Research Article

Method to Screen Substrates of Apical Sodium-Dependent Bile Acid Transporter

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Abstract. Human apical sodium-dependent bile acid transporter (hASBT) is a potential prodrug target under study. Development of prodrugs that target hASBT may yield compounds with low solubility and/or susceptibility to hydrolysis. A transport uptake method is needed that increases compound solubility and avoids NaOH for cell lysis during postexperimental cell sample preparation. The overall goal was to develop an assay method to screen for hASBT uptake of novel compounds. The first objective was to determine the maximum cosolvent concentrations that are compatible with an hASBT active transport assay. The second objective was to develop a NaOH-free cell lysis method to process cell samples from these uptake studies. The following cosolvents were studied: dimethylacetamide (DMAC), dimethylformamide (DMF), dimethylsulfoxide (DMSO), ethanol, methanol, polyethylene glycol-400, propylene glycol, and dioxane. Initial studies included taurocholate flux studies across hASBT-Madin-Darby canine kidney monolayers using up to 10% cosolvent, as well as cytotoxicity studies. The effect of selected cosolvent concentrations on the hASBT Michaelis-Menten kinetic parameters was evaluated. Additionally, two acetonitrile-based cell lysis methods that do not use NaOH were evaluated in terms of percent sample recovery and hASBT kinetic parameters. Results showed that the maximum permissible cosolvent concentrations for hASBT uptake studies, without compromising assay results or causing cytotoxicity, are 1% DMAC, 1% DMF, 2.5% DMSO, 2.5% methanol, and 2.5% ethanol. Additionally, both NaOH-free, acetonitrile-based cell lysis methods provided similar recovery and hASBT results, compared to NaOH method. Hence, an assay method was developed to screen for active transport, allowing for cosolvents that can solubilize compounds and avoid NaOH sample treatment, which can otherwise degrade compound.

KEY WORDS: apical sodium-dependent bile acid transporter; bile acid; screen; solvent; transporter.

INTRODUCTION

The drug discovery and development process often involves screening drug candidates for their pharmacokinetic properties, including active transporter studies. Such screening studies frequently employ *in vitro* cell culture that expresses the transporter of interest, cosolvent (e.g., dimethylsulfoxide (DMSO)) as a solubilizing agent, and cell sample processing for subsequent liquid chromatography/mass spectrometry (LC-MS) quantification to determine compound uptake or permeability.

The human apical sodium-dependent bile acid transporter (hASBT) is a prodrug target (1). A need exists to screen novel compounds for their ability to bind and be translocated by hASBT (2). An hASBT-Madin-Darby canine kidney (MDCK) assay has been previously developed (3). However,

since many novel compounds exhibit low solubility, an assay method that uses a cosolvent to enhance compound solubility without impacting kinetic results would be beneficial. Additionally, after an uptake study, NaOH is frequently used to lyse cells; sample is further processed and subsequently quantified for amount of compound taken up. A cell sample processing method that extracts compound from cells without degrading the compound is needed. Unfortunately, some compounds and prodrugs are susceptible to base-catalyzed hydrolysis (4). Hence, an assay method that does not use NaOH in cell sample preparation from uptake studies would be desirable.

The overall goal was to develop an assay method to screen for hASBT uptake of novel compounds, which was pursued through two objectives. The first objective was to determine the maximum concentrations of cosolvents that are compatible with an *in vitro* active transport assay. Little information is available about cosolvent effect on active transporter function, despite cosolvent use in cell culture screening assays of low solubility compounds. This study was designed to elucidate the maximum allowable concentration of cosolvent that can be used without altering an active transporter activity.

The following cosolvents were studied: dimethylacetamide (DMAC), dimethylformamide (DMF), DMSO, ethanol,

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ABBREVIATIONS: DMAC, dimethylacetamide; DMF, dimethylformamide; DMSO, dimethylsulfoxide; PEG-400, polyethylene glycol-400; PG, polypropylene glycol.

methanol, polyethylene glycol (PEG), propylene glycol, and dioxane. These cosolvents were selected based upon their varying physiochemical properties (e.g., polarity, hydrogen bonding character, volatility, water miscibility), such that this set of cosolvents is expected to serve as possible solubilizing agents for a wide range of compounds. In addition to being useful as solubilizing agents, each cosolvent is a commonly used solvent and readily available. Using taurocholate as a model hASBT substrate, results showed selected cosolvents at suggested concentrations did not compromise hASBT functioning or cause cytotoxicity.

The second objective was to develop a NaOH-free cell lysis method to process cell samples from uptake studies. Two methods based on acetonitrile cell lysis were evaluated, including a method which showed comparable hASBT kinetic results to NaOH lysis without requiring manual cell scraping.

MATERIALS AND METHODS

Materials. ^3H -taurocholic acid (10 $\mu\text{Ci}/\text{mmol}$) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). Sodium taurocholate was purchased from Sigma (St. Louis, MO, USA). Geneticin (G-418), fetal bovine serum (FBS), trypsin, and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen (Carlsbad, CA, USA). WST reagent was purchased from Roche Applied Science (Indianapolis, IN, USA). All other reagents were of highest purity available commercially.

Cell Culture. Stably transfected MDCK-hASBT cells were cultured as described previously (2,3). Briefly, cells were grown at 37°C, 90% relative humidity, and 5% CO_2 atmosphere and fed every 2 days. Media comprised DMEM supplemented with 10% FBS, 50 U/mL penicillin, and 50 $\mu\text{g}/\text{mL}$ streptomycin. Geneticin was used at 1 mg/mL to maintain selection pressure. Cells were passaged every 4 days or after reaching 90% confluence.

In Vitro Uptake of Taurocholate: Cosolvent Screening Study. In order to screen cosolvent effects on taurocholate uptake into hASBT-MDCK cells, uptake studies were performed in 12-well cell culture polystyrene plates (Corning Costar; Corning, NY, USA). The 12-well plates were seeded with MDCK-hASBT at a density of 1.5×10^6 cells/well and induced on day 4 with 10 mM sodium butyrate for 12–15 h prior to uptake experiments. Taurocholate concentration was 2.5 μM spiked with 0.5 $\mu\text{Ci}/\text{mL}$ of ^3H -taurocholate. Cosolvent concentrations in donor solutions for each DMAC, DMF, DMSO, ethanol, methanol, PEG-400, propylene glycol, and dioxane were varied between 0% and 10% v/v.

Uptake studies were initiated by washing the cells three times with warm Hank's balanced salt solution (HBSS) [pH 6.8] and exposing cells to donor solution for 10 min at 37°C and 50 rpm in an orbital shaker (3). To terminate uptake, cells were washed thrice with chilled sodium free buffer. Cells were then subjected to 0.25 mL of 1 M NaOH to lyse cells. After 2 h, 0.25 mL of 1 M HCl acid was added to each well to neutralize NaOH. Of the sample, 0.25 mL was then taken and quantified for taurocholate by liquid scintillation counter (model LS65000, Beckmann Instruments, Inc.; Fullerton, CA, USA). Parallel studies were performed using HBSS buffer without cosolvent, in order to screen for cosolvent effect compared to HBSS buffer. HBSS contains

sodium which is required for hASBT functioning such that these studies reflect total taurocholate uptake, including hASBT-mediated uptake. All studies were performed in triplicate.

Since hASBT is a sodium-dependent transporter, additional identical studies were performed without sodium, in order to measure passive taurocholate uptake. Regardless whether cosolvent was present or absent, these sodium-free studies employed modified HBSS, rather than HBSS. Modified HBSS [pH 6.8] contains 137 mM tetraethylammonium chloride in place of NaCl (3).

Cytotoxicity Studies. Cells were seeded at a density of 50,000 cells per well in 96-well plates and grown for 48 h. Cells were washed thrice with HBSS and incubated with 250 μL of cosolvent system (or HBSS buffer control) system for 10 min to simulate the uptake studies. After 10 min of exposure, cells were washed thrice with HBSS. Of diluted cell proliferation reagent WST-1 (i.e., 10 μL plus 100 μL HBSS), 110 μL was added to each well followed by an incubation period of 4 h. Absorbance was measured at 440 nm using a SpectraMax 384 Plus plate reader (Molecular Devices; Sunnyvale, CA, USA). All studies were performed in triplicate.

In Vitro Uptake of Taurocholate: Kinetic Parameters. Based on the results from screening and cytotoxicity studies, five cosolvents were selected for further evaluation. The five cosolvents were DMAC 1%, DMF 1%, DMSO 2.5%, ethanol (1%, 2.5%, and 5%), and methanol (1%, 2.5%, and 5%). The study was performed using taurocholate to evaluate the effect the cosolvent on hASBT kinetic parameters J_{max} , K_t , and P_p , where J_{max} and K_t are the Michaelis–Menten parameters and P_p is the taurocholate passive permeability (see below Kinetic Analysis section). Uptake studies into hASBT-MDCK cells were conducted by varying the taurocholate concentration (1–200 μM) spiked with 0.5 $\mu\text{Ci}/\text{mL}$ of ^3H -taurocholate, using the methods described above (see *In Vitro Uptake of Taurocholate* section). HBSS and modified HBSS served as the controls (i.e., buffer without cosolvent) for total taurocholate uptake and passive taurocholate uptake, respectively. All studies were performed in triplicate.

Evaluation of Cosolvent to Facilitate Screening. To exemplify the utility of cosolvent, uptake inhibition of taurocholate was measured from buffer (without DMSO) as well as from buffer with 2.5% DMSO, each with and without inhibitor A. Inhibitor A is anthracenyl–glutamyl–chenodeoxycholate. Briefly, inhibitor A was synthesized by conjugation of chenodeoxycholate to glutamic acid through an amide bond using peptide chemistry, which was then coupled to 2-aminoanthracene. Compound identity and purity were confirmed by mass spectrometry, thin-layer chromatography, and nuclear magnetic resonance techniques, including lack of chenodeoxycholate moieties as impurities.

Inhibition studies were performed in the similar manner as uptake studies above (see *In Vitro Uptake of Taurocholate* section). The donor solutions contained 2.5 μM of taurocholate, either with and without 2.5% DMSO in absence of inhibitor A. Additionally, uptake inhibition was studied using 50 μM of inhibitor A, either with or without 2.5% DMSO.

Recoveries from NaOH and Acetonitrile Lysis Methods. With the aim to develop a NaOH-free cell lysis method to process cell samples from uptake studies, ^3H -taurocholate

recovery experiments were performed to compare the efficiency of 1 M NaOH and acetonitrile as cell lysing agents in uptake studies. Stably transfected hASBT-MDCK cells were grown on 12-well plates (3.8 cm², Corning; Corning, NY, USA) and grown under conditions described above (see Cell Culture section). Briefly, cells were seeded at a density of 1.5×10^6 cells/well and induced with 10 mM sodium butyrate 12–15 h at 37°C prior to study on day 4. On the day of the experiment, cells were washed thrice with chilled modified HBSS (pH 6.8). Cell monolayer lysis was initiated by adding 0.25 mL of either 1 M NaOH or chilled acetonitrile, where each lysing agent was spiked with 0.25 μ Ci/mL of ³H-taurocholate. All studies were performed in triplicate.

Cells were subjected to NaOH lysis, and plates were sealed to prevent evaporation. After 2 h, cell lysate was neutralized with 0.25 mL of 1 M HCl. A 0.25-mL sample of lysate was transferred into a scintillation vial and spiked with nonradiolabeled taurocholate to reflect intracellular taurocholate concentration after a 10-min uptake study from 1, 2.5, 25, and 200 μ M taurocholate donor concentration. Scintillation vials were counted for associated radioactivity.

Alternatively, cells subjected to acetonitrile lysis were allowed to stand at room temperature (RT) until complete evaporation of solvent was achieved (about 2 h). Of Dulbecco's phosphate buffered saline (DPBS), 0.25 mL was added to each well, where treated cells remained attached and not as a lysate. Treated cells were scraped from the wells with a spatula. This volume was transferred into a 1.5-mL microcentrifuge tube. Wells were washed with additional 0.25 mL DPBS and added into the tube. Tubes were vortexed for 1 min and then centrifuged at $6,400 \times g$ for 10 min. Of supernatant, 0.25 mL was transferred into a scintillation vial. Vials were spiked with nonradiolabeled taurocholate to reflect an uptake study. Scintillation vials were counted for associated radioactivity.

The percent recoveries of the NaOH lysis method and acetonitrile lysis method were computed by comparing above sample radioactivities to theoretical 100% recovery samples. Theoretical 100% recovery samples were generated by spiking ³H-taurocholate directly into scintillation vials, although vials also included nonradiolabeled cell lysate as matrix from above methods. Briefly, scintillation vials containing 5 mL of scintillation fluid were spiked with 0.25 μ Ci/mL of ³H-taurocholate, along with nonradiolabeled taurocholate to reflect typical intracellular taurocholate amounts after a 10-min uptake study from 1, 2.5, 25, and 200 μ M taurocholate donor concentration. A 0.25-mL cell lysate sample from either NaOH lysis method or acetonitrile lysis method was generated using the above procedure, except sample was not spiked with ³H-taurocholate, and added to the scintillation vial. Hence, for each the NaOH lysis method and acetonitrile lysis method, the percent recovery was calculated by dividing scintillation results from samples by the theoretical 100% recovery samples.

Taurocholate Uptake from NaOH and Acetonitrile Lysis Methods with Radioactivity Quantification. Uptake studies (1–200 μ M taurocholate) were performed where hASBT-MDCK monolayers were lysed using either 1 M NaOH or acetonitrile, in order to evaluate the suitability of the acetonitrile lysis method. Uptake studies were conducted as

described above (see *In Vitro* Uptake of Taurocholate section). Each donor was spiked with 1 μ Ci of ³H-taurocholate. At the end of the assay, cells were lysed with either 1 M NaOH or chilled acetonitrile. Taurocholate uptake was quantified via scintillation counting. All studies were performed in triplicate.

Taurocholate Uptake from Acetonitrile Lysis Method with LC-MS Quantification. Taurocholate uptake studies were conducted as described above (see *In Vitro* Uptake of Taurocholate section), except taurocholate was quantified via LC-MS rather than via scintillation counter. Additionally, sodium-free uptake studies were performed using modified HBSS. Cells were lysed using the above acetonitrile method. These studies collectively assess the utility of the acetonitrile lysis method for uptake studies, where taurocholate is quantified by LC-MS. hASBT kinetic parameters were obtained as described below. All studies were performed in triplicate.

Taurocholate was quantified by LC-MS on an Agilent HPLC system, equipped with an autosampler (CTC Analytics, Zwingen, Switzerland) and a Sciex API4000-Qtrap mass spectrometer (Applied Biosystems; Foster City, CA, USA). The column was a Phenomenex Luna C8 (50 $\times$ 2.0 mm, 3 μ m; Phenomenex; Torrance, CA, USA) heated to 40°C. The flow rate was 0.7 mL/min. The injection volume was 10 μ L. The mobile phase contained a gradient of 1–95% acetonitrile in ammonium formate (pH 3.0). The gradient times (as percent organic phase, B%) were as follows: 0–0.2 (1%), 0.2–3 (1–95%), 3–3.25 (95–1%), and 3.25–5 min (1%). The retention time was 2.0 min. Detection was achieved under negative ion electrospray [M-H][−] tandem mass spectrometry using the 514.4/123.8 amu transition, as negative mode provided the greatest sensitivity. Accuracy for all standards when back calculated from the calibration curve ranged from 93% to 105%.

Modified Acetonitrile Lysis Method. Given the favorable results of the above acetonitrile lysis method, a modified acetonitrile lysis method that does not employ cell scraping was evaluated. Taurocholate uptake studies were performed from 200 μ M taurocholate donor concentration, which was spiked with ³H-taurocholate. After 10-min uptake, cells were lysed by adding 0.25 mL acetonitrile to each well. Following evaporation at RT for about 2 h, 0.5 mL of DPBS was added, without cell scraping. Wells were sealed with parafilm and left overnight (about 12–15 h); 0.25 mL was then counted for associated radioactivity. Results of this modified acetonitrile lysis method were compared to parallel studies that employed the NaOH lysis method, as well as parallel studies using the acetonitrile lysis method that involves cell scraping.

Given favorable results of these uptake studies from 200 μ M taurocholate donor concentration, full kinetic uptake profiles (1–200 μ M taurocholate donor concentration) were performed, using each the modified acetonitrile lysis method and NaOH lysis method. Additionally, studies were performed in parallel using modified HBSS to measure taurocholate passive uptake. Taurocholate was quantified by scintillation counting. $J_{\max}$, K_t , and P_p were estimated via kinetic analysis. All studies were performed in triplicate.

Kinetic Analysis. Flux data across monolayers was fitted to a previously developed modified Michaelis–Menten (5)

model that takes into account aqueous boundary layer resistance:

$$J = \frac{P_{ABL} \left(\frac{J_{\max}}{K_t + S} + P_p \right)}{P_{ABL} + \frac{J_{\max}}{K_t + S} + P_p} \quad (1)$$

$$J = \frac{P_{ABL} P_p S}{P_{ABL} + P_p} \quad (2)$$

where J is taurocholate flux, $J_{\max}$ and K_t are the Michaelis-Menten coefficients for hASBT-mediated transport, S is taurocholate (*i.e.*, substrate) concentration, P_p is the passive taurocholate permeability coefficient, and P_{ABL} is the aqueous boundary layer (ABL) permeability. P_{ABL} was 1.5×10^{-4} cm/s. Equations 1 and 2 were applied simultaneously to

Fig. 1. Taurocholate uptake as a function of cosolvent concentration. **a** Uptake studies were conducted without sodium and reflect passive taurocholate uptake. Each cosolvent reduced passive taurocholate uptake. More notably, no cosolvent caused high passive taurocholate permeability (*i.e.*, no cosolvent caused loss of monolayer passive integrity). **b** Uptake studies were conducted with sodium and reflect total taurocholate uptake, including the dominant hASBT-mediated uptake. Higher cosolvent concentration reduced hASBT activity. DMAC, DMF, DMSO, ethanol, and methanol were subsequently selected for further evaluation at concentrations that would not be expected to reduce total taurocholate uptake by more than 25%

Fig. 2. Cytotoxicity effect of cosolvents. **a** All five cosolvent were noncytotoxic up to 25% but were toxic at 50%. These five cosolvents were selected for further evaluation. **b** Dioxane (red bar) was cytotoxic at 5%. PEG-400 (blue bar) was not cytotoxic at 10%, but not further evaluated due to viscosity effect

with sodium and sodium-free flux data to estimate K_t , $J_{\max}$, and P_p . Data were analyzed using WinNonlin 4.1 (Pharsight Corporation; Mountain View, CA, USA).

RESULTS

Cosolvent Screening and Cytotoxicity Studies. Figure 1 illustrates the uptake of taurocholate into hASBT-MDCK cells as a function of cosolvent concentration. In panel a, passive taurocholate uptake was measured as studies were conducted in the absence of sodium. Cell membrane integrity was generally not compromised in terms of passive taurocholate permeability, since no cosolvent resulted in high taurocholate uptake. General disruption of the passive membrane barrier function of the plasma membrane would cause cosolvent to be unacceptable, but this was not the case. Rather, each cosolvent reduced passive taurocholate uptake by about 20–50%. Although not a major effect here, a possible explanation for this decrease in passive uptake may be reduced partitioning of taurocholate from donor

solution into cell membrane, resulting in decreased passive permeability.

In panel b of Fig. 1, uptake studies were conducted with sodium, resulting in much larger taurocholate uptake, compared to passive uptake in panel a. Hence, panel b reflects total taurocholate uptake, indicating hASBT-mediated uptake to be the dominant uptake mechanism. hASBT activity declined in a cosolvent concentration-dependent fashion. This decrease with cosolvent is beyond the impact of cosolvent on passive permeability (panel a). Hence, each cosolvent reduced hASBT activity.

Figure 2 shows cytotoxicity effect of cosolvents on hASBT-MDCK cells. In panel a, DMAC, DMF, DMSO, ethanol, and methanol were noncytotoxic up to 25% and did not affect the cell viability, compared to buffer without cosolvent. However, each cosolvent was cytotoxic at 50%. Cytotoxicity results of dioxane and PEG are shown in panel b. Dioxane reduced cell viability at 5%. PEG-400 was not cytotoxic at 10%.

Given these cytotoxicity results and the uptake screening results in Fig. 1, DMAC, DMF, DMSO, ethanol, and methanol were selected for further evaluation. DMAC, DMF, and DMSO were selected at concentrations of 1%, 1%, and 2.5%, respectively, since these concentrations were not cytotoxic and did not reduce total taurocholate uptake by more than 25% in Fig. 1b. Ethanol and methanol were each selected at concentrations of 1%, 2.5%, and 5% for further evaluation. Dioxane was not selected for further evaluation since it affected hASBT activity at even 1% concentration. Although PEG-400 at 10% was not cytotoxic and did not reduce hASBT functioning beyond 25%, PEG-400 was not further evaluated. PEG-400 solutions exhibited high viscosities, such that pipetting was not reproducible (data not shown).

In Vitro Uptake of Taurocholate: Kinetic Parameters.

Five cosolvents were further evaluated for their impact on hASBT, by conducting taurocholate uptake studies over a range of taurocholate concentrations and estimating hASBT kinetic parameters J_{max} , K_t , and P_p . As an example, Fig. 3 shows the effect of 5% methanol on taurocholate uptake. Studies were performed using either 5% methanol (in HBSS) or HBSS buffer. Identical studies were performed using sodium-free buffer (*i.e.*, modified HBSS) to measure passive taurocholate uptake. Table I lists kinetic parameters. The taurocholate K_t value of 9.93 μ M from buffer is consistent with historical K_t values of taurocholate for hASBT (2). Studies from 5% methanol and buffer yielded K_t values of 16.7 and 9.93 μ M, respectively. Given this possible reduction in transporter affinity (*i.e.*, increased K_t) in 5% methanol, 5% methanol is conservatively not suggested as an acceptable cosolvent concentration.

Additionally, Fig. 4 shows the effect of the suggested maximum cosolvents on taurocholate uptake. Studies were conducted in presence and absence of sodium for each cosolvent. Total uptake reflects studies with sodium. Passive uptake was measured from studies without sodium. In panel a, uptake media were buffer without any cosolvent. Panels b–f show uptake profiles with DMAC 1%, DMF 1%, DMSO 2.5%, ethanol 2.5%, and methanol 2.5%, respectively. Uptake parameters are listed in Table I. Uptake parameters with cosolvent were practically the same as parameters

Fig. 3. Effect of 5% methanol on taurocholate uptake. Studies were performed using either 5% methanol (*open symbols*) or buffer [0% cosolvent] (*closed symbols*) and either with or without sodium (total or passive taurocholate uptake, respectively). Conservatively, 5% methanol is not suggested as an acceptable cosolvent concentration, in spite of practical profile similarity to buffer. Profiles from 5% methanol and buffer yielded K_t values of 16.7 and 9.93 μ M, respectively, in Table I. Given this possible reduction in transporter affinity (*i.e.*, increased K_t) in 5% methanol, 2.5% methanol was conservatively identified as the maximum methanol concentration

obtained from buffer, such that these cosolvents levels were deemed acceptable. For example, K_t from 2.5% methanol was 6.93 μ M, which is taken to be the same as K_t of 9.93 from the buffer ($p=0.19$). Hence, 2.5% is the maximum permissible methanol concentration for hASBT studies.

From Table I, acceptable concentrations of DMAC, DMF, and DMSO were 1%, 1%, and 2.5%, respectively. K_t values from these cosolvent conditions were taken to be the same as K_t of 4.11 (± 0.89) from buffer ($p=0.28$, 0.003, and 0.014, respectively). Although there was statistically significant difference in the presence and absence of DMF 1% and DMSO 2.5%, these differences were interpreted to be practically unimportant. In our experience, we typically see threefold variations in K_t values, inclusive of values observed here. Results from ethanol mimic methanol results, where 5% ethanol was not deemed to be acceptable. Ethanol 2.5% is the maximum allowable ethanol concentration for hASBT studies ($p=0.63$).

In Table I, taurocholate passive permeability sometimes showed statistical significance, as low permeability values are generally suspect to several-fold variations. These differences were less than threefold and not viewed as practically relevant. Most importantly, cosolvent did not result in leaky membranes. For example, taurocholate passive permeability remained low in presence and absence of DMSO 2.5%.

Figure 5 illustrates the benefit of DMSO in transporter screening, without cosolvent compromising transporter functioning. Figure 5 shows the percent inhibition of taurocholate uptake under three situations, compared to control condition. Control was buffer without either DMSO or inhibitor A. The three test conditions included DMSO 2.5% and/or inhibitor A. Buffer/DMSO (first bar) did not enhance taurocholate inhibition, compared to buffer [$-2.6(\pm 1.7)\%$; $p=0.25$], show-

Table I. Taurocholate Kinetic Parameters as a Function of Cosolvent [mean (SEM)]

Cosolvent	$J_{\max}$ (pmol/cm ² /s)	K_t (μM)	P_p (cm/s)×10 ⁶
Buffer ^a	0.478 (0.017)	9.93 (1.41)	0.720 (0.063)
Buffer ^b	0.448 (0.020)	4.11 (0.89)	0.487 (0.077)
DMAC 1% ^b	0.470 (0.021)	7.66 (1.52)*	0.598 (0.084)*
DMF 1% ^b	0.621 (0.024)	9.83 (1.59)**	0.365 (0.087)**
DMSO 2.5% ^b	0.382 (0.011)	1.52 (0.423)***	0.203 (0.056)**
Ethanol 1% ^a	0.513 (0.029)	13.5 (2.70)*	0.912 (0.096)*
Ethanol 2.5% ^a	0.498 (0.029)	8.73 (2.10)*	0.758 (0.109)*
Ethanol 5% ^a	0.983 (0.099)	22.9 (7.3)*	0.537 (0.257)*
Methanol 1% ^a	0.577 (0.029)	6.34 (1.61)*	0.653 (0.119)*
Methanol 2.5% ^a	0.597 (0.030)	6.93 (1.70)*	0.526 (0.120)*
Methanol 5% ^a	0.428 (0.019)	16.7 (2.5)***	0.375 (0.060)**
Methanol 5% ^b	0.611 (0.024)	18.3 (2.3)***	0.541 (0.0710)*

* $p>0.05$; ** $p<0.01$; *** $0.01<p<0.05$ [when compared to buffer (on same occasion)]
^a Performed on occasion one (buffer, ethanol, and methanol)
^b Performed on occasion two (buffer, DMAC, DMF, DMSO, and methanol 5%)

ing lack of DMSO effect on the transporter. Inhibitor A in buffer (second bar) enhanced taurocholate inhibition by 17.5 (±2.1)% ($p=0.03$), although inhibitor A was not completely dissolved. Inhibitor A in buffer/DMSO (third bar) yielded a

solution and further enhanced taurocholate inhibition to 42.8 (±1.0)% ($p=0.0015$ compared to 17.5% inhibition results; $p=0.0016$ compared to −2.6% inhibition results). DMSO at a 2.5% level did not modulate hASBT functioning and allowed

Fig. 4. Effect of suggested maximum cosolvents on taurocholate uptake. In each panel, profiles were obtained either with or without sodium (*i.e.*, total uptake and only passive uptake, respectively). **a** Uptake media were buffer without any cosolvent. **b–f** Uptake profiles with DMAC 1%, DMF 1%, DMSO 2.5%, ethanol 2.5%, and methanol 2.5%, respectively, yielding uptake parameters in Table I. Uptake parameters with cosolvent were practically similar to parameters obtained from buffer, such that these cosolvents levels are deemed acceptable

Fig. 5. Taurocholate uptake inhibition screening with and without DMSO 2.5%. The *first, second, and third bar* show relative taurocholate inhibition by buffer/DMSO (without inhibitor A), inhibitor A in buffer (without DMSO), and inhibitor A in buffer/DMSO, respectively, compare to buffer without either DMSO or inhibitor A. Buffer/DMSO did not enhance taurocholate inhibition, compared to buffer, showing lack of DMSO effect on the transporter. Inhibitor A in buffer enhanced taurocholate inhibition, although inhibitor A was not completely dissolved. Inhibitor A in buffer/DMSO yielded a solution and further enhanced taurocholate inhibition. These results exemplify cosolvent facilitated screening, without cosolvent compromising transporter functioning

for more inhibitor to dissolve, leading to enhancement in the inhibition potential.

Recoveries from NaOH and Acetonitrile Lysis Methods.

With the aim to develop a NaOH-free cell lysis method to process cell samples from uptake studies, ^3H -taurocholate recovery experiments were performed to compare the efficiency of 1 M NaOH and acetonitrile as cell lysing in uptake studies. Recovery of taurocholate from these two different sample preparation methodologies was computed by comparing scintillation counting of ^3H -taurocholate spiked into lysis solvent (*i.e.*, NaOH or acetonitrile) *versus* scintillation counting of ^3H -taurocholate spiked directly into scintillation fluid.

Figure 6 illustrates percent recoveries from each method at each taurocholate donor concentration. Percent recoveries from these two methods were reproducible and were about

Fig. 6. Recovery of taurocholate from two different sample preparation methodologies that employ different cell lysis methods. Recovery was computed by comparing scintillation counting of ^3H -taurocholate spiked into lysis solvent (*i.e.*, NaOH or acetonitrile) *versus* scintillation counting of ^3H -taurocholate spiked into scintillation fluid. Recovery from NaOH lysis (blue bar) and from acetonitrile lysis (red bar) was reproducible and about 100%, compared to direct spiking into scintillation fluid

100%, compared to directly spiking the scintillation fluid (*i.e.*, compared to theoretical 100% recovery). These acetonitrile lysis method results are favorable.

Taurocholate Uptake from NaOH and Acetonitrile Lysis Methods. Total taurocholate uptake profiles were obtained using the two lysis methods. Figure 7a plots the total taurocholate uptake as a function of taurocholate donor concentration, where taurocholate was quantified using scintillation counting. The uptake profiles are practically the same, regardless of cell lysis method, and support the use of acetonitrile followed by cell scraping as a suitable method for cell lysis in an uptake experiment. The total taurocholate uptake was slightly lower for acetonitrile method, compared to NaOH lysis method, which was consistent with the recovery experiment. More importantly, uptake from the acetonitrile method was as reproducible as from the NaOH method.

In Fig. 7b, taurocholate uptake profiles in the presence and absence of sodium (total and passive, respectively) was measured by LC-MS after acetonitrile lysis. The estimated taurocholate kinetic parameters were $K_t=3.99 (\pm 0.45) \mu\text{M}$, $J_{\max}=0.487 (\pm 0.009) \text{ pmol/cm}^2/\text{s}$, and $P_p=0.339 (\pm 0.041) \times 10^{-6} \text{ cm/s}$. The similarity of these profiles and kinetic estimates to historical profiles and kinetic estimates (2–3) substantiates the use of acetonitrile to lyse the cells and subsequent LC-MS to quantify bile acid.

Modified Acetonitrile Cell Lysis Method. Figure 8a compares the uptake of taurocholate from 200 μM donor solution when sample is prepared via NaOH, acetonitrile, and modified acetonitrile. The modified acetonitrile differs from the acetonitrile method by not involving cell scraping, which is a labor-intensive step (*e.g.*, cell scraping, vortexing, and centrifuging). From Fig. 8a, the measured taurocholate uptake was practically same whether NaOH, acetonitrile with scraping, or acetonitrile without scraping was performed. These results support the suitability of the modified acetonitrile method. An additional potential benefit of the modified

Fig. 7. Suitability of acetonitrile lysis and LC-MS quantification. **a** ^3H -taurocholate uptake profile from NaOH lysis and from acetonitrile lysis were practically the same, supporting the use of the acetonitrile lysis method. **b** Taurocholate uptake profiles in the presence and absence of sodium (total and passive, respectively) was measured by LC-MS after acetonitrile lysis. The similarity of these profiles to historical profiles (e.g., Fig. 5a) supports the use of acetonitrile to lyse the cells and subsequent LC-MS to quantify bile acid

acetonitrile method is its general absence of cell debris, for more facile sample quantification.

Figure 8b further supports the utility of the modified acetonitrile method. In Fig. 8b, studies were conducted with and without sodium (*i.e.*, total or passive taurocholate uptake, respectively). The similarity of these profiles from modified acetonitrile and NaOH validates the use of acetonitrile to lyse the cells without scraping. Kinetic parameters were also similar, regardless of lysis method. From modified acetonitrile, $K_t = 7.34 (\pm 1.26) \mu\text{M}$, $J_{\text{max}} = 0.621 (\pm 0.023) \text{ pmol/cm}^2/\text{s}$, and $P_p = 0.466 (\pm 0.086) \times 10^{-6} \text{ cm/s}$. These values were practically indistinguishable from kinetic parameters from the standard NaOH method, which were $K_t = 5.31 (\pm 0.53) \mu\text{M}$, $J_{\text{max}} = 0.615 (\pm 0.011) \text{ pmol/cm}^2/\text{s}$, and $P_p = 0.530 (\pm 0.046) \times 10^{-6} \text{ cm/s}$.

DISCUSSION

hASBT as a Screening Target. The overall goal of the work was to develop an assay method to screen for hASBT uptake. The first objective was to determine the maximum concentrations of cosolvents that are compatible with an *in vitro* active transport assay. The second objective was to develop a NaOH-free cell lysis method to process cell samples from uptake studies.

hASBT is expressed in the terminal ileum and is a high efficiency and high capacity transporter, such that it can serve

as a potential prodrug target. For example, drugs can be coupled to the natural bile acids (2–4), and such conjugates then evaluated for their hASBT uptake properties, using hASBT-MDCK cells. Often during such screening studies, candidate compounds precipitate at even 100 μM. A cosolvent system that aids in compound solubilization without affecting the transporter's kinetics or impacting cell membrane integrity of the cells would be beneficial.

Additionally, compound degradation during cell sample processing can be problematic. If drug is degraded during extraction from cells, LC-MS quantification underestimates the extent to which compound was taken up into cells during the uptake study. For example, bile acid prodrugs that target hASBT employ esters and amides, which are susceptible to NaOH-catalyzed hydrolysis (4). Hence, an assay method that does not use NaOH in cell sample preparation from uptake studies would be advantageous.

Effect of Cosolvents on Active Transport. Several reports describe surfactant and/or cosolvents in cell culture pharmacokinetic screening studies. A majority of these reports assess surfactant effects, rather than cosolvent effects (6). However, surfactants have potential to reduce apparent passive (and perhaps active) permeability via reduced thermodynamic activity, where drug can largely be associated with micelles (7).

DMAC, DMF, DMSO, ethanol, methanol, PEG, polypropylene glycol, and dioxane are commonly used, readily

Fig. 8. Suitability of acetonitrile lysis without scraping. **a** Measured taurocholate uptake was the same, irrespective of the method of cell lysis. NaOH, acetonitrile with scraping, and acetonitrile without scraping provided the same taurocholate uptake. **b** Studies were performed using either the acetonitrile lysis method without scraping (open symbols) or NaOH lysis (closed symbols) and either with or without sodium (total or passive taurocholate uptake, respectively). The similarity of these profiles support the use of acetonitrile to lyse the cells without scraping

available solvents that possess a range of physiochemical properties. Collectively, this group of cosolvents has potential to solubilizing a wide number of poorly water soluble compounds. Results indicate that acceptable cosolvents are DMAC 1%, DMF 1%, DMSO 2.5%, methanol 2.5%, and ethanol 2.5%. K_t values from these cosolvent conditions were taken to be the same as K_t from buffer, indicating cosolvents did not modulate hASBT function. These concentrations were also not cytotoxic. Above these concentrations, each of these five cosolvents reduced hASBT activity, although were noncytotoxic up to 25%, but cytotoxic at 50%. Dioxane compromised hASBT activity at even 1% dioxane concentration. PEG at 1% was not cytotoxic and did not markedly reduce hASBT functioning but afforded nonreproducible pipetting due to viscosity, which is consistent with observations from Yamashita *et al.* (8)

Little information is available about cosolvent effect on active transporter function, even though cosolvent use in cell culture to screen novel, low solubility compounds for active transport. Since membrane transporters are generally embedded in the lipid bilayer, with the lipid bilayer contributing to transporter structure and function, cosolvents can be expected to impact transporter functioning (9–11).

Among reports that evaluate cosolvent effect on cell culture permeability, the majority focus on passive or apparent permeability (6,12–14). For example, in seeking to optimize assay condition to measure drug candidate permeabilities, Aungst *et al.* (12) measured the effect of the solubilizing agent DMAC on permeability values. DMAC 5% was found to increase drug solubility and did not generally modulate apparent Caco-2 permeability. However, compound DMP 851 had higher apparent absorptive permeability and decreased apparent secretory permeability when using DMAC 5%.

Takahashi *et al.* (15) found PEG-400 5% and propylene glycol 20% reduced Caco-2 electrical resistance, although did not impact apparent absorptive permeability across Caco-2 monolayers of several passive model compounds. Yamashita *et al.* (8) studied the influence of DMSO, ethanol, and PEG-400 on the apical-to-basolateral dexamethasone permeability across Caco-2 monolayers. Dexamethasone was selected as a representative high lipophilicity compound, perhaps more reflective of high passive permeability than active transport. DMSO and PEG-400 reduced dexamethasone permeability, while ethanol had no effect. Rege *et al.* (16) studied the influence of common formulation excipients PEG-400 1.5% and propylene glycol 1.5% on both passive and active transport and found no cosolvent effect.

Flaten *et al.* examined the impact of several surfactants and cosolvents on passive drug permeability across a phospholipid vesicle barrier (14). While designed to screen passive drug permeability, this model consists of a tight barrier of liposomes on a filter support, packed by centrifugation (17), such that cosolvent effects on active transport were not elucidated.

Surfactants were not studied here since they have potential to reduce apparent passive (and perhaps active) permeability via reduced thermodynamic activity, where drug can largely be associated with micelles (7). Additionally, Nerurkar *et al.* showed that surfactants can diminish active transport (18). Potential mechanisms for surfactant-mediated effects on active transport include bilayer fluidity modulation were examined by Rege *et al.* (10).

However, cosolvents can also demonstrate these same or similar effects. For example, DMSO and PEG-400 reduced the permeability of dexamethasone, a model lipophilic drug with high permeability (8). This reduction may be due to cosolvent reducing drug partitioning from medium and into the membrane, or cosolvent effects via osmotic pressure or viscosity. Our results of passive taurocholate permeability provide agreement, in terms of cosolvent effect on reducing passive permeability. Each cosolvent here reduced passive taurocholate uptake by about 20–50%.

Regarding the Mechanism by Which Cosolvents Here Compromised hASBT Function, Further Evaluation Is Necessary. Although DMAC 1%, DMF 1%, DMSO 2.5%, methanol 2.5%, and ethanol 2.5% did not compromise hASBT function, nor caused cytotoxicity, each cosolvent inhibited hASBT at higher concentration, prior to causing cytotoxicity. Cosolvent effects on cell membranes are poorly understood. For example, in spite of DMSO being a common penetration enhancer, cell fusogen, cryoprotectant, and cosolvent *in vitro* screening studies, the underlying molecular mechanisms of DMSO effects on cell membranes are not well understood (9). Cosolvent effect on membrane-associated transporters would seem be even more complex. Nevertheless, results here demonstrate a range of cosolvents can be applied at certain levels to facilitate screening studies of active transport.

NaOH-Free Cell Lysis Method. The second objective was to develop a NaOH-free cell lysis method to process cell samples from uptake studies. Cell culture-based methods typically require cells at the end of the experiment to be lysed, followed by compound quantification (*e.g.*, LC–MS for analysis of nonradiolabeled compounds). The processing of cell samples for analysis represents an important step. NaOH is commonly used to lyse cells, where drug-containing cells are incubated with 1 M NaOH for about 2 h. Drug stability in NaOH is a potential concern as strong base can lead to compound hydrolysis.

Two methods based on acetonitrile cell lysis were evaluated. Acetonitrile was selected since this solvent is widely used to precipitate proteins (19). After acetonitrile exposure, one method entailed scraping of hASBT-MDCK cells to ensure complete cell lysis, followed by vortexing, centrifugation, and quantifying the substrate. A second method, denoted the modified acetonitrile method, did not apply manual cell scraping. Results show all three methods were comparable, including the acetonitrile cell lysis method without scraping was being comparable to NaOH lysis. These results show some similarity to the methodological results of Harlfinger *et al.* (20), who investigated methanol, perchlorate, and Triton X-100 as cell lysis agents. Harlfinger *et al.* observed that uptake of a model substrate for hOCT2 was the same, regardless of lysing agent.

CONCLUSIONS

An assay method was developed to screen for active transport into hASBT-MDCK cells. This assay method can employ 1% DMAC, 1% DMF, 2.5% DMSO, 2.5% methanol, or 2.5% ethanol in order to solubilize compound without having a detrimental affect on hASBT functioning. Since compounds to be screened may also be susceptible to NaOH-

catalyzed hydrolysis, an acetonitrile-based method to lyse hASBT-MDCK cells, without the need for manual scrapping, was developed. These methods may also be useful to compound screening assays for other active transport systems.

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