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Sulfasalazine transport in in-vitro, ex-vivo and in-vivo absorption models: contribution of efflux carriers and their modulation by co-administration of synthetic nature-identical fruit extracts

Raf Mols, Sven Deferme and Patrick Augustijns

Abstract

Sulfasalazine is characterised by low oral bioavailability. In this study, its intestinal transport characteristics were studied in an in-vitro, ex-vivo and in-situ system. The absorptive transport of sulfasalazine across Caco-2 monolayers appeared to be lower than the secretory transport ($P_{app\text{-}abs} = 0.21 \pm 0.02 \times 10^{-6}\,cm\,s^{-1}$ and $P_{app\text{-}secr} = 2.97 \pm 0.30 \times 10^{-6}\,cm\,s^{-1}$, respectively). This polarity in transport of sulfasalazine was not mediated by P-glycoprotein (P-gp), as inclusion of verapamil (100 μM) did not have any effect on the transport polarity of sulfasalazine. However, inclusion of the multidrug resistance-associated protein (MRP) inhibitors benzbromarone (50 μM) and sulfinpyrazone (1 mM), and the glutathione-depleting agent chlorodinitrobenzene (100 μM), resulted in an increased absorptive transport of sulfasalazine in the Caco-2 system ($P_{app\text{-}abs} = 0.64 \pm 0.02$, 0.51 ± 0.04 and $0.60 \pm 0.03 \times 10^{-6}\,cm\,s^{-1}$, respectively). The interference of carriers implies that, during absorption, interactions with food components may occur at the level of this carrier. Therefore, the effect of food extracts was studied in a parallel set of experiments. For two standardized nature-identical fruit extracts (pineapple and apricot extract) a concentration-dependent absorption-enhancing effect could be observed in the Caco-2 system. The functional expression of similar carriers was also demonstrated in rat ileum in the Ussing chamber system. Interaction studies with fruit extracts in the Ussing chamber system, as well as in the in-situ intestinal perfusion study, revealed a 2- to 4-fold increase in the absorptive transport of sulfasalazine. These results indicate that food components in the intestinal lumen can have a significant impact on the intestinal absorption characteristics of sulfasalazine by modulating the biochemical barrier function of the intestinal mucosa.

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Introduction

After oral administration, drug absorption can be limited by the biochemical barrier function of the intestinal mucosa. Both apically located efflux carriers and intestinal metabolism are known to limit the absorption of drugs and function as a source of drug interactions, unwanted side effects and unpredictable pharmacological activity (Fleisher et al 1999; Singh 1999). The co-administration of food may result in food–drug interactions at various levels, including the biochemical barrier function of the intestinal mucosa. Over the last few years, many groups have studied the effect of food and individual food components on the gastrointestinal absorption and metabolism of drugs (Takanaga et al 1998; Eagling et al 1999; Ohnishi et al 2000; Bhardwaj et al 2002; Tian et al 2002). Especially fruit juices have been evaluated for their possible inhibitory effects on P-gp-mediated efflux and intestinal CYP3A4-mediated degradation. Grapefruit juice and several flavonoids present in grapefruit juice (e.g. 6′,7′-dihydroxybergamottin) have been shown to exert P-gp- and CYP3A4-inhibitory activity (Ameer & Weintraub 1997; Takanaga et al 1998; Spahn-Langguth & Langguth 2001).

Sulfasalazine (4-hydroxy-4′-(2-pyridylsulfamoyl)azobenzene-3-carboxylic acid) is commonly used for the treatment of chronic inflammatory bowel diseases, including Crohn's disease and ulcerative colitis (Hasko et al 2001). After oral administration, this compound is metabolized into sulfapyridine and 5-aminosalicylic acid by the intestinal

flora (Khan et al 1982); it is characterized by a low oral bioavailability (approximately 15%) (Van Hees et al 1980; Klotz 1985; Friedman 1986). Caco-2 cell studies have shown that sulfasalazine is a substrate for various efflux carriers, which may, at least partially, explain its low intestinal absorption in-vivo (Yazdanian et al 1998). It was shown that at least two cellular efflux mechanisms, the multidrug resistance-associated protein (MRP) and the anion-sensitive transport systems, are involved in the transport of sulfasalazine across Caco-2 cell monolayers (Liang et al 2000).

As already mentioned, several fruit juices have been evaluated for their interaction with the intestinal absorption of drugs. However, contradictory results have been obtained for most of them. Grapefruit juice has been shown to inhibit P-glycoprotein (P-gp), stimulate P-gp or to have no significant effect on the functionality of P-gp (Becquemont et al 2001; Soldner et al 1999; Spahn-Langguth & Langguth 2001; Tian et al 2002; Takanaga et al 1998). This difference in results might be explained by a multitude of factors, including the use of different models and different concentrations and variability in composition of the fruit juice (Ameer & Weintraub 1997). In previous studies we approached these problems by using synthetic nature-identical fruit extracts that were evaluated for their possible P-gp inhibitory effects (Deferme et al 2002a). More specifically, a standardized apricot extract was shown to diminish the P-gp-mediated efflux of talinolol, a specific P-gp substrate, in different models (Deferme et al 2002b).

The involvement of different carriers in the intestinal transport of sulfasalazine implies that interactions with food components may occur at the level of carriers present in the intestinal mucosa. Therefore, a set of experiments was conducted in which the effect of different food extracts was studied. In this study, the intestinal absorption of sulfasalazine is assessed in different models, including the Caco-2 model, the Ussing chamber system and the in-situ intestinal perfusion technique. Using these different models, the effect of nature-identical fruit extracts (apricot and pineapple) and several known inhibitors of P-gp and MRP/anion-sensitive transporters on the intestinal absorption of sulfasalazine was evaluated.

The high polarity in transport of sulfasalazine observed in the study of Liang et al (2000) prompted us to further explore whether the functional expression of these carrier(s) could also be observed in other absorption systems, and whether interaction would occur at the level of these carrier(s).

Materials and Methods

Materials

All chemicals used for culturing the Caco-2 cells were purchased from Invitrogen (Merelbeke, Belgium). Cell culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 1% Minimum Essential Medium (MEM) non-essential amino acids solution and 100 IU mL^{-1} penicillin–streptomycin. Transport medium (TM) consisted of Hanks' Balanced Salt solution containing 25 mM D-(+)-glucose (Sigma Chemical, St-Louis, MO) and 10 mM HEPES; the pH was adjusted to 7.4 at 37°C with sodium hydroxide (2 M) (BDH, Poole, UK).

Verapamil, sulfinpyrazone, benzbromarone, 1-chloro-2,4-dinitrobenzene, sodium fumarate, sodium pyruvate, sodium glutamate, sulfapyridine and sulfasalazine were purchased from Sigma-Aldrich (St-Louis, MO). Methanol was provided by Fisher Scientific (Leicestershire, UK). Dichloromethane and KCl were obtained from Acros Organics (Geel, Belgium). KH_2PO_4, $CaCl_2.2H_2O$ and $NaH_2PO_4.H_2O$ were purchased from Merck (Darmstadt, Germany). Vel (Leuven, Belgium) supplied us with theophylline, $Na_2HPO_4.2H_2O$, NH_4Cl, NaCl, $MgCl_2.6H_2O$, EDTA and $NaHCO_3$. Indometacin was purchased from Certa (Braine-l'Alleud, Belgium). Sodium fluorescein was provided by UCB (Brussels, Belgium). Pineapple extract (PE036583) and apricot extract (PE036578) and its fractions (based on chemical class of compounds) were a kind gift of Danisco (Louvain-la-Neuve, Belgium). The quantitative and qualitative composition of these extracts is well defined and is nature-identical, meaning that these extracts consist of a mixture of synthetic compounds whose quantitative and qualitative composition is comparable to the composition of natural fruit extracts. The extracts were used as an aqueous solution of 0–1% (v/v%). Fractions of the apricot extract (based on the chemical class of the different ingredients of the extract) were used at lower concentrations, relative to their proportion in the total apricot extract. The qualitative composition and the quantitative composition per chemical class of the pineapple and apricot extracts are shown in Tables 1 and 2, respectively. In the text, we refer to this synthetic nature-identical fruit extracts as fruit extracts.

Caco-2 experiments

Cell culture

Caco-2 cells were purchased from Cambrex Biosciences (Walkersville, MD). 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 1:7. Cells were negative for *Mycoplasma* infection.

Table 1 Qualitative composition and quantitative composition per chemical class of the synthetic pineapple extract (PE036583)

Aromatic esters	52.4%
Allyl hexanoate	
Ethyl heptanoate	
Ethyl butyrate	
Iso-amyl acetate	
Methyl butyrate	
Iso-amyl butyrate	
Amyl hexanoate	
Ethyl hexanoate	
Citrus oils	47.6%
Lemon oil	
Mandarin oil	
Orange oil	

Table 2 Qualitative composition and quantitative composition per chemical class of the synthetic apricot extract (PE036578)

Aromatic esters	14.5%
Ethyl acetate	
Ethyl formate	
Butyl acetate	
Ethyl propionate	
Ethyl butyrate	
Iso-amyl acetate	
Butyl butyrate	
Ethyl hexanoate	
Diethyl malonate	
Benzyl acetate	
Geranyl acetate	
Ethyl decanoate	
2-Hexenyl butyrate	
Ethyl dodecanoate	
Triethyl citrate	
Aromatic acids	12.1%
Methyl butyric acid	
Iso-butyric acid	
Acetic acid	
Hexanoic acid	
Aromatic alcohols	4.0%
Geraniol	
2-Hexenol	
Linalool	
Terpineol	
Benzyl alcohol	
4-Terpineol	
Aldehydes	12.1%
Benzaldehyde	
2-Hexanal	
Lactones	5.3%
4-Ethylbutan-4-olide (gamma-hexalactone)	
4-Butylbutan-4-olide (gamma-octalactone)	
5-Butylpentan-5-olide (delta-nonalactone)	
4-Hexylbutan-4-olide (gamma-decalactone)	
4-Heptylbutan-4-olide (gamma-undecalactone)	
4-Octylbutan-4-olide (gamma-dodecalactone)	
5-Heptylpentan-5-olide (delta-dodecalactone)	
Oil	0.4%
Clary sage oil	
Other	51.7%
Solvent (alcohol)	
Vanillin	
Myrcene	

Transport experiments

For transport experiments, Caco-2 cells were plated at a density of 40 000 cells cm^{-2} on Costar Transwell membrane inserts (0.2 μm pore diameter, 12 mm diameter; Corning Inc., NY). 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. Transepithelial electrical resistance (TEER) values were measured with an EndOhm Voltohmmeter (WPI, Aston, UK). Only monolayers with initial TEER values higher than 250 $\Omega\,cm^2$ were used. All volumes amounted to 0.5 mL at the apical side of the monolayer and 1.5 mL at the basolateral side.

After rinsing the monolayers 3 times with TM, a preincubation step (30 min) with TM (control) was performed. After measuring TEER values, transport was initiated by adding sulfasalazine (500 μM) to the donor compartment in the absence or presence of inhibitors or fruit extracts. All the solutions were adjusted to a pH of 7.4 before administration. Samples were taken from the acceptor compartment after 2 h and TEER values were measured again. TEER values at the end of the experiment were higher than 90% of the initial value. As an additional control of the monolayer integrity, sodium fluorescein flux was measured at the end of the experiment. Briefly, sodium fluorescein (1 mg mL^{-1}) was added to the apical compartment and after 60 min samples were taken from the basolateral compartment, followed by TEER measurement. The amount of sodium fluorescein appearing in the basolateral compartment was measured by UV spectrophotometry (Uvikon 810P spectrophotometer; Kontron Instruments, Watford, UK) at 490 nm. Sodium fluorescein flux values across the monolayers were below 0.6% $h^{-1}\,cm^{-2}$. None of the test conditions affected the integrity of the tight junctions (based on TEER measurements and sodium fluorescein flux).

Animals

The rats used in this study were purpose-bred. The rats were housed, according to the Belgian and European laws, guidelines and policies for animal experiments, housing and care, in the Central Animal Facilities of the university. These facilities have the obligatory accreditation of the authorised Belgian Ministry and are registered under license number LA2210393. Approval for this project was granted by the institutional Ethical Committee for Animal Experimentation.

Ex-vivo experiments

Ussing chamber experiments were performed based on a previously described method (Deferme et al 2002b). Male Wistar rats (±300 g; Elévage Janvier, France) were used. Rats were housed under standard laboratory conditions with free access to water and food. After anaesthetizing the rats with sodium pentobarbital (Nembutal, 60 mg kg^{-1}; Sanofi Santé Animale, Brussels, Belgium), a laparotomy was performed. A segment of ileum (0–20 cm proximal to the ileocaecal junction) was removed, rinsed twice with ice-cold buffer and immediately put for 30 min into ice-cold modified Krebs–Ringer buffer solution (KRB, pH 7.4, composition in mM: NaCl 111.9, KCl 5.0, $CaCl_2$ 1.2, $MgCl_2$ 1.2, NaH_2PO_4 0.4, Na_2HPO_4 1.6, $NaHCO_3$ 25.0, NaGlutamate 4.9, NaPyruvate 4.9, Na_2Fumarate 5.4 and glucose 11.5) that was continuously bubbled with carbogen O_2–CO_2 (95%:5%). The specimen was divided into 2.5-cm segments, which were cut open along the mesenteric border with blunt-end scissors. The serosal layer was stripped off in ice-cold oxygenated buffer and the tissue was mounted in the Ussing diffusion chamber (Physiologic Instruments, Palo Alto, CA) with a 1-cm^2 exposed tissue area.

After mounting, each half-cell was filled with 4 mL KRB (36°C), bathing the bowel segment on both the mucosal and serosal side. The temperature was kept constant at 36°C by a heating block. The KRB was continuously oxygenated with carbogen and circulated by gas lift in the chambers. A 15-min equilibration period was performed to achieve steady-state electrophysiological conditions, after which permeability experiments were initiated by adding sulfasalazine (100 μM) in the absence (reference) or presence of efflux inhibitors or fruit extracts to the donor compartment. Samples were taken from the acceptor compartment every 10 min (1 mL) and replaced by 1 mL of pre-warmed KRB. Experiments were run for 150 min. To characterize the relationship between transepithelial potential difference (PD), short circuit current (I_{sc}) and transepithelial resistance (R), a four-electrode system was used. One pair of Ag/AgCl electrodes imbedded in 3 M KCl/2% agar was used for measurement of PD and one pair of Ag electrodes for current passage. The electrodes were coupled to an external six-channel electronic unit with a voltage-controlled current source (home-made device). Data sampling was computer controlled via an A/D–D/A board by a program developed in LabView. Typical initial PD values were between −2 and −5 mV, with initial I_{sc} values of 90–150 μA and initial TEER values of 30–40 $\Omega\,cm^2$. These values are in correspondence with previously reported data (Polentarutti et al 1999). As an additional control to check tissue viability, theophylline (10 mM) was added to each side of the chambers at the end of the experiment and the change in I_{sc} was observed. I_{sc} typically increased at least 30%, indicating viability of the tissue at the end of the experiment.

In-situ experiments

In-situ intestinal perfusion studies: experimental set-up
In-situ perfusion experiments were performed based on a previously described method (Van Gelder et al 2000). Male Wistar rats (±300 g; Elevage Janvier, France) were used. After anaesthetizing the rats with sodium pentobarbital (60 mg kg^{-1}), the right jugular vein was cannulated with a heparinized (50 IU mL^{-1}) polyethylene cannula (1.02 mm outer diameter; Portex, Kent, UK) for blood supply from a donor rat during the perfusion experiment. A laparotomy was performed and the small intestine was exposed. The mesenteric vein draining the last part of the ileum (0–10 cm proximal to ileocaecal junction) was cannulated using a heparinized (50 IU mL^{-1}) catheter (Insyte-W 0.7 × 19 mm; Beckton Dickinson, Salt Lake City, UT). The cannula was secured with a few drops of cyanoacrylate adhesive. A mesenteric-jugular shunt was constructed to avoid blood loss during further preparation of the perfusion experiment. A segment of the ileum (6–10 cm) was isolated by inserting two glass cannulas (4 mm o.d., 3 mm i.d.) at the proximal and distal end of the segment. Polyethylene tubing (6.5 mm o.d., 3.1 mm i.d.) was connected to the inlet cannula. The intestinal content was removed by perfusing the segment with pre-warmed TM (37°C) at a flow rate of 3 mL min^{-1}. The perfusion pump (HVL Tris, Brussels, Belgium) was placed between the reservoir and the inlet cannula. A 3-way valve placed immediately before the inlet cannula allowed sampling from the perfusion medium.

In-situ intestinal perfusion studies with sulfasalazine (200 μM)
The flow rate of the perfusate amounted to 3 mL min^{-1}. This relatively high flow rate was used to obtain quick steady-state perfusate concentrations and to obtain a homogeneous distribution of the drug in the perfused segment throughout the whole experiment. At the beginning of the perfusion with sulfasalazine (200 μM), the mesenteric–jugular shunt was opened and donor blood supply initiated via the jugular vein at a rate of 0.5 mL min^{-1}. The intestinal segment was perfused with sulfasalazine in the absence or presence of apricot extract (1%). Experiments were performed under a closed-loop (perfusate was recirculated) and open-loop (single-pass perfusion) technique. Blood from the mesenteric vein was collected in heparinized tubes over 5-min time intervals for 30 min. In addition, samples were taken from the perfusion medium in the middle of each time interval.

HPLC analysis of sulfasalazine (Caco-2, Ussing chambers)

Concentrations of sulfasalazine were determined using an HPLC-UV system (Waters Associated Inc., Milford, MA; $\lambda = 357$ nm). The obtained peaks were integrated using a PC running Waters Millennium chromatography software. The column used was a Waters Novapak C-18 column (4 μm). The flow rate amounted to 1 mL min^{-1}, and the mobile phase (pH 3.0) consisted of 35% buffer (KH_2PO_4 50 mM, trifluoroacetic acid 0.06%) and 65% methanol. All water was purified by a Maxima system (Elga Ltd, High Wycombe Bucks, UK). The volume injected was 100 μL. The retention time of sulfasalazine under these conditions was 8.5 min. Before injection, the pH of the samples from the Caco-2 system and the Ussing chamber system was adjusted to 3.0. Intraday repeatability using this HPLC method resulted in relative standard deviations of 1.5% and 5.4% for 1000 nM and 100 nM, respectively.

Extraction of sulfapyridine and sulfasalazine from blood samples

The concentrations of sulfasalazine and sulfapyridine in blood samples obtained from the in-situ perfusion experiments were analysed following protein precipitation. Blood (200 μL) was added to MeOH (400 μL). After vortex-mixing, samples were centrifuged for 5 min at 14 000 *g* (4°C) and the supernatant was transferred to a new test tube. Following a second centrifugation step, 200 μL of supernatant was transferred to a new test tube. After evaporation to dryness under a gentle stream of air, the residue was dissolved in 200 μL of TM, of which 100 μL was injected into the HPLC system. Concentrations were quantified by comparison with a calibration curve made

up with spiked blood samples that were treated in the same way as the samples.

HPLC analysis of sulfasalazine and sulfapyridine (in-situ perfusion)

Concentrations of sulfasalazine and sulfapyridine extracted from blood were determined using an HPLC gradient method. The same system as described in the previous section was used. The solvent flow rate amounted to 1 mL min^{-1}. Mobile phase A consisted of 80% buffer (KH_2PO_4 50 mM, trifluoroacetic acid 0.06%) and 20% methanol. Mobile phase B consisted of 20% buffer (KH_2PO_4 50 mM, trifluoroacetic acid 0.06%) and 80% methanol. Both mobile phase A and B were adjusted to pH 3. The column was initially equilibrated at 90% mobile phase A and 10% mobile phase B. Four minutes after injection, the concentration of mobile phase B was increased to 70% over 3 min. After 15 min, the system was returned to the initial conditions over 4 min and equilibrated for another 4 min before the next injection. The retention times of sulfapyridine and sulfasalazine were 6 and 16 min, respectively. Intraday repeatability using this HPLC method resulted in relative standard deviations of 0.6% and 3.5% for 10 μM and 0.5 μM sulfasalazine, respectively, and of 0.7% and 4.4% for 10 μM and 0.5 μM sulfapyridine, respectively. Samples were always analysed immediately after performing the experiment.

Calculations

Results of the transport experiments with the Caco-2 monolayers and the ex-vivo Ussing chamber experiments are expressed as permeability coefficients (in cm s^{-1}), which were calculated as follows:

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

with $\Delta Q/\Delta t$ the amount of drug appearing in the acceptor compartment in function of time (nmol s^{-1}), C_0 the initial concentration in the donor compartment (μM) and A the surface area (cm^2) across which the transport occurred. Results of the in-situ perfusion experiments are expressed as the cumulative amounts appearing in the mesenteric blood, corrected for length of the perfused segment (nmol cm^{-1}). Values are expressed as mean ± s.d. (n = 3). After performing an analysis of variance using the non-parametric Kruskal–Wallis test with the significance level set at $P < 0.05$, a multiple comparisons test (Dunn's test) was performed to test the null hypothesis of no difference between each of the effects of the different conditions and the control condition.

Results

In-vitro experiments

To confirm the findings by Liang et al (2000), the bidirectional transport of sulfasalazine was assessed in a first set of experiments. Figure 1 shows that the absorptive transport

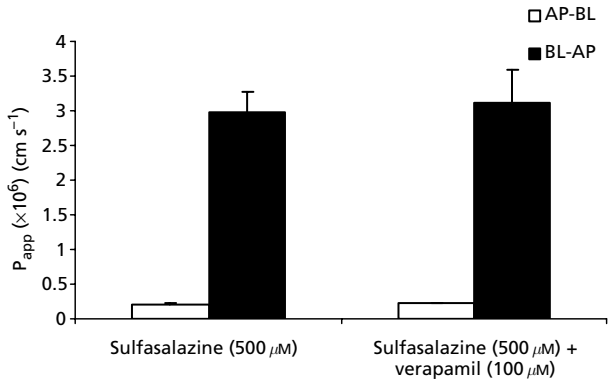

Figure 1 Influence of verapamil (100 μM) on the bidirectional transport of sulfasalazine (500 μM) across Caco-2 monolayers. Results are expressed as $P_{app} \times 10^6$ (cm s^{-1} ± s.d., n = 3). Open bars represent the absorptive transport. Closed bars represent the secretory transport.

(apical → basolateral compartment) of sulfasalazine was much lower than the secretory transport (basolateral → apical compartment) ($P_{app\text{-}abs} = 0.21 \pm 0.02 \times 10^{-6}$ cm s^{-1} and $P_{app\text{-}secr} = 2.97 \pm 0.30 \times 10^{-6}$ cm s^{-1}, respectively). The absorptive permeability for sulfasalazine appeared to be 10-fold higher as compared to the findings by Liang et al (2000). Addition of 100 μM verapamil, a known P-gp inhibitor, to the apical compartment did not result in any significant changes in the polarity in transport of sulfasalazine ($P_{app\text{-}abs} = 0.22 \pm 0.01 \times 10^{-6}$ cm s^{-1} and $P_{app\text{-}secr} = 3.12 \pm 0.48 \times 10^{-6}$ cm s^{-1}, respectively).

The influence of inhibitors of MRP and organic anion transporters, including indometacin, furosemide, sulfinpyrazone and benzbromarone, on the absorptive transport of sulfasalazine was also evaluated in the Caco-2 system (Table 3). Addition of 100 μM indometacin or 1 mM furosemide did not result in any significant increase of the absorptive transport of sulfasalazine ($P_{app\text{-}abs} = 0.30 \pm 0.01$, 0.36 ± 0.03 and $0.31 \pm 0.02 \times 10^{-6}$ cm s^{-1}, for the control condition, indometacin and furosemide, respectively). However, addition of 1 mM sulfinpyrazone, 50 μM benzbromarone or 100 μM chlorodinitrobenzene resulted in an increased absorptive

Table 3 Influence of inhibitors of MRP and anion-sensitive transporters on the absorptive transport of sulfasalazine across Caco-2 monolayers and across rat ileum in the Ussing chambers

Condition	$P_{app\text{-}abs}$ ($\times 10^6$) (cm s^{-1})	
	Caco-2	Ussing chambers
Sulfasalazine[a]	0.30 ± 0.01	3.29 ± 0.20
+ Indometacin (100 μM)	0.36 ± 0.03	3.21 ± 1.66
+ Furosemide (1 mM)	0.31 ± 0.02	4.67 ± 2.30
+ Sulfinpyrazone (1 mM)	0.51 ± 0.04	4.44 ± 1.30
+ Benzbromarone (50 μM)	0.64 ± 0.02*	8.35 ± 2.82
+ Chlorodinotrobenzene (100 μM)	0.60 ± 0.03	7.49 ± 1.77

[a]500 μM Sulfasalazine in the Caco-2 system, 100 μM sulfasalazine in the Ussing chamber model. Results are expressed as $P_{app} \times 10^6$ (cm s^{-1} ± s.d., n = 3). *$P < 0.05$, compared with absence of inhibitor.

transport of sulfasalazine across the Caco-2 monolayers ($P_{app\text{-}abs} = 0.51 \pm 0.04$, 0.64 ± 0.02 and $0.60 \pm 0.03 \times 10^{-6}$ cm s^{-1}, respectively).

In a second set of experiments, the effect of standardized fruit extracts on the absorptive transport of sulfasalazine across Caco-2 monolayers was investigated. After screening the modulatory effect of 48 standardized fruit extracts on the absorptive transport of sulfasalazine in the Caco-2 model (data not shown), a standardized apricot and pineapple extract were selected for further evaluation of their influence on the absorptive transport of sulfasalazine (Deferme 2002). These extracts were selected for further evaluation because they showed the highest effect among the fruit extracts tested and because no interference in the analytical procedure could be observed. Figure 2A illustrates that the inclusion of 0.3% and 0.1% apricot extract resulted in an increased absorption of sulfasalazine ($P_{app\text{-}abs} = 0.71 \pm 0.02$ and $0.22 \pm 0.02 \times 10^{-6}$ cm s^{-1}, respectively, vs $0.12 \pm 0.02 \times 10^{-6}$ cm s^{-1} for the control condition), while the addition of 0.03% of apricot extract did not affect the absorptive transport of sulfasalazine ($P_{app\text{-}abs} = 0.13 \pm 0.02 \times 10^{-6}$ cm s^{-1}). Figure 2B illustrates that the inclusion of pineapple extract (0.03–1%) also resulted in a concentration-dependent effect on the absorptive transport of sulfasalazine in the Caco-2 model. A 4.5-fold increase of the absorptive transport of sulfasalazine was observed after inclusion of 1% pineapple extract in the apical medium ($P_{app\text{-}abs} = 0.93 \pm 0.04$ vs $0.20 \pm 0.01 \times 10^{-6}$ cm s^{-1} for the control condition). Inclusion of lower concentrations of pineapple extract still resulted in an increased absorptive transport of sulfasalazine ($P_{app\text{-}abs} = 0.71 \pm 0.01$, 0.38 ± 0.01 and $0.30 \pm 0.01 \times 10^{-6}$ cm s^{-1}, for 0.3%, 0.1% and 0.03% pineapple extract, respectively).

Ussing chamber experiments

The bidirectional transport of sulfasalazine across rat ileum was assessed in modified Ussing chambers. Although to a lower extent, the polarity in transport of sulfasalazine could be confirmed in this model, as the absorptive transport (mucosal → serosal) was three-fold lower than the secretory transport (serosal → mucosal) ($P_{app\text{-}abs} = 4.79 \pm 0.67$ and $P_{app\text{-}secr} = 17.61 \pm 3.90 \times 10^{-6}$ cm s^{-1}) (Figure 3A). As shown in Table 3, inclusion of 100 μM indometacin, 1 mM furosemide or 1 mM sulfinpyrazone in the mucosal compartment did not result in a significantly increased absorptive transport of sulfasalazine across rat ileum ($P_{app\text{-}abs} = 3.21 \pm 1.66$, 4.67 ± 2.30 and $4.44 \pm 1.30 \times 10^{-6}$ cm s^{-1}, respectively) as compared with the control condition ($P_{app\text{-}abs} = 3.29 \pm 0.20 \times 10^{-6}$ cm s^{-1}). However, addition of 50 μM benzbromarone and 100 μM chlorodinitrobenzene resulted in an increased absorptive transport of sulfasalazine in the Ussing chamber model ($P_{app\text{-}abs} = 8.35 \pm 2.82$ and $7.49 \pm 1.77 \times 10^{-6}$ cm s^{-1}, respectively) (Table 3). Inclusion of 1% apricot extract in the mucosal medium

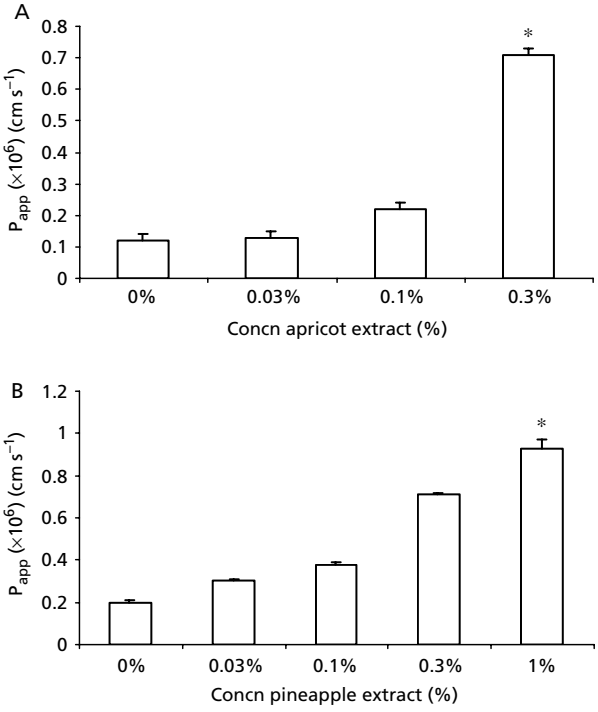

Figure 2 Influence of apricot extract (0–0.3%) on the absorptive transport of sulfasalazine (500 μM) (A) and influence of pineapple extract (0–1%) on the absorptive transport of sulfasalazine (250 μM) (B) across Caco-2 monolayers. Results are expressed as $P_{app} \times 10^6$ (cm s^{-1} ± s.d., n = 3). *$P < 0.05$, compared with 0% extract.

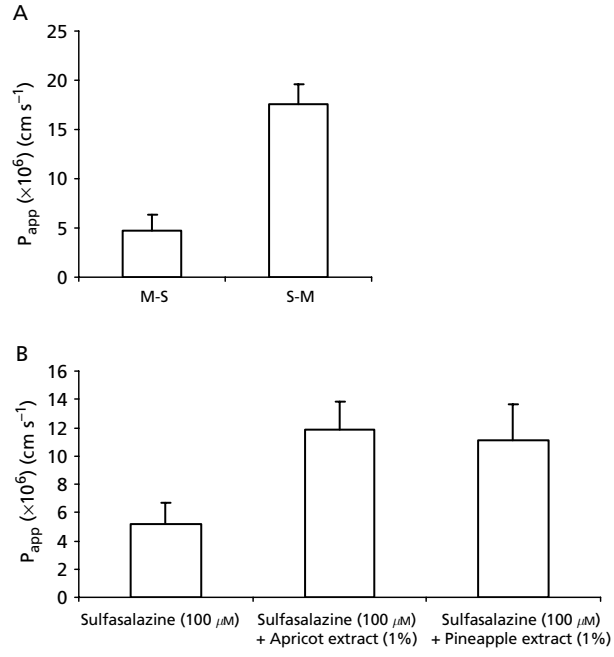

Figure 3 A. Polarity in transport of sulfasalazine (100 μM) across rat ileum in Ussing chamber experiments. Results are expressed as $P_{app} \times 10^6$ (cm s^{-1} ± s.d., n = 3). M-S represents absorptive transport, S-M represents secretory transport. B. Influence of the inclusion of apricot extract (1%) and pineapple extract (1%) on the absorptive transport of sulfasalazine (100 μM) across rat ileum in Ussing chamber experiments. Results are expressed as $P_{app} \times 10^6$ (cm s^{-1} ± s.d., n = 3).

resulted in a 2-fold increase of the absorptive transport of sulfasalazine ($P_{app\text{-}abs} = 11.89 \pm 1.93 \times 10^{-6}$ cm s^{-1}) as compared with the control condition ($P_{app\text{-}abs} = 5.16 \pm 1.50 \times 10^{-6}$ cm s^{-1}) (Figure 3B). Similarly, the inclusion of 1% pineapple extract in the mucosal medium resulted in an increased absorptive transport of sulfasalazine ($P_{app\text{-}abs} = 11.06 \pm 2.55 \times 10^{-6}$ cm s^{-1}).

In-situ experiments

As shown in Figure 4, inclusion of 1% apricot extract in the perfusion medium during in-situ perfusion of an intestinal segment (rat ileum) with sulfasalazine (200 μM) resulted in a 2.2-fold increase in the intestinal absorption of sulfasalazine in the closed loop set-up and a 4.4-fold increase in the open loop set-up. No sulfapyridine could be observed in the chromatograms, suggesting that no metabolism occurred during absorption.

Discussion

The results obtained with the Caco-2 system clearly show the asymmetry in transport of sulfasalazine (Figure 1), the absorptive transport (AP-BL) being lower than the secretory transport (BL-AP), and corroborate the results that have been published by Liang et al (2000). However, the polarity in transport appeared to be lower (14-fold) as compared with the 176-fold difference that was reported previously (Liang et al 2000). This might be explained by a difference in the expression of transporters (due to the use of different passage numbers, or different cell seeding densities) for which sulfasalazine is a substrate (Anderle et al 1998). The results shown in Figure 1 also confirm the lack of P-gp involvement in the efflux of sulfasalazine, as the known P-gp inhibitor verapamil did not affect the polarity in transport of sulfasalazine across the Caco-2 system (Liang et al 2000). Table 3 shows the influence of several inhibitors of MRP/anion sensitive transporters on the absorptive transport of sulfasalazine in the Caco-2 system. The presence of indometacin and furosemide in the apical compartment did not result in an increase of the absorptive transport, in contrast to the results obtained by Liang et al (2000), who showed a 3-fold increased absorptive transport of sulfasalazine upon addition of indometacin. However, inclusion of sulfinpyrazone and benzbromarone resulted in an increased absorptive transport across the Caco-2 monolayers (Table 3). Most compounds become substrates for MRP transporters after phase II metabolism, by which they are conjugated with glutathione, or they can be co-transported with glutathione (Borst et al 2000; Evers et al 2000; Fricker & Miller 2002). The addition of the glutathione-depleting agent chlorodinitrobenzene resulted in an increased absorption of sulfasalazine across the Caco-2 monolayers, probably due to the lower disposition of glutathione, resulting in less glutathione conjugation/co-transport of sulfasalazine.

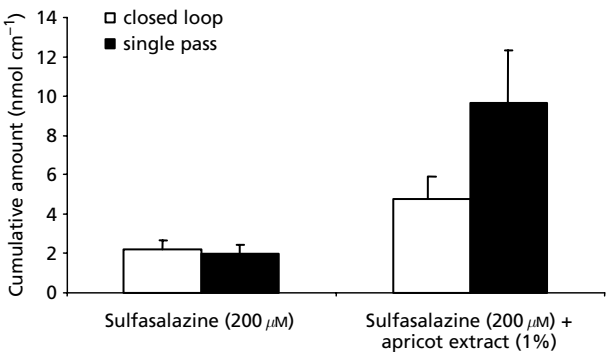

Figure 4 Effect of apricot extract (1%) on the absorptive transport of sulfasalazine (200 μM) in the in-situ intestinal perfusion model. Results are expressed as cumulative amount appearing in the mesenteric blood, corrected for length of the perfused segment (nmol cm^{-1} ± s.d., n = 3). Open bars represent data obtained with the closed-loop set-up. Closed bars represent data obtained with the open-loop set-up.

Not only the effect of inhibitors of MRP/anion sensitive transporters on the absorptive transport of sulfasalazine was investigated in this study, but also the importance of possible food–drug interactions. Of the 48 standardized fruit extracts that were tested in preliminary experiments (data not shown), apricot and pineapple extract were selected for further experiments. As shown in Figures 2A and 2B, the addition of these fruit extracts resulted in a concentration-dependent increased absorptive transport of sulfasalazine across the Caco-2 system. In previous studies with nature-identical apricot extract, an influence on P-gp-mediated transport was shown in the Caco-2 system, the Ussing chamber system and the in-situ intestinal perfusion model, by demonstrating a decrease in polarity in transport of the specific P-gp substrate talinolol in the presence of this extract (Deferme et al 2002b). Since these nature-identical extracts are mixtures of a multitude of compounds belonging to different chemical classes with completely different properties (Deferme 2002), it is not surprising that apricot extract and pineapple extract might contain compounds that interfere with MRP or anion-sensitive efflux mechanisms as well.

The results shown in Figure 3A confirm the polarity in transport of sulfasalazine (100 μM) in an ex-vivo model, more specifically in the Ussing chamber system. The absorptive transport of sulfasalazine across isolated rat ileum in the Ussing chamber system was 3.6-fold lower than the secretory transport, indicating the presence and functionality of an active efflux mechanism in rat intestinal tissue. The polarity in transport was less pronounced than in the Caco-2 system (3.6-fold in the Ussing chamber system compared with 14-fold in the Caco-2 system), which might be due to a different expression or activity of efflux carriers in rat intestine compared with Caco-2 monolayers and to the fact that rat ileum is a more leaky epithelium than Caco-2 monolayers. However, despite this less pronounced polarity, the similarity in results obtained with the MRP/anion-sensitive transporter inhibitors in the Ussing chambers and in the Caco-2 model demonstrate that similar carriers are operational in the ex-vivo and the in-vitro model. Inclusion of apricot extract and pineapple extract (1%) resulted in a 2-fold increase in the absorptive transport of sulfasalazine across rat ileum in the Ussing

chamber system (Figure 3B), suggesting a similar functional expression of carriers in rat ileum. The modulatory effect of apricot extract was confirmed in the in-situ intestinal perfusion system using rat ileum: a 2.2-fold increase of the absorption of sulfasalazine was obtained in the presence of standardized apricot extract (1%) in the closed-loop set-up, while a 4.4-fold increase was observed in the open-loop set-up (Figure 4). The large difference between the results obtained with nature-identical apricot extract in the closed- and open-loop set-up indicates that the active components of this nature-identical fruit extract responsible for the interaction with the efflux carrier were either absorbed or metabolized by the rat ileum during perfusion.

The absorption-enhancing effect of these standardized fruit extracts (pineapple and apricot extract) is not due to inhibition of the metabolism of sulfasalazine. Sulfasalazine is metabolized into sulfapyridine and 5-aminosalicylic acid by the intestinal flora. In our studies with the in-situ perfusion model, no sulfapyridine could be detected, probably due to the lack of bacteria capable of metabolizing sulfasalazine. The clinical relevance of the modulating effect of the carrier(s) observed remains to be explored; a confounding factor is the fact that the effect is concentration dependent and that the local concentration of sulfasalazine remains unknown. The observed absorption-enhancing effect of the used fruit extracts in different absorption models indicates that caution is required when adding flavouring agents to mask the bad taste or odour of drug solutions or suspensions, without knowing the exact effect of this agent on the absorption or metabolism of the drug. In the case of sulfasalazine for example, a formulation for a suspension with Essentia rubi ideai (0.3% w/v) has been described in the Dutch Pharmacists' Formularium (Suspensio Sulfasalazini, FNA). The effect of this fruit essence on the absorption and metabolism of sulfasalazine remains to be investigated.

Conclusion

The combined results of this study confirm that sulfasalazine efflux is not mediated by P-gp, but rather by MRP/anion-sensitive transport mechanisms. A nature-identical pineapple extract, apricot extract and some of its fractions were shown to exert a concentration-dependent effect on the absorptive transport of sulfasalazine in the Caco-2 system. A similar functional expression of carriers in rat ileum was suggested by the observed absorption enhancing effect of these nature-identical fruit extracts in Ussing chambers and the in-situ intestinal perfusion experiments. These results confirm the importance of identifying food–drug interactions at the level of the intestinal biochemical barrier function.

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