Mechanisms of Transport and Structure-Permeability Relationship of Sulfasalazine and Its Analogs in Caco-2 Cell Monolayers

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Purpose. To investigate the mechanisms involved in transport of sulfasalazine in Caco-2 cells.

Methods. Permeability coefficients of sulfasalazine and its analogs across Caco-2 cell monolayers were measured as a function of direction of transport, energy and concentration dependence, and in the presence of inhibitors of various cellular efflux pumps and transporters.

Results. Permeability coefficients of sulfasalazine across Caco-2 cell monolayers were approximately 342-, 261-, and 176-fold higher from basolateral to apical direction (BL→AP) than from apical to basolateral direction (AP→BL) at 100, 200, and 500 μM, respectively. Carrier permeability coefficient, non-saturable membrane permeability coefficient, and Michaelis constant were estimated to be 1.4×10^{-5} cm/s, 1.9×10^{-8} cm/s, and 369 μM, respectively. The efflux of sulfasalazine was completely blocked at 4°C and in the presence of an uncoupler of oxidative phosphorylation. Using cellular efflux inhibitors, the permeability of sulfasalazine was shown to depend on multidrug resistance-associated protein and anion sensitive transport mechanisms. Structure-permeability studies showed that the affinity of sulfasalazine for the cellular efflux pumps and transporters in Caco-2 cells depended strongly on the carboxylic acid functional group.

Conclusions. The permeability of sulfasalazine across Caco-2 cell monolayer is very low due to its strong interaction with multiple cellular efflux pumps and transporters. This may partially explain its low absorption *in vivo*.

KEY WORDS: sulfasalazine; Caco-2 cells; structure-permeability relationship; multiple cellular efflux mechanisms.

INTRODUCTION

Drug absorption can be influenced by a variety of factors including solubility, partitioning, and permeability (1). Permeability, in particular, has drawn the attention of many researchers due to the discovery of increasing number of drugs as substrates for intestinal cellular efflux pumps and transporters that can in turn affect drug absorption (2,3).

Sulfasalazine (Figure 1A) is intended for the treatment of chronic inflammatory bowel diseases including ulcerative colitis and Crohn's disease. When administered orally, sulfasalazine is poorly absorbed (3–12%) (4–7).

Caco-2 cell studies have shown that sulfasalazine may be a substrate for cellular efflux pumps due to its very low cellular permeability (8). This may account for its low absorption *in vivo*. To date, however, there has been no systematic study on the mechanisms involved in the transport of sulfasalazine across cellular membranes. In this report, the interaction between sulfasalazine and Caco-2 cells was studied with respect to direction of transport, concentration, energy dependence, and the influence of cellular efflux pump and transporter inhibitors. In addition, the relationship between structure and permeability was explored to better explain the interaction between sulfasalazine and cellular efflux pumps and transporters in Caco-2 cells.

MATERIALS AND METHODS

Materials

4-Acetamido-4′-isothiocyanato-stilbene-2,2′-disulfonic acid (SITS), 5-aminosalicylic acid, calcein, carbonyl cyanide m-chlorophenyl-hydrazone (CCCP), 4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid (DIDS), indomethacin, N-methyl-nicotinamide, nicotinic acid, probenecid, rhodamine 123, sulfapyridine, sulfasalazine, tetraethylammonium, valproic acid, and verapamil were purchased from Sigma (St. Louis, MO). 2-Hydroxyphenylacetic acid, sodium nitrite, and phenol were bought from Aldrich (Milwaukee, WI). Potassium hydroxide and sodium carbonate were bought from EM Science (Gibbstown, NJ), and salicylamide from Anachemia (Montreal, Canada). Dulbecco's modified Eagle medium (DMEM), Hanks' balanced salt solution (HBSS), fetal bovine serum, non-essential amino acids, penicillin, streptomycin, and trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY). Rat tail collagen type I was purchased from Collaborative Biomedical (Bedford, MA).

Methods

Cell Culture

Caco-2 cells obtained from American Type Culture Collection (Rockville, MD) were grown at 37°C in an atmosphere of 5% CO_2 in DMEM growth medium supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) non-essential amino acids, penicillin (100 Unit/ml), and streptomycin (100 μg/ml). Confluent cell monolayers were subcultured every seven days by treatment with 0.25% trypsin containing 1 mM EDTA. Caco-2 cells were seeded at a density of 80,000 cells/cm^2 in 6-well plates on Transwell® polycarbonate filters (Costar; diameter: 24.5 mm, pore size: 3.0 μm) coated with rat tail collagen type I. They were grown until fully differentiated after 21 days, and all experiments were conducted between 21 and 25 days. Cells of passage numbers 30 to 50 were used throughout.

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ABBREVIATIONS: AET, anion exchange transporter; AP→BL, Apical to basolateral side; (BL→AP, Basolateral to apical side; SITS, 4-acetamido-4′-isothiocyanato-stilbene-2,2′-disulfonic acid; CCCP, Carbonyl cyanide m-chlorophenyl-hydrazone; DIDS, 4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid; DMEM, Dulbecco's modified Eagle medium; HBSS, Hanks' balanced salt solution; MRP, Multidrug-resistance protein; OAT, Organic anion transporter; OCT, Organic cation transporter; P_{Caco-2}, Permeability coefficient through the Caco-2 cell monolayer; PDR, Permeability directional ratio; Pgp, P-glycoprotein.

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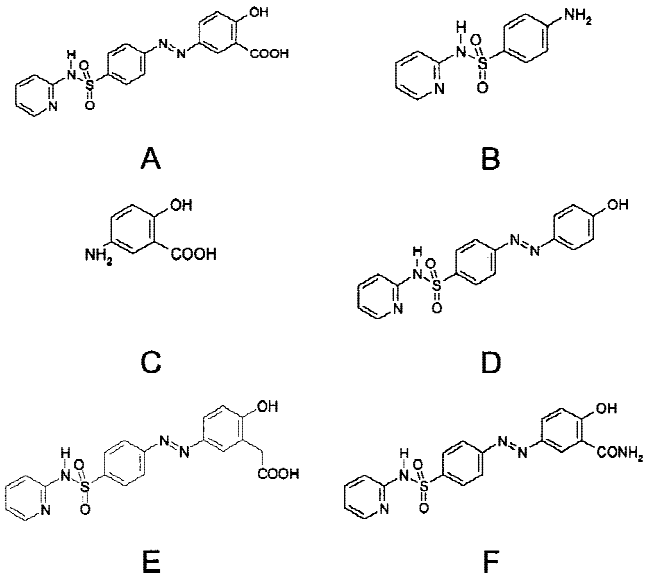

Fig. 1. Chemical Structure of (A) sulfasalazine, (B) sulfapyridine, (C) 5-aminosalicylic acid, (D) descarboxysulfasalazine, (E) homosulfasalazine, and (F) sulfasalamide.

Permeability Studies

Caco-2 cell monolayers were pre-conditioned with HBSS (pH 7.4) consisting of 1.3 mM $CaCl_2$, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.49 mM $MgCl_2$, 0.41 mM $MgSO_4$, 137 mM NaCl, 0.34 mM Na_2HPO_4, 5.5 mM D-glucose, and 4.2 mM $NaHCO_3$ at 37°C for 30 minutes. Sulfasalazine at specified concentrations was added to either apical (AP→BL) or basolateral side (BL→AP) of the Caco-2 cell monolayers. When applicable, inhibitors were present in the transport medium from the pre-incubation period throughout the permeability study. The pH of the transport medium after the addition of tested compounds did not change by more than 0.2 pH unit over the duration of the entire experiment. At various time points, samples were collected from the other side of the cell monolayers for quantification. Unless otherwise stated, all permeability studies were performed at 37°C. Sulfasalazine and its metabolites and analogs were analyzed by isocratic HPLC methods (HP1090, Hewlett Packard). A 150×4.6 mm ODS2 5μ column (Metachem) was used along with a mobile phase consisting of water (0.06% (v/v) trifluoroacetic acid) and acetonitrile with a flow rate of 1.0 ml/min and temperature at 40°C.

Permeability coefficient ($P_{Caco\text{-}2}$) was determined according to equation 1:

$$P_{Caco-2} = \frac{J}{A*C_i} \quad (1)$$

where J is the transport rate determined by plotting cumulative amounts of drug permeated to the receiver chamber as a function of time, A is the surface area of the filter, and C_i is the initial concentration of the solution in the donor chamber.

Sulfasalazine was found to be chemically stable throughout the permeability studies. The mass balance for each experiment was calculated to be more than 90%. The integrity of Caco-2 cell monolayers was assessed by measuring the permeability of mannitol, a paracellular marker. The permeability values for mannitol (AP→BL) in the absence of inhibitors ranged from 1.7 to 3.1 × 10^{-7} cm/s. While in the presence of inhibitors at concentrations up to 2 mM, its permeability values ranged from 1.9 to 4.3 × 10^{-7} cm/s. Therefore, the integrity of the Caco-2 cell monolayers in the presence of inhibitors was not compromised.

Synthesis of Sulfasalazine Analogs

The synthetic pathway to synthesize descarboxysulfasalazine (Figure 1D) was similar to that reported previously for sulfasalazine (9). In brief, sulfapyridine, dissolved in hydrochloric acid solution, was diazotized with an equimolar amount of sodium nitrite in solution aqueous for 10 min. The suspension was then added into an alkaline solution of phenol and left overnight to yield the final product. Homosulfasalazine (Figure 1E) and sulfasalamide (Figure 1F) were synthesized following the same protocol except that phenol was replaced by 2-hydroxyphenylacetic acid or salicylamide, respectively. Crude descarboxysulfasalazine, homosulfasalazine, and sulfasalamide were purified by chromatography over silica gel or by recrystallization. Their structure and purity were confirmed by 1H-NMR, mass spectroscopy, HPLC, and elemental analysis.

Statistical Analysis

All experiments were done in at least triplicates and data are expressed as mean ± standard deviation. Statistical differences between the treatments were determined using analysis of variance where appropriate (SigmaStat 2.03; SPSS Inc., San Rafael, CA) with $p<0.05$ considered statistically significant.

RESULTS AND DISCUSSION

Direction of Transport

To determine whether sulfasalazine was a substrate for the apically polarized efflux systems in Caco-2 cell monolayers, $P_{Caco\text{-}2}$ of sulfasalazine from both AP→BL and BL→AP at three concentrations, 100, 200, and 500 μM, were measured. The $P_{Caco\text{-}2}$ values for sulfasalazine were significantly higher ($p<0.001$) from BL→AP than from AP→BL at all concentrations (Figure 2). Permeability directional ratio (PDR) was calculated according to equation 2.

$$PDR = \frac{P_{Caco-2}(BL \rightarrow AP)}{P_{Caco-2}(AP \rightarrow BL)} \quad (2)$$

For the substrates of cellular efflux pumps, $P_{Caco\text{-}2}$ values are expected to be higher from BL→AP than from AP→BL (10,11). The PDR values were calculated to be 342, 261, and 176 for sulfasalazine at 100, 200, and 500 μM, respectively. These high PDR values indicated that there was a strong association between sulfasalazine and the cellular efflux pumps in Caco-2 cells.

Concentration Dependence

Since efflux permeability is, in general, concentration dependent and saturable, permeability values for sulfasalazine across Caco-2 cell monolayers from BL→AP were measured at concentrations ranging from 1 to 2000 μM. The $P_{Caco\text{-}2}$ values for sulfasalazine were shown to be concentration de-

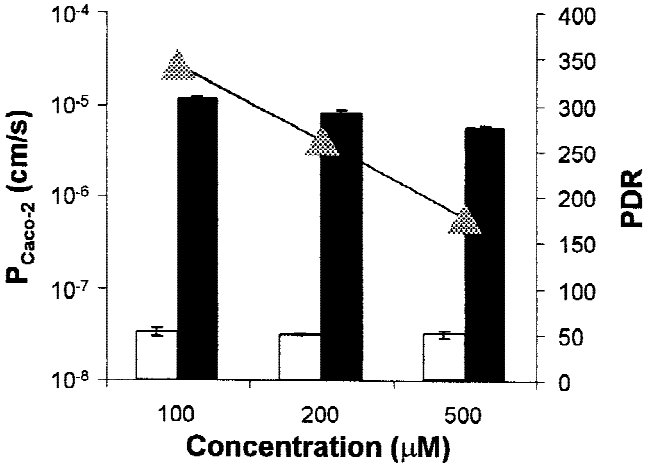

Fig. 2. Permeability coefficients of sulfasalazine (n = 6) when added either from apical side (open bar) or from basolateral side (closed bar). The PDR value (closed triangle) for each concentration of sulfasalazine was also plotted.

pendent (Figure 3). However, due to aqueous solubility limitations of sulfasalazine (2.1 mM), a complete saturation of the cellular efflux systems was not observed.

Kinetic values associated with the efflux (BL→AP) of sulfasalazine through Caco-2 cells were calculated using the Scientist® 2.0 program (Micromath; Salt Lake City, UT) based on equation 3 (12):

$$P_{Caco-2} = \frac{P_c}{1 + \frac{C_i}{K_m}} + P_m \quad (3)$$

where P_c is the carrier permeability coefficient, P_m is the non-saturable membrane permeability coefficient, and K_m is the Michaelis constant. After fitting equation 3 to the data, carrier permeability coefficient, non-saturable membrane permeability coefficient, and Michaelis constant were determined to be 1.4×10^{-5} cm/s, 1.9×10^{-8} cm/s, and 369 μM, respectively. The large ratio between the estimated carrier and non-saturable membrane permeability coefficients not only confirmed the involvement of carrier-mediated system but also demonstrated its dominance over passive diffusion in the cellular transport of sulfasalazine.

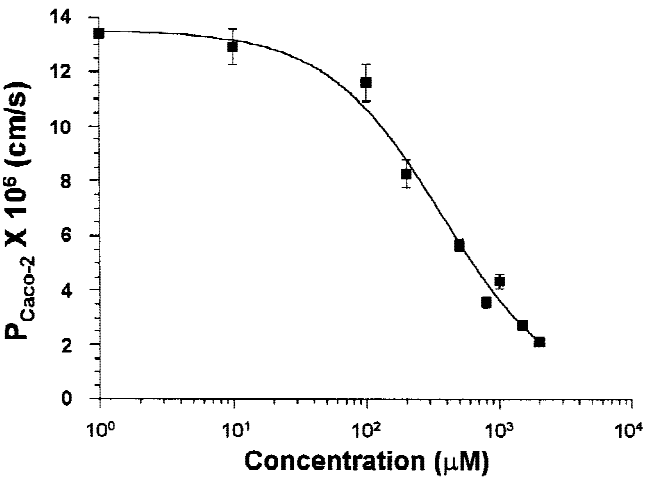

Fig. 3. The effect of concentration on the permeability coefficients of sulfasalazine from BL→AP (n = 6). Fitted curve was based on equation 3.

When sulfasalazine was added from the apical side of the Caco-2 cell monolayers, the P_{Caco-2} values were found to be 3.4±0.4, 3.2±0.1, 3.2±0.2, 3.2±0.3, 3.3±0.2, and 3.7±0.1 cm/s (average±SD, n = 3) at 100, 200, 500, 800, 1000, and 2000 μM, respectively. There was no concentration dependence ($p = 0.153$) in the P_{Caco-2} values in the concentration range used. The average P_{Caco-2} value, 3.3×10^{-8} cm/s, compared well with the above estimated non-saturable membrane permeability coefficient, 1.9×10^{-8} cm/s, from BL→AP. This further corroborated the hypothesis that transport of sulfasalazine from AP→BL was subject to the cellular efflux system and therefore remained at the level for passive diffusion in the concentration range used.

Energy Dependence

Inhibition of active transport at 4°C and in the presence of CCCP, an uncoupler of oxidative phosphorylation in mitochondrial systems, has been reported (13,14). To determine whether the efflux of sulfasalazine at 200 μM was energy dependent, its permeability at 4°C or in the presence of 100 μM CCCP was measured. The P_{Caco-2} values for sulfasalazine from BL→AP and AP→BL changed significantly ($p<0.001$) at 4°C and in the presence of CCCP (Table 1). The PDR values were 11 and 14, respectively. In both cases, although the PDR values decreased by more than 20-fold, the efflux of sulfasalazine was not completely blocked. Combining 4°C and CCCP further prevented membrane transporters from using energy entirely and consequently reduced the PDR value to approximately 1. These results showed that the cellular efflux of sulfasalazine across Caco-2 cell monolayers was strongly energy dependent.

Use of Inhibitors of Cellular Efflux Pump and Transporter

In an attempt to identify the active transporters responsible for the efflux of sulfasalazine, its permeability across Caco-2 cell monolayers in the presence of inhibitors of various cellular efflux pumps and transporters was measured (Table 2). The influence of five different cellular efflux pumps and transporters that have been reported in Caco-2 cells was evaluated (3,12,15-17). These were P-glycoprotein (Pgp), multidrug resistance-associated protein (MRP), organic anion transporter (OAT), anion exchange transporter (AET), and organic cation transporter (OCT). Unless otherwise stated, the inhibitors were used at the same concentration as sulfasalazine at 200 μM or at the limit of their solubility in the transport medium.

Table 1. The Effect of Temperature and CCCP on P_{Caco-2} of Sulfasalazine (200 μM) from Either AP→BL or BL→AP[a]

Treatment	$P_{Caco-2} \times 10^7$ (cm/s)		PDR
	AP→BL	BL→AP	
Control (37°C)	0.32 ± 0.01 (6)	83.0 ± 5.1 (6)	261
4°C	0.24 ± 0.01 (3)	2.6 ± 0.3 (3)	11
CCCP (100 μM)	2.7 ± 0.2 (3)	39.0 ± 4.5 (3)	14
4°C + CCCP (100 μM)	5.3 ± 1.0 (3)	5.6 ± 0.5 (3)	1.1

[a] Number of monolayers used in parenthesis.

Table 2. The Effect of Inhibitors from Various Cellular Efflux Pumps and Transporters on P_{Caco-2} of Sulfasalazine (200 μM) from Either AP→BL or BL→AP[a]

Inhibitor	$P_{Caco-2} \times 10^7$ (cm/s)		PDR
	AP→BL	BL→AP	
Control	0.32 ± 0.01 (6)	83.0 ± 5.1 (6)	261
P-glycoprotein (Pgp)			
Verapamil (200 μM)	0.35 ± 0.02 (3)	81.2 ± 6.1 (3)	231
Rhodamine 123 (200 μM)	0.33 ± 0.04 (3)	79.7 ± 4.9 (3)	242
Multidrug Resistance-Associated Protein (MRP)			
Indomethacin (50 μM)	0.94 ± 0.21 (3)	64.9 ± 3.5 (3)	69
Calcein (200 μM)	0.76 ± 0.14 (3)	67.3 ± 6.0 (3)	89
Organic Cation Transporter (OCT)			
Tetraethylammonium (200 μM)	0.38 ± 0.01 (3)	82.5 ± 4.6 (3)	217
N-methyl nicotinamide (200 μM)	0.40 ± 0.05 (3)	72.9 ± 0.5 (3)	182
Organic Anion Transporter (OAT)			
Probenecid (200 μM)	0.56 ± 0.06 (3)	44.1 ± 4.4 (3)	79
Nicotinic Acid (200 μM)	0.60 ± 0.12 (3)	59.3 ± 5.1 (3)	99
Valproic Acid (200 μM)	0.52 ± 0.02 (3)	59.5 ± 3.1 (3)	114
Lactic Acid (200 μM)	0.48 ± 0.05 (3)	72.1 ± 2.7 (3)	150
Hippuric Acid (200 μM)	0.37 ± 0.04 (3)	78.5 ± 6.9 (3)	212
Anion Exchange Transporter (AET)			
SITS (200 μM)	0.85 ± 0.03 (3)	39.5 ± 4.5 (3)	46
DIDS (200 μM)	1.3 ± 0.1 (3)	41.5 ± 2.7 (3)	32
AET and MRP			
DIDS (200 μM) + Indomethacin (50 μM)	2.3 ± 0.1 (3)	23.7 ± 1.2 (3)	10
OAT and MRP			
Probenecid (200 μM) + Indomethacin (50 μM)	0.79 ± 0.06 (3)	22.9 ± 3.1 (3)	29

[a] Number of monolayers used in parenthesis.

Pgp and MRP Inhibitors

Verapamil and rhodamine 123, substrates for Pgp (18–22), did not affect the transport of sulfasalazine across Caco-2 cell monolayers from either direction. On the other hand, in the presence of indomethacin and calcein, substrates for MRP (20–22), the transport of sulfasalazine from both directions was changed significantly ($p<0.03$), and a 3- to 4-fold decrease in the PDR values was observed. Hence, the efflux of sulfasalazine in Caco-2 cells was dependent on MRP but not on Pgp transporters.

OAT and AET Inhibitors

It is well documented that the transport of some monocarboxylic acids is associated with the anion sensitive cellular efflux systems (2,3). Therefore, the effect of OAT inhibitors on the permeability of sulfasalazine, a monocarboxylic acid, was studied. Probenecid, nicotinic acid, valproic acid, lactic acid, and hippuric acid have been used as inhibitors of OAT (22,23). All OAT inhibitors decreased the PDR values of sulfasalazine. In particular, probenecid, nicotinic acid, and valproic acid changed sulfasalazine permeability from both directions significantly ($p<0.01$) and consequently decreased the PDR values by approximately 2 to 3-fold. This suggested that sulfasalazine had an affinity for OAT in Caco-2 cells.

The anion sensitive transport system associated with the transport of sulfasalazine was then further challenged by inhibitors of AET (24–26). In the presence of SITS and DIDS, the PDR values decreased approximately 6- and 8-fold respectively, indicating the strong interaction between sulfasalazine and AET.

It has also been shown that the transport of many monocarboxylic acids such as benzoic acid and nicotinic acid is affected by the anionic Cl^-/HCO_3^- exchange mechanism (17,27). However, sulfasalazine did not appear to take advantage of this system, as the transport of sulfasalazine did not change from either direction in the absence of bicarbonate ions in the transport medium (data not shown).

OCT Inhibitors

To confirm the specificity of sulfasalazine for anion transport efflux mechanisms, substrates for OCT such as tetraethylammonium and N-methyl nicotinamide were tested (15,16,28,29). As expected, these inhibitors did not affect the transport of sulfasalazine from either direction.

Combination of Inhibitors

Since the cellular efflux of sulfasalazine was modulated by both MRP and the anion sensitive transport systems of AET and OAT, inhibitors for both transporters were combined to determine whether there was an overlap between these two mechanisms. Combination of DIDS and indomethacin or probenecid and indomethacin decreased the PDR values by 26- and 9-fold, respectively. This indicated that the effect of MRP and the anion sensitive transport systems in the transport of sulfasalazine in Caco-2 cells was additive.

Concentration Dependence of Inhibitors

To determine whether the concentration of cellular efflux inhibitors at 200 μM for sulfasalazine transport studies was optimal, one compound from each of the inhibitor classes was selected from Table 2. These were verapamil (Pgp), cal-

cein (MRP), tetraethylammonium (OCT), probenecid (OAT), and DIDS (AET). The permeability of sulfasalazine in the presence of these inhibitors at 20, 200, and 2000 μM was measured (Table 3). For verapamil and tetraethylammonium treated groups, the PDR values were constant between 20 and 2000 μM, indicating an absence of concentration dependence for these inhibitors. However, for calcein, probenecid, and DIDS treated groups, the PDR values decreased substantially from 20 to 200 μM, and then remained the same between 200 and 2000 μM. Hence, it appeared that 200 μM was an appropriate concentration to use in order to determine the effect of all classes of inhibitors on sulfasalazine transport.

The absence of complete inhibition for the transport of sulfasalazine in the presence of all inhibitors used in this study could be due to involvement of other as yet unidentified membrane transporters. It is also possible that higher concentrations of inhibitors might have been effective. However, these studies could not be carried out due to the low aqueous solubility of some of these inhibitors such as indomethacin as well as the potential effect of higher concentrations on Caco-2 cell monolayer integrity. Nevertheless, the specificity and effect of each inhibitor class on sulfasalazine transport was demonstrated.

Structure Permeability Studies

Whether the intact structure of sulfasalazine or any of its different moieties or functional groups was necessary in order for it to serve as a substrate for cellular efflux pumps and transporters was investigated (Table 4). Sulfasalazine derivatives or analogs (Figure 1) were used either at the same concentration as sulfasalazine at 100 μM or at the limit of their solubility in the transport medium.

Table 3. The Concentration Effect of Selected Inhibitors on $P_{Caco\text{-}2}$ of Sulfasalazine (200 μM) from Either AP→BL or BL→AP[a]

Inhibitor (classification)	$P_{Caco\text{-}2} \times 10^7$ (cm/s) AP→BL	$P_{Caco\text{-}2} \times 10^7$ (cm/s) BL→AP	PDR
Control	0.32 ± 0.01 (6)	83.0 ± 5.1 (6)	261
Verapamil (Pgp)			
20 μM	0.35 ± 0.05 (3)	77.6 ± 4.5 (3)	222
200 μM	0.35 ± 0.02 (3)	81.2 ± 6.1 (3)	231
2000 μM	0.38 ± 0.09 (3)	76.8 ± 6.5 (3)	202
Calcein (MRP)			
20 μM	0.40 ± 0.04 (3)	77.3 ± 7.5 (3)	193
200 μM	0.76 ± 0.14 (3)	67.3 ± 6.0 (3)	89
2000 μM	0.81 ± 0.09 (3)	64.8 ± 4.8 (3)	80
Tetraethylammonium (OCT)			
20 μM	0.37 ± 0.04 (3)	87.1 ± 10.9 (3)	235
200 μM	0.38 ± 0.01 (3)	82.5 ± 4.6 (3)	217
2000 μM	0.36 ± 0.09 (3)	80.2 ± 3.8 (3)	223
Probenecid (OAT)			
20 μM	0.39 ± 0.04 (3)	74.1 ± 3.9 (3)	190
200 μM	0.56 ± 0.06 (3)	44.1 ± 4.4 (3)	79
2000 μM	0.53 ± 0.04 (3)	48.2 ± 3.2 (3)	91
DIDS (AET)			
20 μM	0.64 ± 0.03 (3)	60.3 ± 6.5 (3)	94
200 μM	1.3 ± 0.1 (3)	41.5 ± 2.7 (3)	32
2000 μM	1.5 ± 0.2 (3)	40.9 ± 2.0 (3)	27

[a] Number of monolayers used in parenthesis.

Table 4. $P_{Caco\text{-}2}$ of Sulfasalazine Metabolites and Analogs from Either AP→BL or BL→AP Direction[a]

Compound	$P_{Caco\text{-}2} \times 10^7$ (cm/s) AP→BL	$P_{Caco\text{-}2} \times 10^7$ (cm/s) BL→AP	PDR
Sulfasalazine (100 μM)	0.34 ± 0.04 (6)	116 ± 7 (6)	345
Sulfapyridine (100 μM)	215 ± 8 (3)	195 ± 7 (3)	0.9
5-Aminosalicylic acid (100 μM)	3.2 ± 0.2 (3)	5.5 ± 0.9 (3)	1.7
Descarboxysulfasalazine (20 μM)	328 ± 14 (3)	467 ± 37 (3)	1.4
Homosulfasalazine (100 μM)	0.93 ± 0.11 (3)	101 ± 5 (3)	109
Sulfasalamide (20 μM)	19.6 ± 4.4 (3)	325 ± 24 (3)	17

[a] Number of monolayers used in parenthesis.

Sulfapyridine and 5-Aminosalicylic Acid

Sulfapyridine and 5-aminosalicylic acid are the major degradation products of sulfasalazine *in vivo* (Figure 1B and 1C). To determine the contribution of these compounds to the specificity of sulfasalazine for the cellular efflux pumps and transporters, their permeability across Caco-2 cells from both directions was measured. The PDR values for both compounds were approximately 1. This indicated that the specificity of sulfasalazine was due to its intact structure and neither degradation product had an affinity for the efflux pumps.

Descarboxysulfasalazine

Since we have shown that sulfasalazine is a substrate for the anion sensitive transport mechanisms, the importance of the carboxylic acid functional group on its permeability was studied (Figure 1D). The PDR value for descarboxysulfasalazine was 1.4 indicating that it was not a substrate for the membrane transporters in Caco-2 cells. As a result, the carboxylate group on sulfasalazine appeared to be a necessary functional group for the recognition by all cellular efflux systems involved in its transport.

Homosulfasalazine

The importance of the proximity of the carboxylate group to the salicylic portion of the molecule was tested by inserting a methylene spacer between the carboxylate group and the rest of sulfasalazine (Figure 1E). This compound was a strong substrate for cellular efflux pumps and transporters in Caco-2 cells with a PDR value of 109. Like sulfasalazine, homosulfasalazine appeared to be the substrate for MRP and the anion sensitive systems but not Pgp and OCT (Table 5). However, the PDR value for homosulfasalazine was approximately 3-fold less than that observed for sulfasalazine indicating a weaker specificity for the cellular efflux pumps and transporters.

Sulfasalamide

To further understand the importance of the carboxylate functional group and its negative charge at neutral pH, sulfasalamide was synthesized by replacing the carboxylic acid with an amide (Figure 1F). Contrary to descarboxysulfasala-

Table 5. The Effect of Selected Inhibitors of Various Cellular Efflux Pumps and Transporters on P_{Caco-2} of Homosulfasalazine (100 μM) and Sulfasalamide (20 μM) from Either AP→BL or BL→AP[a]

Inhibitor	$P_{Caco-2} \times 10^7$ (cm/s) AP→BL	$P_{Caco-2} \times 10^7$ (cm/s) BL→AP	PDR
Homosulfasalazine	0.93 ± 0.11 (3)	101 ± 5 (3)	109
Verapamil (200 μM)	1.1 ± 0.2 (3)	89.2 ± 4.6 (3)	81
Tetraethylammonium (200 μM)	0.98 ± 0.21 (3)	91.3 ± 14.9 (3)	93
Indomethacin (50 μM)	2.9 ± 0.4 (3)	58.6 ± 1.1 (3)	20
DIDS (200 μM)	2.5 ± 0.4 (3)	45.8 ± 2.2 (3)	18
Sulfasalamide	19.6 ± 4.4 (3)	325 ± 24 (3)	17
Verapamil (200 μM)	18.2 ± 2.8 (3)	374 ± 10 (3)	21
Tetraethylammonium (200 μM)	16.5 ± 1.6 (3)	316 ± 22 (3)	19
Indomethacin (50 μM)	25.2 ± 0.9 (3)	360 ± 13 (3)	14
DIDS (200 μM)	17.7 ± 0.7 (3)	342 ± 7 (3)	19

[a] Number of monolayers used in parenthesis.

zine, the interaction of sulfasalamide with cellular efflux pumps was stronger with a PDR value of 17.

Due to analytical limitations in detection of sulfasalazine from AP→BL at concentrations less than 100μM and the limit of aqueous solubility for sulfasalamide at 20μM, a direct comparison of the PDR values for these two compounds at the same concentration could not be accomplished. However, the PDR values of sulfasalazine were shown to increase as its concentration decreased (Figure 1). Hence, a larger difference in the PDR values between sulfasalazine and sulfasalamide is expected if the comparison is made at 20 μM.

In the presence of indomethacin or DIDS which were shown to be effective in inhibiting cellular transport of sulfasalazine, the PDR values for sulfasalamide did not change (Table 5). As a result, unlike sulfasalazine, sulfasalamide did not seem to employ MRP and the anion sensitive systems for cellular transport. It is known that the pK_a of the carboxylate group on sulfasalazine and that of the aminocarbonyl phenol group on sulfasalamide are approximately 4.8 and 8.9, respectively (30). Therefore, the 20-fold difference in the PDR values between these two compounds could be attributed to the negative charge of sulfasalazine as opposed to the neutral charge of sulfasalamide at pH 7.4.

Based on the above results, the presence of a functional group next to the hydroxyl group on the phenyl ring of the sulfasalazine backbone is necessary for recognition by cellular efflux pumps and transporters in Caco-2 cells. In addition, compounds with a functional group on the phenyl ring possessing a negative charge such as sulfasalazine and homosulfasalazine appear to be much better substrates.

CONCLUSIONS

Sulfasalazine exhibited a very high PDR value at all concentrations studied, indicating its strong interaction with cellular efflux pumps and transporters in Caco-2 cells. Indeed, it has the highest PDR value among more than 50 commercially available drugs we have looked at in our laboratory. The high PDR value for sulfasalazine in Caco-2 cells allows us to differentiate the effect of various cellular efflux inhibitors as well as small structural changes on its permeability. Therefore, sulfasalazine appears to be a good starting compound for establishing structure-permeability relationships for substrate recognition by cellular efflux pumps and transporters.

In this study, we have shown that at least two cellular efflux mechanisms, MRP and the anion sensitive transport systems, are involved with the transport of sulfasalazine across Caco-2 cell monolayers. These findings may partly explain the very low absorption of sulfasalazine in the gastrointestinal tract. Ongoing studies are focused on understanding the significance of other functional groups especially the position of negatively charged groups on the salicylate ring of the sulfasalazine on its permeability in Caco-2 cells.

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