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Identification of Inhibitor Concentrations to Efficiently Screen and Measure Inhibition K_i Values against Solute Carrier Transporters

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Abstract

The objective was to identify inhibitor concentrations to efficiently screen and measure inhibition K_i values of solute carrier (SLC) transporters. The intestinal bile acid transporter and its native substrate taurocholate were used as a model system. Inhibition experiments were conducted using 27 compounds. For each compound, the inhibition constant K_i was obtained from the comprehensive inhibition profile, and referred as the reference K_i . K_i values were also estimated from various partial profiles and were compared to the reference K_i . A screening K_i was estimated from one data point and also compared to the reference K_i . Results indicate that K_i can be accurately measured using an inhibitor concentration range of only 0- K_i via five different inhibitor concentrations. Additionally, a screening concentration of 10-fold the substrate affinity K_t for potent inhibitors ($K_i < 20K_t$) and 100-fold K_t for nonpotent inhibitors ($K_i > 20K_t$) provided an accurate K_i estimation. Results were validated through inhibition studies of two other SLC transporters. In conclusion, experimental conditions to screen and measure accurate transporter inhibition constant K_i are suggested where a low range of inhibitor concentrations can be used. This approach is advantageous in that minimal compound is needed to perform studies and accommodates compounds with low aqueous solubility.

Keywords

Solute carrier transporter; Inhibition; Screen; Inhibitor concentration range

1. Introduction

Solute carrier (SLC) transporters are often involved in drug uptake and can play important roles in drug absorption and distribution (Ayrton and Morgan, 2008). Most drug transporters belong to the SLC family of transporters (You and Morris, 2007). They can impact drug movement into tissues such as intestine, liver, kidney, and brain (Mizuno *et al.*, 2003). Drug bioavailability can be increased by targeting SLC transporters (e.g., Pept1, ASBT) (Balakrishnan and Polli, 2006; Balimane *et al.*, 1998; Han *et al.*, 1998; Katsura and Inui, 2003). Evaluation of drug-transporter interactions commonly employs *in vitro* cell models to better understand transporter characteristics and to elucidate substrate/inhibition specificity,

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with a goal to examine transporter-mediated drug-drug interactions and the drug pharmacokinetic profile.

Inhibition studies are typically the first type of study performed to assess compound affinity for a transporter. Inhibition studies often entail screening studies of a large number of potential inhibitors, followed by comprehensive studies of those compounds that showed preliminary inhibition. Inhibitor constant K_i (or EC_{50}) is usually measured to reflect compound binding activity and inhibitory effect.

Selection of inhibitor concentrations is an important element to accurately determine K_i . Regarding comprehensive profile studies to measure K_i , simulated Eadie-Hofstee plots have suggested that the range of inhibitor concentrations for *in vitro* drug-metabolizing enzymes include the expected K_i values, and span two orders of magnitude (e.g. $0.4K_i$ - $40K_i$) (Madan *et al.*, 2002). Another recommendation is that enzyme inhibitor concentrations need to extend beyond K_i by three-fold on each side, with a minimum of 12 data points (Obach, 2008). However, a high concentration over a 100-fold range may not be feasible for novel compounds in limited availability, or for hydrophobic compounds with low solubility. A suggestion for transport inhibition studies is an equal number of concentrations above and below the K_i (Su and Sinko, 2004). However, the range of inhibitor concentrations and minimum number of data points has not been explained in detail.

Although screening studies and subsequent K_i measurement are commonplace, study methods for transporters are not broadly standardized. Practices across laboratories vary, in terms of applied inhibitor concentration ranges and number of inhibitor concentrations. The objective of this study was to suggest an efficient and resource-sparing approach to measure inhibition constant K_i values for SLC transporters. Experiment conditions in this study build upon the prior literature and focus on identifying minimal inhibitor concentrations that are able to measure K_i value accurately. The human apical sodium-dependent transporter (SLC10A2, ASBT) stably-transfected MDCK cell line was used as a model system. ASBT is a prodrug target to increase oral drug bioavailability (Balakrishnan *et al.*, 2006). This study involved the ASBT inhibition profiles of 27 compounds. Results were further validated with two other solute carrier transporters, the human organic cation/carnitine transporter (SLC22A5, OCTN2) and excitatory amino acid transporters (SLC1A6, EAAT4).

Results indicate that K_i can be accurately measured using an inhibitor concentration range of only 0 to estimated K_i via five data points. Water soluble compounds allowed for even more limited conditions to measure an accurate K_i . K_i can also be accurately screened using concentration $10K_i$ or $100K_i$ for potent ($K_i < 20 K_i$) and nonpotent inhibitors ($K_i < 20 K_i$).

2. Materials and Methods

2.1 Materials

[3H]-taurocholic acid, [3H]-L-carnitine, and [3H]-glutamic acid were purchased from Perkin Elmer (Waltham, MA). Sodium taurocholate was purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS), trypsin, and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen Corporation (Carlsbad, CA). WST reagent was purchased from Roche Applied Science (Indianapolis, IN). All drugs and other chemicals were obtained from Sigma Chemical (St. Louis, MO), Alexis Biochemicals (San Diego, CA), AK Scientific (Mountain View, CA), LKT Labs (St. Paul, MN), Spectrum Chemicals & Laboratory Products (Gardena, CA), Spectrum Pharmacy Products (Tucson, AZ), or TCI America (Portland, OR).

2.2 Cell Culture

ASBT-MDCK and OCTN2-MDCK cells were cultured as described (Balakrishnan *et al.*, 2005; Diao *et al.*, 2009). Briefly, stably transfected ASBT-MDCK cells and OCTN2-MDCK were grown at 37 °C, 90% relative humidity, and 5% CO₂ atmosphere and fed every two days. Media comprised DMEM supplemented with 10% fetal bovine serum, 50 units/mL penicillin, and 50 µg/mL streptomycin. Geneticin was used at 1 mg/mL to maintain selection pressure. Cells were passaged every 4 days or after reaching 90% confluence.

EAAT4 stably transfected EAAT4-HEK cells (Jackson *et al.*, 2001) were grown at 37 °C, 90% relative humidity, and 5% CO₂ atmosphere and fed every two days. Growth media comprised DMEM (include 110 mg/L sodium pyruvate), supplemented with 50 units/mL penicillin and 50 µg/mL streptomycin. Hygromycin was used for selection at 50 µg/mL. Cells were passaged after reaching 70% confluence.

ASBT-MDCK, OCTN2-MDCK, and EAAT4-HEK cells have been previously characterized, including in terms of their transport of taurocholate (Balakrishnan *et al.*, 2005), carnitine (Diao *et al.*, 2009), and glutamate (Jackson *et al.*, 2001), respectively.

2.3 ASBT Inhibition Study

For 15 compounds (atropine, bendroflumethiazide, bumetanide, dibucaine hydrochloride, diltiazem hydrochloride, fluvastatin, isradipine, ketoprofen, latanoprost, pentamidine isethionate, simvastatin, tioconazole, propafenone, thiothixene), inhibition data was obtained from a previous study (Zheng *et al.*, 2009). For 12 other compounds, ASBT cis-inhibition studies of taurocholate uptake were performed as previously described (Diao *et al.*, 2009). Briefly, stably transfected ASBT-MDCK cells were seeded in 12 well cluster plates (Corning; NY) at a density of 1.5 million cells/well. Uptake studies were performed on the fifth day in Hank's Balanced Salt Solution (HBSS) and sodium-free buffer. Since ASBT is a sodium-dependent transporter, studies using sodium-free buffer enabled the measurement of passive permeability of taurocholate. Cells were exposed to donor solution containing 2.5 µM taurocholate (spiked with 0.5 µCi/mL [³H]-taurocholate) in the presence of a drug at different concentration for 10 min. For low water soluble compounds, 1-2.5% DMSO was included in transport buffer, which has been shown to not affect transporter kinetics nor cell viability (Rais *et al.*, 2008). For all 27 compounds, no precipitation was observed at any concentration.

Additionally, inhibition studies were conducted using compounds with solubility issues such as nitrendipine and torsemide. They displayed incomplete solubility at higher drug concentrations.

2.4 OCTN2 Inhibition Study

For four compounds (carvedilol, cerivastatin, daunorubicin hydrochloride, propantheline bromide), inhibition data was obtained from a previous study (Diao *et al.*, 2009). To characterize four other compounds, cis-inhibition studies of L-carnitine uptake were performed as previously described (Diao *et al.*, 2009). Briefly, OCTN2-MDCK cells were seeded at a density of 1.5 million cells/well in 12-well plates. Cells were exposed to donor solution containing 2.5 µM L-carnitine (spiked with 0.5 µCi/mL [³H]- L-carnitine) in the presence of a drug at different concentrations for 10 min.

2.5 EAAT4 Inhibition Study

To characterize EAAT4 binding affinities, cis-inhibition of glutamate uptake was measured in stably transfected HEK 293 cells. Cells were seeded in 12 well Poly-D Lysine coated plates (BD BioCoat; Bedford, MA) at a density of 50,000 cells/well. Uptake studies were

performed after 48 hours, as described (Fang *et al.*, 2006; Liu *et al.*, 2008). Briefly, the cells were incubated with HBSS buffer containing 1 μM glutamate, 0.5 $\mu\text{Ci/mL}$ [^3H]-glutamic acid, and inhibitor at various concentrations for 10 min at 37 $^{\circ}\text{C}$. Glutamate uptake was terminated by rinsing three times with ice-cold sodium free buffer. Cells were lysed using NaOH. Cell lysate was analyzed for radioactivity using a scintillation counter.

2.6 Kinetic Analysis

Inhibition data was analyzed in terms of inhibition constant K_i by using Michaelis-Menten competitive inhibition model (eqn 1).

$$J = \frac{J_{\max} \cdot S}{K_t \left(1 + \frac{I}{K_i}\right) + S} + P_p \cdot S \quad (1)$$

where J is flux of the substrate, $J_{\max}$ is the flux capacity, K_t is the Michaelis-Menten constant for transporter mediated transport, S is substrate concentration, P_p is the passive substrate permeability, I is the inhibitor concentration, and K_i is the inhibition constant. K_t values were measured from uptake studies. K_t for taurocholate, L-carnitine, and glutamate were 5.03 μM , 5.33 μM , and 2.02 μM , respectively. $J_{\max}$ was measured from uptake studies at 200 μM substrate concentrations where transporter was saturated; $J_{\max}$ were corrected for passive substrate flux using sodium-free flux data, where $J_{\max} = J_{\text{with sodium (HBSS)}} - J_{\text{without sodium (sodium-free buffer)}}$. P_p was measured from uptake studies in the absence of sodium. $[S] = \frac{1}{2} K_t$. Concentrations of taurocholate, L-carnitine, and glutamate were 2.50 μM , 2.50 μM , and 1.00 μM , respectively. Typical inhibition studies use $[S] = K_t$, $\frac{1}{2}K_t$, or $\frac{1}{4}K_t$. The transporter may be saturated at concentrations higher than the K_t . Too low a substrate concentration than K_t may not result in a measureable uptake activity. K_t , P_p , and $J_{\max}$ were obtained from substrate uptake studies. K_i was calculated using WinNonlin Professional (Pharsight Corporation; Mountain View, CA).

2.7 Reference K_i Measurement

Reference K_i values were measured for each compound from a comprehensive inhibition profile, using five to eight inhibitor concentrations that covered a concentration range above and below K_i . A presumed accurate K_i value was calculated from the comprehensive inhibition profile and is referred as the reference K_i .

2.8 Partial Inhibition Profile Measurement

Compounds in development, as well as commercial compounds, are often available in limited quantities (low mg). An objective was to identify inhibitor concentrations to efficiently measure K_i by determining the minimum amount of compound needed to perform the studies. Hence, the approach here was to examine the extent that high concentration studies could be eliminated and the efficiency of screening could be increased.

For each compound, using the same comprehensive inhibition data, the highest concentration was removed, and a new K_i value was calculated from the partial inhibition profile. Removal of the highest concentration data and new K_i calculation progressed, until only one inhibitor concentration remained. For each compound, K_i values from partial profiles were compared to its reference K_i value. A K_i value was concluded to be that same as the reference K_i if it was within 30% of the reference K_i . A 30% limit was pre-selected as a less than 30% difference from the reference was taken to be not practically significant. This analysis aims to evaluate the range and the number of inhibitor concentrations that impact the accuracy of K_i .

Standard error of the mean (S.E.) of K_i was estimated using WinNonlin from three replicates. As expected, larger S.E. was obtained when fewer concentration data points were employed.

2.9 Screening Study

For each compound, using the same inhibition data, only one concentration and control (i.e. no inhibitor) data points were used to estimate K_i . These retrospective analyses were conducted to mimic a wide range of screening studies using a wide range of inhibitor screening concentrations. K_i was calculated using eqn 1 and WinNonlin Professional. Estimated K_i values from screening studies were compared to the reference K_i value. This analysis aims to evaluate the screening concentrations that could estimate K_i accurately.

3. Results

3.1 ASBT Inhibition Profile Analysis

Twenty seven compounds were subjected to ASBT inhibition evaluation, using taurocholate as the substrate. Compounds included bile acid analogues and drugs, and were categorized according their potency and aqueous solubility. Inhibitors with K_i values less than 100 μM were considered potent inhibitors. Inhibitors with K_i values higher than 100 μM were considered nonpotent inhibitors. This demarcation of 100 μM was applied since 100 μM is approximately 20-fold greater than taurocholate's K_t of 5 μM . Compound solubility is listed in Supplemental Table S1. Water soluble compounds were taken to be those with at least moderate solubility (30–100 mL/g), while low water soluble compounds exhibit slight solubility or lower solubility.

Table 1 shows K_i values of water soluble, potent inhibitors. Compounds include five bile acid analogues and four drugs. The structures of the bile acid analogues are shown in Figure 1. Table 2 shows K_i values of six drugs that are water soluble, nonpotent inhibitors. Table 3 shows K_i values of low water soluble, potent inhibitors, which were five drugs and one bile acid analogue. Table 4 shows K_i values of six drugs that are low water soluble, nonpotent inhibitors. In each table, compounds are listed in order of potency. The reference K_i value is bolded. Additionally, four to six K_i values were computed from partial inhibition profiles, where the highest inhibitor concentration was sequentially removed. K_i values that differ by more than 30% from the reference K_i were considered practically different than the reference K_i and highlighted in red color in tables.

Figure 2 illustrates the concentration-dependent inhibition profile of taurocholate uptake by indomethacin. The fit to the comprehensive inhibition profile is drawn, along with the fits to all partial profiles. Solid lines depict the partial profiles that provided the same K_i as the reference K_i . Dashed lines are fits to partial profiles that provided a different K_i compared to the reference K_i .

For example, the reference K_i of indomethacin was 86.4 μM (bold in Table 2), which was obtained from a comprehensive profile employing 0, 10, 25, 50, 100, 200, 500, and 1000 μM of indomethacin. Indomethacin K_i was 80.5 μM when only 0, 10, 25, and 50 μM were used (green solid line in Figure 2). This K_i value was considered the same as the reference K_i since the difference was less than 30%. Meanwhile, indomethacin K_i was 52.6 μM (red in Table 2) when only 0, 10, and 25 μM were used (black dash line in Figure 2) and differed from the reference K_i as the difference was greater than 30%.

In Tables 1-4, all partial profiles that yielded inaccurate K_i (red color) inhibited taurocholate by less than 40% at the highest inhibitor concentration. Therefore, from 27 inhibitors of ASBT, K_i was accurately measured from a partial inhibition profile that caused more than

40% inhibition. Excepted diltiazem hydrochloride (Table 2), compound C6 and isradipine (Table 3) needed five data points (exclusive of zero control) to accurately estimate K_i . The remaining 24 compounds needed less than five inhibitor concentrations. Therefore, we concluded only five inhibitor concentrations, exclusive of control (i.e. zero-concentration), were required.

Interestingly, water soluble compounds allowed for even more limited conditions and still generally provided an accurate K_i . In Tables 1 and 2, accurate K_i values for 13 of 15 compounds (i.e. except diltiazem hydrochloride and atropine) were achieved using an inhibitor concentration that caused 25% inhibition. ASBT inhibition studies show that the amount of water soluble compound needed in inhibition studies is over 80% less than conventionally used (i.e. 0-10 K_i).

No difference was observed between potent and nonpotent inhibitors, in terms of minimum necessary inhibitor concentration range. Therefore, regardless of the inhibitor's potency, the minimum necessary inhibitor concentration range is to achieve 40% inhibition at the highest concentration.

3.2 Estimated K_i from ASBT Screening Studies

Using the identical inhibition data that yielded Tables 1-4, K_i was estimated from one inhibitor concentration, along with control. For each compound, such screening K_i values were compared to the reference K_i . Screening results are listed in Supplemental Tables S2-S5, which correspond to the same compounds in Tables 1-4, respectively. For all potent inhibitors (Tables S2 and S4), K_i estimation using 50 μM screening concentration matched the reference K_i with less than 30% differences. For all nonpotent inhibitors (Tables S3 and S5), K_i estimation using 500 μM matched the reference K_i . Screening used 50 μM for potent inhibitors and 500 μM for nonpotent inhibitors, resulting in approximately 50-80% inhibition (Table 5). Since K_t of taurocholate is 5 μM , concentrations 50 μM and 500 μM correspond to 10-fold K_t and 100-fold K_t , respectively.

3.3 Validation using OCTN2 and EAAT4 transporters

To confirm the ASBT observations could be extended to other transporters, inhibition profiles were measured using two other SLC transporters. Eight compounds were subjected to OCTN2 inhibition using L-carnitine as the substrate. Table 6 lists K_i values measured from comprehensive and partial profiles. Two compounds were subjected to EAAT4 inhibition using L-glutamate as the substrate; K_i values are listed Table 7. For all ten compounds, inaccurate K_i measured from partial profiles caused less than 40% inhibition. This result was in agreement with ASBT.

To validate the ASBT screening results, screening studies of OCTN2 and EAAT4 were assessed using the suggested concentrations. OCTN2 and EAAT4 results are listed in Table 8. Since K_t values of L-carnitine and L-glutamate are 5.33 μM and 2.02 μM , the suggested screening concentrations are 50 μM (i.e. 10-fold K_t) and 500 μM (i.e. 100-fold K_t) for OCTN2, and are 20 μM and 200 μM for EAAT4. All OCTN2 inhibitors provided screening K_i estimates that were comparable to the reference values. For the two EAAT4 inhibitors, K_i of D-aspartic acid was accurately estimated. However, the screening K_i of L-aspartic acid was 3.03 μM , which was differed from the reference K_i of 1.92 μM by over 30%.

4. Discussion

4.1 Inhibition Study Design

Inhibition studies entail screening studies of potential inhibitors, followed by comprehensive profile characterization by using a range of inhibitor concentrations of those compounds that show preliminary inhibition. A preliminary estimate of K_i can be calculated from the percent inhibition from screening data using eqn 2.

$$\text{Percent inhibited} = \frac{\text{Flux}_{\text{no inhibitor}} - \text{Flux}_{\text{inhibitor}}}{\text{Flux}_{\text{no inhibitor}}} * 100\% = \frac{[I]/K_i}{1 + \left(\frac{[I]}{K_i}\right) + \left(\frac{[S]}{K_t}\right)} * 100\% \quad (2)$$

From 27 ASBT inhibitors, results suggest that screening studies employ an inhibitor concentration of 10-fold K_t and 100-fold K_t , with the expectation that the lower concentration will accurately estimate K_i if the compound is potent (i.e. $K_i < 20K_t$, since 100 μM is 20 times the K_t of substrate taurocholate 5.03 μM), and that the higher concentration will accurately estimate K_i if the compound is nonpotent ($K_i > 20K_t$). OCTN2 and EAAT4 screening results support these observations, since all except onescreened compounds provided an accurate K_i estimate.

Compounds that show inhibition from initial screening study are typically subjected to comprehensive profile characterization to measure K_i . The results show K_i can be accurately measured from a partial inhibition profile that cause 40% inhibition. Using eqn 2, when $[S] = \frac{1}{2}K_t$, $[I] = K_i$ would cause 40% inhibition. ASBT results suggest that the inhibitor concentration range only need extend from 0 to the estimated K_i from initial screening study. An inhibitor concentration higher than K_i was not necessary, although it also yielded an accurate K_i . This result was further validated using eight OCTN2 and two EAAT4 inhibition profiles. The suggested approach has implications for designing inhibition studies in an efficient and resource sparing approach. The obtained *in vitro* kinetic parameters have not been correlated with *in vivo* data to date.

The Hill coefficients of three transporters were estimated using their substrate uptake data. For ASBT, OCTN2, and EAAT4, Hill coefficients were 1.03 ($r^2=0.926$), 0.947 ($r^2=0.979$), and 1.12 ($r^2=0.976$), respectively, supporting a single-binding site and using a Michaelis-Menten model for their substrates. Therefore, IC_{50} can be calculated from K_i values using the Cheng-Prusoff equation. Under the condition $[S] = \frac{1}{2}K_t$, $\text{IC}_{50} = 1.5K_i$.

4.2 Comparison to Murphy's considerations

Figure 3 shows a simulated comprehensive inhibition profile, where the profile curve is divided into three regions. This illustration reflects three regions identified by Murphy (Copeland, 2005; Murphy, 2004). Through simulations based upon the Morrison equation, Murphy provided suggestions for enzyme concentrations to be used in the study of tight-binding enzyme inhibitors (i.e. $K_i < K_t$).

Region A is the area where substrate flux decreases nearly linearly. In this segment, substrate is inhibited approximately 0 to 50%. Our results showed only data in this region are needed in determining K_i . Figure 2 shows only a few data points in this region are needed to define the entire inhibition profile, which is consistent with the near linearity of region A. The importance of region A is supported by our experimental results of three different transporters using a total of 37 inhibitors with a 1000-fold difference in potency. This finding was different from Murphy's simulation results of tight-binding enzyme inhibitors, which favored data in region B for accurate K_i determination.

Region B is the area where the profile displays high curvature. In this region, the substrate is inhibited approximately 50-80%. Murphy defined region B as the region where the total inhibitor concentration is equivalent to total enzyme (or transporter) concentration, and therefore is the most informative region to determine K_i (Copeland, 2005; Murphy, 2004). Since our objective was to suggest an efficient and resource sparing approach, the accurate K_i measurement analysis was not preferentially focused on region B, which entails high concentrations. However, the screening analysis used single concentrations over all three regions (Table S2-S5); region B yielded the best K_i prediction from a single screening concentration, indicated the importance of region B in K_i estimation from a single screening concentration. K_i can be estimated just from one datum point that causes 50-80% inhibition. However, since K_i measurement results show five data points in region A were sufficient to measure K_i accurately, data in region B are not suggested.

Region C is the area at high inhibitor concentrations, where the substrate is inhibited approximately over 80%. Transporter activity is low and changes minimally with additional inhibitor. Since flux is low, a small error can significantly affect the K_i estimate. Therefore, data in this region was not recommended by Murphy (Copeland, 2005; Murphy, 2004). Our screening results (Tables S2-S5) followed this expectation, where high inhibitor concentrations yielded high error in K_i estimation.

4.3 Comparison to Other Approaches

Based on experimental analysis, two studies have suggested efficient inhibition approaches to study drug-drug interactions that involved cytochrome P450. The objective of Gao *et al.* was to suggest a reliable approach to screen IC_{50} , which was similar to our study (Gao *et al.*, 2002). From single concentration screening using 1 μ M, 3 μ M, and 10 μ M, Gao *et al.* found both 3 μ M and 10 μ M provided good IC_{50} predictions (correlation $r = 0.99$). The authors suggested 3 μ M was preferable because less compound is required than for 10 μ M. Additionally, analytical precision at this concentration was also comparable to the 3 and 10 point inhibition screens. This work is different from the present study which concerns SLC transporters. Additionally, a 1000-fold screening concentration range was examined here. Furthermore, our suggested approaches are in terms of K_t and K_i , rather than absolute concentrations, which may be applied to all SLC transporters. Atkinson *et al.* suggested two automated, time-dependent inhibition assays to accurately measure K_{inact}/K_i , to predict enzyme inactivation (Atkinson *et al.*, 2005). Their approach was specific for irreversible inhibitors.

Based on simulation, Kakkar *et al.* examined a minimal experimental design for obtaining reliable V_{max} , K_m , and K_i . They suggest enzyme studies involving three substrate concentrations and one substrate-inhibitor pair (Kakkar *et al.*, 2000). However, they did not suggest an inhibitor concentration to measure or screen K_i .

4.4 Resource-sparing approach solves solubility issue

The efficient and resource-sparing suggestions may circumvent solubility issues for compounds with limited water solubility. Insufficient solubility is a common issue in conducting inhibition studies. Compound aqueous solubility determines the highest inhibitor concentration that can be studied. Small amount of co-solvents can be used without influencing transporter kinetics, but co-solvents have limitations (Rais *et al.*, 2008). The resource-sparing approach provides a lower inhibitor concentration range, such that transporter binding affinities of hydrophobic compounds can be evaluated.

For example, nitrendipine was a potent ASBT inhibitor with low water solubility. Figure 4 shows the concentration-dependent inhibition of taurocholate uptake by 0-200 μ M

nitrendipine. No precipitation was observed at 50 μM of nitrendipine, but was observed above it. At 50 μM , 39.7% of taurocholate uptake was reduced; no further inhibition was observed at 100 μM and 200 μM concentrations. As a result, the inhibition concentration range of nitrendipine was only extended up to 50 μM , and beyond 50 μM the drug is not soluble. Using only the drug soluble concentration range of 0–50 μM , nitrendipine K_i was 43.9 μM . This scenario for nitrendipine exemplifies the utility of the suggested conditions that accommodate a drug with low solubility.

A second example is torsemide, which, unlike nitrendipine, was found to be a nonpotent ASBT inhibitor. Figure 5 shows the inhibition profile of taurocholate uptake by 0–2500 μM torsemide. No precipitation was observed at 1000 μM torsemide, but was observed above 1000 μM . At 1000 μM , 58.4% of taurocholate uptake was reduced; no further inhibition was observed at 2500 μM . Consequently, the inhibition profile of torsemide can only be obtained up to 1000 μM . Using only the drug soluble concentration range of 0–1000 μM , torsemide K_i was 460 μM . Again, the suggested resource-sparing conditions allowed K_i of a low solubility drug to be measured.

In conclusion, experimental conditions to screen and measure accurate transporter inhibition K_i are suggested, where a low range of inhibitor concentrations can be used. This study provides a reliable recommendation for future SLC transporter inhibition study design. The approach is useful in studies where a large number of drugs are evaluated. It is advantageous in that minimal compound is needed to perform studies, especially for compounds with limited availability. In addition, this approach facilitates the evaluation of compounds with low aqueous solubility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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	Bile acid analogues	R ₁	R ₂
C1	CDCA-glu-4-amino-2-methylpyridine	-H	
C2	CDCA-glu-2-aminopyridine	-H	
C3	CDCA-glu-3- amino-6-methylpyridine	-H	
C4	CDCA-glu-3-amino-4-methylpyridine	-H	
C5	CDCA-glu-4-aminopyridine	-H	
C6	CDCA-glu-α-benzyl-2-amino-3-hydroxypyridine	-CH ₂ C ₆ H ₅	

Figure 1.
Structure of bile acid analogues. Analogues were pyridines conjugates of glutamyl-chenodeoxycholic acid.

Figure 2.

Concentration-dependent inhibition of taurocholate uptake into ASBT-MDCK monolayers by indomethacin. The reference K_i was derived from the entire inhibition profile of 0-1000 μM (black solid), and was 84.6 μM . The K_i values from the following partial inhibition profiles yielded K_i that were comparable to the reference K_i : 0-500 μM (pink solid), 0-200 μM (light blue solid), 0-100 μM (red solid), and 0-50 μM (green solid). The K_i values from the following partial inhibition profiles yielded K_i that differed from the reference K_i : 0-25 μM (black dash) and 0-10 μM (dark blue dash).

Figure 3. Comprehensive inhibitor profile to illustrate the three regions described by Murphy (Murphy, 2004). Dotted line indicates that Region A is linear.

Figure 4.

Concentration-dependent inhibition of taurocholate uptake into ASBT-MDCK monolayers by nitrendipine. Cis-inhibition studies were carried out at varying concentrations of nitrendipine (0-200 µM). Closed circles indicate observed data points, where inhibitor was soluble. Open circles indicate data points where the inhibitor was insoluble. The solid line indicates model fit to data point where inhibitor was soluble (0-50 µM). Taurocholate uptake into ASBT-MDCK cells was reduced 39.7% at 50 µM, where $K_i = 43.9 \pm 6.3$ µM.

Figure 5.

Concentration-dependent inhibition of taurocholate uptake into ASBT-MDCK monolayers by torsemide. Cis-inhibition studies were carried out at varying concentrations of torsemide (0-2500 µM). Closed circles indicate observed data points, where inhibitor was soluble. Open circle indicates datum point where the inhibitor was insoluble. The solid line indicates model fit to data point where inhibitor was soluble (0-1000 µM). Taurocholate uptake into ASBT-MDCK cells was reduced 58.4% at 1000 µM, where $K_i = 460 \pm 45$ µM.

Table 1

K_i values (μM) of water soluble, potent ASBT inhibitors as a function of inhibitor concentration range.

Concentration range (μM)	K _i values (μM)								
	C1 ^a	C2	C3	C4	C5	Fluvastatin	Cerivastatin	Mesordiazine besylate	Pitavastatin
0-5000						24.8±1.3 (99.8%)		63.5±6.6 (94.0%)	
0-1000						24.8±1.4 (96.2%)		64.9±7.3 (79.8%)	67.6±4.8 (78.6%)
0-500						24.9±1.6 (95.5%)		63.6±8.2 (62.2%)	64.9±4.8 (64.6%)
0-200						25.1±1.6 (82.9%)		52.2±8.5 (72.4%)	
0-100	4.84±0.32 ^b (93.0%) ^c	7.23±0.66 (88.8%)	12.4±1.4 (79.9%)	16.5±1.5 (77.2%)	25.8±2.0 (69.9%)	24.9±1.9 (77.0%)	52.8±10.5 (58.7%)	59.6±9.9 (52.5%)	60.9±5.3 (52.4%)
0-50	4.85±0.35 (87.4%)	7.25±0.74 (79.3%)	11.8±1.5 (70.1%)	16.0±1.7 (67.5%)	25.7±2.4 (56.8%)	27.4±2.0 (52.6%)	57.7±16.5 (40.0%)	69.9±19.5 (31.3%)	59.8±7.5 (36.5%)
0-25	4.89±0.40 (78.3%)	7.17±0.86 (66.8%)	10.7±1.5 (62.2%)	16.4±2.2 (49.7%)	26.9±3.6 (36.1%)	24.5±2.4 (40.2%)	54.5±26.1 (25.9%)		54.1±10.9 (22.7%)
0-10	5.01±0.49 (59.4%)	6.94±1.07 (48.6%)	10.7±2.2 (43.7%)	16.5±3.4 (27.8%)	22.5±4.4 (23.2%)		31.1±21.0 (16.9%)		29.7±5.9 (14.4)
0-5	5.55±0.77 (35.0%)	7.40±1.87 (30.9%)	13.7±5.4 (24.0%)	15.4±5.5 (17.6%)	29.6±22.0 (5.27%)		14.7±10.5 (15.4%)		27.9±7.3 (10.4%)
0-2.5	4.42±0.81 (27.4%)	8.41±3.64 (16.0%)	11.8±8.9 (16.4%)	16.1±12.4 (8.63%)	10.3±5.5 (5.83%)		9.34±10.17 (14.5%)		
0, 1	5.06±1.46 (11.6%)		6.38±9.3 ^d (13.9%)	17.2±41.8 (3.23%)					

For each compound, K_i values were calculated over differ ranges of inhibitor concentrations. Results are expressed as mean±S.E. and based on triplicate determinations.

^aStructures are listed in Figure 1.

^bThe reference K_i value (bold) was taken to be the K_i value obtained from the comprehensive inhibition profile.

^cValue in parentheses is the percent taurocholate inhibited at the highest inhibitor concentration.

^dThe K_i values in red were different than the reference K_i value over 30%.

Table 2

K_i values of water soluble, nonpotent ASBT inhibitors a function of inhibitor concentration range.

Concentration range (μ M)	K_i (μ M)					
	Dibucaine hydrochloride	Rosuvastatin	Pentamidine Isethionate	Diltiazem hydrochloride	Atropine	Anastrozole
0-1000	101$\pm$6 (86.7%)	117$\pm$8 (71.0%)	174$\pm$19 (74.4%)	301$\pm$28 (66.1%)	553$\pm$53 (51.6%)	745$\pm$74 (48.3%)
0-500	101 $\pm$ 7 (73.7%)	115 $\pm$ 10 (53.3%)	164 $\pm$ 20 (62.8%)	273 $\pm$ 30 (52.4%)	492 $\pm$ 118 (43.6%)	686 $\pm$ 99 (35.6%)
0-200	96.2 $\pm$ 7.7 (53.9%)	118 $\pm$ 15 (37.8%)	147 $\pm$ 23 (52.7%)	221 $\pm$ 28 (36.4%)	432 $\pm$ 171 (27.3%)	700 $\pm$ 196 (18.0%)
0-100	85.8 $\pm$ 7.6 (43.8%)	148 $\pm$ 33 (19.4%)	189 $\pm$ 40 (27.9%)	174 $\pm$ 26 (26.5%)	339 $\pm$ 213 (12.0%)	451 $\pm$ 163 (13.2%)
0-50	93.2 $\pm$ 12.5 (27.6%)	147 $\pm$ 67 (8.9%)	253 $\pm$ 126 (10.7%)	124 $\pm$ 16 (19.5%)	84.6 $\pm$ 14.2 (30.2%)	231 $\pm$ 87 (12.0%)
0, 25	140 $\pm$ 17 (10.5%)	46.7 $\pm$ 14.4 (11.7%)	134 $\pm$ 82 (10.9%)	86.4 $\pm$ 11.9 (16.0%)	155 $\pm$ 33 (9.6%)	153 $\pm$ 79 (9.73%)
0-10		196 $\pm$ 423 (1.54%)				
0, 5						

Table 3
 K_i values of low water soluble, potent ASBT inhibitors a function of inhibitor concentration range.

Concentration range (μM)	K_i (μM)					
	Latanoprost	Simvastatin	C6	Tioconazole	Isradipine	Indomethacin
0-1000	13.3$\pm$1.9 (96.2%)			34.1$\pm$2.7 (87.2%)		86.4$\pm$6.0 (89.5%)
0-500	13.6 $\pm$ 1.9 (90.0%)			33.6 $\pm$ 2.8 (87.1%)		87.8 $\pm$ 6.8 (76.4%)
0-200		19.9$\pm$2.4 (83.8%)		33.7 $\pm$ 3.1 (74.0%)	36.4$\pm$4.2 (82.5%)	84.2 $\pm$ 7.3 (59.1%)
0-100	14.3 $\pm$ 2.0 (79.4%)	19.5 $\pm$ 2.5 (75.8%)	25.2$\pm$2.6 (67.6%)	32.8 $\pm$ 3.6 (63.7%)	38.8 $\pm$ 5.1 (68.1%)	77.9 $\pm$ 8.4 (46.8%)
0-50	15.1 $\pm$ 2.4 (60.3%)	18.8 $\pm$ 2.9 (67.3%)	22.4 $\pm$ 2.4 (57.4%)	32.7 $\pm$ 4.7 (47.7%)	42.7 $\pm$ 7.4 (49.7%)	80.5 $\pm$ 13.7 (27.3%)
0-25	14.0 $\pm$ 2.8 (46.9%)	19.9 $\pm$ 4.3 (39.7%)	19.4 $\pm$ 2.5 (46.6%)	32.3 $\pm$ 6.3 (32.2%)	60.4 $\pm$ 19.6 (25.7%)	53.9 $\pm$ 8.9 (23.2%)
0-10	12.2 $\pm$ 2.9 (34.3%)	10.8 $\pm$ 1.6 (37.6%)	16.7 $\pm$ 3.2 (25.0%)		53.4 $\pm$ 35.1 (12.1%)	52.6 $\pm$ 13.6 (11.0%)
0-5	19.3 $\pm$ 6.7 (28.1%)	10.2 $\pm$ 2.8 (23.8%)	9.1 $\pm$ 1.1 (25.8%)		17.5 $\pm$ 6.6 (14.4%)	
0-2.5		9.49 $\pm$ 4.48 (14.7%)	7.91 $\pm$ 6 (17.2%)		9.55 $\pm$ 3.67 (14.5%)	
0, 1			9.15 $\pm$ 4.79 (6.65%)			

Table 4
 K_i values of low water soluble, nonpotent ASBT inhibitors as a function of inhibitor concentration range

Concentration range (μM)	K_i (μM)					
	Lansoprazole	Bendroflumethiazide	Propafenone	Ketoprofen	Bumetanide	Thiothixene
0-5000						
0-1000	120±16 (91.9%)	149±18 (79.4%)	162±15 (82.4%)	310±28 (98.0%)	341±22 (65.6%)	917±111 (83.3%)
0-500	132±19 (73.6%)	144±20 (63.6%)	169±18 (68.9%)	328±24 (64.7%)	1105±136 (11.0%)	1105±136 (11.0%)
0-200	134±25 (44.4%)	121±18 (46.6%)	186±26 (44.3%)	305±26 (49.7%)	338±28 (48.0%)	913±115 (25.7%)
0-100	86.9±14.7 (39.4%)	96.4±16.2 (37.6%)	247±58 (19.9%)	255±14 (34.2%)	299±33 (32.1%)	1030±344 (34.6%)
0-50	62.1±11.3 (29.5%)	84.3±20.7 (30.5%)	236±91 (9.53%)		343±75 (15.6%)	
0-25	40.9±7.09 (30.2%)	166±115 (8.86%)	140±71 (10.4%)		232±60 (12.2%)	
0, 10	103±37 (5.99%)		1970±29900 (-2.53)		211±108 (7.1%)	

Table 5

K_i values of ASBT inhibitors estimated from one concentration.

Potent inhibitor	Reference K_i^a (μ M)	0, 50 b (μ M)	Nonpotent inhibitor	Reference K_i (μ M)	0, 500 c (μ M)
C1	4.84	4.52 $\pm$ 0.58 (87.4%)	Dibucaine hydrochloride	101	116 $\pm$ 3 (73.7%)
C2	7.23	7.79 $\pm$ 0.75 (79.3%)	Rosuvastatin	117	123 $\pm$ 11 (71.0%)
C3	12.4	14.8 $\pm$ 5.2 (70.1%)	Lansoprazole	120	116 $\pm$ 2 (73.6%)
C4	16.5	15.6 $\pm$ 2.6 (67.5%)	Bendroflumethiazide	149	188 $\pm$ 21 (63.6%)
Simvastatin	19.9	15.8 $\pm$ 3.4 (67.3%)	Propafenone	162	146 $\pm$ 27 (68.9%)
Latanoprost	13.3	17.3 $\pm$ 2.7 (60.3%)	Pentamidine isethionate	174	194 $\pm$ 27 (62.8%)
C6	25.2	24.1 $\pm$ 2.3 (57.4%)	Diltiazem hydrochloride	301	296 $\pm$ 22 (52.4%)
C5	25.8	23.8 $\pm$ 2.7 (56.8%)	Ketoprofen	310	336 $\pm$ 26 (49.7%)
Fluvastatin	24.8	29.7 $\pm$ 2.4 (52.6%)	Bumetanide	341	354 $\pm$ 35 (48.0%)
Tioconazole	34.1	33.0 $\pm$ 6.2 (47.7%)	Atropine	535	427 $\pm$ 29 (43.6%)
Isradipine	36.4	32.7 $\pm$ 2.7 (49.7%)	Anastrozole	745	601 $\pm$ 69 (35.6%)
Cerivastatin	52.2	47.4 $\pm$ 22.5 (40.0%)	Thiothixene	917	905 $\pm$ 105 (25.7%)
Pitavastatin	67.6	56.1 $\pm$ 4.4 (36.5%)			
Mesoridazine besylate	63.5	59.5 $\pm$ 7.3 (31.3%)			
Indomethacin	86.4	86.9 $\pm$ 12.5 (27.3%)			

^aThe reference K_i value was from Table 1-4.

^bBased on analysis of 15 ASBT potent inhibitors, result showed K_i estimation using one inhibitor screening concentration 50 μ M yield an accurate K_i values that were less than 30% difference from the reference K_i value.

^cBased on analysis of 12 ASBT potent inhibitors, result showed K_i estimation using one inhibitor screening concentration 500 μ M yield an accurate K_i values that were less than 30% difference from the reference K_i value.

K_i estimations using other concentrations are in supplementary information Table 1-4.

Table 6

K_i values of OCTN2 inhibitors as function of inhibitor concentration range.

Concentration range (μ M)	K_i values (μ M) of water soluble compound							K_i values (μ M) low water soluble compound		
	D-carnitine	Vincristine sulfate	Propantheline bromide	Cetirizine dihydrochloride	Daunorubicin hydrochloride	Cerivastatin	Carvedilol	Propafenone		
0-1500										
0-1000				80.7±8.1 (87.4%)	496±41 (66.4%)	422±43 (55.5%)				74.4±6.1 (92.5%)
0-500		15.9±1.4 (95.1%)		79.6±8.8 (79.2%)	484±48 (56.0%)	340±30 (49.9%)				76.1±6.7 (84.0%)
0-200	12.2±0.5 (89.9%)	15.9±1.5 (87.2%)	20.3±4.0 (84.1%)	77.6±10.1 (68.1%)	443±56 (40.8%)	326±42 (30.0%)				79.0±7.8 (61.7%)
0-100	12.1±0.5 (83.7%)	15.7±1.6 (75.0%)	19.5±4.3 (76.9%)	87.1±15.2 (43.1%)	375±63 (31.2%)			10.7±1.6 (86.5%)		77.3±10.4 (47.2%)
0-50	12.0±0.6 (73.3%)	14.6±1.5 (69.4%)	18.4±4.9 (66.7%)	75.5±16.8 (29.1%)	424±122 (13.7%)	300±107 (10.2%)	10.6±1.8 (72.1%)			87.4±20.4 (28.7%)
0-20	11.8±0.7 (58.5%)	14.5±1.2 (48.4%)	17.1±6.3 (42.0%)	47.7±12.8 (21.9%)		156±66 (7.87%)	9.32±1.83 (57.5%)			138±81 (8.71%)
0-10	11.5±0.9 (36.4%)	14.9±2.0 (31.1%)								
0-5	10.2±1.1 (24.4%)						7.29±1.98 (28.1%)			
0-2.5	9.0±1.5 (15.8%)						3.85±1.07 (23.9%)			
0-1	8.9±4.0 (6.98%)						3.00±1.33 (18.7%)			

Table 7

Inhibition K_i values of EAAT4 inhibitors as function of inhibitor concentration range.

Concentration range (μM)	K_i (μM)	
	L-Aspartic acid	D-Aspartic acid
0-200	1.92±0.23 (85.8%)	9.54±0.75 (83.7%)
0-100	1.88±0.18 (86.1%)	9.21±0.57 (82.2%)
0-50	1.83±0.14 (84.8%)	8.95±0.57 (72.0%)
0-20	1.77±0.12 (77.4%)	8.58±0.59 (61.3%)
0-10	1.72±0.14 (70.2%)	8.72±0.76 (34.8%)
0-5	2.01±0.13 (45.9%)	9.06±1.51 (15.0%)
0-2.5		

Table 8

K_i values of OCTN2 and EAAAT1 inhibitors estimated from one concentration.

Transporter	Reference K_i^a (μ M)	0, 50 (μ M)	Reference K_i^a (μ M)	0, 500 (μ M)		
OCTN2	Carvedilol	10.7	12.9 $\pm$ 3.8 (72.1%)	Cerivastatin	422	340 $\pm$ 46 (49.9%)
	D-carnitine	12.2	12.2 $\pm$ 0.5 (73.3%)	Daunorubicin hydrochloride	496	488 $\pm$ 47 (40.8%)
	Vincristine sulfate	15.9	14.7 $\pm$ 3.1 (69.4%)			
	Propantheline bromide	20.3	16.7 $\pm$ 4.5 (66.7%)			
	Cetirizine dihydrochloride	80.7	82.2 $\pm$ 11.3 (29.1%)			
	Propafenone	74.4	83.2 $\pm$ 16.8 (28.7%)			
EAAT4		Reference K_i^a (μ M)	0, 20 (μ M)			
	L-aspartic acid	1.92	3.03 $\pm$ 0.30 (84.8%)			
	D-aspartic acid	9.54	8.40 $\pm$ 0.81 (61.3%)			

^aThe reliable K_i value was from Table 6.

^bThe reliable K_i value was from Table 7.