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Title page

Inhibition of bile acid transport across NTCP and BSEP co-expressing LLC-PK1 cells by cholestasis-inducing drugs.

Sachiko Mita, Hiroshi Suzuki, Hidetaka Akita, Hisamitsu Hayashi, Reiko Onuki, Alan F. Hofmann and Yuichi Sugiyama

Graduate School of Pharmaceutical Sciences (SM, HS, HA, HH, RO and YS), The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan., Present address: Graduate School of Pharmaceutical Sciences (HA), Hokkaido University, Nishi 6, Kita 12, Kita-ku, Sapporo 060-0812, Japan., Department of Medicine (AH), University of California, La Jolla CA, CA 92093-0813.

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Running title page:

Running title: Inhibition of transport across NTCP/BSEP co-expressing cells

Corresponding author : Yuichi Sugiyama

Address: Department of Molecular Pharmacokinetics, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Phone: +81-3-5841-4770

Fax: +81-3-5841-4667

E-mail: sugiyama@mol.f.u-tokyo.ac.jp

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Abbreviations: NTCP, Na^+/taurocholate cotransporting polypeptide (SLC10A1); BSEP, bile salt export pump (ABCB 11); OATP, organic anion transporting polypeptide; MRP, multidrug-resistance protein; MOI, multiplicity of infection; GFP, green fluorescent protein; TEER, transepithelial electrical resistance; PBS, phosphate buffered saline; PS, permeability surface area product

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Abstract

Vectorial transport of bile acids across hepatocytes is a major driving force for bile flow, and bile acid retention in the liver causes hepatotoxicity. The basolateral and apical transporters for bile acids are thought to be targets of drugs that induce cholestasis. Previously, we constructed polarized LLC-PK1 cells that express both a major bile acid uptake transporter human NTCP and the bile acid efflux transporter human BSEP and showed that monolayers of such cells can be used to characterize vectorial transcellular transport of bile acids. In the present study, we investigated whether cholestasis-inducing drugs could inhibit bile acid transport in such cells. Because fluorescent substrates allow the development of a high throughput screening method, we examined the transport by NTCP and BSEP of fluorescent bile acids as well as taurocholate. The aminofluorescein-tagged bile acids, CDCGamF and CGamF, were substrates of both NTCP and BSEP, and their basal-to-apical transport rates across co-expressing cell monolayers were 4.3-4.5 times those of the vector control, although smaller than for taurocholate. The well-known cholestatic drugs, rifampicin, rifamycin SV, glibenclamide and cyclosporin A, reduced the basal-to-apical transport and the

apical efflux clearance of taurocholate across NTCP and BSEP co-expressing cell monolayers. Further analysis indicated that the drugs inhibited both NTCP and BSEP. Our study suggests that such co-expressing cells can provide a useful system for the identification of inhibitors of these two transport systems, including potential drug candidates.

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Introduction

Hepatotoxic adverse effects, often indicated by cholestasis, are a concern for every drug, and severe hepatotoxicity may cause a drug to be withdrawn from the market. Biliary excretion of bile acids is one of the principal driving forces for bile formation by generating an osmotic driving force favoring influx of water and electrolytes through the paracellular space (Wheeler et al., 1968) (Wheeler, 1972). The transcellular transport is mediated by transporter proteins located on the sinusoidal (basolateral) and canalicular (apical) membrane of hepatocytes (Meier and Stieger, 2002; Trauner and Boyer, 2003). The basolateral Na^+-taurocholate cotransporting polypeptide (NTCP/SLC10A1) transports bile acids from the space of Disse into hepatocytes (Hagenbuch et al., 1991; Boyer et al., 1994). Human NTCP accepts most physiological bile acids and some organic anions, such as estrone-3-sulfate and bromosulfophthalein (Meier et al., 1997). Sodium-independent uptake of bile acids is carried out by members of the organic anion transporting polypeptide family, such as rat Oatp1a1 and human OATP1B1. Although there are several carrier proteins capable of transporting bile acids, much evidence suggests, at least in the rodent, that

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NTCP-mediated transport accounts for a large part of the total bile acid uptake (Wolkoff and Cohen, 2003). At the canalicular membrane, the efflux of bile acids by the bile salt export pump (BSEP/ABCB11) mediates concentrative excretion (Boyer et al., 1994; Gerloff et al., 1998; Noe et al., 2002). Mutations of BSEP in humans causes primary familial intrahepatic cholestasis type II (PFICII), a fatal condition. (Strautnieks et al., 1998).

One mechanism for cholestasis is thought to be inhibition of hepatocyte transport systems for bile acids and other organic anions by drugs. The inhibitory effects of such drugs on the uptake and efflux of bile acids have been studied using isolated and primary cultured hepatocyte or canalicular membrane vesicles (Kukongviriyapan and Stacey, 1991), as well as the isolated perfused liver (Bolder et al., 1999). Recently, NTCP and BSEP which generate bile salt-dependent bile flow, have been shown to be possible target molecules for cholestatic drugs (Kim et al., 1999; Stieger et al., 2000; Akita et al., 2001; Bohan and Boyer, 2002).

Previously, we constructed NTCP and BSEP co-expressing LLC-PK1 cells as an *in vitro* model of the vectorial transcellular transport of bile acids in hepatocytes

(Mita et al., 2006). This approach is useful for the screening of choleretic bile acids, which are good substrates of these transporters. A second use of this system is to identify inhibitors of these transporters which might have cholestatic effects in vivo. The method should also be useful for defining structure-transport activity relationships of bile acids. In the present study, we assessed the inhibitory effects of cholestasis-inducing drugs on transport across co-expressing cells with the aim of developing a screening system for cholestatic compounds. We compared the transport of fluorescent bile acid derivatives with that of taurocholate in the hope that such fluorescent compounds would be efficiently transported and thereby permit the development of a high throughput screening method for detecting the inhibitory effects of drug candidates.

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Materials and Methods

Chemicals

[^{3}H]taurocholic acid (2 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA). Unlabeled taurocholic acid was obtained from Sigma Chemical Co. (St. Louis, MO). Unlabeled cholic acid was purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Unlabeled ursodeoxycholic acid, tauroursodeoxycholic acid and glycoursodeoxycholic acid were kindly provided by Mitsubishi Pharma (Osaka, Japan). Fluorescent bile acids (cholylglycylamidofluorescein (CGamF), cholylamidofluorescein(CamF), chenodeoxycholylglycylamidofluorescein(CDCGamF), ursodeoxycholyl-(Nε-NBD)-lysine(UDC-L-NBD), 7β-NBD-cholyltaurine(7β-NBD-NCT)) were synthesized in the laboratory of Alan F. Hofmann as described previously (Sorscher et al., 1992; Holzinger et al., 1998). The following compounds were obtained from Sigma-Aldrich (St. Louis, MO): cyclosporin A, rifampicin, rifamycin SV and glibenclamide. All other chemicals used were commercially available and of reagent grade.

Cell culture and transfection

Human NTCP- and human BSEP-expressing LLC-PK1 cells were established and maintained as described previously (Mita et al., 2006). Briefly, parental LLC-PK1 cells were grown in M199 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1 % antibiotic-antimycotic (Gibco; 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B) at 37 C under 5 % CO_2. Full length human NTCP cDNA was subcloned into pcDNA3.1 (Invitrogen) and transfected into LLC-PK1 cells with FuGENE 6 (Roche Diagnostics Corporation, Indianapolis, IN) according to the manufacturer's instructions. Transfectants expressing NTCP were selected with G418 (800 µg/ml) and the clone with the highest NTCP activity was screened by the uptake activity for taurocholate. The BD Adeno-X™ Adenoviral Expression System (BD Biosciences, Palo Alto, CA) was used to establish the recombinant adenovirus encoding human BSEP (Hayashi et al., 2005). 48 hrs before each experiment, LLC-PK1 cells were infected by the recombinant adenoviruses or control viruses containing green fluorescent protein (GFP) at a multiplicity of infection (MOI) of 100.

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Transport Studies

NTCP- or mock- transfected LLC-PK1 cells were seeded on Transwell membrane inserts (pore size of 3 μm; Falcon, Bedford, MA) in 12-well plates at a density of 1.4×10^5 cells per insert for transcellular transport studies, cultured at confluence for 2 days, and infected by recombinant adenovirus containing cDNAs for BSEP or GFP (100 MOI). For uptake studies, NTCP- or mock- transfected LLC-PK1 cells were seeded on 12-well plates and cultured without viral infection. Cells were harvested 48 hrs after infection, and expression of NTCP was induced by 10 mM sodium butyrate for 24 hrs (Cui et al., 1999). To evaluate the integrity of the monolayer, transepithelial electrical resistance (TEER) was measured using a Millicell-ERS (Millipore Co., Bedford, Mass.). The monolayers' TEERs before the experiments were 200-300 Ω cm2. Then, cells were washed with transport buffer (118 mM NaCl, 23.8 mM $NaHCO_3$, 4.83 mM KCl, 0.96 mM KH_2PO_4, 1.20 mM $MgSO_4$, 12.5 mM HEPES, 5 mM glucose, and 1.53 mM $CaCl_2$ adjusted to pH 7.4). Subsequently, ^3H labeled taurocholate or fluorescent bile acids were added to the transport buffer in the basal compartment (950 μl) for transcellular transport studies or 12-well plates for

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uptake studies. After the times indicated, the amount of substrates in the opposite apical compartment was measured by the radioactivity for taurocholate, or by the absorbance at 490nm for fluorescent bile acids using a Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA). Potential inhibitors were added to both apical and basal compartments 30 minutes before the transport study. The accumulated radioactivity in the cell was determined at the end of the experiments by lysing the cells with 500 µl 0.2N NaOH and measuring the radioactivity in the cell lysates. Aliquots (50 µl) of cell lysate were used to determine protein concentrations by the method of Lowry (Lowry, 1951) with bovine serum albumin as a standard. The apparent intracellular concentration of taurocholate (C_{cell}) was determined by assuming that the cellular volume per mg cellular protein was 4 µl.

Data Analysis

The kinetic parameters were defined as follows: PS_{b-a} (µl/min/mg protein) is the permeability-surface area product (PS) for the basal-to-apical clearance defined for the ligand concentration in the medium [C_{med} (pmol/µl)] (Fig. 1). PS1 (µl/min/mg protein) is the PS product for the influx of ligand across the basal membrane, which is defined

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for C_{med}, PS3 (μl/min/mg protein) is the PS product for the efflux of ligand across the apical membrane, which is defined for the ligand concentration in the cells [(C_{cell} (pmol/μl)], PS2 (μl/min/mg protein) is the PS product for the efflux of ligand across the basal membrane from the cell to the basal compartment, which is defined for C_{cell}, respectively. PS_{b-a} is given as a hybrid parameter consisting of PS1, PS2 and PS3 (Mita et al., 2005): $PS_{b-a} = PS1 \cdot PS3 / (PS2 + PS3)$

In this study, PS_{b-a} and PS3 of taurocholate were calculated as follows:

$$PS_{b-a} = V_{apical} / C_{med}$$

$$PS3 = V_{apical} / C_{cell}$$

where V_{apical} (pmol/min/mg protein) is the increasing velocity of taurocholate in the apical compartment. V_{apical} was determined by analyzing the transcellular transport for 1 h. Since the amount of taurocholate transported increased linearly as a function of time over the 2 h period and the intracellular concentration was constant during the incubation periods (Mita et al., 2006), we hypothesized that the initial transport velocity could be determined from the slope over the period 0-1 h.

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Results

Transcellular transport of fluorescent bile acids

To identify a good substrate of NTCP and BSEP for the functional probe in the inhibition study, the basal-to-apical transport clearance (PS_{b-a}) of taurocholate and fluorescent bile acids across NTCP and BSEP co-expressing LLC-PK1 cells (LLC-NTCP/BSEP) was compared (Fig. 1, 2 and TABLE 1). The PS_{b-a} of [^{3}H] taurocholate (1 μM) and aminofluorescein-tagged glycochenodeoxycholate and glycocholate (CDCGamF and CGamF) (10 μM), in LLC-NTCP/BSEP were significantly greater than that in control LLC-PK1 cells and NTCP-expressing LLC-PK1 (LLC-NTCP) cells, indicating that these bile acids are good substrates of BSEP. On the other hand, for aminofluorescein-tagged cholate (CamF), lysine-NBD-tagged ursodeoxycholate (UDC-L-NBD) (10 μM) and 7β-NBD-NCT (10 μM), transport by NTCP and BSEP was barely detectable.

The value of PS_{b-a} in LLC-NTCP/BSEP was the highest when [^{3}H] taurocholate was used. The absolute PS_{b-a} value of all the fluorescent bile acids was less than 1/6 that of [^{3}H] taurocholate. The ratio of the PS_{b-a} value of LLC-NTCP/BSEP to that of

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LLC-NTCP was 4.8-fold for [^{3}H] taurocholate, 2.6-fold for CGamF and 1.8-fold for CDCGamF (Fig.2 bottom graph). These results indicate that taurocholate is a better substrate for the subsequent inhibition studies. Furthermore, labeled compounds are better tools for measuring the intracellular content of the compounds which is important for this study because it is needed to calculate the efflux clearance across the apical membrane (PS3).

Inhibitory effects of a series of cholestasis-inducing drugs

Next, the inhibitory effect of cholestasis-inducing drugs on the basal-to-apical transport clearance PS_{b-a} of taurocholate was examined (Fig. 3). PS_{b-a} was reduced by 100 μM rifampicin and rifamycin SV to 50 % of the control level and 10 μM glibenclamide reduced it to 70 % (Fig. 3A). The intracellular concentration (C_{cell}) of taurocholate was determined for each compound at the end of the experiment (Fig.3B). The C_{cell} of taurocholate was increased by 100 μM rifampicin to 160 % of that of control cells (no inhibitor added). However, 100 μM rifamycin caused a 10 % reduction in the apparent cellular concentration of taurocholate and 10 μM glibenclamide led to a 30 % reduction in C_{cell} compared with control cells. Calculation

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of the efflux clearance across the apical membrane PS3 using the measured C_{cell} showed that 100 μM rifampicin, rifamycin SV and glibenclamide produced a 70%, 44% and 63% inhibition of PS3, respectively, indicating that the drugs inhibited the efflux of taurocholate by BSEP located in the apical membrane (Fig. 3C). When the efflux process is the only target of inhibition, C_{cell} should be increased by the drugs compared with untreated LLC-NTCP/BSEP cells. However, as mentioned above, C_{cell} was reduced by rifamycin SV and glibenclamide. This means that not only BSEP but also NTCP was inhibited in this experiment as far as rifamycin SV and glibenclamide were concerned. Of course, from these data, we cannot exclude the possibility that inhibition of NTCP is also involved in the case of rifampicin. 100 μM of Captopril and Cimetidine did not affect the transport and C_{cell} of taurocholate significantly (Fig. 3A-C).

Kinetics of the inhibition by cyclosporin A

In order to evaluate the inhibition kinetics involved in the transcellular transport when both the uptake and efflux processes are affected, cyclosporin A, an inhibitor of both NTCP and BSEP, was also examined (Fig. 4). The basal-to-apical

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transport clearance PS_{b-a} of taurocholate was inhibited by cyclosporin A (and its metabolites) with a K_i value of 1.0 ± 0.2 (μM) (Fig. 4A). The intracellular concentration C_{cell} determined at the end of each experiment was also reduced by cyclosporin A, suggesting that uptake of taurocholate by NTCP was inhibited by cyclosporin A treatment. The inhibition of the uptake process was confirmed by evaluating the inhibitory effect of cyclosporin A on the uptake of taurocholate into only NTCP-expressing LLC-PK1 cells. The K_i value was determined as 0.27 ± 0.06 (μM) (Fig. 4C). At the same time, the calculated PS3 showed a reduction depending on the concentration of cyclosporin A, probably because of inhibition of BSEP by cyclosporin A (and/or its metabolites)(Fig. 4B). These results showed that both uptake and efflux processes are affected by 1-10 μM cyclosporin A.

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Discussion

In the present study, we assessed the inhibitory effects of cholestasis-inducing drugs on bile acid transport across LLC-NTCP/BSEP cells. Our hope was to develop a rapid screening system for drugs that inhibit these transporters.

Initially, we focused on the fluorescent bile acids as a probe of NTCP and BSEP function and investigated whether they were substrates of NTCP and BSEP using LLC-NTCP/BSEP. The fluorescent derivatives of bile acids used in this study were originally synthesized for the functional analysis of bile salt transport systems in isolated hepatocytes, immortalized cell lines derived from hepatocytes or in vivo (Holzinger et al., 1998; Cantz et al., 2000). Direct demonstration of the transport of these bile acids via NTCP or BSEP has not yet been carried out, although sodium-dependent uptake for CGamF has been observed (Maglova et al., 1995).

Basal-to-apical transport across LLC-NTCP/BSEP was observed in a rank order of taurocholate > CGamF > CDCGamF and no significant transport was observed for UDC-L-NBD, CamF and 7β-NBD-NCT (Fig. 2). This order was similar to that of the maximum output rate of the bile acids in an isolated liver perfusion study:

taurocholate 22.7 > CGamF 14.1 > CamF 7.7 > UDC-L-NBD 1.1 (nmol/g liver/min) (Holzinger et al., 1998). This result supports the hypothesis that the transport of fluorescent derivatives of cholic acid in hepatocytes is mainly mediated by NTCP and BSEP, and showed that our *in vitro* system can reflect the physiological function of these transporters as far as transcellular transport is concerned. As for UDC-L-NBD, although uptake by LLC-NTCP inhibited by taurocholate was observed using fluorescent microscopy (data not shown), no significant transcellular transport across LLC-NTCP/BSEP was observed. This might be because of the nature of this bile salt which is sequestered in the cells (Holzinger et al., 1998; Cantz et al., 2000). Nonetheless, fluorescent bile acids were transported in this system. Better fluorescent bile acids which will be transported as efficiently as taurocholate will make excellent tools for high-throughput screening.

Inhibition of BSEP by cholestasis-inducible drugs is one of the most frequently reported mechanisms of drug-induced cholestasis (Bohan and Boyer, 2002). Among such drugs, rifampicin, rifamycin SV, glibenclamide and cyclosporin A (Stieger et al., 2000) (Byrne et al., 2002) were used in this study. As shown in figure 3, PS3, the

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efflux clearance that reflects the function of BSEP, was reduced by all the drugs examined. The concentration needed for 50% inhibition of PS3 is between 10 and 100 μM for rifampicin and glibenclamide and approximately 100 μM for rifamycin SV. The reported *Ki* values for the inhibition of taurocholate uptake into human BSEP-expressing membrane vesicles are 31 μM for rifamycin SV and 31 μM for glibenclamide (Byrne et al., 2002). For rifampicin, only the *Ki* value of 12 μM for rat Bsep is available (Stieger et al., 2000). Compared with these values, the inhibitory concentration was higher in our LLC-NTCP/BSEP cells than in other studies that used vesicles. One possible explanation for this is that the protein unbound concentrations of the drugs in cytoplasm are lower than in the medium because the drugs may not penetrate the plasma membrane efficiently and the drugs may also bind to intracellular proteins.

Inhibition of BSEP in the transcellular transport of taurocholate should be accompanied by an increase in the intracellular concentration of taurocholate. However, the increase was observed only in the case of rifampicin. This means that rifamycin SV and glibenclamide also inhibited NTCP-mediated uptake at the same time.

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Recently, it has been reported that 100 μM rifampicin or rifamycin SV can reduce the uptake of taurocholate by rat Ntcp to 60% of the total uptake (Fattinger et al., 2000). However, in this study, following incubation with 100 μM rifampicin and rifamycin SV, the reduction in C_{cell} was not as much as 60%. An increase by rifampicin and only a slight decrease by rifamycin SV were observed (Fig. 3). If we hypothesize there is no species difference in the inhibitory effect of these drugs between humans and rats, this result indicates that the inhibition of NTCP and BSEP balanced each other.

Captopril and cimetidine are reported to cause cholestasis (Mohi-ud-din and Lewis, 2004). However, their interactions with bile acid transporters have not been reported (Cimetidine does not have a significant inhibitory effect on BSEP (Wang et al., 2003)) and other pathways are postulated as a possible mechanism. Corresponding to this, both captopril and cimetidine did not affect the transcellular transport and C_{cell} of taurocholate at 100 μM (Fig. 3A-C).

The inhibitory effect of cyclosporin A, an inhibitor of both NTCP and BSEP, was also examined as well as the inhibition kinetics of the transcellular transport when both the uptake and efflux processes are affected (Fig. 4). The basal-to-apical

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transport clearance PS_{b-a} was inhibited with a value K_i of 1.0±0.2 (μM). The efflux clearance PS3 was inhibited depending on the medium concentration of cyclosporin A. Although estimation of the exact *Ki* value is difficult, it appeared to be close to the reported *Ki* value for the inhibition of the uptake of taurocholate into human BSEP expressing membrane vesicles by cyclosporin A (9.5μM) (Byrne et al., 2002).

The question which we must consider here is to what extent inhibition of the uptake and efflux process affects the net transcellular transport. It was estimated that the K_i value for the inhibitory effect of cyclosporin A on the uptake of taurocholate into human NTCP expressing LLC-PK1 cells was 0.27±0.06 (μM) (Fig. 4). This value is similar to the K_i value for PS_{b-a}, which suggests that the inhibition of PS_{b-a} reflects the inhibition of the uptake process mediated by NTCP. Although we do not know whether NTCP or BSEP is important for the cyclosporin A-induced cholestasis in physiological situations, the result of this study and the following aspects support the importance of NTCP. The transcellular transport clearance can be expressed as the hybrid of each transmembrane transport clearance as described in the Data Analysis section: $PS_{b-a} = PS1 \cdot PS3 / (PS2 + PS3)$. If the efflux clearance across the apical

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membrane, PS3, is far greater than that across the basal membrane, PS2, PS_{b-a} is nearly equal to PS1. Thus, inhibition of the uptake process, PS1, can lead to inhibition of transcellular transport more easily than inhibition of the efflux process, PS3. The effect of inhibition of the uptake and/or efflux process on the net transcellular transport is simulated in Fig.5. The ratio of PS2:PS3 is substituted by the measured value in the isolated rat liver perfusion studies PS3:69.2 ± 6.3 (μl/min/g liver) PS2:8.4 ± 0.6 (μl/min/g liver)(Akita et al., 2002). If the efficacy of the inhibitory effect of the drug is similar for the uptake and efflux processes, inhibition of uptake is more effective than that of efflux as far as the net transcellular transport is concerned.

BSEP has been extensively studied as a target molecule of drug-induced cholestasis because it plays a role in the regulation of the concentration of bile acids in hepatocytes. Inhibition of BSEP leads to an intracellular accumulation of bile acids resulting in cellular damage because of their cytotoxic effects. However, there should be some cases where the inhibition of NTCP plays a major role in drug-induced cholestasis considering the importance of the uptake process in the overall transcellular transport of bile acids as described above. Cyclosporin A induced cholestasis may be

the one of those. The plasma bile salt concentration was increased in rats following administration of cyclosporin A 10mg/kg (Stone et al., 1987) indicating that cyclosporin A inhibits the uptake of bile acids from the portal blood into hepatocytes. Moreover, there was no change in liver histology in the cholestasis caused by cyclosporin A (Kukongviriyapan and Stacey, 1991) suggesting the cytotoxicity brought about by intracellular bile acids here is not very severe. These facts indicate the importance of the inhibition of NTCP at least in the case of cyclosporin A induced cholestasis.

In conclusion, LLC-NTCP/BSEP cells were used for the detection of the inhibitory effect of drugs on NTCP and/or BSEP although the quantitative evaluation of the inhibitory effect on BSEP appears to be difficult at the present time compared with transport studies using membrane vesicles. Furthermore, in order to predict the effect of drugs under physiological conditions, we must consider the drug metabolites which sometimes significantly inhibit BSEP (Funk et al., 2001). As there is only a minor quantity of hepatic enzymes involved in drug metabolism in LLC-PK1 cells (Gonzalez and Tarloff, 2004), the inhibitory effects observed in this study are speculated to be those produced by drugs in their unchanged forms. The additional expression of such

enzymes and uptake transporters of drugs, such as OATP1B1 and OATP1B3, will provide a more useful tool for quantitative measurement of the inhibitory effect on BSEP.

References

Akita H, Suzuki H, Ito K, Kinoshita S, Sato N, Takikawa H and Sugiyama Y (2001) Characterization of bile acid transport mediated by multidrug resistance associated protein 2 and bile salt export pump. *Biochim Biophys Acta* **1511:**7-16.

Akita H, Suzuki H and Sugiyama Y (2002) Sinusoidal efflux of taurocholate correlates with the hepatic expression level of Mrp3. *Biochem Biophys Res Commun* **299:**681-687.

Bohan A and Boyer JL (2002) Mechanisms of hepatic transport of drugs: implications for cholestatic drug reactions. *Semin Liver Dis* **22:**123-136.

Bolder U, Trang NV, Hagey LR, Schteingart CD, Ton-Nu HT, Cerre C, Elferink RP and Hofmann AF (1999) Sulindac is excreted into bile by a canalicular bile salt pump and undergoes a cholehepatic circulation in rats. *Gastroenterology* **117:**962-971.

Boyer JL, Ng OC, Ananthanarayanan M, Hofmann AF, Schteingart CD, Hagenbuch B, Stieger B and Meier PJ (1994) Expression and characterization of a functional rat liver Na+ bile acid cotransport system in COS-7 cells. *Am J Physiol* **266:**G382-387.

Byrne JA, Strautnieks SS, Mieli-Vergani G, Higgins CF, Linton KJ and Thompson RJ (2002) The human bile salt export pump: characterization of substrate specificity and identification of inhibitors. *Gastroenterology* **123:**1649-1658.

Cantz T, Nies AT, Brom M, Hofmann AF and Keppler D (2000) MRP2, a human conjugate export pump, is present and transports fluo 3 into apical vacuoles of Hep G2 cells. *Am J Physiol Gastrointest Liver Physiol* **278:**G522-531.

Cui Y, Konig J, Buchholz JK, Spring H, Leier I and Keppler D (1999) Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. *Mol Pharmacol* **55:**929-937.

Fattinger K, Cattori V, Hagenbuch B, Meier PJ and Stieger B (2000) Rifamycin SV and rifampicin exhibit differential inhibition of the hepatic rat organic anion transporting polypeptides, Oatp1 and Oatp2. *Hepatology* **32:**82-86.

Funk C, Ponelle C, Scheuermann G and Pantze M (2001) Cholestatic potential of troglitazone as a possible factor contributing to troglitazone-induced hepatotoxicity: in vivo and in vitro interaction at the canalicular bile salt export pump (Bsep) in the rat. *Mol Pharmacol* **59:**627-635.

Gerloff T, Stieger B, Hagenbuch B, Madon J, Landmann L, Roth J, Hofmann AF and Meier PJ (1998) The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver. *J Biol Chem* **273:**10046-10050.

Gonzalez RJ and Tarloff JB (2004) Expression and activities of several drug-metabolizing enzymes in LLC-PK1 cells. *Toxicol In Vitro* **18:**887-894.

Hagenbuch B, Stieger B, Foguet M, Lubbert H and Meier PJ (1991) Functional expression cloning and characterization of the hepatocyte Na+/bile acid cotransport system. *Proc Natl Acad Sci U S A* **88:**10629-10633.

Hayashi H, Takada T, Suzuki H, Akita H and Sugiyama Y (2005) Two common PFIC2 mutations are associated with the impaired membrane trafficking of BSEP/ABCB11. *Hepatology* **41:**916-924.

Holzinger F, Schteingart CD, Ton-Nu HT, Cerre C, Steinbach JH, Yeh HZ and Hofmann AF (1998) Transport of fluorescent bile acids by the isolated perfused rat liver: kinetics, sequestration, and mobilization. *Hepatology* **28:**510-520.

Kim RB, Leake B, Cvetkovic M, Roden MM, Nadeau J, Walubo A and Wilkinson GR (1999) Modulation by drugs of human hepatic sodium-dependent bile acid transporter (sodium taurocholate cotransporting polypeptide) activity. *J Pharmacol Exp Ther* **291:**1204-1209.

Kukongviriyapan V and Stacey NH (1991) Chemical-induced interference with hepatocellular transport. Role in cholestasis. *Chem Biol Interact* **77:**245-261.

Lowry OH, Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265-275.

Maglova LM, Jackson AM, Meng XJ, Carruth MW, Schteingart CD, Ton-Nu HT, Hofmann AF and Weinman SA (1995) Transport characteristics of three fluorescent conjugated bile acid analogs in isolated rat hepatocytes and couplets. *Hepatology* **22:**637-647.

Meier PJ, Eckhardt U, Schroeder A, Hagenbuch B and Stieger B (1997) Substrate specificity of sinusoidal bile acid and organic anion uptake systems in rat and human liver. *Hepatology* **26:**1667-1677.

Meier PJ and Stieger B (2002) Bile salt transporters. *Annu Rev Physiol* **64:**635-661.

Mita S, Suzuki H, Akita H, Hayashi H, Onuki R, Hofmann AF and Sugiyama Y (2006) Vectorial transport of unconjugated and conjugated bile salts by monolayers of LLC-PK1 cells doubly transfected with human NTCP and BSEP or with rat Ntcp and Bsep. *Am J Physiol Gastrointest Liver Physiol* **290:**G550-556.

Mita S, Suzuki H, Akita H, Stieger B, Meier PJ, Hofmann AF and Sugiyama Y (2005) Vectorial transport of bile salts across MDCK cells expressing both rat Na+-taurocholate cotransporting polypeptide and rat bile salt export pump. *Am J Physiol Gastrointest Liver Physiol* **288:**G159-167.

Mohi-ud-din R and Lewis JH (2004) Drug- and chemical-induced cholestasis. *Clin Liver Dis* **8:**95-132, vii.

Noe J, Stieger B and Meier PJ (2002) Functional expression of the canalicular bile salt export pump of human liver. *Gastroenterology* **123:**1659-1666.

Sorscher S, Lillienau J, Meinkoth JL, Steinbach JH, Schteingart CD, Feramisco J and Hofmann AF (1992) Conjugated bile acid uptake by Xenopus laevis oocytes induced by microinjection with ileal Poly A+ mRNA. *Biochem Biophys Res Commun* **186:**1455-1462.

Stieger B, Fattinger K, Madon J, Kullak-Ublick GA and Meier PJ (2000) Drug- and estrogen-induced cholestasis through inhibition of the hepatocellular bile salt export pump (Bsep) of rat liver. *Gastroenterology* **118:**422-430.

Stone BG, Udani M, Sanghvi A, Warty V, Plocki K, Bedetti CD and Van Thiel DH (1987) Cyclosporin A-induced cholestasis. The mechanism in a rat model. *Gastroenterology* **93:**344-351.

Strautnieks SS, Bull LN, Knisely AS, Kocoshis SA, Dahl N, Arnell H, Sokal E, Dahan K, Childs S, Ling V, Tanner MS, Kagalwalla AF, Nemeth A, Pawlowska J, Baker A, Mieli-Vergani G, Freimer NB, Gardiner RM and Thompson RJ (1998) A gene encoding a liver-specific ABC transporter is mutated in progressive familial intrahepatic cholestasis. *Nat Genet* **20:**233-238.

Trauner M and Boyer JL (2003) Bile salt transporters: molecular characterization,

function, and regulation. *Physiol Rev* **83:**633-671.

Wang EJ, Casciano CN, Clement RP and Johnson WW (2003) Fluorescent substrates of sister-P-glycoprotein (BSEP) evaluated as markers of active transport and inhibition: evidence for contingent unequal binding sites. *Pharm Res* **20:**537-544.

Wheeler HO (1972) Secretion of bile acids by the liver and their role in the formation of hepatic bile. *Arch Intern Med* **130:**533-541.

Wheeler HO, Ross ED and Bradley SE (1968) Canalicular bile production in dogs. *Am J Physiol* **214:**866-874.

Wolkoff AW and Cohen DE (2003) Bile acid regulation of hepatic physiology: I. Hepatocyte transport of bile acids. *Am J Physiol Gastrointest Liver Physiol* **284:**G175-179.

Footnotes

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To whom correspondence should be addressed:

Department of Molecular Pharmacokinetics, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

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Legends to figures

Fig. 1. Schematic diagram illustrating transcellular transport across LLC-PK1 monolayers. PS_{b-a} (μl/min/mg protein) is the permeability-surface area (PS) product for the basal-to-apical clearance defined for the ligand concentration in the medium (C_{med} (pmol/μl)). PS1 (μl/min/mg protein) is the PS product for the influx of ligand across the basal membrane, which is defined for C_{med}. PS3 (μl/min/mg protein) is the PS product for the efflux of ligand across the apical membrane, which is defined for the ligand concentration in the cells (C_{cell} (pmol/μl)). PS2 (μl/min/mg protein) is the PS product for the efflux of ligand across the basal membrane from the cell to the basal compartment, which is defined for C_{cell}, respectively.

Fig. 2. Transcellular transport of labeled and fluorescent bile acids across NTCP and BSEP co-expressing LLC-PK1 cells. [^{3}H]taurocholate (1 μM), cholylglycylamidofluorescein (CGamF), cholylamidofluorescein(CamF), chenodeoxycholylglycylamidofluorescein(CDCGamF), ursodeoxycholyl-(Nε-NBD)-lysine(UDC-L-NBD) and

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7β-NBD-cholyltaurine(7β-NBD-NCT) (10μM) across the LLC-PK1 cell monolayers was determined. *Open*, *hatched* and *closed bars* represent the basal-to-apical transcellular transport across the control (LLC), LLC-NTCP and LLC-NTCP/BSEP monolayers, respectively. *Vertical bars* represent the S.E. of three determinations. At the bottom is a graph where the transcellular transport data are expressed on the same scale.

Fig. 3. Inhibitory effects of cholestasis-inducing drugs on the transport of taurocholate across NTCP and BSEP co-expressing LLC-PK1 cells. Basal-to-apical transport clearance $PS_{b\text{-}a}$ (A), intracellular concentration C_{cell} (B) and apical efflux clearance PS3 (C) of taurocholate in LLC-PK1, LLC-NTCP and LLC-NTCP/BSEP cell monolayers were determined at 60 min (*closed bars*). Inhibitory effects of 10 or 100 μM concentrations of various drugs on LLC-NTCP/BSEP were studied (*open and hatched bars*). The drugs were added to the apical and basal compartment 30 minutes before applying taurocholate.

Fig. 4. Inhibition of the transport of taurocholate by cyclosporin A. Various concentrations of cyclosporin A were added to the apical and basal compartment of LLC-NTCP/BSEP (A, B). After 30 minutes, the inhibitory effects of cyclosporin A (*closed circles*) and an excess (500 μM) of taurocholate (*open circle*) on the basal-to-apical transport of [^{3}H]taurocholate (1 μM) for 1 h across LLC-NTCP/BSEP cell monolayers were studied (A). Apical efflux clearance PS3 of taurocholate was calculated versus the intracellular concentration of taurocholate determined at the end of the experiments (B). The inhibitory effects of cyclosporin A (*closed circles*) and an excess (500 μM) of taurocholate (*open circle*) on the uptake of [^{3}H]taurocholate (1 μM) for 1 min into LLC-NTCP cells were studied (C).

Fig. 5. Simulation of the inhibitory effect of the uptake and/or efflux process on the net basal-to-apical transport of bile taurocholate across hepatocytes. The basal-to-apical clearance $PS_{b\text{-}a}$ of taurocholate across hepatocytes was calculated in the cases where the influx clearance, PS1, the efflux clearance, PS3, or both were affected by the inhibitor under the following conditions: The K_m values of PS1 and PS3 are = 30

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μM and 6 μM, respectively, according to the reported Km values for rat Ntcp and rat Bsep (Schroeder et al., 1998, Hagenbuch et al., 1991, Gerloff et al., 1998 and Akita et al., 2001). The unbound concentration of taurocholate in the basal compartment and the intracellular compartment are smaller than these Km values (Fixed at 1μM). The inhibition constant K_i for both the influx and efflux clearance is , K_i = 1 μM, PS2:PS3 = 1:8.2, . $PS_{b-a} = PS1 \cdot PS3 / (PS2 + PS3)$, according to the equation in data analysis. $PS_{b-a} = PS1 \cdot PS3 / (PS2 + PS3)$ The ratio of PS1:PS2:PS3 is substituted by the measured value (PS1:PS2:PS3 = 1.0:0.7:6.0) in the isolated rat liver perfusion studies cited from Akita et al., 2002.

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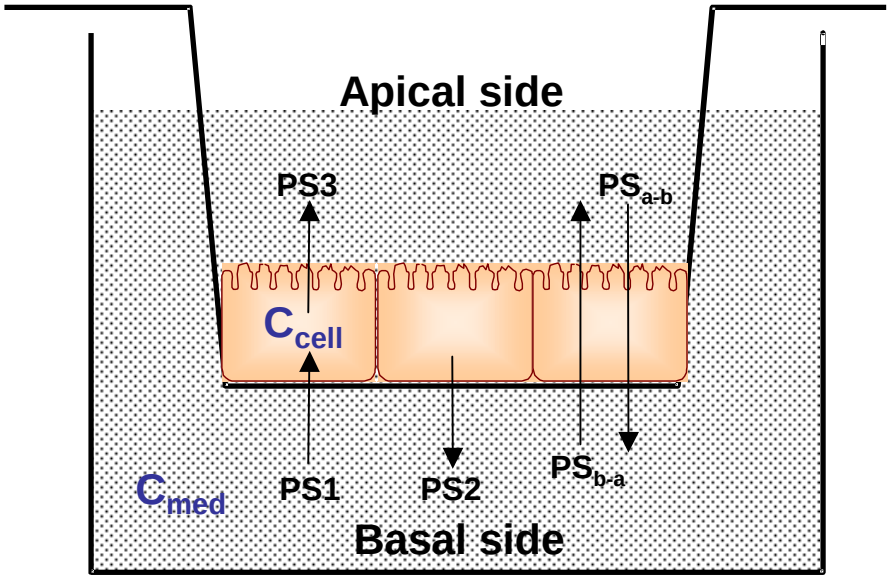
Table 1

Transcellular transport of labeled and fluorescent bile acids across NTCP and BSEP co-expressing LLC-PK1 cells. Data are expressed as mean ±SE.

	Basal-to-apical transport (l/min/mg protein)		Ratio (NTCP/BSEP/control)
	LLC-PK1 (control)	LLC-NTCP/BSEP	
TC	0.36 ± 0.00	7.62 ± 0.26	20.94
CGamF	0.28 ± 0.08	1.19 ± 0.13	4.32
CDCGamF	0.27 ± 0.18	1.24 ± 0.22	4.61
CamF	0.66 ± 0.06	0.89 ± 0.07	1.33
7 -NBD-NCT	1.38 ± 0.35	1.63 ± 0.13	1.18
UDC-L-NBD	1.16 ± 0.07	1.83 ± 0.00	1.58

Fig. 1



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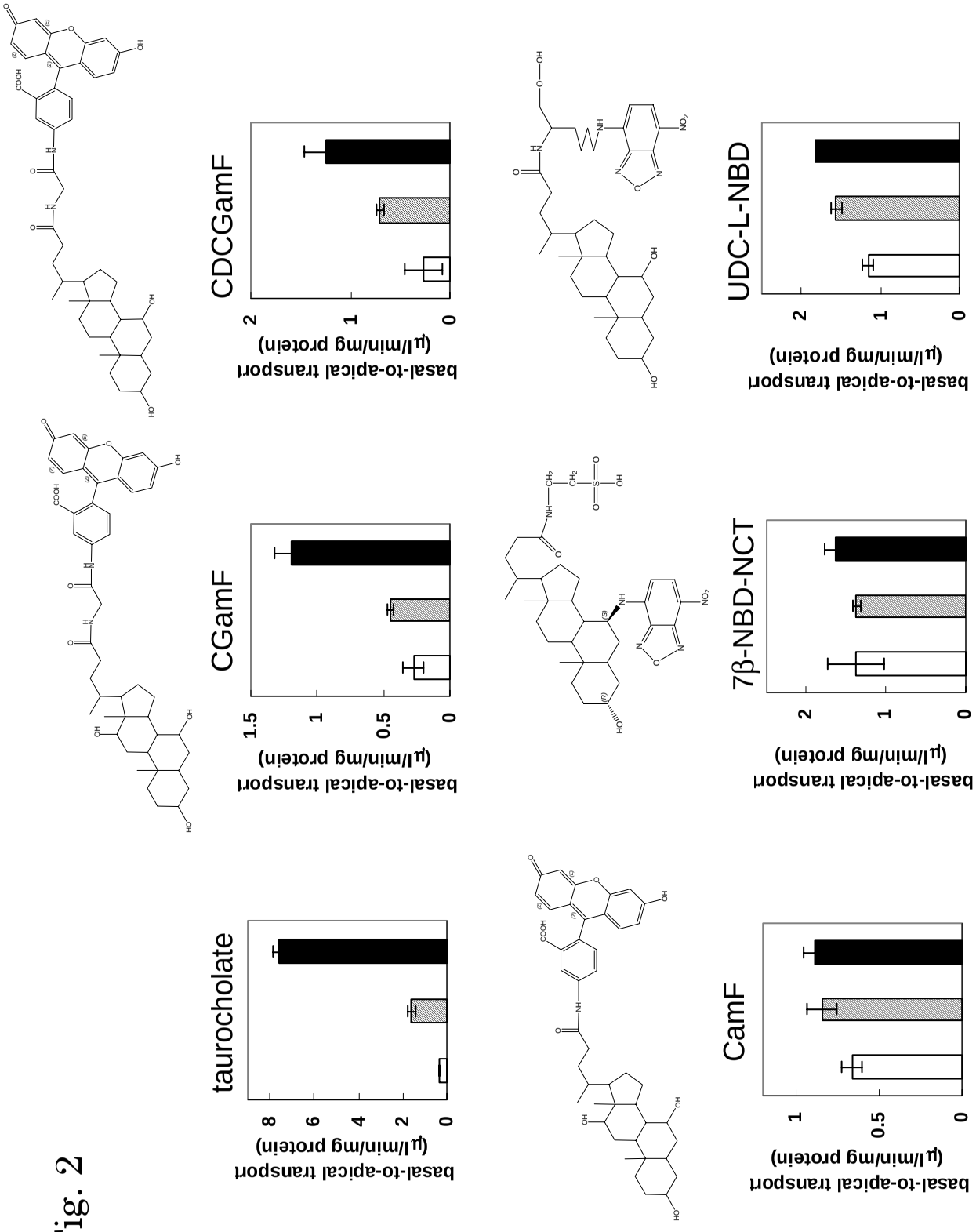

Fig. 2

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Fig. 2 continued

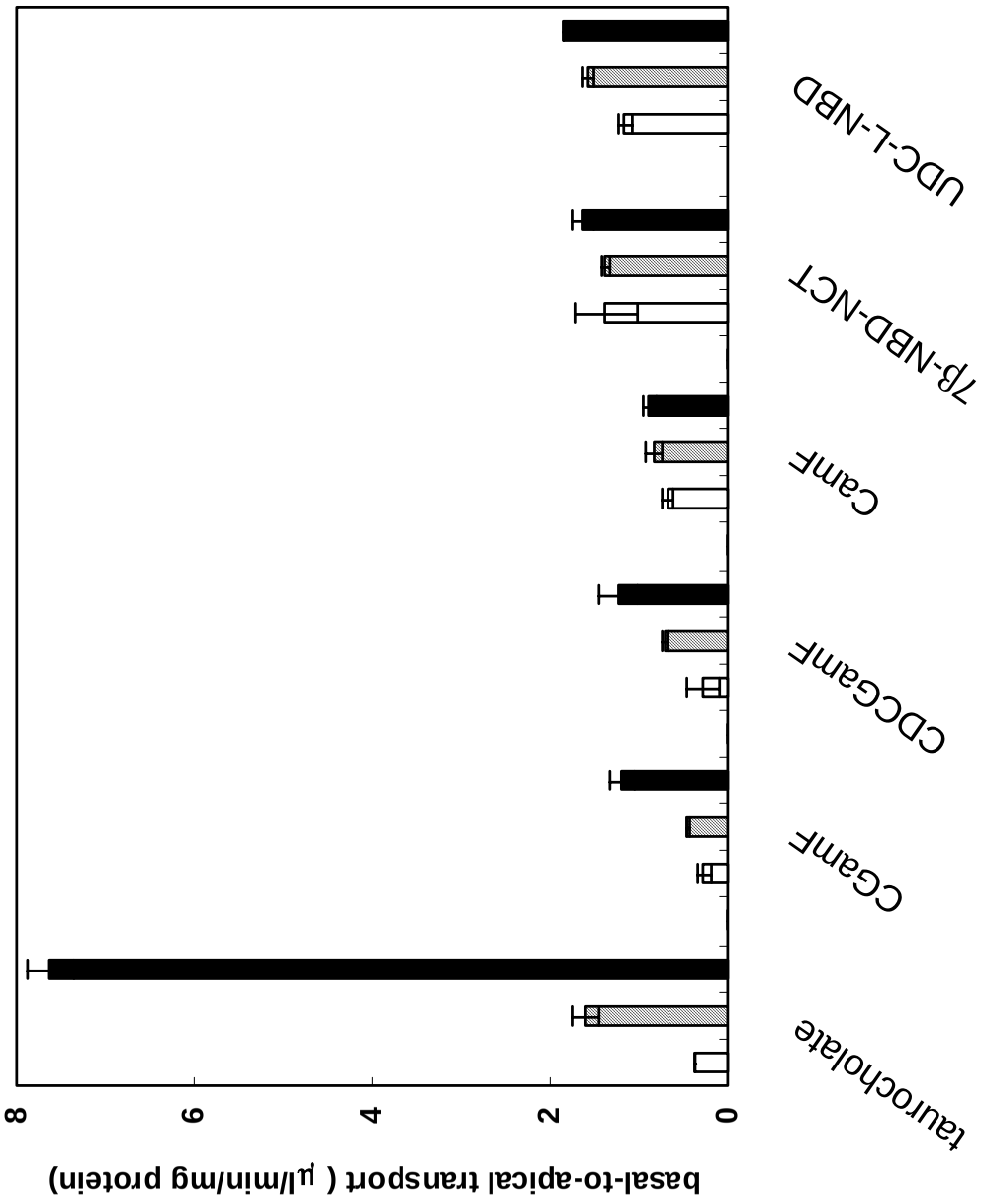
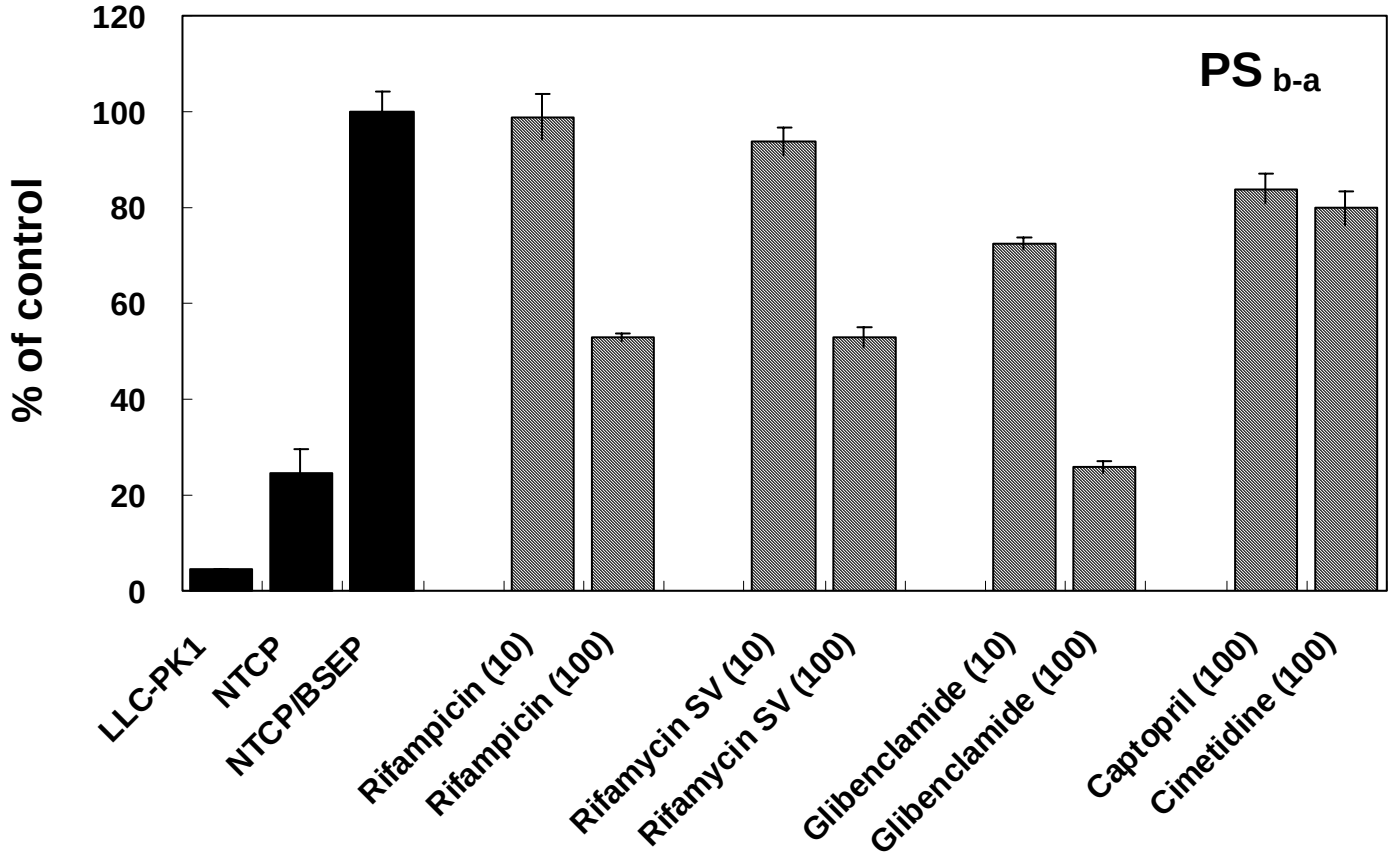


Fig. 3



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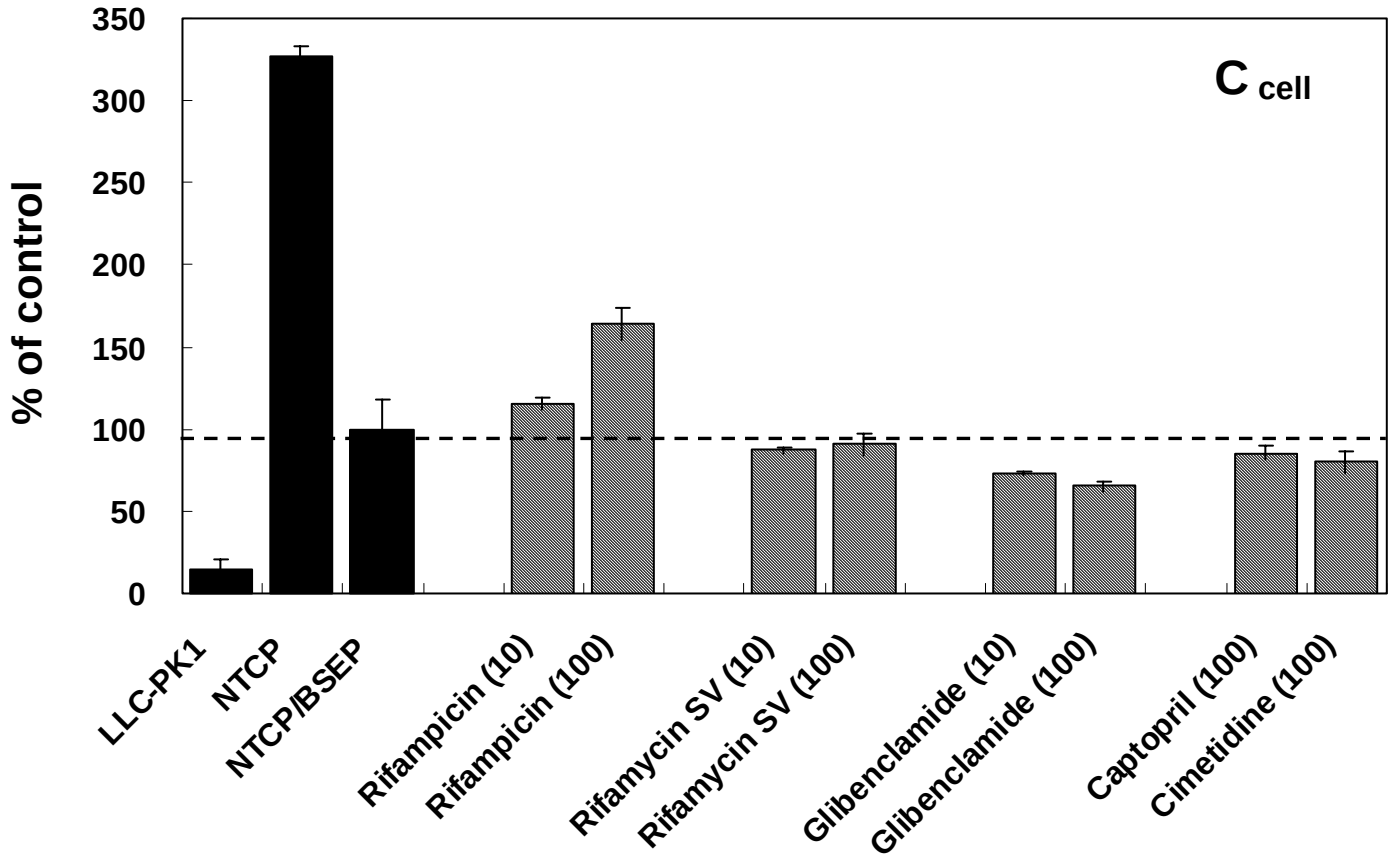

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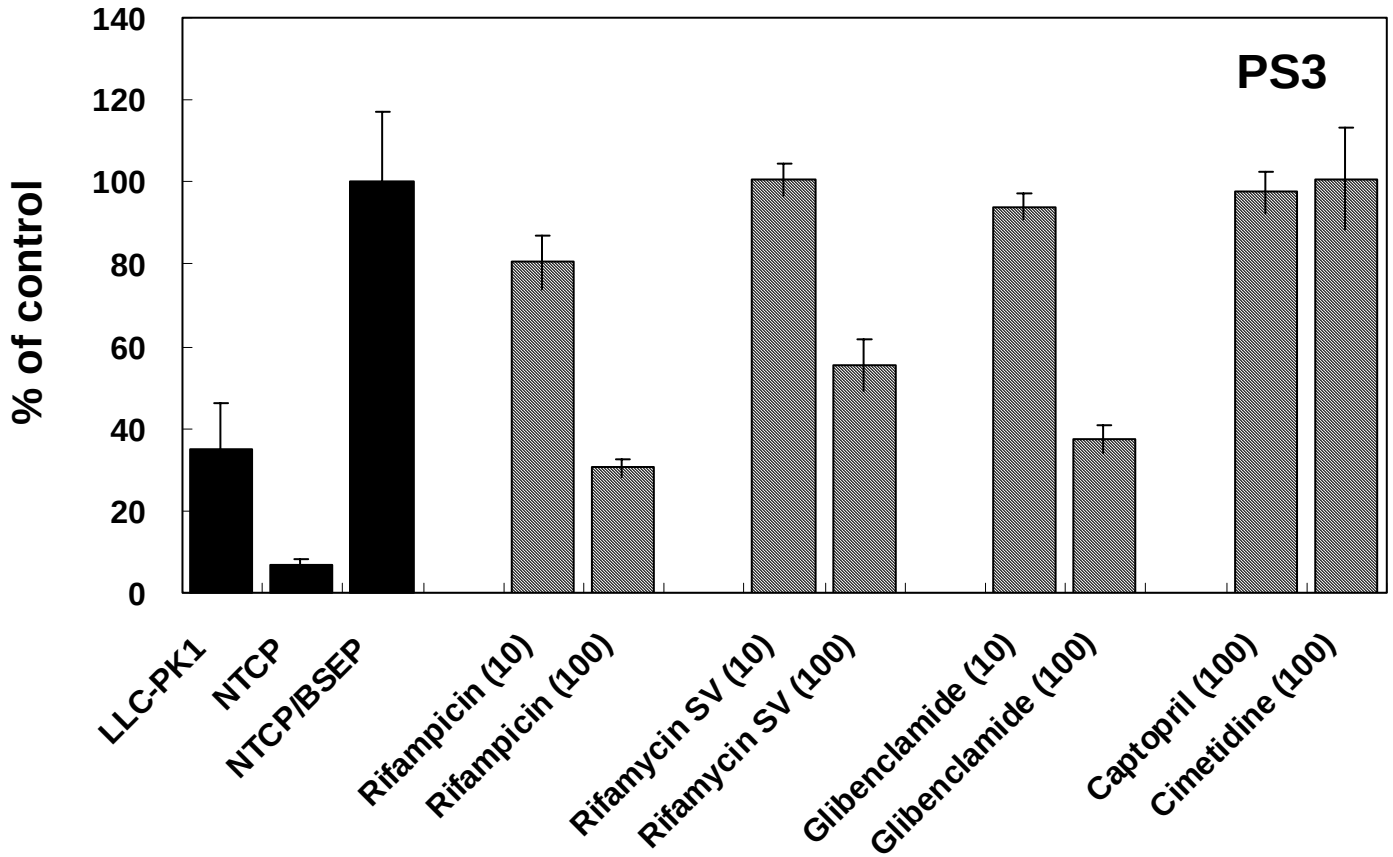

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Fig. 4

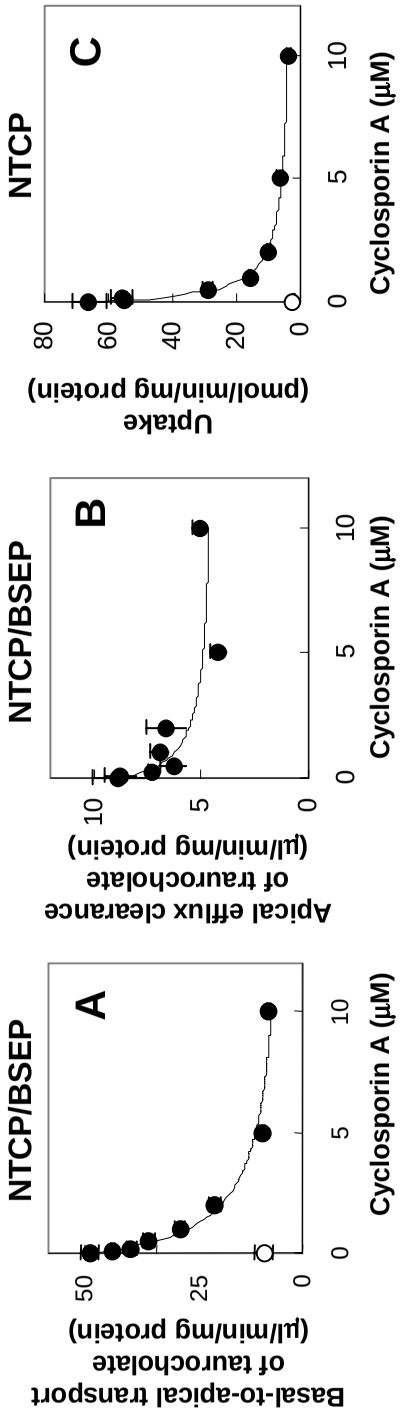
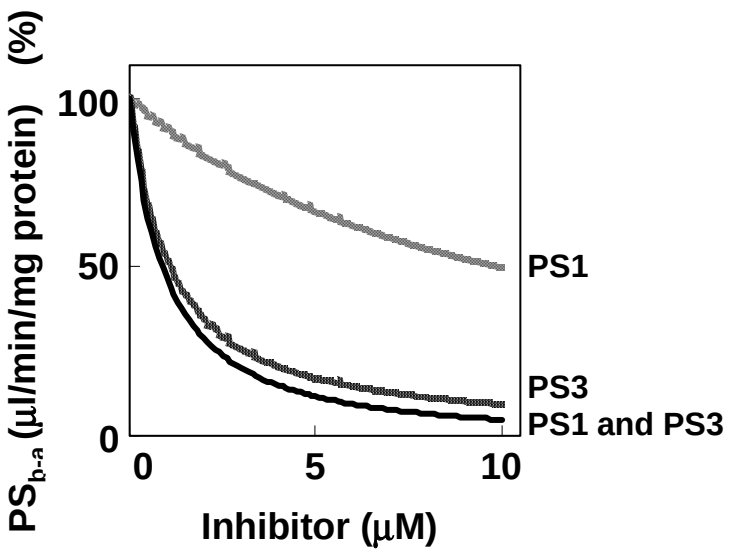


Fig. 5



PS3 : PS2 = 1 : 0.12

K_i = 1μM