Fluorescent Substrates of Sister-P-Glycoprotein (BSEP) Evaluated as Markers of Active Transport and Inhibition: Evidence for Contingent Unequal Binding Sites

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Purpose. Although sister-P-glycoprotein (SPGP, BSEP) is closely related to P-glycoprotein, it is much more selective in distribution and substrate recognition. Moreover, because inhibition or lack of BSEP function has severe consequences including cholestasis, hepatotoxicity, exposure to toxic xenobiotics, and drug interactions, *in vitro* methods are necessary for quantifying and characterizing specific inhibition of BSEP. Therefore, the objective is to discern a method and quantitatively characterize several example BSEP inhibitors.

Methods. With fluorescent markers having been used successfully to evaluate and quantify inhibition of P-gp-mediated transport, this study evaluates several compounds for specific cell retention caused by BSEP inhibitors. In addition to the several compounds asserted to be BSEP inhibitors, the compounds suggested to be BSEP substrates might also inhibit BSEP competitively. Retained fluorescence of possible BSEP substrates was measured by a flow cell cytometer using transfected cells presenting the BSEP transporter specifically and abundantly.

Results. Several compounds were shown to inhibit BSEP active transport of the fluorescent substrates dihydrofluorescein and bodipy. The inhibition potency was quantified (i.e., cyclosporin A $IC_{50} \sim 7\ \mu M$), revealing incongruent relative sensitivities among the substrate markers, with H_2FDA generally the most sensitive of the series of substrate markers evaluated.

Conclusions. The inconsistent sensitivities of the transport markers (H_2FDA and bodipy) were reminiscent of the apparent multiple binding site behaviors observed for P-gp and could indicate opposing and unequal yet interacting binding sites akin to those of P-gp. Nonetheless, notable differences between P-gp and BSEP in marker substrate recognition/transport were apparent despite the observed overlap in xenobiotic recognition and transport. Thus far the most potent inhibitors seem to be cyclosporin, tamoxifen, and valinomycin. There are likely to be much more potent inhibitors, and other substrates also may be more sensitive to inhibition of transport.

KEY WORDS: sister-P-glycoprotein; BSEP; ABC-B11; drug interaction; inhibition; cholestasis.

INTRODUCTION

Members of the ABC transporter family exhibit the ability to relocate xenobiotics from the interior of the cell to the exterior. P-glycoprotein remains the best studied and is apparently the most ubiquitous in tissue distribution as well as most able to recognize a wide range of molecule types (1,2). The closely related ABC transporter sister-P-gp (SPGP), also known as the bile salt export pump (BSEP, ABC-B11), is significantly more selective with respect to substrate recognition. Expressed exclusively in the liver (3), BSEP appears to have a role in efflux of endogenous compounds (bile acids) and exogenous compounds (xenobiotics) into the bile (4,5). Some examples of endogenous substrates thought to be exported by BSEP include: taurocholate, estradiol-17β-glucuronide, cholic acid, muricholates, and other monoanionic bile salts. Canalicular secretion of bile acids from the liver in the form of bile facilitates the emulsification of dietary lipids and fat-soluble vitamins. Defective bile secretion results in cholestasis with accumulation of bile salts and other toxic bile constituents within hepatocytes and blood plasma. Mutations in the *BSEP* gene can result in the absence of BSEP expression and are the cause of certain forms of progressive familial intrahepatic cholestasis (PFIC-2) (6,7). PFIC manifestations are jaundice, fibrosis, and cirrhosis (caused by <1% of normal biliary bile salts), hyperbilirubinemia, suppressed lipid and cholesterol metabolism, and intestinal malabsorption of fat and fat-soluble vitamins.

BSEP has recently been characterized as a transporter that interacts with drugs and xenobiotics, including vinblastine, ditekiren, troglitazone, troglitazone sulfate, cyclosporin, rifamycin, glibenclamide (8), sulindac, and taxol (9). Moreover, the administration of troglitazone (10,11), cyclosporin, rifampicin, and bosentan (12)—all inhibitors of BSEP—has been linked with cholestasis. Xenobiotic-induced cholestasis is a significant clinical problem, though drug interactions mediated by BSEP may also have dangerous consequences. Because P-gp and BSEP are both expressed in the liver, it is clear that the extent of overlap between P-gp and BSEP drug substrates and inhibitors needs to be established to estimate the role BSEP plays in drug disposition. The importance of BSEP interactions at the level of hepatobiliary export processes should be considered in the evaluation of drug interactions.

In order to discern the contribution of BSEP to drug interactions, it is critical to explore and demonstrate a method for characterizing and quantifying the inhibition of BSEP-mediated transport. Fluorescent substrates of ABC transporters that have served as excellent markers of active transport, with their retention (increased cytosolic fluorescence) indicating inhibition (13), should be similarly valuable for BSEP evaluations (with novel fluorescent substrates). Such potential fluorescent substrates must be available to the cytoplasmic side of the cell membrane and be specifically affected by inhibitors of the particular transporter. It has recently been shown for P-gp that particular inhibitor/substrate combinations can exhibit dramatically different quantitative potencies of (or sensitivities to) inhibition, apparently because of an asymmetric interdependent pair of substrate binding sites (14). Indeed, a sole marker substrate of P-gp active transport can be inadequate, as different inhibition parameters are commonly observed with different markers. Therefore, several potential fluorescent substrates should be evaluated for active transport and for specific effects by BSEP inhibitors.

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ABBREVIATIONS: P-gp, P-glycoprotein; BSEP, sister-P-glycoprotein; BSEP, bile salt export pump; ABC, ATP-binding cassette; MDR, multidrug resistance; DNR, daunorubicin; Rho, rhodamine 123; H_2FDA, dihydrofluorescein; NBD, nucleotide binding domain.

This paper reports an evaluation of several fluorescent compounds for interaction with BSEP and shows at least one exhibiting valuable qualities as a marker of BSEP function. This marker should prove useful for the quantification of BSEP inhibition and allow for assessment of the BSEP-mediated contribution to drug interactions. Significantly, we show evidence for substrate/inhibitor pair-unique interactions that are consistent with unequal yet contingent substrate binding sites for BSEP.

MATERIALS AND METHODS

Chemicals

Daunorubicin (DNR), rhodamine 123 (Rho), vinblastine, verapamil, (−)-epigallocatechin gallate (EGCG), quinine, reserpine, paclitaxel, progesterone, tamoxifen, taurolithocholic acid, valinomycin, ketoconazole, quinidine, cimetidine, indomethacin, and probenecid were purchased from Sigma Chemical Co. (St. Louis, MO). LDS 751 (LDS), calcein AM (CAM), tetramethyl rosamine (TMR), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarboxyanine iodide (JC-1), fluo-3-AM (FAM), bodipy® FL C_1-IA or *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*S*-indacene-3yl)methyliodoacetamide (BOD), dihydrofluorescein (H_2FDA), SYTO (SYTO® 11), 2′,7′-bis-(2-carboxyethyl)-5- and -6-carboxyfluorescein acetoxymethyl ester (BCE), fluorescein (FRS), 5- and 6-carboxy-2′,7′-dichlorofluorescein diacetate (DCF), 5-iodoacetamidofluorescein (IAF), *N*-(2-(iodoacetoxy)-*N*-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole (IAN), doxorubicin (DOX), Hoechst 33342 (H33), and cholesteryl bodipy® FL C_{12} (CBO) were purchased from Molecular Probes (Eugene, OR). Troglitazone was obtained from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). Glibenclamide, chlopromazine, clarithromycin, clofazimine, and rifamycin SV were obtained from ICN (Costa Mesa, CA). Cyclosporin A (CSA) was purchased from Alexis Biochemicals (San Diego, CA). Hanks' balanced salt solution, Alpha Minimum Essential Medium, DMEM, penicillin/streptomycin, geneticin (G418), fetal bovine serum (FBS), and trypsin-EDTA were obtained from Life Technologies, Inc. (Rockville, MD). All other reagents were of the highest grade commercially available.

Cell Lines

The SK-E2 cell line (presenting the gene product from Human ABC-B11/BSEP) was licensed from Dr. V. Ling's lab at Vancouver, BC, Canada. These cells were maintained in α-Minimum Essential Medium (α-MEM) supplemented with 15% FBS and geneticin (800 ng/ml) in a 5% CO_2–95% air atmosphere at 37°C. Cells were grown to 80–90% confluency and treated with trypsin-EDTA before subculturing. SK-OV-3 cells were purchased from ATCC (Manassas, VA) and maintained in α-MEM supplemented with 15% FBS.

FACS Flow Cytometry

Fluorescence measurements of individual cells were performed using a Becton-Dickinson FACScalibur fluorescence-activated cell sorter (San Jose, CA) equipped with an ultraviolet argon laser (excitation at 488 nm, emission at 530/30 and 570/30 nm band-pass filters). Analysis was gated to include single viable cells on the basis of forward and side light scatter and was based on acquisition of data from 10,000 cells. Log fluorescence was collected and displayed as single-parameter histograms. A modified inhibition assay for the BSEP efflux pump in viable transfected cells was performed with the flow cytometer (13). The inhibitors and the fluorescent substrate marker are preincubated (30 min) with the cells during the "accumulation" incubation. The fluorescent substrate marker concentrations are 0.2 μM except DNR (at 2 μM). The "efflux" incubation is terminated at 15 min by ice bath, and the cells are analyzed within 30 min. Data from 10,000 cells were accumulated for each data point.

Cell Viability Test

Cell viability was assessed using propidium iodide staining. Dead cells in which propidium iodide was bound to double strands of DNA or RNA were detected in certain regions of the cytometry dot plots and not included in the final data calculations. Cells that are nonviable as a result of exposure to high concentrations of test compound are identified as a different quality of light scattering (in a different region of the dot plot) and are excluded from that assay.

Calculation of Relative Fluorescence

The fluorescence intensity of individual cells was recorded on histograms. The mean fluorescence intensity of 10,000 cells was used for comparison among different conditions. For the initial inhibition screening assay the fluorescence intensity was compared to control cells. Cyclosporin A was selected as a positive control and used to normalize the measurements because it can significantly inhibit the BSEP-mediated active efflux of fluorescent substrate markers. Relative fluorescence was used for quantitation and comparison among different compounds. The relative fluorescence (% maximal or reference inhibition) represents a ratio obtained through the following formula: the geometric mean fluorescence of a discrete sample divided by the geometric mean fluorescence in the presence of 50 μM cyclosporin A, times 100.

The resulting values represented the percentage inhibition of BSEP normalized for the reference (standard) inhibition and were used to compare the test drugs for inhibition potential.

Stability of Marker Retention

To test the stability of fluorescent marker substrate retention within the cell under experimental conditions, an experiment relating retention at 0°C to time was performed. Experiments conducted at time points over a 40-min period showed that at approximately 0°C (in the presence of the inhibitor cyclosporin A or chlorpromazine), the tested marker H_2FDA was well retained for a sufficient time to assure a stable signal for the duration of the fluorescence assay phase (data not shown).

RESULTS

As fluorescent substrates transported by mammalian P-gp, DNR and Rho and others serve as markers for active transport function simply by measurement of retained fluo-

rescence per cell (13). Several typical P-gp and BSEP substrates/inhibitors were quantitatively evaluated for their interaction with BSEP. The efflux or retention of the fluorescent compounds DCF, IAF, IAN, DOX, H33 JC1, LDS, Rho, TMR, DNR, CBO, CAM, and FAM was not affected by the positive controls (verapamil and cyclosporin, previously reported to inhibit BSEP) in the SK-E2 cell line (BSEP transfected, Fig. 1). Furthermore, of those fluorochromes affected, some were also affected by the positive control inhibitors in the parent cell line (not presenting BSEP; i.e., BOD, SYTO, BCE, FAM; Fig. 2). This apparent nonspecific effect—inhibition of passive efflux or other transporters—renders them potentially dubious as quantitative markers of BSEP active transport without proper controls. It is H_2FDA and FRS (and perhaps bodipy in many cases) that stand out as useful indicators of transport and therefore of retention (inhibition). The usefulness of CAM is very suspect, as it is only very slightly affected by the positive controls, though it may be significantly more affected by ditekiren (16).

Passive diffusion of H_2FDA is very slow (Fig. 3). Steady-state accumulation requires about 10–20 min under normal incubation procedures (30 min with inhibitor). More notably, active efflux of H_2FDA is slow and has an apparent lag of about 8–10 min. Therefore, in the presence of a test compound, the efflux incubation should be conducted for a minimum of about 20–25 min to maximize the difference from control. Bodipy appears significantly faster than H_2FDA for active transport, achieving steady state in only minutes (2–3 min) for the efflux phase (data not shown).

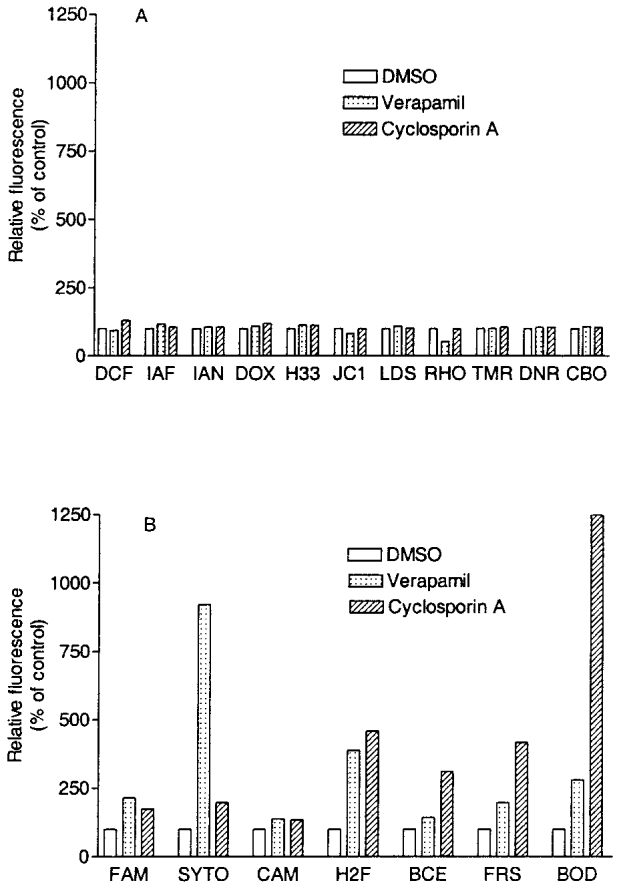

Fig. 1. Relative retention of various fluorescent substrate transport markers in the presence of two positive controls (inhibitors), verapamil and cyclosporin A, within SK-E2 cells (transfected with the BSEP gene). The fluorescence measured is of each potential marker noted in axis. A level of 100% is established for the control response (based on fluorescence emissions) without inhibitor present. The legend is noted on the figure.

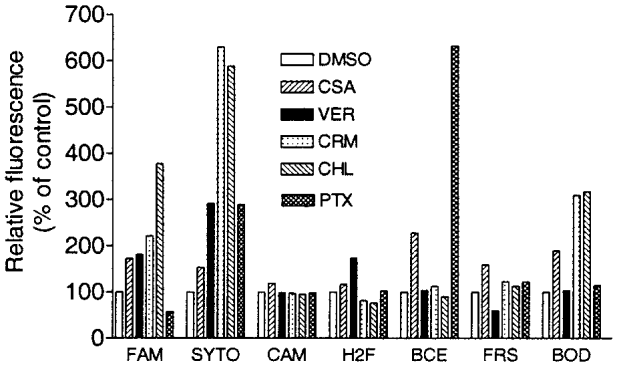

Fig. 2. Relative retention of fluorescent substrate transport markers in the presence of various positive controls (shown in legend) within the parent cells (nontransfected with the BSEP gene). The fluorescence measured is of each potential marker noted in the axis. A level of 100% is established for the control response (based on fluorescence emissions) without P-gp inhibitor present. The legend is noted on the figure.

The IC_{50} (concentration at half-maximum inhibition) was determined from a simple function, as shown in Fig. 4, where the retained fluorescence was measured for samples of

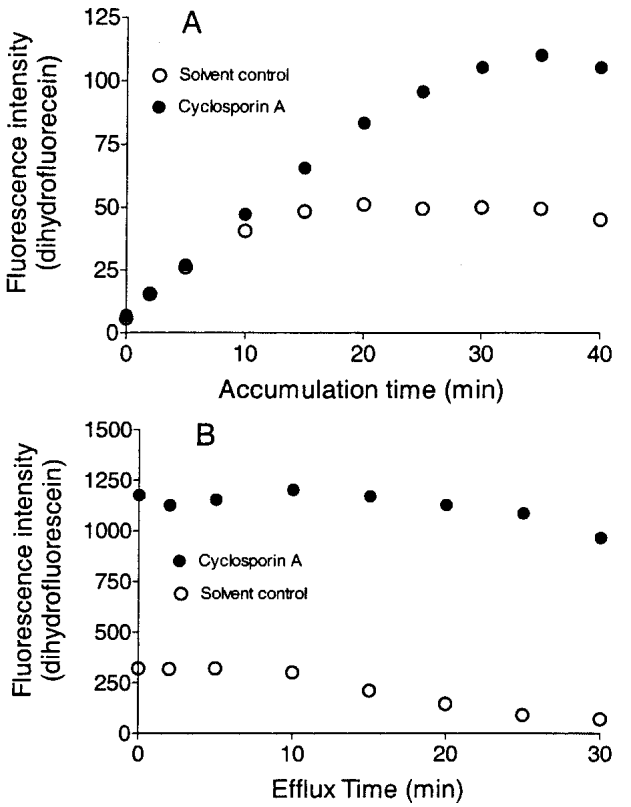

Fig. 3. Temporal retention of fluorescent substrate transport marker H_2FDA during accumulation incubation (A) and efflux incubation (B) with and without a positive control inhibitor, cyclosporin A. The legend is noted on the figure.

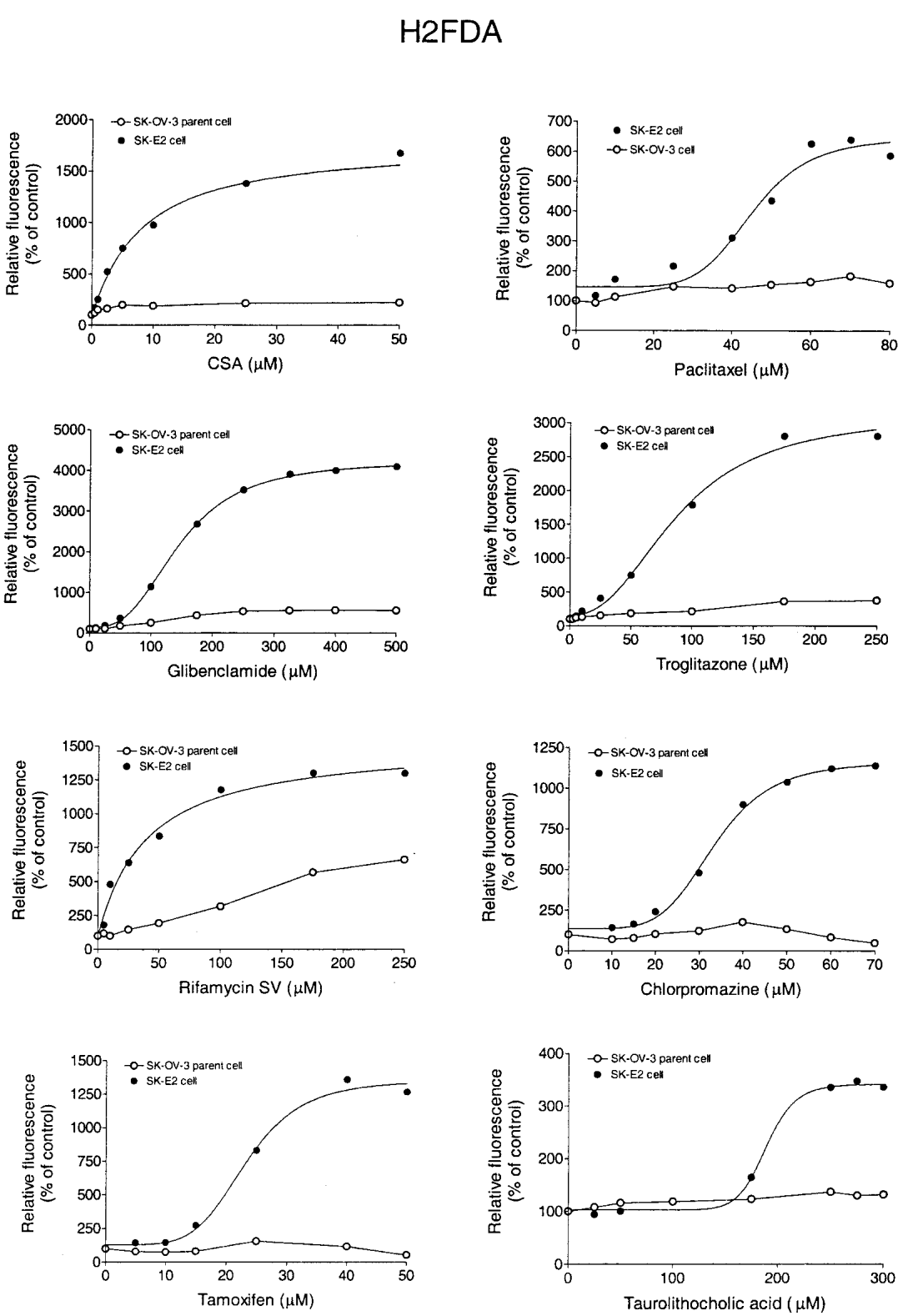

Fig. 4. Intracellular retention of dihydrofluorescein (H_2FDA) in SK-E2 cells vs. competing inhibitor concentration. Fluorescence intensity is expressed as relative fluorescence. The average number of cells per assay was 10,000. The function for the line through the data is the Hill equation: $I = I_{max}S^n/(K' + S^n)$. The parameters IC_{50} and the Hill coefficient along with the standard deviation are shown in Table I. Potential nonspecific effects were tested by also exposing the nontransfected parent cells (negative control) to the inhibitors (Fig. 3). Because of the effect on marker efflux from the parent cell line, the apparent "background" is subtracted from the raw observed data for the determination of IC_{50} shown in the tables.

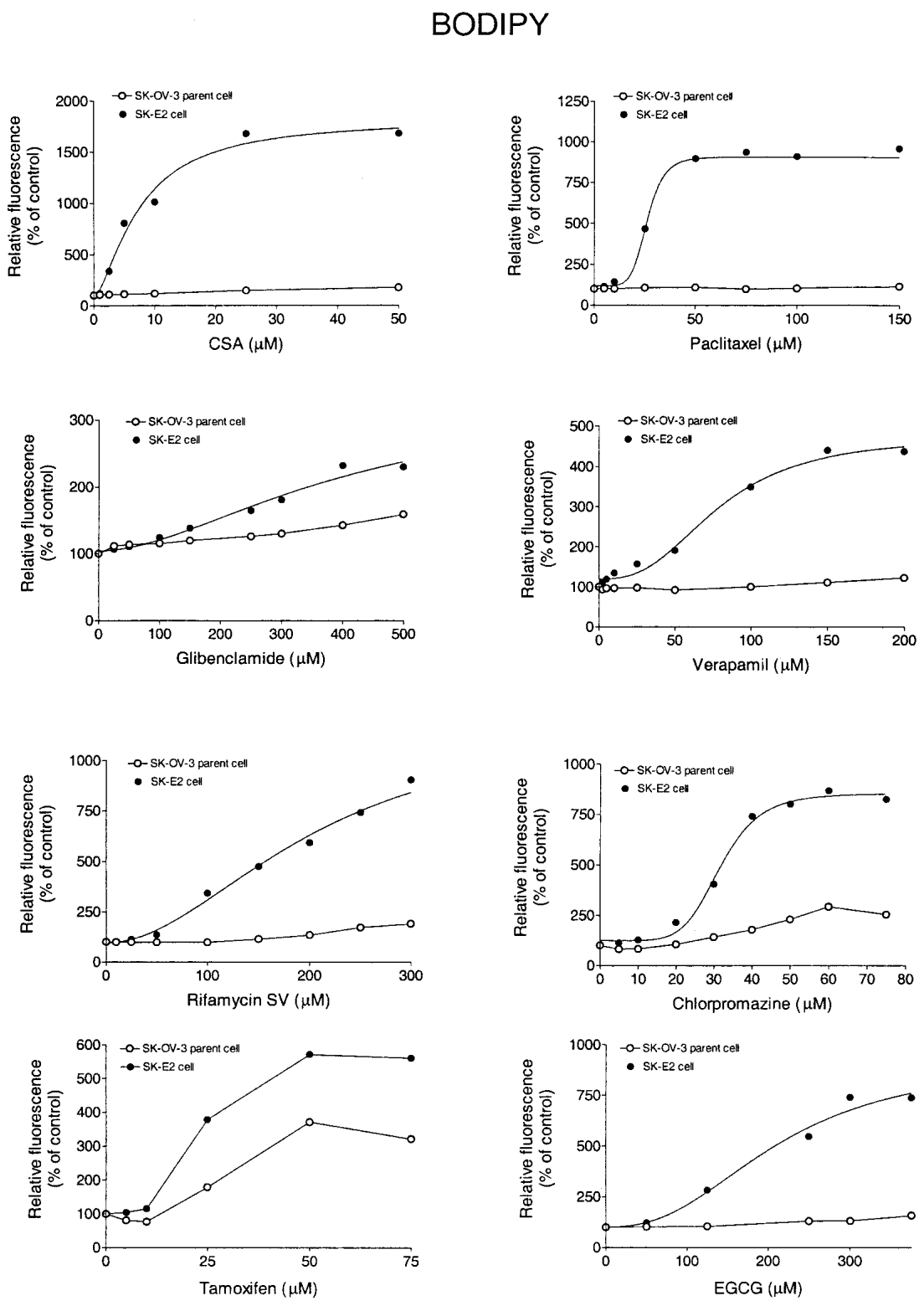

Fig. 4. Continued.

viable cells by a flow cytometer at varying concentrations of compound (13). The concentration dependence of inhibition displayed a sigmoidal response curve (Fig. 4), a consequence of cooperativity (15), with the Hill equation for allosteric interaction enzymes therefore being the appropriate function for fitting to the data: $I = I_{max}S^n/(K' + S^n)$. The IC_{50} for cyclosporin A on H_2FDA transport in the SK-E2 cell line (which overexpresses the human BSEP gene product) was ≈7.8 μM. Potential nonspecific effects were tested by also exposing the nontransfected parent cells (negative control) to the inhibitors (Fig. 3). Because of the effect on the marker efflux from the parent cell line, the apparent "background"

retention of fluorescence is subtracted from the raw observed data for the determination of IC_{50} shown in the tables.

Once noting that H_2FDA and bodipy stood out as the most sensitive to inhibition among the fluorescent substrate markers tested, we extended the quantitative evaluation to other putative BSEP substrates/inhibitors for effecting the active transport of these markers (Tables I and II). Both of these fluorescent substrate markers provide similar IC_{50}s for some of the most potent inhibitors among this set: cyclosporin A, tamoxifen, paclitaxel, and chlorpromazine. Dramatic differences still appear, however, in the trend of the effect on a given marker. For example, verapamil, EGCG, and quinine have less potency to inhibit H_2FDA transport than bodipy transport despite the often similar sensitivities of the two transport markers (EGCG and quinine do not even affect specific BSEP-mediated H_2FDA transport). Conversely, progesterone, valinomycin, reserpine, rifamycin, vinblastine, glibencamide, and taurolithocholic acid exert a more potent effect on H_2FDA than on bodipy. Indeed, valinomycin, reserpine, and taurolithocholic acid apparently do not affect bodipy active transport, yet valinomycin is as potent as cyclosporin A for the inhibition of H_2FDA active transport, similar to reserpine. Moreover, progesterone and rifamycin also exhibit dramatic differences in potency toward the two markers' active specific transport (to wit, H_2FDA is significantly more sensitive). Examples of negative controls include cimetidine and probenecid, which show no effect on any of the tested transport markers. Most compound pairs exhibit allostery in the form of a Hill coefficient of ~2–5, which is consistent with previous observations for P-gp (15).

DISCUSSION

Although active BSEP-mediated transport of some of the markers evaluated was affected by several of the inhibitors, the concentrations required for inhibition were often quite different. Additionally, the hydrophobic linear hexapeptide ditekiren has been reported to inhibit the BSEP-mediated calcein-AM transport, although it was not affected by cyclosporin, reserpine, etc. (16), consistent with our observations. The substrate/inhibitor/transporter interaction might be more complex than competition for a single, or two equivalent, binding sites. A substrate may be preferentially recognized by (and transported from) a binding site that is less affected by some inhibitors than another opposing binding site. Two substrate binding sites with distinct affinities for a given substrate or inhibitor would be analogous to particular observations of P-gp. This difference between binding sites may render the transport of a marker substrate relatively underaffected by certain inhibitors that favor binding to the other substrate binding site.

BSEP inhibition of active transport may be unique to a given pair of substrates (or substrate and inhibitor) regardless of raw binding affinity or interactions the inhibitor may have with other substrates and explain the different behaviors of H_2FDA and bodipy. An IC_{50} determined using one substrate marker may be quite different from that for transport inhibition of another substrate. Such a substrate/inhibitor pair-dependent outcome would be similar to P-gp's apparent two unequal substrate binding sites (2,17,18) that are linked allosterically (15).

Indeed, the three-dimensional structure for the closest bacterial homology mimic to P-gp, MsbA, shows opposing pseudosymmetric "chamber" openings (~25 Å) predominantly into the inner leaflet of the lipid bilayer (19). The type and tightness of association of an inhibitor at one site can have distinctly differing transduced effects on the other binding site that may be favored by a transport marker. This effect may be much weaker (or even greater), depending on the particular pair and their unique influence on protein conformation, and may lead to unpredictable transport marker responsiveness to inhibition. Additionally, if the binding site

Table I. BSEP Inhibition Parameters for Several Compounds Assessed with Dihydrofluorescein as the Fluorescent Marker

Compounds	I_{max} (% control)	IC_{50} (μM)	*n* (Hill coefficient)
Chlorpromazine	1164.0 ± 32.2	33.5 ± 0.8	5.1 ± 0.6
Clofazimine	1170.0 ± 247.0	28.7 ± 11.6	1.4 ± 0.5
Cyclosporin A	1757.0 ± 116.4	7.8 ± 1.5	1.1 ± 0.2
Glibenclamide	4224.0 ± 40.1	146.5 ± 2.0	2.9 ± 0.1
Ketoconazole[a]	435.2 ± 70.4	65.4 ± 14.1	2.6 ± 1.0
Paclitaxel	646.8 ± 63.5	28.9 ± 3.0	3.0 ± 1.1
Progesterone[a]	195.4 ± 29.7	17.3 ± 13.7	1.0 ± 0.5
Reserpine[a]	1142.0 ± 68.3	10.2 ± 0.9	4.7 ± 3.3
Rifamycin SV[a]	836.3 ± 75.5	12.2 ± 4.5	1.6 ± 0.8
Tamoxifen	1354.0 ± 68.8	23.3 ± 1.2	5.1 ± 1.4
Taurolithocholic acid	341.7 ± 9.9	189.2 ± 10.4	13.7 ± 8.8
Troglitazone	3133.0 ± 216.7	66.4 ± 8.7	2.1 ± 0.5
Valinomycin	267.9 ± 40.6	7.2 ± 3.2	1.0 ± 0.4
Verapamil	2536.0 ± 281.8	178.9 ± 23.5	2.6 ± 0.7
Vinblastine	3012.0 ± 199.4	62.0 ± 6.6	1.9 ± 0.3
Cemetidine	NE	ND	ND
Quinidine	NE	ND	ND
EGCG	NE	ND	ND

Note: H_2FDA (1 μM) as a marker.
NE, no effect; ND, not determined.
[a] Recalculated by substracting data of parent cell from data of SK-E2 cell.

Table II. BSEP Inhibition Parameters for Several Compounds Assessed with Bodipy as the Fluorescent Marker

Compounds	I_{max} (% control)	IC_{50} (μM)	*n* (Hill coefficient)
Chlorpromazine[a]	677.8 ± 21.1	30.9 ± 0.9	11.0 ± 4.9
Cyclosporin A	1831.0 ± 98.0	7.5 ± 1.3	1.5 ± 0.4
EGCG	925.3 ± 297.0	214.0 ± 76.8	2.5 ± 1.2
Glibenclamide[a]	194.4 ± 28.8	260.1 ± 70.9	2.7 ± 1.5
Paclitaxel	910.6 ± 25.8	26.8 ± 0.7	4.2 ± 0.7
Progesterone	579.8 ± 44.8	224.6 ± 16.0	3.5 ± 0.6
Rifamycin SV	1200.0 ± 195.7	206.5 ± 41.6	2.0 ± 0.4
Tamoxifen[a]	321.8 ± 17.0	15.4 ± 3.1	4.1 ± 1.8
Verapamil	475.5 ± 35.0	79.1 ± 9.1	2.7 ± 0.6
Vinblastine	766.0 ± 39.4	114.7 ± 7.3	3.4 ± 0.6
Reserpine	NE	ND	ND
Cimetidine	NE	ND	ND
Valinomycin	NE	ND	ND
Taurolithocholic acid	NE	ND	ND

Note: Bodipy (0.2 μM) as a marker.
NE, no effect; ND, not determined.
[a] Recalculated by substracting data of parent cell from data of SK-E2 cell.

is large enough to accommodate both a transport marker and an inhibitor, an interaction between the two may produce similar results (20). Nonetheless, H_2FDA appears to be the superior marker because of its lack of inhibitor interactions in the parent cell line as well as the generally greater sensitivity to BSEP inhibitors. Bodipy is apparently faster than H_2FDA in active efflux.

BSEP is a primary route of bile salt export from hepatic canalicular cells and has been linked genetically to a biliary flow disease, intrahepatic cholestasis PFIC-2. Consequently, the molecular inhibition of BSEP can lead to, among other things, clinical cholestasis, a condition that can lead further to various physiologic maladies. Several cholestatic drugs have already been shown to potently inhibit BSEP: cyclosporin A, rifampicin, glibenclamide, estradiol-17β-glucuronide, bosentan, troglitazone, and sulindac all can cause increased bile salt concentrations in serum and eventually cholestatic liver injury and are BSEP inhibitors (8,10,21) and probably substrates (22). Indeed, BSEP shares some predictable qualities with P-gp as a multixenobiotic efflux transporter, although BSEP is seemingly more selective than P-gp for substrates and apparently recognizes only a subset of the multitude of P-gp substrates. With more study, however, many more P-gp substrates/inhibitors may be observed to interact to some degree with BSEP. BSEP, with its apparent bile flow role, is likely much more selective than P-gp, and a dramatic difference between the two is illustrated by the contrasting interactions with these fluorescent substrate markers. Although rhodamine, daunorubicin, and LDS-751 (also rhodamine analogues JC-1 and CAM, to some extent) are very good substrates for P-gp, they are essentially unaffected by BSEP. Furthermore, a different set of fluorescent substrate markers is shown to be efficiently ejected from the cell specifically by BSEP (e.g., bodipy, H_2FDA). Clearly, this distinction of substrate recognition indicates a significant difference in the binding sites of these two important transporters, consistent with a more selective role for BSEP as a monoanionic bile salt transmembrane export enzyme. Nonetheless, BSEP can be appreciated as a multixenobiotic transport protein in the fashion of P-gp, MRP1, and perhaps other multispecific ABC transporters. On the other hand, P-gp, MRP-2, MRP-3, and MRP-4 appear to recognize several bile salts and are up-regulated during cholestasis and/or BSEP suppression (23).

BSEP and P-gp share about 50% identity in amino acid sequence and about 70% similarity. An adjunct role for BSEP as a xenobiotic defense transporter is not surprising, and even monoanionic bile salts include a variety of amphipathic structures. Consequently, BSEP may confer cytotoxin resistance at the hepatocyte as well as affect the disposition of drugs (and therefore be operative in drug interactions). Anticancer drugs such as tamoxifen, vinblastine, and taxol may be removed from target cells by BSEP, in analogy to P-gp. Because a variety of drugs are now shown to interact with BSEP, it must be addressed when one is considering the effect of ABC transporters on drugs as well as a drug's effect on BSEP function. These markers of active BSEP-mediated efflux (particularly H_2FDA) provide the tools for evaluating potential BSEP-mediated drug interaction or intrahepatic cholestasis.

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REFERENCES

1. S. V. Ambudkar, S. Dey, C. A. Hrycyna. M. Ramachandra, I. Pastan, and M. M. Gottesman. Biochemical, cellular, and pharmacological aspects of the multidrug transporter. *Annu. Rev. Pharmacol. Toxicol.* **39**:361–398 (1999).
2. F. J. Sharom, R. Liu, Q. Qu, and Y. Romsicki. Exploring the structure and function of the P-glycoprotein multidrug transporter using fluorescence spectroscopic tools. *Semin. Cell Dev. Biol.* **12**:257–265 (2001).
3. S. Childs, R. L. Yeh, E. Georges, and V. Ling. Identification of a sister gene to P-glycoprotein. *Cancer Res.* **55**:2029–2034 (1995).
4. R. Thompson and S. Strautnieks. BSEP: function and role in progressive familial intrahepatic cholestasis. *Semin. Liver Dis.* **21**:545–550 (2001).
5. P. J. Meier and B. Stieger. Bile salt transporters. *Annu. Rev. Physiol.* **64**:635–661 (2002).
6. S. S. Strautnieks, L. N. Bull, A. S. Knisely, S. A. Kocoshis, N. Dahl, H. Arnell, E. Sokal, K. Dahan, S. Childs, V. Ling, M. S. Tanner, A. F. Kagalwalla, A. Nemeth, J. Pawlowska, A. Baker, G. Mieli-Vergani, N. B. Freimer, R. M. Gardiner, and R. J. Thompson. A gene encoding a liver-specific ABC transporter is mutated in progressive familial intrahepatic cholestasis. *Nat. Genet.* **20**:233–238 (1998).
7. R. Wang, M. Salem, I. M. Yousef, B. Tuchweber, P. Lam, S. J. Childs, C. D. Helgason, C. Ackerley, M. J. Phillips, and V. Ling. Targeted inactivation of sister of P-glycoprotein gene (BSEP) in mice results in non-progressive but persistent intrahepatic cholestasis. *Proc. Natl. Acad. Sci. USA* **98**:2011–2016 (2001).
8. B. Stieger, K. Fattinger, J. Madon, G. A. Kullak-Ublick, and P. J. Meier. Drug- and estrogen-induced cholestasis through inhibition of the hepatocellular bile salt export pump (Bsep) of rat liver. *Gastroenterology* **118**:422–430 (2000).
9. S. Childs, R. L. Yeh, D. Hui, and V. Ling. Taxol resistance mediated by transfection of the liver-specific sister gene of P-glycoprotein. *Cancer Res.* **58**:4160–4167 (1998).
10. C. Funk, C. Ponelle, G. Scheuermann, and M. Pantze. 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 (2001).
11. V. E. Kostrubsky, M. Vore, E. Kindt, J. Burliegh, K. Rogers, G. Peter, D. Altrogge, and M. W. Sinz. The effect of troglitazone biliary excretion on metabolite distribution and cholestasis in transporter-deficient rats. *Drug Metab. Dispos.* **29**:1561–1566 (2001).
12. K. Fattinger, C. Funk, M. Pantze, C. Weber, J. Reichen, B. Stieger, and P. J. Meier. The endothelin antagonist bosentan inhibits the canalicular bile salt export pump: a potential mechanism for hepatic adverse reactions. *Clin. Pharmacol. Ther.* **69**: 223–231 (2001).
13. E. J. Wang, C. N. Casciano, R. P. Clement, and W. W. Johnson. *In vitro* flow cytometry method to quantitatively assess inhibitors of P-glycoprotein. *Drug Metab. Dispos.* **28**:522–528 (2000).
14. E. J. Wang, C. N. Casciano, R. P. Clement, and W. W. Johnson. Active transport of fluorescent P-glycoprotein substrates: evaluation as markers and interaction with inhibitors. *Biochem. Biophys. Res. Commun.* **289**:580–585 (2001).
15. E. J. Wang, C. N. Casciano, R. P. Clement, and W. W. Johnson. Cooperativity in the inhibition of P-glycoprotein-mediated daunorubicin transport: evidence for "half-of-the-sites reactivity. *Arch. Biochem. Biophys.* **383**:91–98 (2000).
16. V. Lecureur, D. Sun, P. Hargrove, E. G. Schuetz, R. B. Kim, L. B. Lan, and J. D. Schuetz. Cloning and expression of murine sister of P-glycoprotein reveals a more discriminating transporter than MDR1/P-glycoprotein. *Mol. Pharmacol.* **57**:24–35 (2000).
17. S. Dey, M. Ramachandra, I. Pastan, and M. M. Gottesman. Evidence for two nonidentical drug-interaction sites in the human P-glycoprotein. *Proc. Natl. Acad. Sci. USA* **94**:10594–10599 (1997).
18. E. J. Wang, C. N. Casciano, R. P. Clement, and W. W. Johnson. Two transport binding sites of P-glycoprotein are unequal yet

contingent: initial rate kinetic analysis by ATP hydrolysis demonstrates intersite dependency. *Biochim. Biophys. Acta* **1481**:63–74 (2000).
19. G. Chang and C. B. Roth. Structure of MsbA from *E. coli*: a homolog of the multidrug resistance ATP binding cassette (ABC) transporters. *Science* **293**:1793–1800 (2001).
20. T. W. Loo and D. M. Clarke. Determining the dimensions of the drug-binding domain of human P-glycoprotein using thiol cross-linking compounds as molecular rulers. *J. Biol. Chem.* **276**:36877–36880 (2001).
21. U. Bolder, N. V. Trang, L. R. Hagey, C. D. Schteingart, H. T. Ton-Nu, C. Cerre, R. P. Elferink, and A. F. Hofmann. Sulindac is excreted into bile by a canalicular bile salt pump and undergoes a cholehepatic circulation in rats. *Gastroenterology* **117**:962–971 (1999).
22. M. Torok, H. Gutmann, G. Fricker. and J. Drewe. Sister of P-glycoprotein expression in different tissues. *Biochem. Pharmacol.* **57**:833–835 (1999).
23. E. G. Schuetz, S. Strom, K. Yasuda, V. Lecureur, M. Assem, C. Brimer, J. Lamba, R. B. Kim, V. Ramachandran, B. J. Komoroski, R. Venkataramanan, H. Cai, C. J. Sinal, F. J. Gonzalez, and J. D. Schuetz. Disrupted bile acid homeostasis reveals an unexpected interaction among nuclear hormone receptors, transporters, and cytochrome P450. *J. Biol. Chem.* **276**:39411–39418 (2001).