European Journal of Pharmaceutics and Biopharmaceutics 70 (2008) 531–538

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Transport evaluation of salicylic acid and structurally related compounds across Caco-2 cell monolayers and artificial PAMPA membranes

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ARTICLE INFO

Article history:
Received 10 October 2007
Accepted in revised form 19 May 2008
Available online 6 June 2008

Keywords:
Caco-2
PAMPA
Transport mechanisms
Anionic compounds
pH-dependency

ABSTRACT

The purpose of this study was to evaluate passive vs. proton-dependent active transport mechanisms of salicylic acid (SA) and four structurally related anions. Transport was studied across Caco-2 cell monolayers and artificial lipid membranes (PAMPA) under pH-gradient and iso-pH conditions. Kinetic permeability parameters were provided by bidirectional Caco-2 experiments and concentration-dependency measurements. The transport route and putative transporters involved in SA transport were studied using EDTA and several inhibitors. SA and lipophilic 5-chlorosalicylic acid and 2-hydroxy-1-naphthoic acid reached saturation with increasing compound concentration indicating active transport. Permeation of 5-hydroxysalicylic acid and 5-hydroxyisophthalic acid was not saturated indicating passive transport. PAMPA with pure passive diffusion underestimated the transport of SA compared to Caco-2. Opening up the paracellular tight junctions by EDTA did not increase the transport of SA under the pH-gradient conditions confirming the hypothesis of pure transcellular transport of SA. Active transport of SA remained concentration-dependent even without the pH-gradient, and was reduced by the known MCT1 and OATP-B inhibitors and structurally related anions. Overall, several permeability test protocols are needed to obtain a more complete picture of transport properties of salicylic acid and structurally related compounds.

1. Introduction

Many naturally occurring monocarboxylates, such as acetate, propionate and butyrate, which are fermentation products of carbohydrates in the colon, are reported to be transported by monocarboxylic acid transporters (MCT) [1–2]. In addition to these physiological molecules, many drugs and food-derived substances possess a carboxylic acid moiety in their structure and are ionised at the prevailing pH conditions in the intestine. According to the pH partition theory and the high degree of ionisation of carboxylic acids at physiological pH, it would be expected that these compounds are poorly permeable. Despite this, many of them are highly permeable compounds and, hence, well absorbed. It has been suggested that these molecules are actively transported, presumably by MCTs (monocarboxylic acid transporter family, SLC16), OATPs (organic anion transporting polypeptide family, SLC21) or other proton-dependent transport systems. Examples of such anionic compounds are salicylic acid [3–4], naproxen, ketoprofen, diflunisal, diclofenac [5], atorvastatin [6], pravastatin, fexofenadine [7–8], nateglinide [9–10] and telmisartan [11]. Additionally, merely passive, pH-dependent mechanism has been suggested for the acids, where the high gradient of the permeable protonated form between the acidic extracellular and intracellular (pH 7.5) compartments facilitates the rapid permeation and then dissociation of the protonated form until the intracellular pH decreases to the level of the outside [12].

The monocarboxylate cotransporter family comprises nowadays 14 members, of which MCT1 (SLC16A1) is the most investigated [13]. MCT1 is localised in the apical membranes of human intestine, and its expression increases along the length of the intestine [14]. OATP represents a family of transporters, which are expressed in several tissues and organisms. One of them, the OATP-B (SLCO2B1; SLC21A9), is localised at the apical membrane of intestinal epithelial cells in humans [15], and has been suggested to participate in the transport of anionic compounds such as estrone-3-sulfate [16], pravastatin, fexofenadine [7] and salicylic acid [4]. For both MCT1 and OATP-B, the driving force for compound transport has been linked to the presence of a proton gradient across the cell membrane. MCT1 functions as a proton-coupled transporter [17], but the importance of a proton gradient for OATP-B is still unclear, as it has been shown to be active even without the pH-gradient [7].

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doi:10.1016/j.ejpb.2008.05.017

Recently published results revealed that the Caco-2 cells express similar transporters as the human jejunum, MCT1 and OATP-B among them [18], and are thus useful for screening purposes and, in particular, for mechanistic studies. In order to avoid the formation of subpopulations, the cells should be used at a well-defined passage range. Bidirectional studies across the Caco-2 cells under pH-gradient (acidic apical pH) and non-gradient conditions are valuable when evaluating the role of active transport [19]. However, in the case of acidic drugs, bidirectional studies under pH-gradient conditions can give misleading information of the transport mechanism, since the high influx ratio under pH-gradient conditions may be interpreted falsely as active transport [4]. Kinetic studies with increasing compound concentration under different pH conditions and the use of inhibitors/substrates of putative active transporters give more detailed information of the transport mechanism and of the pH-dependency.

Permeation experiments across an artificial lipid membrane (PAMPA, parallel artificial membrane permeability assay) are used as a rapid screening method for new drug candidates. Even though it is a fast and good predictor of passive permeation, PAMPA membrane lacks the paracellular spaces and active transporters and, thus, may underestimate the permeation of actively transported compounds. Therefore, it has been suggested that PAMPA and Caco-2 permeation methods should be used parallel for the evaluation of drug permeation mechanism(s) in drug discovery [20].

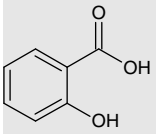
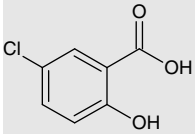
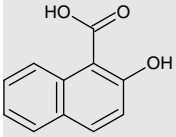
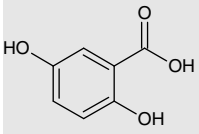
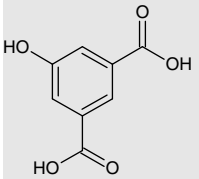
The aim of our study was to probe a range of permeation methods in the evaluation of passive vs. proton-dependent active transport mechanisms of salicylic acid (SA) and four structurally related anions. Firstly, the applicability of bidirectional and concentration dependency studies in the evaluation of permeation mechanisms (active and passive) was explored with Caco-2 cell monolayers. Passive permeation was evaluated also using the PAMPA method in order to utilize the information obtained from the Caco-2 and PAMPA experiments in a complementary fashion. Salicylic acid (SA) was used as a model compound, as present evidence suggests that it is a substrate of both MCT1 and OATP-B [4]. The other structurally related anions have similar ionisation behaviour, but differ from each other by their substituents, amount of ionisable/polar groups and lipophilicity (Table 1). These physico-chemical characteristics of the model anions were considered in relation to their potential active and passive transport. Second, the feasibility of paracellular disruption (by EDTA) in elucidating the contribution of paracellular and transcellular routes was explored using SA and known markers for transcellular (antipyrine) and paracellular (mannitol) permeation. Finally, transport inhibition studies of SA were performed using MCT1 and OATP-B inhibitors as well as other structurally related compounds, and proton-dependency of the active transport examined.

2. Materials and methods

2.1. Compounds

Salicylic acid (SA), 5-chlorosalicylic acid (5-Cl-SA), 2-hydroxy-1-naphthoic acid (2-OH-NA), 5-hydroxysalicylic acid (5-OH-SA), 5-hydroxyisophthalic acid (5-OH-IPA) (Table 1), as well as the inhibitors, pravastatin sodium and probenecid, were purchased from Sigma–Aldrich Chemie (Steinheim, Germany). Passive transcellular marker molecule antipyrine, the inhibitors α-cyano-4-hydroxycinnamic acid (CHC) and 2-hydroxy-3-isopropylbenzoic acid (2-OH-3-IPBA) were from Aldrich Chemical Company Inc. (Milwaukee, WI), and the paracellular membrane integrity marker

Table 1
Structures and physico-chemical parameters of the studied compounds

Compound		pK_a	Log P	Log $D_{5.5}$	log $D_{7.4}$
	Salicylic acid (SA)[a] (MW 138)	2.90	2.23	−0.37	−2.27
	5-Chlorosalicylic acid (5-Cl-SA)[a] (MW 173)	2.68	2.96	0.20	−0.62
	2-Hydroxy-1-naphthoic acid (2-OH-NA) (MW 188)	3.08	3.28	0.88	−0.29
	5-Hydroxysalicylic acid (5-OH-SA)[a] (MW 154)	2.72 (COOH) 10.07 (5-OH)	1.66	−1.12	−3.02
	5-Hydroxyisophthalic acid (5-OH-IPA) (MW 182)	3.44 (1-COOH) 4.16 (3-COOH) 9.35 (5-OH)	1.54	−1.69	−4.02

[a] pK_a and log P values from Ref. [21].

D-[1-^{14}C]mannitol (specific activity 56,0 mCi/mmol) from Amersham Pharmacia Biotech (England).

Physico-chemical properties (pK_a, log P and log D) of 2-OH-NA and 5-OH-IPA were determined using a computerized potentiometric titrator (GLpKa, firmware v.1.114, Sirius Analytical Instruments Ltd., Forest Row, U.K.) at controlled temperature (25 ± 1 °C) under nitrogen flow, and the data were calculated by pKaLOGP software (pKaLogP V5.2; Sirius Analytical Instruments) (Table 1). The respective parameters for the other probe compounds SA, 5-Cl-SA and 5-OH-SA have previously been determined in our laboratory and presented in the paper of Hänninen et al. [21], where also the used methodology is described in detail.

2.2. Cell culture

The Caco-2 cell line was from American Type Culture Collection (Rockville, MD). Caco-2 cells were grown in tissue culture flasks (Corning Costar Corp., Cambridge, MA) (75 cm^2) in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS (foetal bovine serum), 1% NEAA (non-essential amino acids), 2 mM L-glutamine, penicillin G 100 U/ml and streptomycin sulfate 100 μg/ml. FBS was from Gibco Invitrogen Corp. (Life Technologies Lts., Paisley, Scotland), and the other constituents of the growth medium were from Euroclone Ltd. (England). Growth medium was changed every 2–3 days. Cells were trypsinised at 80% confluency and seeded on polycarbonate Transwell® inserts (Corning Costar Corp., Cambridge, MA, pore size 0.4 μm, growth area 1.1 cm^2) at a density of 68,000 cells/cm^2. The cells used in transport studies were at passages 32–41 and they were grown on the filters for 23–27 days before the experiments. The culturing conditions were 37 °C in an atmosphere of 5% CO_2 and 90% relative humidity.

2.3. Permeability experiments across Caco-2 monolayers

The transport medium was HBSS (Gibco Invitrogen Corp., Life Technologies Lts., Paisley, Scotland) containing 10 mM Hepes (pH adjusted to 7.4) or MES (pH adjusted to 5.5). In general, the transport experiments were performed as follows: Prior to starting the experiments, fully differentiated cell monolayers were washed twice with pre-warmed HBSS/Hepes pH 7.4, and the cells were equilibrated under the pH conditions of the experiment for 30 min at 37 °C. The pH-adjusted and pre-warmed drug solutions in transport medium (0.55 ml) were added on the apical side to start the AP-BL (apical to basolateral) experiment, and a sample of 50 μl was taken immediately from the apical side to obtain individual initial donor concentrations. The basolateral side contained 1.5 ml of fresh HBSS. Basolateral samples (4 or 5) were taken at 2–60 min time intervals depending on the compound studied by transferring the cell inserts into new wells containing fresh HBSS. The sampling intervals were selected by considering the maintenance of sink conditions and analytical sensitivity. For SA, 5-Cl-SA and 2-OH-NA fast sampling (2 min) under the pH-gradient conditions was necessary in order to maintain the sink conditions. For the slowly permeating compounds (5-OH-SA and 5-OH_IPA) the sampling interval of 30–60 min was required in order to get quantifiable results. The BL-AP (basolateral to apical) experiment was started by adding 1.5 ml of drug solution on the basolateral side. When BL-AP experiments were performed, samples of 0.4 ml (out of the total of 0.5 ml) were withdrawn at 5–60 min time intervals from the apical side and the volume replaced with fresh HBSS. At the end of the experiment, samples were taken from the donor sides to calculate the recovery of the compound. The sum of the cumulatively transported amount and the amount of the remaining compound on the donor side were always >90% of the initial donor amount. The integrity of the monolayers was ensured by TEER-(transepithelial electrical resistance) measurements before and after the experiments by Millicell®-ERS (Millipore, USA). The average TEER-values were 417 ± 40 Ωcm^2 under iso-pH 7.4 conditions and 450 ± 24 Ωcm^2 under pH-gradient conditions. Monolayers exhibiting TEER-values under 200 Ωcm^2 were discarded. All the experiments were performed at least in triplicate. In addition to TEER-measurements, permeability of radiolabelled mannitol was used as an internal standard for paracellular integrity in some experiments. The sample of 100 μl was taken from the basolateral side, and the radioactivity was determined in 4 ml of HiSafe 2 (Wallac Scintillation Products, Fisher Chemicals, Loughborough, UK) by liquid scintillation counting (Wallac, Turku, Finland).

The bidirectional studies were performed under non-gradient (pH 7.4 on both sides; iso-pH 7.4) and under pH-gradient (pH 5.5 apically, pH 7.4 basolaterally) conditions at a concentration of 250–500 μM and also at a considerably higher concentration (50- to 125-fold). In order to calculate the kinetic parameters of transport, flux values at several concentration levels [5–10] were determined.

2.4. Permeability experiments across artificial phospholipid membrane (PAMPA)

PAMPA experiments for all the five compounds, as well as for the permeability standards ketoprofen (high permeability, pH-dependent acidic marker molecule) and ranitidine (low permeability molecule) were performed with the Double-Sink™ -method [22] using 96-well microtitre plate and 96-well filter plate mounted as "sandwich" (PAMPA sandwich, P/N, 110163, pION Inc., Woburn, MA, USA). The stock solutions of the compounds were dissolved in DMSO (Sigma–Aldrich, Germany) at 50 mM concentration, of which the donor solutions of 250 μM were prepared in diluted buffer solution (System Solution Concentrate, P/N 110151, pION Inc., Woburn, MA, USA). The pH-values of the buffer solutions were adjusted to 5.0, 5.5, 6.2 and 7.4 with 0.5 M NaOH. The upper acceptor plate was filled with a buffer solution ASB-7.4 (Acceptor Sink Buffer, P/N 110139, pION Inc., Woburn, MA, USA), and the filters (poresize 0.45, thickness 125 μm) were coated with 5 μl of lipid (GIT-0, P/N 110669, pION Inc., Woburn, MA, USA). The PAMPA sandwich was incubated in a Gut Box™ (P/N, 110205 pION Inc., Woburn, MA, USA) for 4 h without stirring.

2.5. Mechanistic permeation studies with salicylic acid

The transport mechanisms of salicylic acid (SA) were investigated in more detail. The transport route of SA was investigated by opening up the paracellular tight junctions of Caco-2 cells with a calcium chelator EDTA. The EDTA study was performed both under iso-pH 7.4 and under pH-gradient conditions. Cell monolayers were pre-incubated first with HBSS for 15 min, after which the integrity was checked by TEER-measurement. The monolayers were further incubated for 30 min with and without 2.5 mM EDTA solution (both apical and basolateral chambers) prior to permeability experiments. In addition to salicylic acid, the test solution contained the transcellular marker molecule antipyrine and also the paracellular marker mannitol.

In order to investigate the role of the transporters involved in SA absorption, several other (competing) monocarboxylic acids and substrates/inhibitors of MCT1 and OATP-B were used. For inhibition studies, the cell monolayers were pre-incubated for 30 min with the inhibitor (added only on the apical side, probenecid on both sides). The concentration of salicylic acid was 50 μM. The inhibitors used were 5-chlorosalicylic acid, 2-hydroxy-1-naphthoic acid, 5-hydroxyisophthalic acid, 2-hydroxy-3-isopropylbenzoic acid (2-OH-IPBA) and benzoic acid at 10 mM concentration. α-Cyano-4-hydroxycinnamic acid (CHC) (1 mM) was used as an

inhibitor of MCT1 and pravastatin (5 mM) as a substrate of OATP-B (SLC21A9) [6,15]. Probenecid (2 mM) was used as an inhibitor of MRP-efflux [23]. Finally, the role of the proton-dependency of the potential active transporters was studied at increasing compound concentration also under iso-pH 7.4 and iso-pH 6.5 conditions.

2.6. Analytical procedures

The compounds were analysed by HPLC (Thermo Separation Products Inc, San Jose, USA) with the method developed by Hänninen et al. [21]. The HPLC system consisted of a vacuum membrane degasser, a Spectra System P2000 binary pump, a Spectra System AS3000 autosampler and a Spectra Focus UV-detector. The column was reversed phase Discovery® column C18 (150 × 4.6 mm, 5 μM) (Supelco, PA, USA) used with Supelguard™ Discovery® guard column (5 μm, 2 cm × 4.0 mm, Supelco, PA, USA). The mobile phase (flow rate 1 ml/min) for all the studied compounds composed of acetonitrile (Rathburn, Walkerburn, Scotland) and of 0.1% TFA pH 2.1 (trifluoroacetic acid) (Sigma–Aldrich Chemie, Steinheim, Germany) at different ratios [21]. For antipyrine and 2-hydroxy-1-naphthoic acid (not presented in Ref. [21]), the ratios of ACN and TFA were 25:75 and 65:35, respectively and the wavelengths were 238 and 235 nm, respectively. The goodness of fit was >0.999 for all the analysed compounds.

PAMPA samples were analysed by a UV-microplate reader (Spectramax 190, Molecular Devices, Sunnyvale, CA, USA) measuring the UV-spectrum of each well at the wavelength range 200–500 nm. Before the experiment, the UV-absorption of buffer/DMSO and drug solutions was measured to find out the background and initial level of starting samples, respectively. After 4 h of incubation, spectra from both donor and receiver sides were measured and compared to the spectra of the initial level. The data were processed and the effective permeability values (P_e) were calculated by PAMPA Explorer-program (version 2.2 pION Inc., Woburn, MA, USA).

2.7. Data analysis

The flux of the compound across the Caco-2 monolayers was determined from the slope of the plot of the cumulatively transported amount (nmol) versus time (min) curve. The apparent permeability coefficients (P_{app}, cm/s) were calculated according to

$$P_{app} = \frac{(dQ/dt)}{A \times C_0 \times 60}, \quad (1)$$

where dQ/dt is the amount of compound transported within a given time period (nmol/min), A is the surface area of the insert (cm^2) and C_0 is the initial drug concentration (μM or nmol/ml). Permeability values (P_e) in PAMPA method were calculated as described in Ref. [22], except that the filter area was multiplied by the apparent porosity of 0.76.

The kinetic parameters, V_{max} (maximum velocity, nmol/min/cm^2), K_m (Michaelis constant, mM) and k_d (rate constant for passive transport, 10^3 cm/min, expressed as cm/s in the results) of the concentration-dependency experiments were calculated by fitting the transport rate (V, nmol/min/cm^2) and concentration of the compound (C, mM) into Eq. (2). This equation takes into account also the non-saturable part of the transport, in addition to saturable transport.

$$V = \frac{V_{max} * C}{K_m + C} + k_d * C \quad (2)$$

Statistical analysis was done with one-way ANOVA and Student's two-tailed t-test considering $p < 0.05$ statistically significant.

3. Results and discussion

3.1. Concentration dependency of transport

In order to evaluate the overall permeability of the studied compounds, bidirectional studies were performed under iso-H 7.4 and under pH-gradient (5.5/7.4) conditions at low concentrations (200–500 μM). AP-BL permeation of salicylic acid (SA), 5-chlorosalicylic acid (5-Cl-SA) and 2-hydroxy-1-naphthoic acid (2-OH-NA) was extremely high, and the AP-BL/BL-AP ratios (influx ratios) were 27, 35 and 57, respectively, under the pH-gradient conditions, but around 0.7 at iso-pH 7.4 (Table 2). This slightly higher BL-AP permeation under iso-pH 7.4 (influx ratio <1) is a normal phenomenon in our Caco-2 cells, and might be due to the sensitivity of the basolateral side to the added compounds. Similar behaviour was observed also for 5-hydroxysalicylic acid (5-OH-SA), with, however, much lower permeation (2.6×10^{-6} cm/s) and influx ratio (2.5) under the pH-gradient conditions. For 5-hydroxyisophthalic acid (5-OH-IPA), the permeation was low, and the influx ratios remained unchanged (2.2) under both the pH conditions, which further supported the result that this compound was transported passively. 5-OH-IPA is almost completely di-ionised under both the pH-conditions applied and, thus, the partitioning into the membrane as a whole is poor and apparently independent of pH. In spite of the high influx ratios of SA, 5-Cl-SA and 2-OH-NA under the pH-gradient conditions, the assumption of their active transport mechanism by a proton-dependent transporter cannot be based merely on the results of these bidirectional studies and pH comparisons, since similar behaviour has been observed for other acidic compounds with pure passive permeation resulting from the higher amount of protonated form under acidic apical pH (4). More thorough investigations than bidirectional studies were, thus, needed to ensure the involvement of active transporters.

Hypothesis of passive permeation is based on the fact that the permeation is linearly dependent on drug concentration, hence leading to permeation coefficients being independent thereof. Thus, the permeation experiments were performed at clearly higher (25–30 mM) concentration in order to reveal if the permeation is saturable. The experiments were performed under the pH-gradient conditions to mimic the physiological state of the intestine, and assuming that the possible transporters involved are proton-dependent. The permeation coefficients of the more lipophilic SA, 5-Cl-SA and 2-OH-NA decreased by 64%, 79% and 74%, when the concentrations were increased from 250 μM to 25 mM, respectively (Fig. 1). The permeability values of the more hydrophilic compounds, 5-OH-SA and dicarboxylic acid 5-OH-IPA, were overall much lower than that of the lipophilic compounds, and independent of concentration (Fig. 1 insert). These preliminary results confirmed the assumption that SA is indeed actively transported like the two other structurally related lipophilic compounds. 5-OH-SA is also structurally closely related to SA, but because of its lower lipophilicity and/or because of its OH-substituent, it is unable to bind to the active site of the transporter protein. The dicarboxylic

Table 2
Permeability coefficients (P_{app}) (10^{-6} cm/s) in the apical (AP) to basolateral (BL) and basolateral to apical directions under pH-gradient (pH 5.5/7.4) and iso-pH 7.4 conditions

Compound	pH-gradient		iso-pH 7.4	
	AP-BL	BL-AP	AP-BL	BL-AP
SA	106 ± 13	3.89 ± 0.08	10.1 ± 0.4	13.9 ± 0.8
5-Cl-SA	151 ± 8	4.21 ± 0.53	10.5 ± 1.1	18.1 ± 0.8
2-OH-NA	202 ± 5	3.54 ± 0.27	25.6 ± 0.9	32.4 ± 3.6
5-OH-SA	2.61 ± 0.16	1.04 ± 0.05	0.28 ± 0.04	0.91 ± 0.03
5-OH-IPA	0.24 ± 0.06	0.11 ± 0.01	0.25 ± 0.08	0.12 ± 0.01

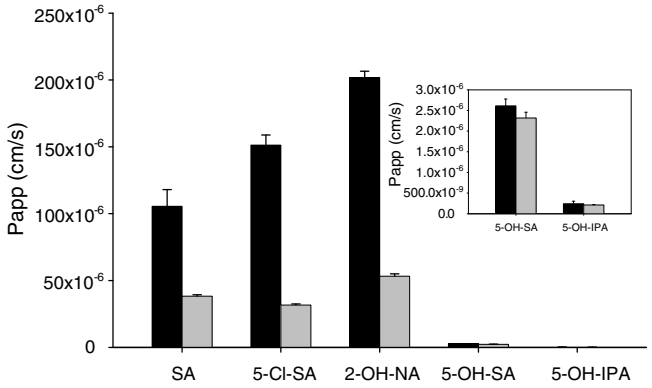


Fig. 1. Permeability coefficients (P_{app}) of the studied compounds in the apical to basolateral (AP-BL) direction with low (200–500 μM) (black bars) and 50- to 125-fold higher (25–30 mM) (gray bars) concentration across Caco-2 monolayers under pH-gradient conditions ($n = 3 \pm SD$).

acid structure of 5-OH-IPA does not support the involvement of monocarboxylic acid transporter and permeation was, as expected, not dependent on the concentration.

3.2. Kinetics of transport

In order to calculate the kinetic parameters of the actively transported compounds, flux values at several concentrations were determined under the pH-gradient conditions (Fig. 2). The calculated maximum velocity (V_{max}) for SA was 38.3 ± 3.0 nmol/min/cm^2 and the Michaelis–Menten constant (K_m) was 4.9 ± 0.5 mM. In addition to this saturable transport, SA was diffusing also by a passive mechanism with a calculated rate constant (k_d) of 17.9 ± 1.4 × 10^{-6} cm/s. The K_m-value of our study matches well with the previously published K_m-values of salicylic acid [3–4]. For 5-Cl-SA and 2-OH-NA, the K_m-values were 5.9 ± 1.7 mM and 7.9 ± 3.4 mM, respectively. These slightly higher K_m-values indicated that the affinity of the compounds for the transporters is similar to that of SA, whereas their higher V_{max}-values (67.4 nmol/min/cm^2 for 5-Cl-SA and 109 nmol/min/cm^{2}for 2-OH-NA) might be due to their higher lipophilicity and passive permeability, or due to the higher turnover rate of the substrate and transporter.

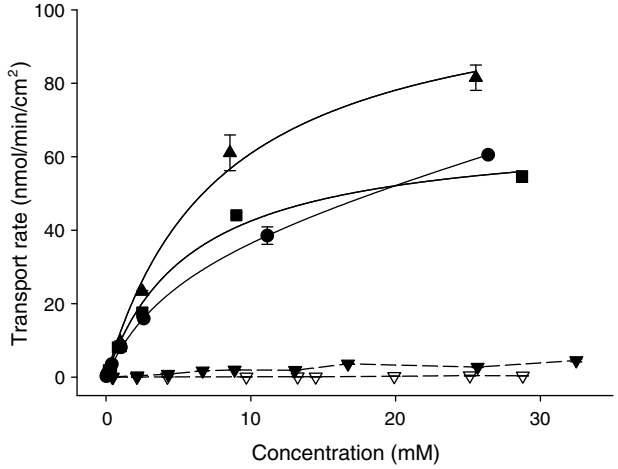


Fig. 2. Concentration dependency of the studied compounds (● SA, ■ 5-Cl-SA, ▲ 2-OH-NA, ▼ 5-OH-SA and ▽ 5 OH-IPA) across Caco-2 cells under pH-gradient conditions (pH 5.5/7.4) ($n = 2–3 \pm SD$).

Transport of 5-OH-SA and 5-OH-IPA was also studied with several concentrations (0.5–30 mM). The concentration increase did not decrease the permeability coefficients of these compounds, as was already shown by the preliminary studies (Figs. 1 and 2). The permeability coefficients varied between 2.6 and 4.3 × 10^{-6}cm/s for the 5-OH-SA and 0.11–0.26 × 10^{-6}cm/s for the 5-OH-IPA, without any indication of concentration dependency, which refers to passive permeation.

3.3. Permeability in the PAMPA model

The kinetic studies discussed above revealed that there is a considerable passive component in the salicylic acid transport, but the non-saturable component of the more lipophilic anions could not be computed from the data. In order to evaluate the properties of passive transport further, PAMPA experiments were performed, as this permeability model describes only the passive transcellular route of permeation [22].

The rank order of the compound permeability in the PAMPA model was similar to that obtained in the Caco-2 model, and correlated nicely with the partition/distribution coefficients of the anions (Table 1). However, the difference in permeability between SA and the most lipophilic molecule, 2-OH-NA, was clearly higher in PAMPA (8-fold) than in Caco-2 cells (2-fold) under the pH-gradient conditions (Fig. 3a and 3b). The permeability coefficients of SA, 5-Cl-SA and 2-OH-NA across the Caco-2 cells were clearly higher than 1 × 10^{-6} cm/s, which is regarded as a boundary value for highly permeable compounds under the pH-gradient conditions in our laboratory [19]. In contrast, the PAMPA permeability of SA, even under the pH-gradient conditions, was only at the level of

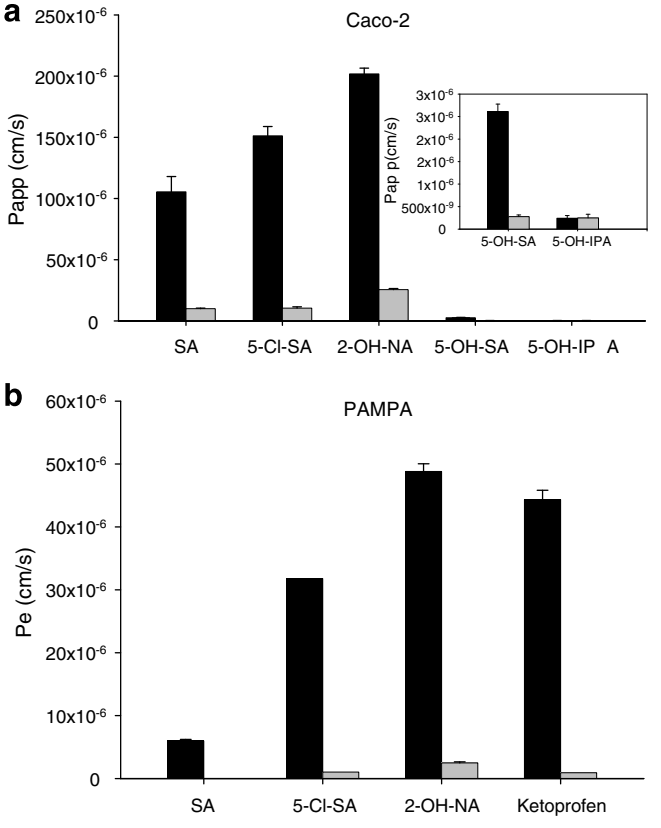


Fig. 3. Permeability coefficients (P_{app} and P_e) of the studied compounds in the apical to basolateral direction across Caco-2 monolayers (a) and artificial membrane (PAMPA) (b) under pH-gradient (black bars) and iso-pH 7.4 (gray bars) conditions ($n = 3 \pm SD$).

moderately permeable compounds (P_e for the moderately permeable compounds is 1–20 $\times$ 10^{-6}cm/s). This suggests that SA is indeed transported actively, which is in accordance with the previous findings that the permeation of actively transported compounds may be underestimated in PAMPA compared to the experiments using biological membranes or cell-based models [20,24].

The apparent partition coefficients (log$D$) at pH 5.5 are positive for 2-OH–NA and 5-Cl-SA (Table 1) and, thus, their passive permeability is predictably higher than for the other studied molecules. Even though these molecules might be transported by the same transporters as SA in vivo, their higher passive permeability is sufficient to position them among highly permeable compounds when low donor pH is used in PAMPA experiments (Fig. 3b).

Under iso-pH 7.4 in PAMPA, the transport of SA was almost undetectable and classified as low ($P_e < 1 \times 10^{-6}$) (Fig. 3b). Higher, but still only moderate permeation was observed for 5-Cl-SA and 2-OH-NA, as well as for the acidic reference molecule ketoprofen (Fig. 3b). It has been observed that the best correlation between the PAMPA and in vivo data is achieved by selecting the PAMPA results under the pH conditions, which provide the highest permeation of the compound [25]. This assumption holds true also for the presently studied anions (including ketoprofen), as their low permeation under the iso-pH 7.4 conditions is not in accordance with in vivo data. Also in the Caco-2 model, acidic conditions on the apical side mimicking the in vivo situation are preferred for the permeability classification of compounds, since the permeability coefficients under the iso-pH 7.4 were lower than the limit (30×10^{-6} cm/s) of high absorption [19] and, hence, give misleading information on the transport properties of acidic drugs (Fig. 3a) [4].

The PAMPA results of 5-OH-SA and 5-OH-IPA were only suggestive, as the 4 h incubation was too short for quantitative analysis. The transport of 5-OH-SA was under the detection limit at the iso-pH 7.4, but increased with the decreasing pH being the highest, but still very low ($0.81 \times 10^{-6} \pm 0.12$ cm/s), at the donor pH of 5.0. For 5-OH-IPA, permeability was undetectable across the studied pH range in the 4 h PAMPA experiments. The results obtained suggest paracellular transport in Caco-2, as the permeability of 5-OH-SA and 5-OH-IPA was extremely low under the iso-pH 7.4 (Table 2, Fig. 3a inset), even lower than the transport of the paracellular marker molecule mannitol (permeability 0.35–0.45 $\times$ 10^{-6} cm/s). The cumulative curves of 5-OH-IPA across each individual monolayer profiled exactly the curves of mannitol when they were applied in the same donor solutions. Therefore, it was concluded that 5-OH-IPA was transported paracellularly even though the paracellular route is favourable to neutral and cationic compounds. The acidic pH 5.5 on the apical side increased the transport of 5-OH-SA about 10-fold, but, interestingly, did not affect the transport of the dicarboxylic acid 5-OH-IPA (Table 2, Fig. 3a inset).

3.4. Evaluation of salicylic acid transport route using EDTA chelation

In order to investigate the role of paracellular and transcellular transport of SA further, the paracellular spaces of Caco-2 cell monolayers were opened by the Ca^{2+}-chelator EDTA, which regulates the cell–cell adhesion and junctional integrity by lowering the concentration of extracellular calcium [26]. After 30 min pre-incubation with EDTA on both sides of the monolayers, the TEER-values decreased approximately 65% under both the pH conditions, which demonstrated that the paracellular spaces were opened. The transport of SA in the presence of EDTA remained unchanged under the pH-gradient conditions, which confirmed the hypothesis that SA is transported purely transcellularly, either passively or actively (most probably in both ways), under the pH-gradient conditions (Table 3). Instead, under iso-pH 7.4 the transport of SA increased 4-fold (from $12.2 \pm 0.4 \times 10^{-6}$ to $52.3 \pm 1.9 \times 10^{-6}$cm/s) with EDTA, pointing out that part of the SA transport occurred via the paracellular route, but a substantial amount of SA permeation still took place via the transcellular route, even though the molecule is completely ionised. The internal standards used in these experiments were antipyrine and mannitol. In the presence of EDTA, the paracellular transport of mannitol increased significantly under both the pH conditions, even though the increase was clearly higher at the iso-pH 7.4. The chelating capacity of EDTA is dependent on the pH and, hence, it is possible that EDTA is not as effective at acidic pH, when some of the ionisable groups are protonated. The transport of transcellularly diffusing antipyrine was increased only slightly under both the pH conditions confirming the effect of EDTA on the paracellular pathway only.

Table 3
The effect of EDTA (2.5 mM) on salicylic acid (250 μM), antipyrine (250 μM) and mannitol transport in apical to basolateral direction

	pH-gradient	Iso-pH 7.4
Salicylic acid	0.9 ± 0.2	4.3 ± 0.2
Antipyrine	1.7 ± 0.3	1.3 ± 0.1
Mannitol	16 ± 3.3	78 ± 6.6

The results are expressed as ratios of permeability coefficients P_{app} obtained in the presence of EDTA vs. without EDTA.

3.5. Effect of inhibitors on salicylic acid transport

In order to probe the role of the known transporters and other competing anions on the transport of SA, inhibitors of the known substrate specificity and other structurally related compounds were applied separately in the same mixture with SA. α-Cyano-4-hydroxycinnamic acid (CHC), an inhibitor of MCT1 and anion exchanger AE1 [13], pravastatin, an inhibitor of OATP-B [16], and probenecid, a common inhibitor of MRP efflux proteins [23], each decreased the transport of SA slightly (20–30%) (Fig. 4). In combination, the inhibitory effect of these compounds was additive, which pointed out that at least MCT1 and OATP-B transporters were involved in the SA transport, as has been previously suggested by Neuhoff et al. [4]. Probenecid is a known inhibitor of MRPs, but it has been shown to affect the transport of OATP-B substrates [16], and also the overall cellular metabolism.

The dicarboxylic acid, 5-OH-IPA, had only a slight inhibitory effect (15%) on SA transport. The dicarboxylic structure does not support the involvement of MCT1, but as an organic anion it might inhibit the OATP-B in a similar fashion to pravastatin. In the paper of Kobayashi et al. [15], a significant inhibitory effect of phthalic acid on the uptake of estrone-3-sulfate was reported, indicating that the substrate specificity of OATP-B is more a question of size of the molecule than the number of carboxylic acid moieties. In the paper of Goto and co-workers [11], phthalic acid did not affect the uptake of angiotensin II inhibitor telmisartan that has been reported to be transported by MCTs, but not by MCT1-4. Similar observations have been observed for fluorescein and the hypoglycemic agent nateglinide, for which a fluorescein transporter has been suggested [9–10]. SA, on the contrary, has been shown to inhibit the uptake of telmisartan and nateglinide, as well as that of estrone-3-sulfate [16]. This broad inhibitory effect of SA on these various compounds further reveals that there might be several transporters involved in SA transport. Whether these interactions with other active pharmaceuticals are clinically important is well worth of finding out.

The most effective inhibitors of SA transport were the lipophilic and structurally related compounds, 5-Cl-SA, 2-OH-NA and 2-OH-3-IPBA, which inhibited the transport of SA by more than 50% (Fig. 4). Benzoic acid was also a potent inhibitor (75%) at 10 mM concentration. When SA and benzoic acid were applied at equimolar

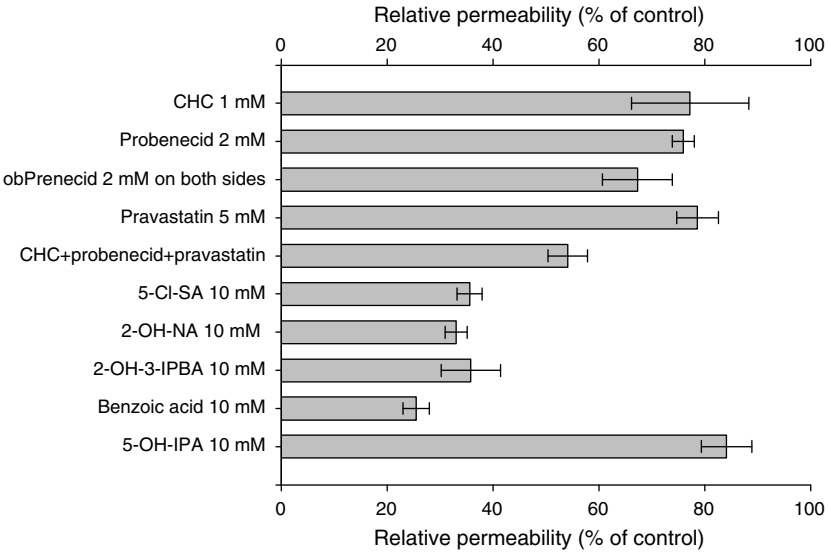


Fig. 4. Inhibitory effect of various compounds on salicylic acid (50 μM) transport under pH-gradient (pH 5.5/7.4) conditions ($n = 3–4 \pm SD$). Differences were statistically significant ($p < 0.05$) in comparison with control (100%).

concentrations of 250 μM, the transport of neither compound was affected (data not shown), which was expected according to the relatively high K_m-values (5 mM) of both the compounds [27]. 5-OH-SA was not used as an inhibitor in this study, but the paper by Konishi et al. [28] suggests that an inhibitory effect by 5-OH-SA is unlikely, as no decrease in the transport was observed in the case of actively transported fluorescein that shares the same transporters with SA.

3.6. Evaluation of proton-dependency of active transport

As previously mentioned and as the inhibitor studies showed, the active transporters of SA (MCT1 and OATP-B) have been reported to be driven by a proton gradient. In order to evaluate this aspect, experiments with an increasing SA concentration were performed under iso-pH 6.5 and under iso-pH 7.4 conditions. Additionally, the experiment under the pH-gradient conditions (pH 5.5/7.4) was repeated with another batch of Caco-2 cells in order to evaluate the behaviour of an actively transported compound between the two batches. The graphs on transport rates at increasing concentrations under pH-gradient conditions followed each other remarkably closely and also the kinetic parameters matched each other (Fig. 5). The K_m-value of this second batch was slightly lower 3.1 ± 0.8 mM (vs. 4.9 mM), the V_{max} about the same 39.9 ± 5.1 nmol/min/cm^2 (vs. 38.3 nmol/min/cm^2). The passive permeation rate constant was $10.0 \pm 2.1 \times 10^{-6}$ cm/s. In addition to the evaluation of passively transported compounds, Caco-2 cells provided, thus, reliable results of active transport of SA from one batch to another.

The transport rate under iso-pH 6.5 conditions (pH 6.5 on both sides) was much lower than under the pH-gradient, as expected according to pH partition theory and proton-dependent transport, and the transport was even lower when the pH 7.4 was used on both the sides of the Caco-2 monolayers (Fig. 5). The Papp-value under the iso-pH 7.4 at a low SA concentration (250 μM) was $10.1 \pm 0.4 \times 10^{-6}$ cm/s, which is close to the calculated passive permeation rate constant under the pH-gradient conditions. Interestingly, the transport of SA was attenuated with increasing compound concentration also under the iso-pH 6.5 (81%), as well as the iso-pH 7.4 (43%) conditions. This saturation phenomenon without the pH-gradient strengthens the concept of carrier-mediated transport for SA, and questions the theory of pH-dependent

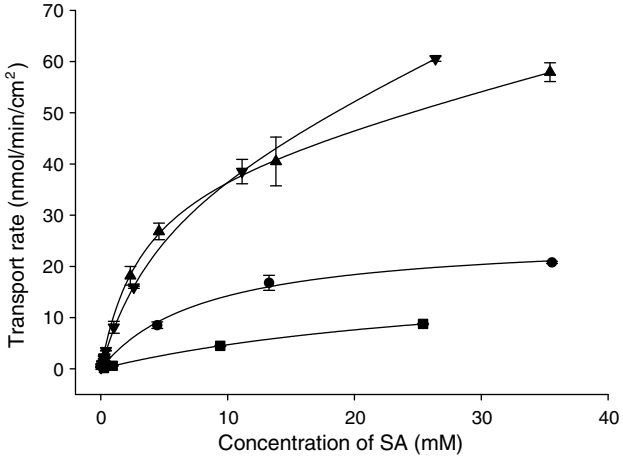


Fig. 5. Concentration dependency of salicylic acid transport in two different Caco-2 cell batches and under pH-gradient (pH 5.5/7.4) (▼ batch 1, ▲ batch 2) and under iso-pH 6.5 ● or iso-pH 7.4 conditions ■ ($n = 3 \pm SD$).

but not carrier-mediated transport by Takagi et al. [12] even though the pH-dependency is evident for SA permeation. In addition, it disagrees with the study by Neuhoff et al. [4], where the transport of SA was not dependent on concentration under iso-pH (7.4) conditions indicating passive transport. The authors also reported that transporters involved in SA transport require proton gradient over the entire cell monolayer, not only between the apical and intracellular compartments. However, our results revealed that the transport of SA was saturable even without the pH-gradient. The transport activity of OATP-B has been shown to work even without the pH-gradient for some compounds [7], which might explain the saturation, or there exists some unknown, pH-independent transporters.

4. Conclusions

The results of this study showed that SA and structurally related lipophilic anions (5-Cl-SA and 2-OH-NA) have similar pH- and concentration-dependent behaviour in their transport across the Caco-2 cell monolayers. High influx ratio under the pH-gradient condi-

tions and a notable difference in apical to basolateral transport between the iso-pH 7.4 and pH-gradient (pH 5.5/7.4) conditions across the Caco-2 cells are supportive, but not conclusive of active transport. Instead, saturation of the transport with increasing compound concentration gave evidence of the active transport mechanism(s). Transport of the more hydrophilic molecule, 5-OH-SA, was similar but much lower than that of the lipophilic compounds, and the transport of 5-OH-SA was not saturated with increasing concentration. Transport of the dicarboxylic acid, 5-OH-IPA, was extremely low, independent of the concentration and pH conditions, and followed the profile of mannitol permeation, which supported the fact that it is transported paracellularly, even though 5-OH-IPA slightly inhibited the transport of SA.

The rank order of the compound permeation was similar in PAMPA and Caco-2 models, but the low permeation of SA in PAMPA compared to Caco-2 permeation supported the theory of active transport for SA. Thus, PAMPA and Caco-2 methods used in parallel are good tools in the evaluation of passive and active transport mechanisms.

Inhibition studies further supported the theory of active transport for SA. 5-Cl-SA and 2-OH-NA inhibited the transport of salicylic acid efficiently, indicating that they share common transporters. According to the inhibitory effect of the MCT1 (CHC) and OATP-B inhibitors (pravastatin), it can be concluded that the transport of SA was mediated by these transporters. The published results of broad and overlapping inhibitory effect of SA on some active pharmaceutical ingredients support the possibility that there are other, still unknown transporters involved. Interestingly, the transport of SA was saturated at high concentrations, even when the pH-gradient was not applied over the whole cell monolayer (iso-pH 6.5), or even without the gradient (iso-pH 7.4), indicating additional pH-independent active transport mechanism(s).

Acknowledgments

The authors thank Katja Suhonen (M. Sc.) for her skillful technical assistance. Orion Corporation Orion Pharma is acknowledged providing the facilities for PAMPA-experiments. National Technology Agency Finland (Tekes, Grant 40186/04) is acknowledged for financial support.

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