P-Glycoprotein Attenuating Effect of Human Intestinal Fluid

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Purpose. To evaluate the effect of human intestinal fluid (HIF) on P-glycoprotein (P-gp)-mediated efflux.
Methods. HIF was obtained from eight healthy volunteers by duodenal aspiration. HIF was applied at different concentrations (0–75%) to the apical compartment of the Caco-2 system. Cyclosporin A (CsA) was used as a model compound for P-gp mediated efflux.
Results. When the bidirectional transport of CsA across Caco-2 monolayers was assessed, a significant polarity in transport could be observed, the absorptive transport being much lower than the secretory transport. Inclusion of HIF resulted in a moderate increase of the absorptive transport, as well as a significant concentration dependent decrease of the secretory transport, without compromising the integrity of the monolayer. Interestingly, a possible gender difference could be detected as inclusion of HIF obtained from female subjects resulted in a decreased absorptive transport of CsA, whereas inclusion of HIF obtained from male subjects resulted in an increased absorptive transport. The P-gp modulating effect of HIF is not caused by a lack of glucose as an energy source for the efflux mechanism when high concentrations of HIF were present in the buffer used.
Conclusions. The results of this study indicate that the contribution of P-gp efflux carriers may be overestimated when using salt buffer solutions as transport media. Additionally, it can be concluded that (presently unidentified) components of HIF may attenuate the P-gp mediated intestinal efflux. The clinical significance of this modulating effect remains to be investigated.

KEY WORDS: P-glycoprotein; bile salts; intestinal fluid; drug absorption; efflux.

INTRODUCTION

The use of a classic buffered salt solution (e.g., HEPES-buffered Hank's balance salt solution (HBSS) supplemented with glucose) is far from the physiological conditions and one can question whether potential permeability problems encountered when using HBSS in the Caco-2 cell culture model will be representative for the situation observed *in vivo*. Especially the presence of surfactants (e.g., bile salts) might affect the outcome of *in vitro* transport experiments. To evaluate the use of more representative buffer solutions in the Caco-2 system, the influence of the application of simulated intestinal fluid buffers as transport medium in the apical compartment was investigated in our Laboratory (1). Fassif (Fasted state simulated intestinal fluid) and Fessif (Fed state simulated intestinal fluid) were introduced by Dressman as bile salt containing dissolution media to simulate the *in vivo* dissolution behavior of drugs (2,3). The application of Fessif buffer in the Caco-2 system seriously compromised the integrity of the monolayers, probably due to the high amounts of bile salts (15 mM of sodium taurocholate) and the high osmolarity (600 mOsm) of this buffer solution (1). The use of Fassif buffer in the Caco-2 system did not result in any toxic effect as measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide cytotoxicity test and evaluation of Transepithelial electrical resistance (TEER). Application of Fassif buffer to the apical compartment of the Caco-2 system did not result in any significantly different transport of several model compounds (for passive and active transport), except for cyclosporin A (CsA), a known P-gp substrate (1). Fassif, and more specifically sodium taurocholate, the bile salt contained in this simulated intestinal fluid, was shown to modulate the P-gp mediated efflux of CsA (1). In this study, the possible P-gp modulating effect of real human intestinal fluid (HIF) was evaluated in the Caco-2 system, using CsA as a model compound for P-gp mediated transport.

MATERIALS AND METHODS

Materials

All chemicals used for culturing the Caco-2 cells were purchased from Invitrogen (Merelbeke, Belgium). Mes (2-[N-Morpholino]ethane-sulfonic acid) was purchased from Sigma Chemicals (St. Louis, MO, USA). Unlabeled Cyclosporin A (CsA; INN: ciclosporin) was obtained from GlaxoSmithKline (Research Triangle Park, NC, USA), whereas tritium-labeled CsA ([^{3}H]-CsA, 7 Ci/mmol) was purchased from Amersham Life Science (Ghent, Belgium).

Caco-2 Monolayers

Caco-2 cells were purchased from Cambrex Biosciences (Walkersville, MD, USA). Caco-2 cells were grown in 75-cm^2 culture flasks at 37°C in an atmosphere of 5% CO_2 and 90% relative humidity. Cells were passaged every 3–4 days (at 70–80% confluence) at a split ratio of 1 to 7. Cell culture medium consisted of Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids solution and 100 IU/mL penicillin–streptomycin. Transport medium (TM) consisted of HBSS containing 25 mM D-(+)-glucose (Sigma Chemical, St-Louis, MO, USA) and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethenesulfonic acid), or 10 mM Mes; pH was adjusted to 7.4 (HEPES) or 6.5 (Mes) at 37°C with sodium hydroxide (1 N; BDH, Poole, UK).

Transport Experiments

For transport experiments, Caco-2 cells were plated at a density of 60,000 cells/cm^2 on Costar® Transwell membrane inserts (3.0-μm pore diameter, 12-mm diameter; Corning Inc., NY, USA). Confluence was reached within 3–4 days after seeding and the monolayers were used for the experiments 21–25 days post-seeding. Cell passages between 85 and 110 were used in the experiments. As interbatch variation was relatively high, data were always compared within one batch;

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a control/reference condition was always included. Only monolayers with TEER values higher than 250 $\Omega.cm^2$ were used.

Transport experiments with CsA were performed following a previously described method (4). Briefly, the monolayers were rinsed three times with TM, and cells were incubated for 30 min with TM prior to the TEER measurement. A 30 min preincubation step with TM was performed and TEER values were measured again. HIF was applied to the apical compartment of the Caco-2 system (0–25–50–75%, pH 6.5). Subsequently, transport was initiated by adding [^{3}H]-CsA (0.1 μCi) together with unlabeled CsA to the donor compartment to obtain a final concentration of 1 μM. TEER values were measured at the end of the incubation step. The results of experiments for which TEER values of monolayers dropped to less than 70% of the initial values were excluded. Following incubation (60 min), the samples in the acceptor compartment were removed via multiple pipetting with 200 μL of disposable pipette tips and placed (along with the tips) in scintillation vials (16 mL of scintillation liquid, Ready Safe®, Beckman, Fullerton, CA, USA) for liquid scintillation counting (Liquid Scintillation Counter, Wallac 1410, Beckman, Fullerton, CA, USA). All solutions of CsA were made in siliconized glass tubes to decrease adsorption of CsA. At the end of the experiment, the inserts were withdrawn and 10 μL of a 1 mM unlabeled CsA solution in DMSO and 200 μL DMSO were added to the wells for one hour before collecting the samples to assure total recovery of [^{3}H]-marked CsA from the basolateral acceptor compartment.

Collection of HIF

HIF was obtained from eight healthy volunteers (up to 20 mL), after an overnight fast, by duodenal aspiration with a catheter, which was fluoroscopically positioned in the descending part of the duodenum. Volunteers were notified of the sampling procedures, the purpose of the study and the possible risks by a written informed consent form. Samples were centrifuged for 10 min at a speed of 5000 g using an ALC PJ180R centrifuge (Winchester, VA, USA), after which pH was measured and adjusted to 6.5. Samples were stored at –40°C until use. Approval for this project was granted by the Institutional Committee for Medical Ethics and Clinical Research.

Determination of Bile Salts and Phospholipids

For determination of total bile salt levels, the 3α-hydroxysteroid dehydrogenase assay was used (5). Biliary phospholipid levels were determined enzymatically, using phospholipase D and choline oxidase (6,7), employing a kit from Wako Chemicals (Neuss, Germany).

Calculations

Results of the bidirectional transport experiments in the Caco-2 system are expressed as permeability coefficients (in cm/s), which were calculated as follows:

$$P_{app} = \frac{\Delta Q}{\Delta t} \times \frac{1}{A \times C_0}$$

with ΔQ the amount of drug appearing in the acceptor compartment (nmol), Δt the incubation period (s), C_0 the initial concentration in the donor compartment (μM) and A the surface area (cm^2) across which transport occurred. All values are expressed as mean ± SD. Experiments were performed in triplicate (n = 3).

After performing an analysis of variance with the significance level set at $p < 0.05$, a multiple comparisons test (Dunnett test) was performed to test the null hypothesis of no difference between each of the effects of the different conditions with the control.

Table I. Influence of Human Intestinal Fluid (0–75% in Apical Compartment) on the Absorptive Transport (Apical to Basolateral) of Cyclosporin A

HIF	0%	25%	50%	75%
1	2.7 ± 0.8	3.1 ± 0.4	1.3 ± 0.1	2.3 ± 0.1
2	2.1 ± 0.6	3.8 ± 0.2*	5.6 ± 0.7*	5.6 ± 0.6*
3	1.6 ± 0.2	2.2 ± 0.5	2.9 ± 0.6*	6.0 ± 0.9*
4	1.6 ± 0.3	1.9 ± 0.2	2.3 ± 0.5*	2.9 ± 0.5*
5	0.7 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
6	1.2 ± 0.1	0.8 ± 0.2	0.3 ± 0.1	0.7 ± 0.1
7	1.3 ± 0.1	0.4 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
8	1.4 ± 0.1	1.7 ± 0.3	2.8 ± 0.6*	4.4 ± 0.3*
Average ± SD	1.6 ± 0.7	1.8 ± 1.2	1.9 ± 1.8	2.8 ± 2.3

Note: Results are shown as $P_{app\ AP\text{-}BL}$ values × 10^6 ± SD (cm/s) (n = 3, *p < 0.05).

RESULTS AND DISCUSSION

In a preliminary experiment, the toxicity of HIF towards Caco-2 monolayers was studied by assessing the integrity for a period of 60 min (measurement of TEER values). Several concentrations of HIF (0-100%, pH 6.5) were applied to the apical side of the monolayers (data not shown). In contrast to the data obtained with Fassif (1), the use of 100% of HIF resulted in a drop of TEER values (lower than 20% of the initial TEER values), impeding the use of this concentration. Application of lower concentrations of HIF in the apical compartment of the Caco-2 system did not compromise the integrity of the Caco-2 monolayers. As the ratio of phospholipids and bile salts in HIF samples (Table III) is comparable to the one of Fassif (phospholipids/bile salts = 0.25 in Fassif; Refs. 1,2), this ratio can not be invoked to explain the more toxic effect of HIF compared with Fassif. A possible explanation might be the effect of bile salts (or other components) present in HIF, but not in Fassif (only contains sodium tau-

Table II. Influence of Human Intestinal Fluid (0–75% in Apical Compartment) on the Secretory Transport (Basolateral to Apical) of Cyclosporin A

HIF	0%	25%	50%	75%
1	13.6 ± 1.5	13.0 ± 1.2	10.3 ± 0.7*	8.7 ± 0.6*
2	15.0 ± 0.4	17.6 ± 0.5	12.5 ± 2.0*	9.7 ± 0.8*
3	15.2 ± 2.3	15.6 ± 0.2	13.6 ± 1.2	11.2 ± 0.2*
4	22.4 ± 4.5	12.5 ± 0.8*	12.4 ± 2.5*	8.5 ± 0.9*
5	18.6 ± 1.2	16.6 ± 1.4	11.6 ± 1.0*	10.2 ± 1.0*
6	18.0 ± 1.9	15.3 ± 2.2	6.8 ± 0.2*	6.0 ± 0.3*
7	15.6 ± 2.1	14.7 ± 1.4	9.1 ± 1.5*	6.6 ± 0.6*
8	14.9 ± 2.7	15.3 ± 2.1	11.2 ± 1.2*	8.2 ± 1.3*
Average ± SD	16.6 ± 3.4	15.1 ± 2.4	11.0 ± 2.5*	8.6 ± 1.8*

Note: Results are shown as $P_{app\ BL\text{-}AP}$ values × 10^6 ± SD (cm/s) (n = 3, * = p < 0.05).

Table III. Age and Gender of the Donor Subjects and Composition of Aspirated Human Intestinal Fluid (HIF) (pH and Osmolarity of the Aspirate (in mOsm), Total Amount of Bile Salts (BS), and Phospholipids (PL) in HIF

HIF	Age	Gender	pH	Osmolarity (mOsm)	BS (μM)	PL (μM)	PL/BS
1	22	F	6.7	ND[a]	1440	39	0.1
2	24	M	6.2	193	800	3	0.1
3	35	M	6.3	238	2760	148	0.1
4	25	M	6.4	201	2420	636	0.3
5	23	F	6.5	ND	2120	738	0.4
6	24	F	6.7	260	1400	216	0.2
7	25	F	6.7	301	800	384	0.5
8	24	M	6.1	220	1300	530	0.4
Average ± SD	27 ± 5		6.5 ± 0.2	236 ± 40	1630 ± 730	337 ± 278	0.2 ± 0.2

Note: Results are expressed as concentrations (in μM) and the ratio of PL/BS.
[a] ND, not determined.

rocholate as bile salt) on the integrity of the Caco-2 monolayers. Tables I and II show the influence of HIF on the absorptive and the secretory transport of CsA across Caco-2 monolayers, respectively. As the interbatch variability for the Caco-2 experiments is relatively high, a control condition (0% HIF) was included for each subject. No general statistically significant effect on the absorptive transport of CsA could be observed. However, the addition of HIF (50% and 75% in the apical compartment) obtained from male subjects (2, 3, 4, and 8) all resulted in a significantly increased absorptive transport. Addition of HIF obtained from female subjects (1, 5, 6, and 7) resulted in a decreased absorptive transport of CsA. This might be caused by either a different hormonal profile or to a change in the composition of bile caused by intake of oral contraceptives (8). Indeed, it has been shown in nonobese healthy young women that the relative size of the cholic acid pool in the total bile acid pool (of which the size did not change) was increased with 36% after a daily intake of 30 μg of ethinyl oestradiol. This predominance of more hydrophilic bile acid conjugates (likely to interact less with P-gp) might be responsible for the observed decreased absorptive transport of CsA. However, because the design and the size of this study did not allow us to draw conclusions on possible gender effects, more research is required.

In contrast to the results of the absorptive transport studies, the effect of HIF on the secretory transport of CsA is more explicit: a statistically significant effect could be observed for HIF present in the apical compartment at a concentration of 50% (all subjects, except for subject 3) and 75% (Table II). No gender difference could be observed for the effect on the secretory transport.

In an effort to correlate the composition of HIF and Fassif with their effect on the bidirectional transport of CsA, the composition of the HIF samples was determined (pH, osmolarity, bile salts and phospholipids; Table III). In agreement with earlier reports (9–11), the pH of HIF varied between 6 and 7 (average of 6.5 ± 0.2). Osmolarity values for HIF were in the same range as reported earlier (from 193 to 301 mOsm, with an average of 236 ± 40 mOsm; Ref. 9). The bile salt content in the HIF samples ranged from 800 to 2760 μM (average of 1630 ± 730 μM). These concentrations are lower than the concentrations that were reported earlier for duodenal bile fluids (12–14). A possible explanation might be that, in contrast with other studies in which cholecystokinin or ceruletide was administered before bile aspiration, gall bladder contraction was not stimulated in our study. The concentration of phospholipids covered a wide range from 3 to 738 μM. These values are also significantly lower than earlier reported values for duodenal bile (13,14). Especially the samples of subject 1 and 2 contained very low concentrations

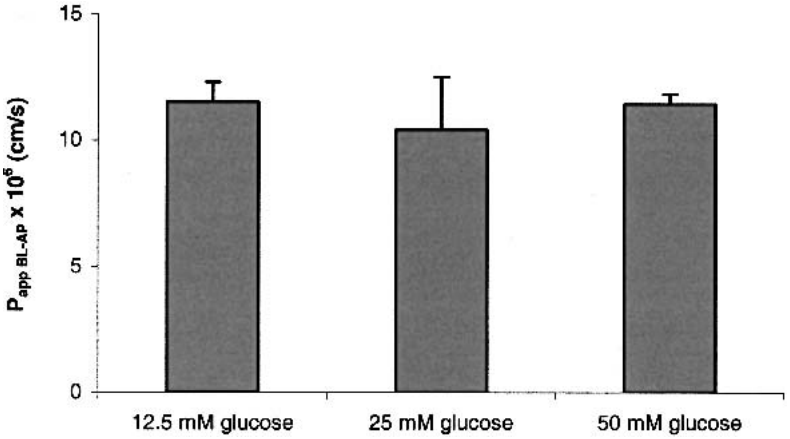

Fig. 1. Influence of the glucose concentration in human intestinal fluid on the P-glycoprotein-mediated efflux of cyclosporin A. Results are shown as $P_{app\ BL\text{-}AP}$ values $\times 10^6$ ± SD (cm/s) (n = 3).

of phospholipids (39 and 3 μM, respectively), which might be the result of inherited or acquired biliary phospholipid deficiency (15). No correlation could be observed between the concentration of bile salts and phospholipids in HIF. In addition, a correlation between the amounts of bile salts, phospholipids or the ratio phospholipids/bile salts and the effect of HIF on the bidirectional transport of CsA across Caco-2 monolayers could not be detected. Although the bile salt concentrations that could be detected in the HIF samples were lower than the concentrations of bile salts used in Fassif (3 mM sodium taurocholate; Refs. 1,2), a modulating effect of the P-gp mediated efflux of CsA could still be observed.

One of the major differences between classic TM and HIF as buffer solution, besides the presence of bile salts and phospholipids, is the lack of supplemented glucose. A shortage of glucose may result in lack of ATP, which is used by P-gp as an energy source to pump its substrates from the intracellular side to the extracellular side of the cell membrane. A lack of energy may result in a decreased secretory transport of CsA across the Caco-2 monolayers. To check this possibility, a comparative study was performed using a dilution of HIF (50% TM, 50% HIF) containing different concentrations of glucose (12.5–50 mM). The results of this experiment are shown in Fig. 1. Changing the total concentration of glucose from 12.5 to 50 mM did not result in any significantly different effect on the secretory transport of CsA, indicating that a shortage of glucose is not responsible for the P-gp attenuating effect of HIF.

Another possibility to explain the attenuating effect of HIF on P-gp mediated efflux is the presence of other (presently unidentified) components in HIF which show a possible direct or indirect inhibitory effect on P-gp. These compounds might be small peptides or conjugated steroids, of which several are known to be substrates/inhibitors for P-gp mediated efflux (e.g., progesterone, aldosterone, cortisol, corticosterone; Ref. 16). However, more research is required to identify the P-gp inhibiting components present in HIF.

CONCLUSION

Inclusion of up to 75% HIF as an apical medium in the Caco-2 system did not compromise the integrity of the monolayers. A concentration dependent effect of HIF on the bidirectional transport of CsA was shown. The results of this study illustrate that (not yet identified) components present in HIF may attenuate the effect of P-gp like efflux carriers on drug absorption. Additionally, it can be concluded that the contribution of P-gp efflux carriers may be overestimated when using HBSS-like salt buffer solutions. The clinical relevance of this attenuating effect remains to be investigated.

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