al., 2008).

Several literature findings suggest that the presence of an unidentified digoxin transporter on the basolateral membrane of polarized cells mediates the uptake of digoxin. Acharya et al. (2008) used kinetic analysis to demonstrate that a basolateral uptake transporter is required to fit P-gp-mediated digoxin efflux across MDCK-hMDR1 cells in the basolateral to apical direction. The kinetically identified basolateral uptake transporter was not inhibited by a series of known inhibitors of OATPs, organic cationic transporters, and organic anion transporters. Although the current findings may link digoxin active uptake to sodium-dependent transporters such as SMVT or ASBT, there are reports that digoxin is transported by the heteromeric organic solute transporter-α (OSTα) and organic solute transporter-β (OSTβ)
In summary, we have generated conclusive data demonstrating that digoxin is not a substrate for human OATP1A2, OATP1B1, and OATP2B1. Although digoxin is not a substrate for a sodium-dependent transporter that is facilitated by a sodium-dependent transporter expressed in renal proximal tubule cells (Ballatori, 2005). In the liver and kidney, these transporters have been reported to facilitate digoxin efflux from the organ to the blood circulation, thus contributing to the systemic exposure of digoxin (Seward et al., 2003). At this time, it is not clear whether certain drugs modulate the function of O斯塔 and O斯塔, and further investigations are required to appropriately assess the role of organic solute transporters in digoxin transport.

Although digoxin was shown not to be a substrate for the OATPs investigated herein, it does appear to inhibit OATP1B1 and OATP1B3, and at very high concentrations (>300 μM) it inhibits OATP2B1 but not OATP1A2. Yamaguchi et al. (2010) showed that digoxin can partially inhibit estrone-3-sulfate transport with an IC50 of 119 μM in MDCK-OATP4C1 cells, a value much greater than the reported Km value of 7.8 for digoxin binding to OATP4C1 (Mikkaichi et al., 2004). Alternatively, the lack of inhibition of digoxin uptake by estrone-3-sulfate suggests that, in the MDCK cell line, uptake of digoxin may be facilitated by an endogenous canine transporter. If so, this would be similar to the observations of Acharya et al. (2008).

In summary, we have generated conclusive data demonstrating that digoxin is not a substrate for human OATP1A2, OATP1B1, OATP1B3, and OATP2B1. Although digoxin is not a substrate for these OATP transporters, it does modestly inhibit OATP1B1 and OATP1B3, although at concentrations that greatly exceed maximal therapeutic plasma concentrations. It remains a possibility that active uptake of digoxin into the human kidney and liver may be facilitated by OATP4C1, and perhaps a transporter that has yet to be identified, respectively, as supported by recent literature reports (Acharya et al., 2008; Kimoto et al., 2010). Intriguingly, we have demonstrated that digoxin is a substrate for a sodium-dependent transporter that is endogenously expressed in a human kidney cell line, HEK293. Thus, although digoxin is not a substrate for human OATPs, in addition to P-gp-mediated efflux, renal clearance of digoxin may be partially facilitated by a sodium-dependent transporter expressed in renal proximal tubular cells. Future studies will determine which sodium-dependent transporter, or possibly family of transporters, recognize digoxin and facilitate its cellular uptake.


Address correspondence to: Caroline A. Lee, Pfizer Worldwide Research and Development, 10646 Science Center Drive, San Diego, CA 92121. E-mail: clee032001@yahoo.com