

Short Communication

Hepatocellular Disposition and Transporter Interactions with Tolvaptan and Metabolites in Sandwich-Cultured Human Hepatocytes [□]

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ABSTRACT

Tolvaptan is a selective V₂-receptor antagonist primarily metabolized by CYP 3A. The present study investigated the hepatocellular disposition of tolvaptan and the generated tolvaptan metabolites, DM-4103 and DM-4107, as well as the potential for drug-drug interactions (DDIs) with metabolic and transport proteins in sandwich-cultured human hepatocytes (SCHH). Tolvaptan was incubated with SCHH and quantified by liquid chromatography–tandem mass spectrometry. Pioglitazone, verapamil, MK-571, and elacridar were used as inhibitors to investigate mechanisms of transport and metabolism of tolvaptan and metabolites. Taurocholate (TCA), pravastatin, digoxin, and metformin were used as transporter probes to investigate which transport proteins were inhibited by tolvaptan and metabolites. Cellular accumulation of tolvaptan (0.15–50 μM), DM-4103, and DM-4107 in SCHH was concentration-dependent.

Tolvaptan accumulation (15 μM) in SCHH was not altered markedly by 50 μM pioglitazone, verapamil, MK-571, or 10 μM elacridar. Coincubation of tolvaptan with pioglitazone, verapamil, MK-571, and elacridar reduced DM-4107 accumulation by 45.6, 79.8, 94.5, and 23.0%, respectively, relative to control. Coincubation with increasing tolvaptan concentrations (0.15–50 μM) decreased TCA (2.5 μM) cell+bile accumulation and the TCA biliary excretion index (BEI; from 76% to 51%), consistent with inhibition of the bile salt export pump (BSEP). Tolvaptan (15 μM) had no effect on the cellular accumulation of 2.5 μM pravastatin or metformin. Digoxin cellular accumulation increased, and the BEI of digoxin decreased from 23.9 to 8.1% in the presence of 15 μM tolvaptan, consistent with inhibition of P-glycoprotein. In summary, SCHH studies revealed potential metabolic- and transporter-mediated DDIs involving tolvaptan and metabolites.

Introduction

Tolvaptan is an orally available selective V₂-receptor antagonist used to treat hypervolemic and euvolemic hyponatremia in patients with heart failure and refractory ascites in cirrhosis (Berl et al., 2010; Sakaida, 2014). After oral administration, tolvaptan was absorbed readily from the gastrointestinal tract with an absolute bioavailability of ~50% after a 30-mg dose, and was metabolized extensively, with ~1% of the dose excreted in the urine unchanged (Shoaf et al., 2007, 2012a). CYP3A is the main enzyme involved in tolvaptan metabolism, primarily forming dehydrogenated and hydroxylated metabolites (Shoaf et al., 2012b). DM-4103 and

DM-4107 are two major metabolites of tolvaptan primarily excreted in urine and feces, respectively (Tammara et al., 1999). When [¹⁴C]tolvaptan was administered orally to rats, biliary excretion was a predominant route of elimination for tolvaptan and metabolites (Furukawa et al., 2011).

Hepatocyte cultures preserve whole cellular architecture and function and have been useful for understanding and estimating metabolic clearance and hepatocellular transport (Chiba et al., 2009). In particular, sandwich-cultured human hepatocytes (SCHH) have become a prominent tool to evaluate hepatobiliary drug disposition owing to their ability to retain *in vivo*-like metabolic and excretory properties, some of which are not preserved using conventional culture conditions (Swift et al., 2010). Transport proteins, including basolateral and biliary efflux transporters, have been shown to properly localize on the basolateral and canalicular domains of SCHH; this allows for accurate correlation between *in vitro* intrinsic biliary clearance and *in vivo* biliary clearance (Liu et al., 1999; Swift et al., 2010).

Despite research characterizing the metabolism of tolvaptan, the hepatobiliary disposition and potential interactions with hepatic transporters have not been fully explored. Previous studies demonstrated that tolvaptan is a substrate and an inhibitor of P-glycoprotein (P-gp) (Shoaf et al., 2011). Our recent studies in membrane vesicles and transfected cells revealed that tolvaptan and metabolites could inhibit several transporters, including sodium taurocholate (TCA) cotransporting polypeptide (NTCP), the bile salt export pump

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ABBREVIATIONS: BEI, biliary excretion index; BSEP, bile salt export pump; BSA, bovine serum albumin; DDIs, drug-drug interactions; HBSS, Hanks' balanced salt solution; MK-571, 3-[[3-[(E)-2-(7-chloroquinolin-2-yl)ethenyl]phenyl]-[3-(dimethylamino)-3-oxopropyl]sulfanyl]methyl]sulfanylpropanoate; MRP, multidrug resistance-associated protein; NTCP, sodium taurocholate cotransporting polypeptide; P-gp, P-glycoprotein; SCHH, sandwich-cultured human hepatocytes; TCA, taurocholate.

(BSEP), the multidrug resistance-associated protein 2 (MRP2), MRP3, and MRP4 (Slizgi et al., 2016). However, the role of hepatic transporters in the disposition of tolvaftan and metabolites has not been identified in hepatocytes under physiologic conditions. The ability to accurately predict the potential for transporter-mediated drug-drug interactions (DDIs) of tolvaftan and metabolites at the cellular level solely on the basis of membrane vesicle data remains to be determined. The purpose of this study was to investigate the hepatobiliary disposition of tolvaftan and generated metabolites, and possible DDIs with hepatic metabolic and transport proteins using SCHH.

Materials and Methods

Materials. Tolvaftan, DM-4103, and DM-4107 (purity > 99%) were provided by Otsuka Pharmaceutical Co. (Tokyo, Japan). Stably labeled TCA (d_8 -TCA) was purchased from Martex Inc. (Minnetonka, MN). Elacridar, digoxin, metformin, MK-571, and pravastatin were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals were of analytical grade.

Cell Culture and Transport Studies in SCHH. Transporter-Certified sandwich-cultured human hepatocytes (SCHH) were purchased from Triangle Research Laboratories (lot no. HUM4059; Caucasian female; age = 17 year; body mass index = 25.5; no tobacco, alcohol, or drug use; see Supplemental Data) and prepared by Qualyst Transporter Solutions (Durham, NC) using cryopreserved hepatocytes as described by Slizgi et al. (2016). On day 5 of culture, the media was aspirated and the SCHH were rinsed with 0.5 ml/well of standard or Ca^{2+} -free Hanks' balanced salt solution (HBSS). After the second rinse, HBSS buffers were aspirated completely and SCHH were incubated with 0.5 ml of standard or Ca^{2+} -free HBSS buffers for 10 minutes. After 10 minutes, HBSS buffers were aspirated and the cells were incubated with standard HBSS buffer containing tolvaftan (0.15–50 μ M) and 4% bovine serum albumin (BSA) for 10 minutes. For studies investigating possible DDIs with tolvaftan and drug-metabolizing enzymes and transporters, tolvaftan (15 μ M) was coincubated with pioglitazone (50 μ M), verapamil (50 μ M), MK-571 (50 μ M), or elacridar (10 μ M). For studies investigating possible DDIs of tolvaftan with hepatic transporters, tolvaftan (15 μ M) was coincubated with d_8 -TCA (2.5 μ M), pravastatin (2.5 μ M), digoxin (2.5 μ M), or metformin (2.5 μ M). Dimethyl sulfoxide was used to solubilize all compounds; the final dimethyl sulfoxide concentration was 0.2% v/v. After incubation, the HBSS buffer was aspirated from all wells and each well was rinsed three times with ice-cold standard HBSS buffer. Plates were sealed and stored at -80°C until analysis.

Sample Preparation and Analysis. Cell samples from each well were lysed with 500 μ l 70% methanol containing internal standard, placed on a shaker for 15 minutes, filtered through a Whatman 96-well Unifilter 25- μ m MBPP/0.45- μ m PP filter plate, evaporated to dryness, and reconstituted in sample diluent (200 μ l, 70% methanol containing 0.1% formic acid). Reconstituted samples were filtered through a Millipore 0.45- μ m filter plate and 5- μ l aliquots were analyzed by liquid chromatography–tandem mass spectrometry as described previously (Slizgi et al., 2016). In brief, tolvaftan, DM-4103, and DM-4107 were separated chromatographically on a Shimadzu high-performance liquid chromatography system (LC-10ADVP pumps, SIL-HTc autosampler and a CTO-10 AVP column oven; Shimadzu, Columbia, MD) with gradient elution. Then tandem mass spectrometry with positive ion electrospray ionization was conducted with a Thermo Electron TSQ Quantum Discovery MAX (Thermo Fisher, Waltham, MA) with an Ion Max electrospray ionization source. The transitions monitored (parent m/z > product m/z) at unit resolution for tolvaftan, DM-4103, and DM-4107 were 449.2 > 252.0, 479.0 > 252.0, and 481.0 > 252.0, respectively. The assay range for tolvaftan, DM-4103, and DM-4107 was 0.0025–25 pmol/ μ l (0.0125–125 pmol injected on column).

Data Analysis. Protein content was determined by Pierce BCA acid protein assay kit according to the manufacturer's protocol (ThermoScientific, Rockford, IL). Cellular accumulation determined in Ca^{2+} -free HBSS (Accumulation Ca^{2+} -free HBSS) represents the total mass of analyte in the hepatocyte at the end of the incubation time period. Total accumulation determined in standard HBSS (Accumulation_{standard HBSS}) represents the total mass of compound taken up and excreted (cells+bile). The biliary excretion index (BEI) was

calculated using the following equation as reported previously by our laboratory (Liu et al., 1999):

$$\text{BEI (\%)} = \frac{\text{Accumulation}_{\text{standard HBSS}} - \text{Accumulation}_{\text{Ca}^{2+}\text{-free HBSS}}}{\text{Accumulation}_{\text{standard HBSS}}} \times 100 \quad (1)$$

The intracellular concentration of each test compound was calculated by dividing the mass of compound in cells (Accumulation_{standard HBSS}) by the intracellular fluid volume for human hepatocytes (7.6 μ l/mg protein; Qualyst Transporter Solutions Technical Application Bulletin, 2011).

Results and Discussion

Accumulation of Tolvaftan and Metabolites in SCHH. The accumulation of tolvaftan, DM-4103, and DM-4107 was investigated in SCHH. Accumulation of tolvaftan was concentration-dependent after a 10-minute incubation over the tolvaftan concentration range of 0.15–50 μ M (Fig. 1A). The total (cells+bile) accumulation ranged from ~13 to 3900 pmol/mg protein, whereas the biliary excretion of tolvaftan was negligible (Fig. 1A). Metabolism of tolvaftan to DM-4103 and DM-4107 was observed at tolvaftan incubation concentrations greater than 15 μ M and 1.5 μ M, respectively (Fig. 1B). Cellular concentrations of tolvaftan, DM-4103, and DM-4107 also showed a concentration-dependent increase after a 10-minute incubation of 0.15–50 μ M tolvaftan (Fig. 1B). The total cellular concentration of tolvaftan ranged from ~2 μ M to 500 μ M and was greater than the respective incubation concentration (0.15–50 μ M). DM-4103 could not be detected at low tolvaftan incubation concentrations (0.15–1.5 μ M). At higher tolvaftan incubation concentrations (15–50 μ M), cellular concentrations of DM-4103 ranged from ~0.16 to 0.19 μ M. Concentrations of DM-4107 were higher than DM-4103 and

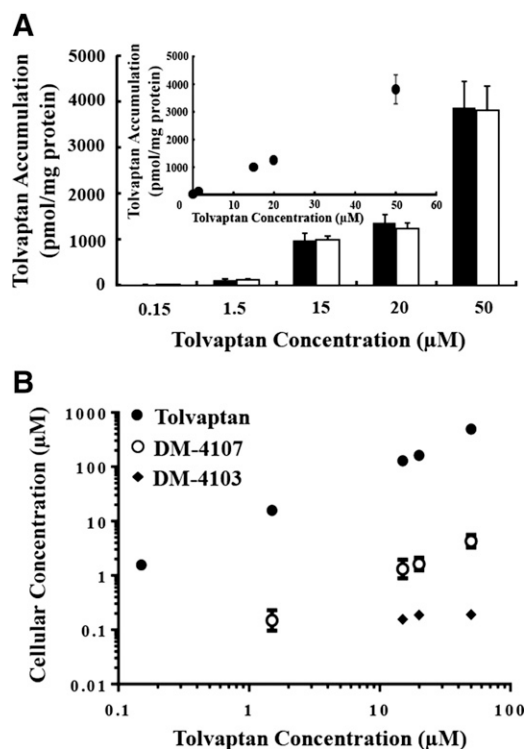


Fig. 1. Accumulation of tolvaftan, DM-4103, and DM-4107 in SCHH after a 10-minute incubation in the presence of 4% BSA. (A) Concentration-dependent accumulation of tolvaftan in standard HBSS (black bars) and Ca^{2+} -free HBSS (white bars and inset). (B) Hepatocellular concentrations of tolvaftan, DM-4103, and DM-4107 after a 10-minute incubation of 0.15–50 μ M tolvaftan in standard HBSS. Data are expressed as mean \pm S.D. ($n = 1$ human liver in triplicate).

ranged from ~ 0.15 to $4.26 \mu\text{M}$ at tolvaptan incubation concentrations of 1.5 to $50 \mu\text{M}$, respectively, after a 10-minute incubation (Fig. 1B).

Effect of Inhibitors on the Disposition of Tolvaptan and Metabolites. The hepatobiliary disposition of tolvaptan, DM-4103, and DM-4107 was evaluated in SCHH in the presence and absence of various inhibitors. For these studies, a tolvaptan concentration of $15 \mu\text{M}$ and incubation time of 10 minutes were selected. Pioglitazone, verapamil, MK-571, and elacridar at the concentrations tested were selected as inhibitors of canalicular transporters (e.g., P-gp, MRP2, BCRP) (Achira et al., 1999; Englund et al., 2014). These compounds also inhibit CYP3A (IC_{50} : 12.3, 20, 11.3, and $4.9 \mu\text{M}$, respectively) (Achira et al., 1999; Sahi et al., 2003; Englund et al., 2014). Tolvaptan accumulation was not markedly altered by $50 \mu\text{M}$ pioglitazone, verapamil, MK-571, or $10 \mu\text{M}$ elacridar (data not shown), consistent with the finding that the biliary excretion of tolvaptan was negligible in the present study design. Pioglitazone, verapamil, MK-571, and elacridar reduced mean DM-4107 accumulation by 45.6, 79.8, 94.5, and 23.0% relative to control, respectively (Fig. 2), consistent with CYP3A inhibition. DM-4103 concentrations were below the limit of detection when tolvaptan was coincubated with pioglitazone, verapamil, MK-571 and elacridar, probably due to reduced tolvaptan metabolism.

Effect of Tolvaptan on the Disposition of Transporter Probes. The effects of tolvaptan coincubation for 10 minutes on the hepatobiliary disposition of select transporter substrates in SCHH were evaluated. TCA cell+bile accumulation and BEI were decreased as tolvaptan concentrations increased (Fig. 3A). These data are consistent with recent findings that tolvaptan and DM-4103 can inhibit BSEP transport in membrane vesicles from Sf9 insect cells expressing human BSEP (IC_{50} of 31.6 and $4.15 \mu\text{M}$, respectively; Slizgi et al., 2016). Although the cellular accumulation of tolvaptan is high when $15 \mu\text{M}$ tolvaptan is incubated with SCHH for 10 minutes in the presence of 4% BSA in the incubation medium (Fig. 1), the cellular unbound concentration of tolvaptan should be less than the reported IC_{50} values for BSEP inhibition considering that the protein binding of tolvaptan is 98.5% in human plasma (Furukawa et al., 2011). On the basis of these data, tolvaptan-mediated BSEP inhibition should be low at typical clinical tolvaptan doses in patients with normal bile acid homeostasis. However, in patients with disease or polymorphisms that disrupt bile acid homeostasis, BSEP inhibition at very high clinical doses of tolvaptan may result in an adverse outcome.

Although tolvaptan can inhibit NTCP-mediated TCA uptake at high concentrations, unbound tolvaptan concentrations in the present studies

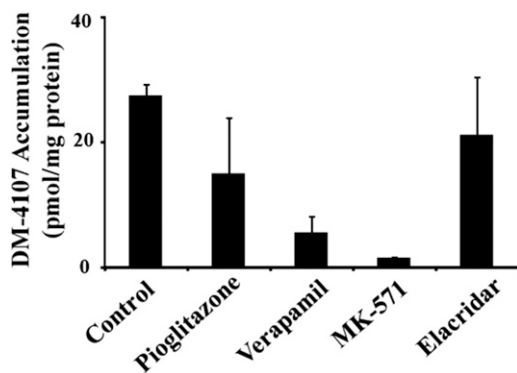


Fig. 2. Effect of inhibitors on generated DM-4107 disposition in SCHH. Total accumulation (cell + bile) of DM-4107 was quantified after coincubation of $50 \mu\text{M}$ pioglitazone, verapamil, and MK-571 or $10 \mu\text{M}$ elacridar with $15 \mu\text{M}$ tolvaptan for 10 minutes in the presence of 4% BSA in SCHH. Data are expressed as mean \pm S.D. ($n = 1$ human liver in triplicate).

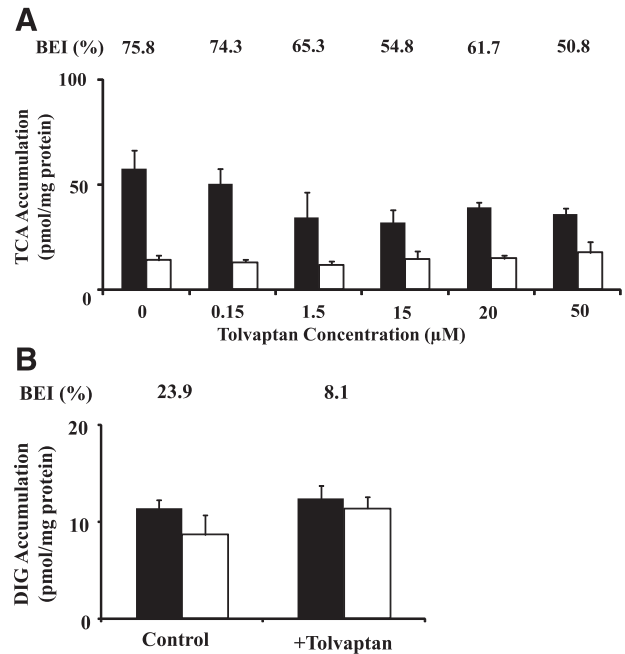


Fig. 3. Effect of tolvaptan on the disposition of transporter probes in sandwich-cultured human hepatocytes. Total accumulation (cell+bile; black bars) and cellular accumulation (white bars) in SCHH of (A) taurocholate (TCA; $2.5 \mu\text{M}$) coincubated for 10 minutes in the presence of 4% BSA with tolvaptan (0 – $50 \mu\text{M}$), or (B) digoxin (DIG; $2.5 \mu\text{M}$) coincubated for 10 minutes in the presence of 4% BSA with tolvaptan ($15 \mu\text{M}$). Data are reported as mean \pm S.D. ($n = 1$ liver in triplicate).

were well below the IC_{50} value of $41.5 \mu\text{M}$ reported by Slizgi et al. (2016). Tolvaptan ($15 \mu\text{M}$) compared with control had no effect on the cell+bile accumulation of pravastatin (3.66 ± 0.50 versus 3.89 ± 0.50 pmol/mg protein) or metformin (6.41 ± 0.49 versus 6.34 ± 0.45 pmol/mg protein), or on the cellular accumulation of these probe substrates. While the cell+bile accumulation of digoxin was not affected by tolvaptan, cellular accumulation appeared to increase; the BEI of digoxin decreased by 65.9% compared with control (Fig. 3B), consistent with P-gp inhibition as reported previously (Shoaf et al., 2011).

In summary, the present study investigated the hepatobiliary disposition of tolvaptan and metabolites in SCHH, and relevant DDIs. Tolvaptan was metabolized in SCHH with accumulation of the generated metabolites DM-4107 and DM-4103. Tolvaptan metabolism in SCHH was impaired by known CYP3A inhibitors. Tolvaptan and/or generated metabolites were capable of inhibiting BSEP and P-gp function on the basis of altered hepatobiliary disposition of probe substrates in SCHH. These results demonstrate the utility of SCHH for investigating DDIs in which both the parent compound and generated metabolites may be involved. However, the in vitro data always need to be interpreted within the context of clinical data. For tolvaptan, although the results of the present series of experiments suggest the possibility of DDIs at higher tolvaptan concentrations, DDIs have not been reported to date on the basis of clinical data (Bhatt et al., 2014).

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References

- Achira M, Suzuki H, Ito K, and Sugiyama Y (1999) Comparative studies to determine the selective inhibitors for P-glycoprotein and cytochrome P4503A4. *AAPS PharmSci* 1:E18 Abstract.
- Berl T, Quitnat-Pelletier F, Verbalis JG, Schrier RW, Bichet DG, Ouyang J, and Czerwiec FS; SALTWATER Investigators (2010) Oral tolvaptan is safe and effective in chronic hyponatremia. *J Am Soc Nephrol* 21:705–712.
- Bhatt PR, McNeely EB, Lin TE, Adams KF, and Patterson JH (2014) Review of tolvaptan's pharmacokinetic and pharmacodynamic properties and drug interactions. *J Clin Med* 3: 1276–1290.
- Chiba M, Ishii Y, and Sugiyama Y (2009) Prediction of hepatic clearance in human from in vitro data for successful drug development. *AAPS J* 11:262–276.
- Englund G, Lundquist P, Skogastierna C, Johansson J, Hoogstraate J, Afzelius L, Andersson TB, and Projean D (2014) Cytochrome p450 inhibitory properties of common efflux transporter inhibitors. *Drug Metab Dispos* 42:441–447.
- Furukawa M, Umehara K, and Kashiyama E (2011) Nonclinical pharmacokinetics of a new nonpeptide V₂ receptor antagonist, tolvaptan. *Cardiovasc Drugs Ther* 25 (Suppl 1): S83–S89.
- Lee JK and Brouwer KR (2010) Determination of hepatocellular volume in rat and human sandwich-cultured hepatocytes. (Abstract ID 1595). *The Toxicologist. Supplement to Toxicol Sci* 114:339.
- Liu X, LeCluyse EL, Brouwer KR, Gan LS, Lemasters JJ, Stieger B, Meier PJ, and Brouwer KLR (1999) Biliary excretion in primary rat hepatocytes cultured in a collagen-sandwich configuration. *Am J Physiol* 277:G12–G21.
- Qualyst Transporter Solutions Technical Application Bulletin TAB Biol 005. (2011). TAB Biol 005v2.
- Sahi J, Black CB, Hamilton GA, Zheng X, Jolley S, Rose KA, Gilbert D, LeCluyse EL, and Sinz MW (2003) Comparative effects of thiazolidinediones on in vitro P450 enzyme induction and inhibition. *Drug Metab Dispos* 31:439–446.
- Sakaida I (2014) Tolvaptan for the treatment of liver cirrhosis oedema. *Expert Rev Gastroenterol Hepatol* 8:461–470.
- Shoaf SE, Bricmont P, and Mallikaarjun S (2012a) Absolute bioavailability of tolvaptan and determination of minimally effective concentrations in healthy subjects. *Int J Clin Pharmacol Ther* 50:150–156.
- Shoaf SE, Bricmont P, and Mallikaarjun S (2012b) Effects of CYP3A4 inhibition and induction on the pharmacokinetics and pharmacodynamics of tolvaptan, a non-peptide AVP antagonist in healthy subjects. *Br J Clin Pharmacol* 73:579–587.
- Shoaf SE, Ohzone Y, Ninomiya S, Furukawa M, Bricmont P, Kashiyama E, and Mallikaarjun S (2011) In vitro P-glycoprotein interactions and steady-state pharmacokinetic interactions between tolvaptan and digoxin in healthy subjects. *J Clin Pharmacol* 51: 761–769.
- Shoaf SE, Wang Z, Bricmont P, and Mallikaarjun S (2007) Pharmacokinetics, pharmacodynamics, and safety of tolvaptan, a nonpeptide AVP antagonist, during ascending single-dose studies in healthy subjects. *J Clin Pharmacol* 47:1498–1507.
- Slizgi JR, Lu Y, Brouwer KR, St. Claire RL, Freeman KM, Pan M, Brock WJ, and Brouwer KLR (2016) Inhibition of human hepatic bile acid transporters by tolvaptan and metabolites: contributing factors to drug-induced liver injury? *Toxicol Sci* 149:237–250.
- Swift B, Pfeifer ND, and Brouwer KLR (2010) Sandwich-cultured hepatocytes: an in vitro model to evaluate hepatobiliary transporter-based drug interactions and hepatotoxicity. *Drug Metab Rev* 42:446–471.
- Tammara BK, Sekar KS, and Brumer SL (1999) The disposition of a single dose of 14C OPC-41061 in healthy male volunteers. 1999 American Association of Pharmaceutical Scientists Annual Meeting and Exposition Abstract 2025. New Orleans, LA.

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