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Master's Thesis

Permeation mechanisms of salicylic acid and derivates across Caco-2 monolayers: involvement of transporter proteins

Hradec Králové 2006 **Jakub Čierný**

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Contents

1. Souhrn

Práce se zabývá transportem kyseliny salicylové a jejích derivátů přes Caco-2 buněčné kultury a vlivem možných inhibitorů buněčného transportu. Dále se zabývá vlivem látek rozvolňujících těsné spoje (tight junctions) mezi Caco-2 buňkami. Hlavním cílem bylo zjistit typ transportu, jakým je kyselina salicylová přenášena, výsledky porovnat s transportem kyseliny gentisové a 5-hydroxyisoftalové, posoudit vliv ethylendiamintetraoctové kyseliny na průnik kyseliny salicylové a porovnat možný inhibiční účinek α-kyano-4-hydroxyskořicové kyseliny, probenecidu, pravastatinu, kyseliny benzoové a 2-hydroxy-3-isopropylbenzoové. Předpokládal jsem, že na transportu kyseliny salicylové se podílí i pasivní difúze i transportní proteiny.

Teoretická část práce stručně shrnuje vlastnosti chemických látek, které mají vliv na transport přes buněčné membrány a představuje Caco-2 buňky jako prostředek výzkumu vstřebávání a distribuce léčiv v lidském organismu. Definuje jednotlivé typy transportu přes buněčné membrány, popisuje typy transportních proteinů, které se nacházejí na membránách buněk lidského adenokarcinomu a podává stručný přehled souvisejících témat.

Experimentální část práce popisuje techniku kultivace Caco-2 buněk a metodiku transportních experimentů. Většina experimentů byla prováděna při teplotě 37°C s pH gradientem – pH 5.5 na apikální a pH 7.4 na basolaterální straně membrány. Integrita buněčných vrstev byla testována měřením transepiteliální elektrické resistence a pomocí radioaktivně značené sloučeniny. Vzorky odebrané v průběhu experimentu byly analyzovány vysokoúčinnou kapalinovou chromatografií. Přenesené množství zkoušené látky bylo kvantifikováno pomocí zdánlivého permeačního koeficientu.

Výsledky potvrdily, že se na transportu kyseliny salicylové přes Caco-2 membrány zčásti podílí aktivní transport. Otevření těsných spojů mezi Caco-2 buňkami přineslo výrazné zvýšení transportu pasivně přenášených sloučenin. U kyseliny salicylové nebyl rozdíl tolik markantní, což podporuje myšlenku zapojení aktivních přenašečů. Vliv inhibičních činitelů byl patrný, nejvyšší inhibiční vliv vykazovala kyselina 2-hydroxy-3-isopropylbenzoová.

2. Summary

The aim of this work is to study properties of the transport of salicylic acid and its derivates across Caco-2 cell monolayers, an influence of possible inhibitors of cell transportation and a brief review of related issues. The influence of substance, which opens tight junctions between Caco-2 cells, so called absorption enhancers, is also examined. The main goal is to find out, what kind of transport takes the main part in salicylic acid transport and to compare the results to the data obtained using gentisic and 5-hydroxyisophthalic acid, and, at the same time, to study the influence of ethylenediaminetetraacetic acid as an absorption enhancer and to compare possible inhibitive effect of several compounds. It was assumed that both passive and active transport take part in the transport of salicylic acid.

The theoretical part of the thesis briefly summarizes chemical substances properties, which have an influence on their transport across cell membranes, introduces Caco-2 cells as an important instrument of drug absorption and distribution research, defines the individual types of cell transports and describes the types of transporter proteins which can be localized on the Caco-2 cell membrane.

The experimental part of the work describes the technique of Caco-2 cells cultivation and the process of transport experiments. Most of experiments were done at 37°C using a pH gradient – pH 5.5 on the apical side and pH 7.4 on the basolateral side of the membrane. The cell monolayer integrity was tested by measuring transepithelial electrical resistance and by using radio-labeled compound. The samples taken during experiment were analyzed by the high performance liquid chromatography. The amount of transported compound was quantified by the permeation coefficient and by the flux.

We can see from the results that both active and passive transport takes part in transport of salicylic acid and its derivates across the Caco-2 cell monolayer. The opening of tight junctions brought a noticeable increase of transfer of passively transported compounds; the difference was not so clear in salicylic acid results that support the hypothesis of transporter proteins involvement. The influence of inhibitive agents was obvious; the highest influence was achieved using 2-hydroxyisopropylbenzoic acid.

3. Preface

This thesis was worked out on the Division of Pharmaceutical Technology of Pharmaceutical faculty, University of Helsinki and was linked to the long-term research which is in progress at this Department.

These days, Caco-2 cells are widely used as an instrument of prediction of intestinal drug absorption both in pharmaceutical industry and academic research. The transport of salicylic acid across Caco-2 cell monolayers is being discussed in several articles and lot of results has been already published. On the other hand, gentisic acid and 5-hydroxyisophthalic acid has not been explored yet and some results might be very interesting. Since these compounds are structurally very close to salicylic acid, we can observe properties of their transport and obtain some new information about transporter proteins function in Caco-2 cells membranes.

4. Aims of the thesis

The objective of the thesis was to acquire deeper knowledge of Caco-2 cells membrane anion transporter proteins and the way of transport which deals with transport of salicylic acid and its derivates across Caco-2 cell monolayers.

The specific aims were:

1. To repeat the experiments investigating transport of salicylic acid across Caco-2 cell monolayers with the aim to confirm that the active transport is still evident and to assess the variability in the functionality in the anion transport of Caco-2 cells.

2. To perform transport experiments across Caco-2 cell monolayers with gentisic acid and 5-hydroxyisophthalic acid and to confront the obtained data to the already known results of salicylic acid transport. To assess the relative importance of the different transport mechanisms potentially involved.

3. To find out a transport mechanism of salicylic acid using absorption enhancer (EDTA).

4. To investigate the influence of several substances with presumed inhibitive effect on salicylic acid transport.

5. Introduction

5.1. Cell Culture

5.1.1. Caco-2

The use of intestinal cells has increased dramatically in the pharmaceutical research and all other fields during last years (Artursson *et al.* 2001). The main aim of the cell using research is to explore ways and types of transport across the intestinal epithelium and to identify the relevant carrier.

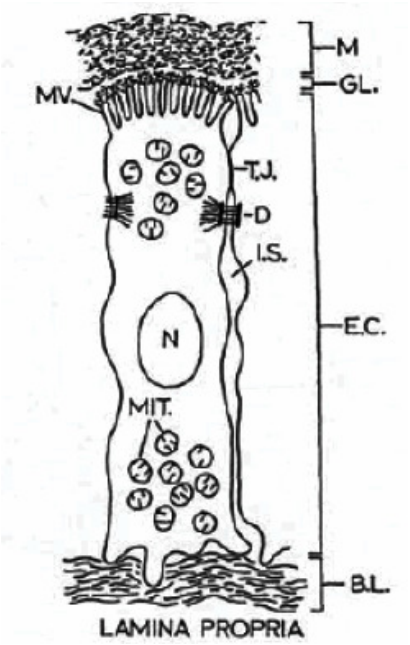


Figure 1: *Enterocyte and its structure;*
M – Mucus, GL – Glycocalix,
MV – Microvilli, TJ – tight junctions,
D – Desmosome, IS – Intracellular space,
N – Nucleus, MIT – Mitochondria,
E.C. – Enterocyte, B.L – Basal layer
(Hillgren *et al.* 1995)

The cell line is derived from well differentiated colon adenocarcinoma in 72-year-old patient (Fogh *et al.* 1977). A lot of effort was focused on this type cell lines because tumors of large intestine are very frequent, unfortunately, as opposite to small intestine tumors (Leibovitz *et al.* 1976; Zweibaum *et al.* 1985). Although originated from large intestine cells, Caco-2 cells have many properties of small intestine cells, including microvilli, intercellular junctions and many of enzymes, nutrient transporters and efflux transporters that present them similar to small intestinal cells (Ward *et al.* 2000). Caco-2 cells also express organic anion (OAT) and organic cation transporters

(OCT), mainly uptake transporters. Also, they express ATP-dependent efflux pump, such as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and several multidrug resistance associated proteins (Taipalensuu *et al.* 2001). In dependence on the time and conditions of cultivation and cell passage number, expression of transporters and enzymes can be various (Briske-Anderson *et al.* 1997).

The important reason for such a wide use of Caco-2 cells is that they are easy to cultivate and they are quite resistant to adverse cultivate conditions. They can grow on plastic surfaces, nitrocellulose filters and on Transwell® polycarbonate membranes as well (Hidalgo *et al.* 1989). When Caco-2 cells grow, they show the contact inhibition (Ward *et al.* 2000). It causes a formation of confluent monolayers necessary for repeatable results of transport experiments. When the forming of monolayer is done, cells start to differentiate. Cultivated for about 21 days, they differentiate into polarized cells with expressed mucosal (apical) and serosal (basolateral) cell membrane domains.

The integrity check of Caco-2 cell monolayers can be provided by assaying monolayer permeability for paracellulary transported compounds (e.g. mannitol). The paracellular permeability of Caco-2 cells appears to be comparable to permeability of rat and human intestinal cells in view of the throughput of tight junctions (Artursson *et al.* 1993). Another possibility of checking the integrity of the monolayer is measuring of transepithelial electrical resistance (TEER) with special electrodes. Nevertheless, TEER values might significantly differ depending on the passage number, the used equipment and the temperature. In general, Caco-2 cell monolayers show TEER properties, which are closer to colonic epithelium then to small intestine epithelium (Grasset *et al.* 1984).

To avoid non-flexible three week period of cell growth, some experiments has been performed to accelerate cell cultivation (Lentz *et al.* 2000). Nowadays it is possible to purchase 3 days Caco-2 cells system (BIOCOAT®, Becton Dickinson Labware, Franklin Lakes, NJ). Lentz *et al.* achieved interesting results of cell cultivation acceleration using 2% iron supplemented calf serum.

5.1.2. MDCK Cells

Madin-Darby canine kidney cells are frequently used in pharmaceutical industry. Their advantage in contrast to most of clones of Caco-2 cells is that they reach full dif-

ferentiation in 3 – 7 days which allows more flexible experiment schedules (Cho *et al.* 1989; Irvine *et al.* 1999). There are two possible clone lines of these cells with very different TEER values, high-resistance MDCK Strain I (~4000 $\Omega \cdot cm^2$) and low-resistance MDCK Strain II (~200-300 $\Omega \cdot cm^2$). The mannitol permeability is as equally low as across Caco-2 cell monolayers. Irvine reported that the correlation to oral fraction absorbed is comparable in both cell systems. Since MDCK cells are derived from dog kidneys, the transporter expression might be very different to human intestinal cells. It is possible to estimate passive epithelial transport with MDCK cells. They were also used to study P-gp expression and to study OCT expression regulated by steroid hormones (Horio *et al.* 1989; Shu *et al.* 2001). MDCK cells are not sufficient for predicting active uptake or efflux across the intestinal epithelium.

5.1.3. HT29 Cells

HT29 cells are other well-examined carcinoma cell line (Collett *et al.* 1996). When cultured in standard culturing conditions with high glucose medium, it forms monolayers with non-differentiated cells, no tight junctions and lack of functional polarity. Culturing in specific cultivating condition (e.g. glucose-free medium) brings significant changes in cell properties. In presence of sodium butyrate in medium cells are produced clones which shows similar morphological signs as both enterocytic cells (junctional complex) and goblet cells (mucosal secretion) (Augeron *et al.* 1984). Deeper investigation of this phenomena shows that HT29 cell cultures contain less then 5% of cells which differentiate spontaneously into both type of cells, enterocytic and goblet (Lesuffleur *et al.* 1990). HT29 cells also show extraordinary adaptation abilities when cultured in presence of cytotoxic compounds such as 5-fluorouracil or methotrexate.

Co-cultures of Caco-2 cells and HT29-H or H29-MTX (methotrexate-induced cells) are used for paracellular transport study with additional presence of a mucus layer (Wikman *et al.* 1993).

5.1.4. 2/4/A1 Cells

2/4/A1 cell line originates from fetal rat intestine and it is conditionally immortalized by temperature-sensitive mutant for the growth promoting oncogene simian virus (SV40) large T (Tavelin *et al.* 1999). Cells are supposed to be cultured in 33°C. In this temperature oncogene is fully active and enables the cells to grow rapidly. The cultivating in 37°C ceases the activity of this oncogene, stops proliferation and provides cell differentiation.

TEER values of 2/4/A1 cells has been reported to be ~25 $\Omega \cdot cm^2$ and apparent permeable coefficient of mannitol 15.5 $cm \cdot s^{-1}$ x 10^{-6}. This value is about 65-fold higher then with Caco-2 cells. Thus, 2/4/A1 cells form monolayers much more penetrable that can be seen both from TEER values and Papp values of mannitol.

Using 2/4/A1 cells means valuable alternation compared to using very tight Caco-2 cells. However, lack of important enzyme systems and low morphological differentiation predetermines 2/4/A1 cell line for studies of passive transcellular transport. Moreover, culturing conditions for this cell line are quite specific, compared to relatively simple techniques used with Caco-2 and MDCK cells. Their use in the research is very limited.

5.1.5. Other Cell Lines

T84 is colonic cancer cell line, which shows typical characteristics for colonic crypt cells (Dharmsathaphorn *et al.* 1984). They are supposed to be cultured from 5 to 7 days, then they polarize and develop monolayers of high TEER (~ 1000 $\Omega \cdot cm^2$) and they are able to grow on both plastic and collagen-coated support. Cells are not well differentiated. T84 are being used for study of Cl^- secretion, the transport event responsible for mucosal hydration of digestive tract. This cell line is not considered to be an adequate instrument to study carrier-mediated transport processes.

IEC-18 is the cell line originating from rat intestinal epithelium (Duizer *et al.* 1997). It has been evaluated as a model for study of small intestine epithelium permeability. This cell line forms monolayers with very low TEER (~ 50 $\Omega \cdot cm^2$) and mannitol permeability 8.0 $cm \cdot s^{-1}$ x 10^{-6}. The goal of developing this cell line was to make a

better model for small intestinal epithelium permeability studies then Caco-2 cell line. It was focused on hydrophilic molecules transport studies; however, the leakier paracellular pathway is connected with poor cell differentiation and low expression of transporters. Therefore, IEC-18 cells are not suitable for carrier-mediated transport studies.

5.2. Absorption Prediction by Caco-2 Cells

Oral administration is recently the most frequent and probably the safest drug delivery route (Neuhoff 2005). Almost all drugs administrated orally are absorbed in small intestine through series of different barriers. From mucosal side it means mucus gel layer, intestinal epithelial cells, lamina propria and capillary endothelium. The barrier with the most significant influence on drug absorption is small intestine epithelium layer. Thus, it is possible to study intestinal drug absorption using intestinal epithelium cell monolayers.

Good correlation between small intestine absorption data and data obtained by initial studies using Caco-2 cell monolayers was shown (Artursson 1990). However, similar results both in small intestine epithelium and Caco-2 cell monolayers is structure related (Table 1)

Table 1: *Summary of structural properties and absorption characteristics of the drugs. Papp means apparent permeability coefficient (cm · s^{-1}) x 10^{-6} (pH is not mentioned), log D is octanol/water partition coefficient at pH 7.4, % Abs. means percent absorbed of an orally administered dose (Artursson et al. 1991). Different values published in Yee 1997.*

Drug	Papp	log D	% Abs.	Mw
Corticosterone	54.5	1.89	100	346
Testosterone	51.8	3.31	100	288
Propranolol	41.9	1.54	90	259
Alprenonol	40.5	1.00	93	249
Warfarin	38.3	0.12	98	308
Metoprolol	27.0	0.07	95	267

Felodipine	22.7	3.48	100	384
Hydrocortisone	21.5	1.53	89	362
Dexamethazone	12.5	1.74	100	392
Salicylic acid	11.9	-2.14	100	138
Acetylsalicylic acid	2.4	-2.57	100	180
Practolol	0.90	-1.4	100	266
Terbutaline	0.38	-1.4	73	255
Atenolol	0.20	-2.14	50	266
Mannitol	0.18	-3.10	16	182
Arginin-vasopressin	0.14	-2.73	0	1084
Sulphasalazine	0.13	-0.13	13	398
1-deamino-8-D-arginin-vasopressin	0.13	-1.95	1	1071
Olsalazine	0.11	-4.5	2	302
Polythylenglycol	0.052	-5.1	0	4000

Usually the most common way to predict absorption coefficient is to determine the drug lipophilicity (Artursson *et al.* 1991). The assumption is that more lipophilic drug should partition faster into lipid cell membrane. However, this prediction gives only approximate rate of drug absorption. Anyway, coarse correlation between octanol/water partition coefficient and absorption rates has been observed.

From results published by Lennernäs *et al.* 1996 it ensues that the passive transcellular drug transport rate in human jejunum can be predicted by experiments with Caco-2 cell monolayers *in vitro*. In general, compounds with Papp < 1 cm $\cdot$ s^{-1} x 10^{-6} are poorly absorbed (0-20%), compounds with Papp 1-10 cm $\cdot$ s^{-1} x 10^{-6} are moderately absorbed (20-70%) and compounds with Papp > 10 cm $\cdot$ s^{-1} x 10^{-6} are well absorbed (Yee 1997). Excellent correlation has been found for a variety of compounds encompassing transcellular, paracellular and carrier-mediated transport. Nevertheless, certain compounds (e.g. L-dopa) might be transported approximately 340-fold slower *in vitro* then *in vivo* (Hu *et al.* 1990).

Therefore, the prediction of human active drug transport is possible after characterization of each transport system.

5.3. Transport Pathways across the Intestinal Epithelium

The limiting factor for the absorption of drugs in small intestine seems to be unstirred water layer (aqueous boundary layer, ABL) (Artursson and Karlsson 1991). The thickness of this layer was determined to be 200-800 μm with anesthetized experimental animals. More recent studies performed with conscious rat shows the thickness of ABL about 100 μm. In both studies the dependence on luminal stirring has been shown.

Transport rate of rapidly transported drugs is influenced by properties of ABL, while slowly transported drugs are not affected by ABL changes (Artursson *et al.* 1991). Another barrier is mucus layer, which consists of 90% water and usually 0.5-1.0% of mucin (Carlstedt *et al.* 1985). After penetrating the mucus layer, the drug is exposed to acidic microclimate at the luminal surface of the intestinal epithelium (Neuhoff 2005). There are more possibilities of drug transport across small intestinal epithelium. Two main pathways are either through epithelial cells (transcellular), or between cells via tight junctions (paracellular). Certain drugs are substrates for transporter proteins localized on luminal side of epithelium cells and can be exposed to carrier-mediated transport (uptake, efflux, antiport, symport). Another possibility limited for some macromolecules is transcytosis which is a variation of the passive transcellular transport.

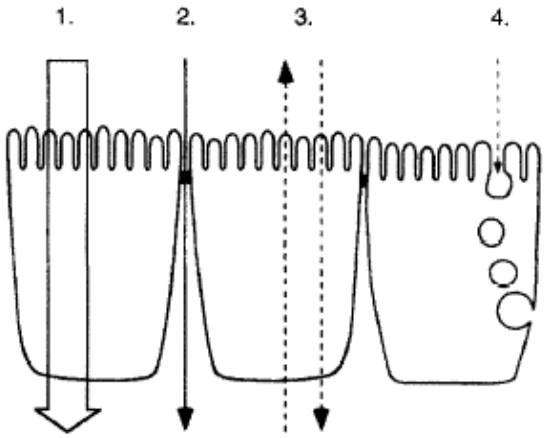


Figure 2: *Schematic drawing of an intestinal epithelium. The arrows indicate the four different transport routes: 1. the passive transcellular; 2. the passive paracellular; 3. the active carrier-mediated transcellular; 4. the transcytosis routes* (Artursson *et al.* 2001)

5.3.1. Paracellular Transport

Paracellular transport leads in between cells through tight junctional complex (Neuhoff 2005). The tightness of this complex and the concentration gradient of the solution therefore is the rate-limiting factor which regulates the transport. Some papers show that pores of tight junctional complex carry negative net charge. Thus, it should be more permeable for cationic compounds then for anionic or neutral compounds (Adson *et al.* 1994).

The permeability of tight junctional complex can be influenced by intra- or extracellular Ca^{2+} ion concentration, protein kinase activators and inhibitors, or absorption enhancers (Quan *et al.* 1998). However, paracellular transport is mainly influenced by molecular weight and net charge of transported compound (Tavelin 2003).

Paracellular transport is also preferred by drugs that are poorly distributed into cell membranes, which mean hydrophilic compounds or peptides. Some compounds are transported both by paracellular and transcellular transport, it is possible that even very hydrophilic compounds, even thought they should be transported via tight junctional complex, might by transported also by transcellular pathway. The reason is that the luminal cell surface area is 1000-fold larger then paracellular space (Pappenheimer *et al.* 1987). This significant different compensate non-suitable physico-chemical properties.

Even thought paracellular transport is considered to be passive, it has been also shown to be saturable (Gan *et al.* 1998) and indirectly linked with intracellular and tight junctional processes (Zhou *et al.* 1999; Lee *et al.* 2002). Therefore, paracellular pathway requires further investigation.

Compounds, which are significantly transported via tight junctions, are all of moderate molecular weight and quite hydrophilic. Among others these are furosemide (Flanagan *et al.* 2002), atenolol (Adson *et al.* 1995), cimetidine (Zhou *et al.* 1999), ranitidine and famotidine (Lee *et al.* 1999).

5.3.2. Passive Transcellular Transport

Physico-chemical properties of involved compounds and properties of the membrane are critical factor of passive transcellular transport (Neuhoff 2005). In general, compound has to be lipophilic in appropriate way. Certain degree of lipophilicity is

necessary to enter the lipidic membrane; on the other hand, too lipophilic molecules might be trapped in the membrane. If transported compound has suitable properties for penetrating lipidic membranes, it is transported mainly transcellulary (however passively or actively), because the membranous surface is 1000-fold larger then intracellular spaces. The driving force of passive transcellular transport is the concentration gradient.

Very simple and very reasonable concept of prediction of the transcellular transport is 'Rule of 5' (Lipinski *et al.* 2001); poor absorption or permeation appears when in the molecule are more than 5 H-bond donors, 10 H-bond acceptors, the molecular weight is greater than 500 and the calculated Log P is greater than 5.

The membrane composition may vary between different cell types, it varies also between apical and basolateral side, both by composition (phospholipids, cholesterol, proteins) and transporter expression. Apical membrane properties mean the limiting factor of transcellular transport in polarized cells (Hayton 1980). Most approved drugs, which are rapidly and completely absorbed after oral administration, are transported by passive transcellular transport (Artursson *et al.* 2001).

5.3.3. Active Transcellular Transport

Active transcellular transport (carrier-mediated transport) is reserved exclusively for certain type of molecules (among others vitamins, glucose) (Neuhoff 2005). It works simultaneously with passive transport. Because of limited amount of binding place, active transport might be saturated by extreme concentrations of the substrate. This route might be inhibited or blocked by structurally similar compounds or energy depletion. Active transport against concentration gradient is energy dependent. This is provided by hydrolysis of high-energy bonds in some compounds (such as ATP) or by coupled transport with another molecule such as H^+ or Na^+.

Naturally, carrier-mediated transport might also work in opposite direction – provide cell efflux and this can be reason for low absorbance of some compounds. When are these efflux mechanisms saturated, it brings sudden increase of absorption.

Some conditions might change properties of membranes (e.g. fluidity) and influence the transport. From this reason all active transport are dependent on condition such as pH or temperature or presence of other compounds

5.3.4. Transcytosis

Because of transcytosis slowness and low capacity, it is less attractive for drug transport. By this pathway are transported practically exclusively only compounds, which are excluded from other routes due to their size, such as peptide antigens (de Aizpurua *et al.* 1988). Another disadvantage is, that transcytosis is performed by transport in membrane vesicles, which contain high amount of proteolytic enzymes, thus, many transported exogenous proteins might be degraded. This effect is obvious both *in situ* and *in vitro* (Heyman *et al.* 1990). Probably the best example of natural compound transported by transcytosis route is vitamin B_{12}, which is transported from mucosal to serosal direction (Dix *et al.* 1990). This transport has very low capacity and requires specific binding to the intrinsic factor.

5.4. Membrane Transporters

Membrane transporters play very important role in intestinal drug absorption, since they provide active molecule transport across intestinal epithelium (Lee 2000). The study of their functions seems to be necessary for understanding mechanisms of absorption and development of what can offer us optimized drug structures with possibility of targeted delivery. The investigation of genetic polymorphism of transporters may be useful for personalization of administered drug.

As mentioned above, most of lipophilic molecules are absorbed by passive diffusion; nevertheless, it is accessible only for lipophilic molecules (Tamai 1997). Many water-soluble compounds might be transported by specialized carrier-mediated transport mechanisms. This transport is available for peptide analogues, nucleosides, amino acids, sugars, monocarboxylic acids, bile acids, fatty acids, organic anions and cations, phosphates and water-soluble vitamins.

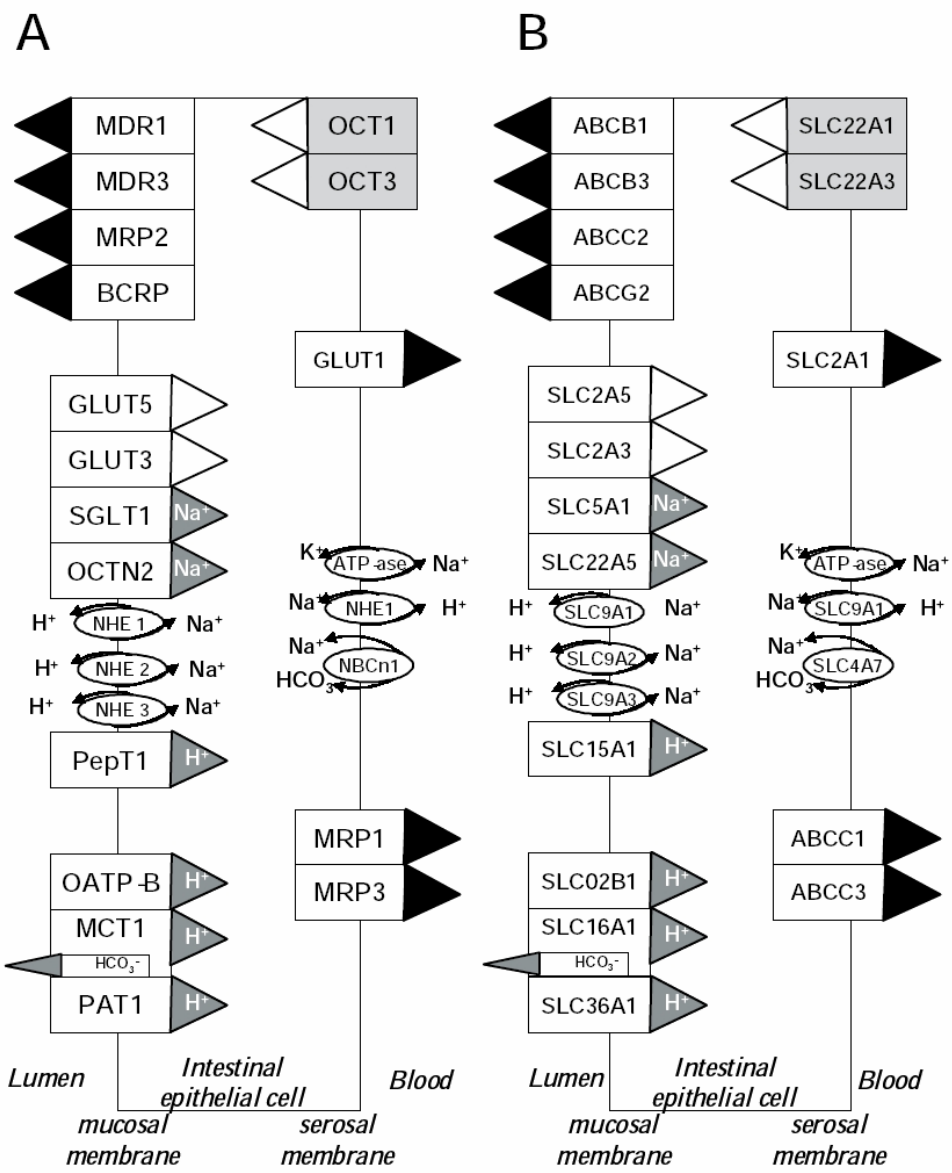


Figure 3: *Schematic illustration of some intestinal epithelial transporters verified to be expressed in the Caco-2 cell line. (A) Transporters shown as a square demonstrate active or facilitated transport. Ion-exchangers are shown as oval shapes. The name of the corresponding transporter for the process is shown within the square or oval shape. For active transporters, the black triangles represent an efflux transporter, the white triangles an uptake transporter and the grey triangles represent symport or antiport of the substrate and the driving force. Grey marked squares represent an unverified location for the transporter. (B) The corresponding gene names of the transporters are shown in the right illustration. SLC: solute carrier, ABC: adenosine triphosphate binding cassette. Transporters are described in the text* (Neuhoff 2005).

5.4.1. Monocarboxylate Transporters (MCTs)

The monocarboxylate transporters are members of SLC16 gene family. Their subfamily includes 14 members nowadays and every isoforms appears to have slightly different substrate and inhibitor (Tamai *et al.* 1995; Halestrap *et al.* 1999; Halestrap *et al.* 2004). They are involved in transporting metabolically important monocarboxylates such as pyruvate, lactate or keton bodies and other monocarboxylates. MCTs were so far identified e.g. in small intestine epithelium cells, skeletal muscle cells, heart muscle cells, in brain and in inner ear. Clear evidence of lactate and pyruvate transport has been shown with MCT1-MCT4.

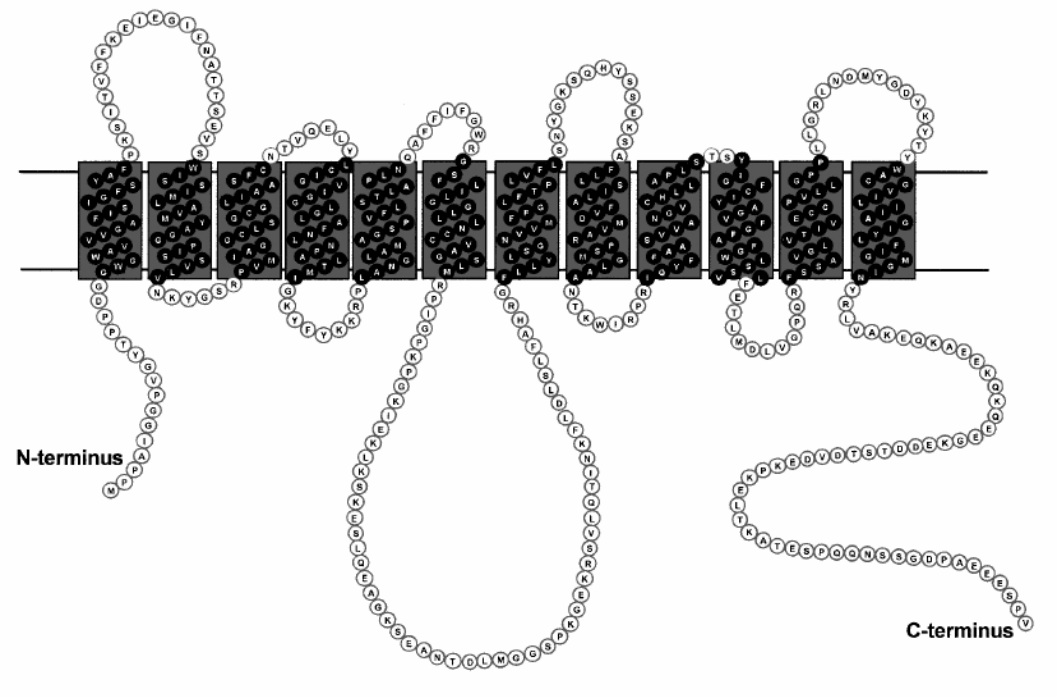


Figure 4: *Proposed membrane topology of the MCT family* (Halestrap *et al.* 1999)

The regulation of expression of individual isoforms of these transporters is still unrevealed in general. However, in skeletal muscle it is possible to observe up-regulation of MCT1 expression connected with chronic stimulation (Bonen *et al.* 2000). On the other hand, decrease of MCT1 and MCT4 expression can be observed in diabetic rats (Py *et al.* 2001).

MCTs transport monocarboxylates with one proton or as an exchange for other monocarboxylate. Transport of monocarboxylates can be increased by decreasing pH from 8 to 6 on one side or by increasing pH on the other side; it means that this transport is pH dependent (Garcia *et al.* 1994). Detailed kinetic analysis is well known for MCT1, other family members are not investigated yet.

Typical substrates for MCTs is among others salicylic, benzoic and lactic acid (Tamai *et al.* 1999)

The function of MCTs is strongly inhibited by α-cyano-hydoroxycinnamate (CHC) (Deuticke 1982).

5.4.2. Organic Anion Transporting Polypeptides (OATPs)

OATPs are one of three major families in the group of anion transporters together with organic anion transporters (OATs) and multidrug resistance proteins (MRPs) (Kusuhara *et al.* 2002). OATPs are involved in transporting many both endogenous substances and xenobiotics, such as conjugated metabolites of steroid hormones, thyroid hormones, bile acids, bilirubin, pravastatin, benzylpenicillin, and digoxin (Kanai *et al.* 1996; Kobayashi *et al.* 2003; Hagenbuch *et al.* 2004; Nozawa *et al.* 2004). This family is classified as the solute carrier family 21A (SLC21A). Recently 9 members of this family were identified in human organisms. Newly they are classified within the OATP/SLCO superfamily and divided into subfamilies.

OATPs are sodium independent transporter proteins localized among other on apical side of human enterocytes. They provide anion exchange transport, coupling the cellular uptake of organic compounds with the efflux of e.g. bicarbonate or glutathione. Small intestine OATPs shows pH sensitive transport with higher activity at acidic pH then at neutral pH. Weak organic acids meant to be transported passively across intestinal epithelium according to the pH-partition hypothesis but it has been proven that they undergo carrier mediated pH-dependent transport; some compounds are transported at lowered pH conditions only.

The pH dependency of transport has been shown in many studies, e.g. clear correlation between pH and OATP function has been published in transportation studies

with pravastatin and estrone-3-sulphat (Nozawa *et al.* 2004) and it was first experimental demonstration that human OATP has pH dependent functionality.

It has been shown as well, that transport of the substrate is lowered in presence of structurally similar compounds. Certain inhibitive influence has been also shown with substances, which are not similar to candidate substrates of OATP (e.g. probenecid (Yasui-Furukori *et al.* 2005).

5.4.3. Other Transporters

Peptide transporters have been identified from the various mammalian tissues. There is lot of peptide transporters in human small intestine, such as PEPT1 (peptide transporter-1), PEPT2, hPT-1 (humane peptide transporter-1) or ATP-binding cassette peptide transporter. PEPT1 and hPT-1 appears mainly in brush border membrane of human intestine, PEPT2 appears mainly in kidneys (Adibi 1997; Yang *et al.* 1999). Probably the most important is PEPT1 which plays the critical role in absorption of peptide-like compounds from the intestine. It uses an electrochemical proton gradient as its driving force. It also shows stereoselectivity. Surprisingly, protein bond is not the essential condition for PEPT1 substrates, e.g. acyclovir prodrugs shows interaction with this transporter.

Nucleoside transporters are divided into two major groups: Na^+-independent equilibrative transporters (ENT family) and Na^+-dependent concentrative transporters (CNT family) (Wang *et al.* 1997; Balimane *et al.* 1999). Nucleoside analogues are used to treat cancer and viral infection and study of function of nucleoside transporters is necessary to possibility of affecting absorption of these compounds. CNT family seems to be restricted both in their distribution and substrate selectivity. They are expressed mainly in small intestine. On the other hand, ENT family is expressed widely and has broad specificity.

Amino acid transporters classification is well defined according to their substrate specificity and tissue distribution (Oxender 1972; Palacin *et al.* 1998). Since amino acids are hydrophilic molecules, carrier-mediated transport is the only possibility of their transport across intestinal epithelium. Neutral, cationic and anionic amino acids are all transported by both Na^+-dependent and Na^+-independent systems. So far, about

30 of amino acids transporters have been cloned from mammalian cells. They have highly restrictive substrate specificity.

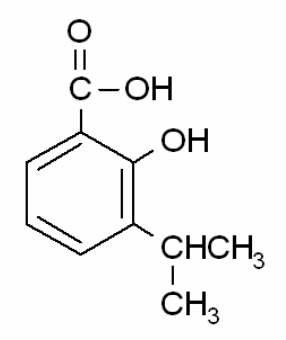
Organic cation transporters expressed in the liver, kidney and intestine are critical for absorption and elimination of both endogenous substances and toxins (Zhang *et al.* 1998). They are divided into two main groups OCT1-3 and OCTN1-3 (organic cation/carnitine transporters). They differ in transported substrate. Since over fifty percents of all drugs has cationic character, they play important role in intestinal absorption.

Glucose transporters are involved of transporting final product of carbohydrate digestion (Mueckler *et al.* 1985; Olson *et al.* 1996). There are two main groups of glucose transporters: Na^+-dependent secondary active transporters (SGLTs) and Na^+-independent facilitated diffusion transporters (GLUTs). Until now, 13 GLUTs has been recognized and this number is growing rapidly. In general, SGLTs are located in the brush-border membrane and GLUTs are located in basolateral membrane. Glucose transporters are specific for galactose, fructose and glucose, mainly for D-form. L-form is also transported but it has 1000 times less affinity for transporters. Different transporters in GLUT and SGLT group have different affinity for different substrates. Glucose transporters transport also some drugs with structure similar to sugars.

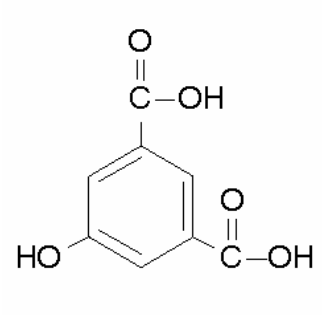
Vitamin transporters transport water-soluble vitamins, it seems that carrier-mediated transport for fat-soluble vitamins is negligible (Prasad *et al.* 1998; Tsukaguchi *et al.* 1999). Vitamin C is transported by two different Na^+-dependent transporters, SVCT1 and SVCT2. Na^+-dependent multivitamin transporter (SMVT) is involved in transport of pantothenat, biotin and lipoate. Nicotinic acid is transported by transporters for monocarboxylic acids.

6. Materials and Methods

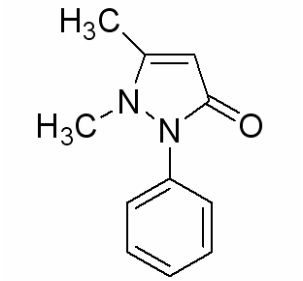
6.1. Drugs and Chemical Material



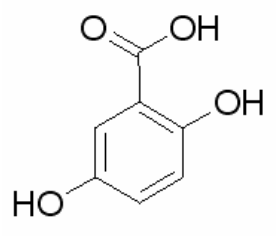
2-hydroxy-3-isopropylbenzoic acid
(Aldrich Chemical Company Inc., USA)



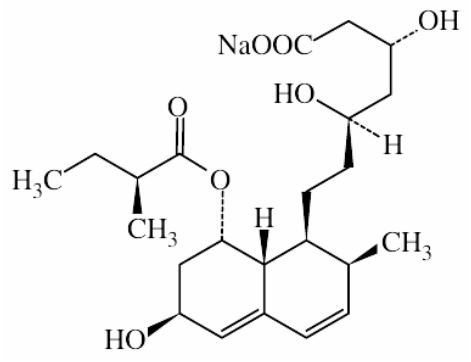
5-hydroxyisopthalic acid
(Sigma-Aldrich Chemie GmbH, Germany)



Antipyrine
(Aldrich Chemical Company Inc., USA)

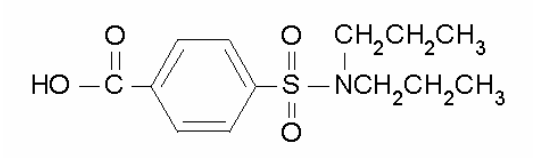


Gentisic acid
(Sigma-Aldrich Chemie GmbH, Germany)



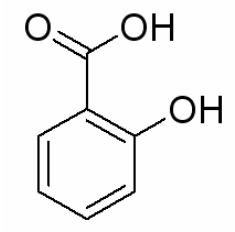
Pravastatin sodium

(Sigma-Aldrich Chemie GmbH, Germany)



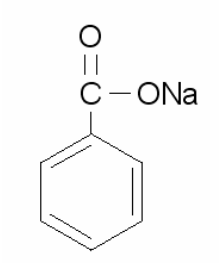
Probenecid

(Sigma-Aldrich Chemie GmbH, Germany)



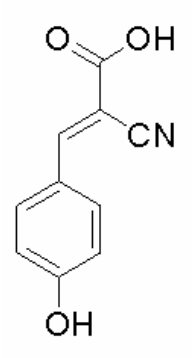
Salicylic acid

(Sigma-Aldrich Chemie GmbH, Germany)



Sodium benzoate

(Aldrich Chemical Company Inc., USA)



α-Cyano-4-hydroxycinnamic acid

(Aldrich Chemical Company Inc., USA)

6.2. Prepared Reagents

Medium 10% consisted of DMEM (EuroClone Ltd., England); 10% of HIFBS; 1% of Penicillin/Streptomycin 100x (EuroClone Ltd., England); 1% of NEAA (EuroClone Ltd., England) and 1% of L-Glutamine (EuroClone Ltd., England).

Medium 20% consisted of DMEM; 20% of HIFBS, 1% of Penicillin/Streptomycin 100x; 1% NEAA and 1% of L-Glutamine.

HBSS buffer solution consisted of 10% of 10x HBSS concentrate (with $CaCl_2$ + $MgCl_2$) (Gibco Invitrogen Corp, Scotland); 1% of MES/HEPES buffering solution (final concentration 10 mmol/l) and sterilized water.

0.1 M EDTA solution consisted of EDTA (EDTA tetrasodium salt hydrate, Sigma-Aldrich Chemie GmbH, Germany) and sterilized water.

PBS-EDTA solution consisted of 0.1M EDTA solution; 10% of PBS concentrate (without $CaCl_2$ and $MgCl_2$) and sterilized water. Final EDTA concentration was 0.5 mmol/l.

Trypsin-PBS-EDTA solution consisted of 10% of 2.5% trypsin solution (EuroClone Ltd., England) and 90% of PBS-EDTA solution. Final trypsin concentration was 0.25%.

1 M MES buffering solution consisted of MES (Sigma-Aldrich Chemie GmbH, Germany) and sterilized water. This solution was used to buffer HBSS at pH 5.5.

1 M HEPES buffering solution consisted of HEPES (Sigma-Aldrich Chemie GmbH, Germany) and sterilized water. This solution was used to buffer HBSS at pH 7.4.

0.1% TFA pH 2.1 consisted of trifluoracetic acid (Sigma-Aldrich Chemie GmbH, Germany) and redistilled water.

6.3. Cell Culture Conditions

6.3.1 Cell Cultivating

Caco-2 cells originated from a human colorectal carcinoma were obtained from American Type Culture Collection (ATCC), Rockville, USA. Cells at passage number from 31 to 40 were used. Cells were cultivated in incubator (HERACell 240 and BB16,

Heraeus Instruments GmbH, Germany) at 95% relative humidity and 5% CO_2, at 37°C in cell culture flasks (Corning, New York, USA). Cells were fed three days per week, with high glucose DMEM medium (DMEM, 10% HIFBS, 1% penicillin/streptomycin sol., 1% NEAA and 1% L-Glutamine). All manipulations with cells were conducted under aseptic condition in laminar air flow cabinet (HeraSafe KS12, Heraeus Instruments GmbH, Germany; KR125 Safety, Kojair, Turku, Finland).

6.3.2. Cell Passaging

Cell culture was passaged when it reached at about 80% confluences, usually once in seven days. Medium was removed using Pasteur pipette attached to the suction hose. At about 10 ml of PBS-EDTA solution was added for 4 minutes. Solution was removed and 2.5 ml of trypsin-PBS-EDTA (trypsin concentration 0.25%) solution was added, cell layer was moistened, the excess (2 ml) of trypsin solution was removed afterwards; cell culture was incubated for other 5 minutes. Then medium was added to cease the effect of trypsin. Suspension of cells and medium was mixed several times by aspirating and dispensing the whole volume and the suspension was dispensed into a Falcon® tube. Cell suspension was centrifuged at 1500 rpm for 5 minutes (Labofuge 400, Heraeus Instruments GmbH, Germany). Supernatant was removed; cells were dispersed in fresh medium and about 250 000 cells were seeded into at least two flasks. Desired cell density was achieved by diluting of liquid according to the result of viable cell count.

6.3.3. Viable Cell Count and Seeding Cells on Transwell Inserts

Viable cell count is based on Trypan blue dye, which is able to penetrate dead cells membrane and colour them.

Cell suspension sample was mixed in 96 well plate well and 50 µl of suspension was pipetted into another well, where 50 µl of Trypan blue solution (0.4% sol.; ICN biomedicals) was added and mixed it by pipetting. Sufficient amount of sample was introduced into two hemocytometer chambers. Cells were counted under the microscope (microscope T041, Olympus, Japan) using a counter, systematically in 5 selected

squares. The volume of medium necessary to achieve the right concentration can be calculated from following formula.

$$Volume = \frac{(cells / squares) \times 2 \times 10000 \times A}{Cell\_concentration} \qquad \text{Eq.2}$$

where

Volume = added medium (ml)
cells = total count from counter
squares = number of the counted squares
2 = dilution factor (cell suspension/colour)
10000 = correction factor for the sample chamber volume (volume = 0.0001 ml)
A = total volume of cell suspension from the pipette scale (ml)
Cell_concentration = desired concentration of the seeding suspension (cells/ml)

After achieving the intended cell concentration, the cells were seeded on Transwell® inserts (Corning, NY, USA). 12 well plates were used; the filter area was 1.13 cm^2. 0.5 ml of cell suspension was added at concentration 150 000 cells/ml to the apical compartment and 1.5 ml of pure medium to basolateral compartment. Cells were grown at least for three week, all transport experiments were done from 21 to 28 days of cultivation.

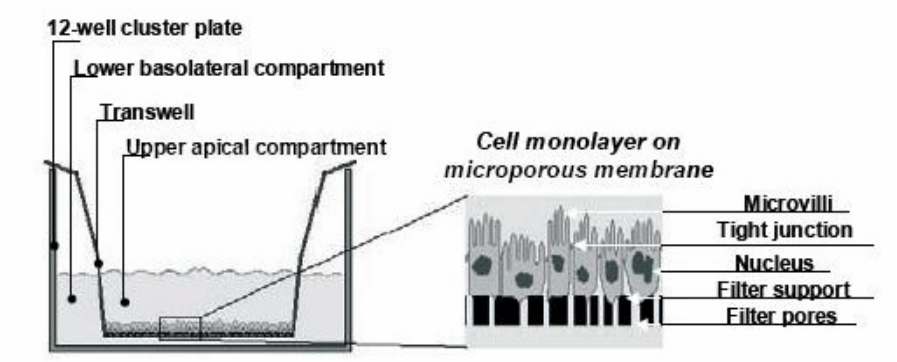


Figure 5: *Schematic illustration of an epithelial monolayer grown on a permeable support, e.g. Transwell®* (Neuhoff 2005)

6.4. Transport Experiments

6.4.1. Preparation of Cells for the Experiment

Before experiment cell monolayers were washed with pre-warmed (37°C) HBSS (pH 7.4) two times. This washing step eliminated residual enzyme activity from serum. After washing, HBSS pH 7.4 was added to basolateral compartment and HBSS pH 5.5/7.4 was added to apical compartment depending on the experimental conditions. Each plate was pre-incubated 30 minutes at 37°C.

6.4.2. Experiment Conditions

Experiments were performed at 37°C, during experiment plates were placed in orbital incubator at 50 rpm (orbital incubator S150, Stuart, Bibby Sterilin Ltd.). All manipulations with plates during experiments were performed on hotplate set to 37°C (AG Multitherm 6, H+P Laboratortechnik).

Experiments with and without pH gradient were performed. With pH gradient conditions pH on apical side was pH 5.5 and basolateral pH was 7.4. Without pH gradient pH 7.4 was on both sides.

All studies were performed at least as triplicates.

6.4.3. Transport Study apical-basolateral (a-b)

HBSS pH 7.4 was pipetted to the basolateral sides of new plate. Then pre-incubated inserts were emptied and placed into the new plate. Drug solution containing the compound of interest was dispensed into the apical side of empty insert and the start sample from the donor side was taken.

Inserts were transferred to new wells containing fresh HBSS according to planned time intervals. At the end of experiment inserts were transferred to the original plate, end sample was taken and fresh HBSS added into the insert.

The sample from each well for HPLC analysis was taken.

6.4.4. Transport Study basolateral-apical (b-a)

Fresh HBSS (pH 5.5/7.4) was pipetted to the apical side of monolayers. Drug solution containing the compound of interest was dispensed to basolateral chambers of the new plate and inserts containing fresh HBSS were placed into it. The start sample from basolateral side was taken.

The sample was taken each interval from apical side and replaced with fresh HBSS. At the end of the experiment inserts were transferred to the original plate and end sample from basolateral side was taken.

All samples taken during experiment were analyzed by HPLC.

6.5. Checking Cell Monolayer Integrity

TEER measurement

The integrity of monolayer and tight junctions were checked by measuring trans-epithelial electrical resistance (TEER), using electrode Millipore Millicell®. The resistance of monolayer was multiplied by the effective growing area (1.1 cm^2) and expressed as $\Omega \cdot cm^2$.

Monolayers were equilibrated for 30 minutes before measurement and three measurements for each well were performed. During experiments with inhibitors TEER were measured after 30 minutes incubation with used inhibitor. All used cells had TEER values over 300 $\Omega \cdot cm^2$.

Radioactivity monitoring

As a simultaneous method to check monolayer integrity was used ^{14}C-Mannitol (2.18 GBq/mmol, Amersham Pharmacia Biotech, England), which is transported only via paracellular pathway.

During experiment sample of 100 µl was taken from acceptor side, each time the sample of transported compound was taken. Each sample was mixed with 4 ml of Opti-Phase 'HiSafe' 2 (Wallac Scintillation Products, Fisher Chemicals, Loughborough, UK) and analyzed on liquid scintillation counter Winspectral 1414 (Wallac, Turku, Finland).

Cumulative transport of ^{14}C-Mannitol was calculated. The transport rate should not exceed the limit value of 0.5%/h.

6.7. HPLC Methods

HPLC configuration with diode array detector with column Supelco Discovery C18 504955 (5 µm particle size, length; 15 cm × 4.6 mm) was used. Different method for each compound (Table 2) was used.

HPLC set consisted of auto sampler Spectrasystem AS3000, Spectrasystem gradient pumps P4000, Spectrasystem SN4000, UV600LP detector, vacuum membrane degasser SCM1000 (Thermo Separation Product).

Experiment samples were analyzed directly after experiment (mainly unstable gentisic acid) or stored at -18°C and analyzed afterwards. After thawing samples were mixed and centrifuged for 5 minutes at 13.2 rcf (5451D, Eppendorf AG, Hamburg, Germany) to avoid any solid particles in analyzed liquid.

Table 2**:** *The composition of eluents, wavelengths and retention times that were used in HPLC analyses of salicylic acid and derivates*

Compound	Acetonitrile (%)	0.1 % TFA pH 2.1 (%)	Wavelength (nm)	Retention time (min)
Salicylic acid	50	50	240	2.9
Gentisic acid	30	70	240	2.9
5-OH-isophthalic acid	20	80	220	2.9

6.8. Data Analysis

Cumulatively transported amount (nmol) was calculated and the cumulative amount over time was plotted. When transported amount did not get over 10 % of starting amount (sink conditions) the flux rate was evaluated from the slope of the initial linear portion of plots of the amount transported against the time, calculated by linear

regression analysis. Apparent permeability coefficient Papp (cm · s^{-1}) was also calculated.

$$Papp = \frac{dQ}{dt} \times \frac{1}{AC_0} \qquad \text{Eq.3}$$

where

dQ/dt = permeability rate
C_0 = the initial concentration in the donor compartment
A = surface area of the monolayer

Also the substrate recovery (mass balance) was checked, there was added cumulatively transported amount to the amount left in the donor side at the end of the experiment and this value was compared to the initial amount in the donor side.

All data were gained from HPLC analysis using calibration curve of each compound measured in advance. Calibration curves with goodness of fit at least R^2=0.999 was used.

Michaelis-Menten constant was calculated for salicylic acid.

$$v = \frac{V_{\max} \times [S]}{K_m + [S]} + k_d[S] \qquad \text{Eq.4}$$

where

V_{max} = maximum velocity of the reaction
K_m = Michaelis-Menten constant
v = reaction velocity
[S] = substrate concentration
k_d = non-saturable permeation rate.

7. Results and Discussion

My goal was to study mechanism of transport of salicylic acid and its derivates across Caco-2 cell monolayers in order to find out transport mechanism of salicylic acid using absorption enhancers and to compare the influence of interaction of salicylic acid and similar compounds, which means possible competitive inhibition of this transport.

7.1. Salicylic Acid Transport

All experiments that examined transportation of salicylic acid across Caco-2 cell monolayers have been performed at 37°C. The goal of this part of experiments was to investigate type of transport which takes part in salicylic acid transportation. All experiments were made as triplicates. The structure of experiments was following:

Table 3: *Salicylic acid experiment review; concentration means salicylic acid initiatory concentration in mmol/l, direction might be apical-basolateral (a-b) or basolateral-apical (b-a), pH means pH apical/basolateral and SI means sampling interval in minutes.*

No.	concentration	direction	pH	SI	cell passage
1	0.048	a-b	5.5/7.4	2	37
2	0.25	a-b	5.5/7.4	2	32
3	0.25	a-b	7.4/7.4	2	32
4	0.25	b-a	5.5/7.4	2	32
5	0.25	b-a	7.4/7.4	2	32
6	2.5	a-b	5.5/7.4	2	39
7	5.0	a-b	5.5/7.4	2	39
8	15.0	a-b	5.5/7.4	2	39
9	40.0	a-b	5.5/7.4	2	39
10	0.25	a-b	6.5/6.5	4	40
11	5.0	a-b	6.5/6.5	4	40
12	15.0	a-b	6.5/6.5	4	40
13	40.0	a-b	6.5/6.5	4	40

In the very first part salicylic acid transportation at 0.25 mmol/l was examined and results were compared to experiments made in apical-basolateral direction and vice versa. Results of experiments performed at iso-pH conditions (pH 7.4 both on apical and basolateral side) and with pH-gradient (pH 5.5 on apical side and pH 7.4 on baso-lateral side were also compared. Results are shown in following table and figure.

Table 4: *Results of transport experiments No. 2-5; avg. slope means average slope (flux), Papp means apparent permeability coefficient (cm · s^{-1}) x 10^{-6}, rel. SD means standard deviation in % (n=3).*

No.	Direction	pH (a/b)	Avg. Slope	rel. SD	Papp	rel. SD
2	a-b	5.5/7.4	2.4385	12.73	175.0	12.82
3	a-b	7.4/7.4	0.2323	3.68	12.2	3.41
4	b-a	5.5/7.4	0.0945	1.95	4.54	1.95
5	b-a	7.4/7.4	0.4073	3.16	19.6	3.16

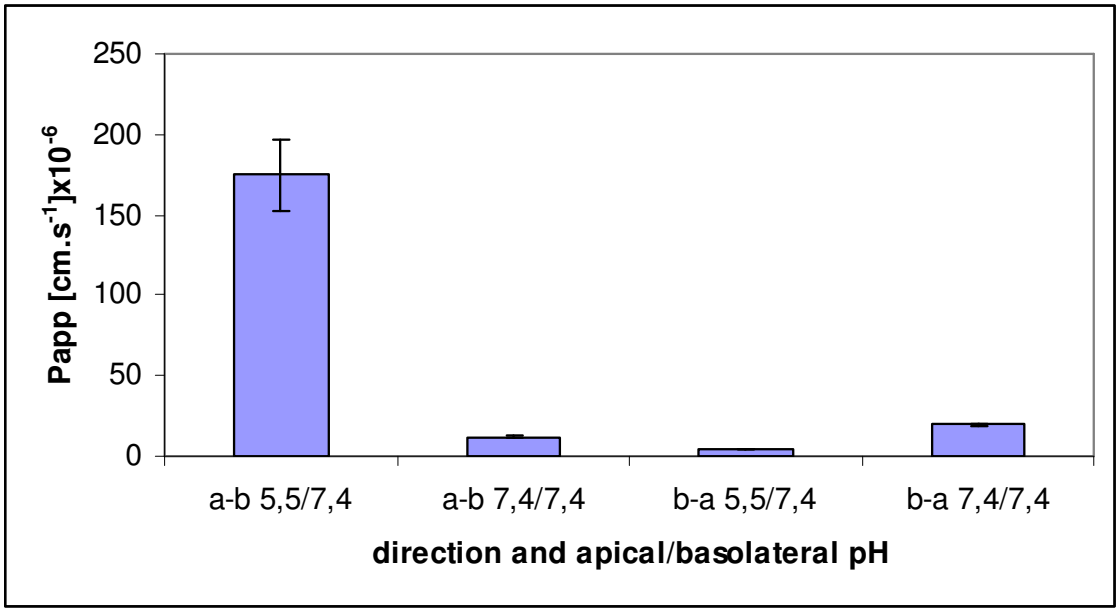


Figure 6*: The apparent permeability coefficients (Papp) across Caco-2 cell monolayers of salicylic acid (0.25 mmol/l) were almost equal on both transport directions, using buffer pH of 7.4 on both sides. In the presence of the pH gradient (apical pH 5.5; baso-lateral 7.4), a-b transport exceeded the transport in the b-a direction. Each bar indi-cates mean ± SD (n=3).*

It is obvious from shown data that at iso-pH condition Papp of transports both a-b and b-a are practically the same. In pH gradient condition are results completely different from each other. In b-a experiment is transportation rate much lower compare both to a-b pH gradient experiment and b-a iso-pH experiment. This might be caused by possible lower expression of transporter proteins on basolateral side of the cell membrane. The experiment with a-b direction in pH gradient conditions shows considerable increase compare to a-b direction experiment in iso-pH condition. The reason might be lower fraction of ionization of salicylic acid at pH 5.5 and by H^+ dependent co-transport by transporter proteins.

In the second part of this experiment set repetitions of some experiments with salicylic acid in a-b direction and in pH gradient conditions (5.5 apical and 7.4 basolateral) were performed and an idea of concentration dependency of this transport was made. Experiments were preformed with salicylic acid concentrations (mmol/l) 2.5; 5.0; 15.0 and 40.0. To help form this notion data acquired from earlier experiments was also used and added to the table transport rate with concentrations of salicylic acid (mmol/l) 0.048 and 0.25.

Table 5: *Results of transport experiments No. 1,2,6-9. All experiments were performed in a-b direction with pH gradient (apical pH 5.5; basolateral 7.4); conc. means planned salicylic acid concentration (mmol/l), real conc. means start sample concentration (mmol/l), avg. slope means average slope (flux), Papp means apparent permeability coefficient ($cm \cdot s^{-1}$) x 10^{-6}, rel. SD means standard deviation in % (n=3).*

No.	conc.	real conc.	passage	Avg. Slope	rel. SD	Papp	rel. SD
1	0.048	0.044	37	0.55	4.82	189.08	4.99
2	0.25	0.21	32	2.44	12.73	174.76	12.82
6	2.5	2.34	39	19.98	9.97	130.12	13.74
7	5.0	4.59	39	29.52	6.07	97.47	5.30
8	15.0	13.81	39	44.55	11.77	48.90	12.67
9	40.0	35.42	39	63.73	3.17	27.26	3.77

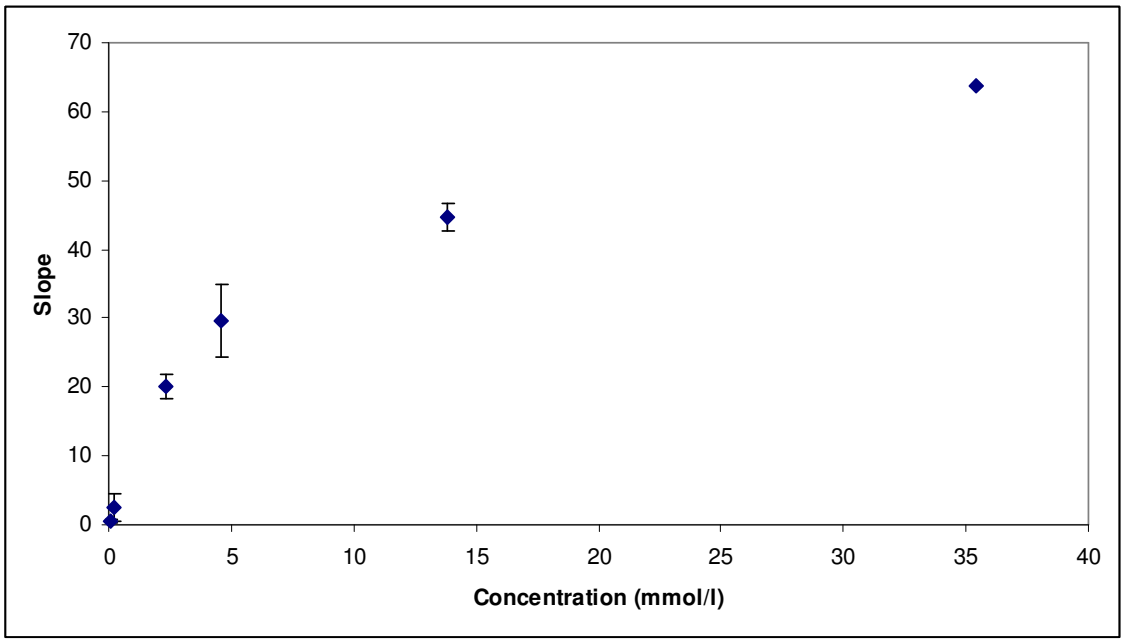


Figure 7*: Slope values dependency on concentration of salicylic acid in a-b direction, apical pH 5.5; basolateral pH 7.4. Slope values are growing non-linearly with increasing salicylic acid concentration. Each point indicates mean ± SD.*

We can see clear concentration dependence of salicylic acid transportation. With increasing concentration of salicylic acid the permeation of Caco-2 cells was down by 85 % (Table 5).

The calculated pH dependent Michaelis-Menten constant was K_m = 3.15 mmol/l. (apical pH 5.5; basolateral pH 7.4), which is reasonably lower than earlier published K_m calculated at apical pH 5.0; basolateral pH 7.4; these values were 5.28 ± 0.72 mmol/l (Takanaga *et al.* 1994) and 5.4 ± 0.7 mmol/l (Neuhoff *et al.* 2005).

From Figure 7 it is possible to see non-linear dependence of slope values on time. Compared to drugs transported strictly by passive diffusion (e.g. antipyrin) where this dependence is linear we might say that salicylic acid is transported across Caco-2 cell monolayers by both passive and active transport.

Next part of my experiment dealt with investigation of apical-to-basolateral transport in iso-pH conditions (apical pH 6.5; basolateral pH 6.5). Experiments were performed with four salicylic acid concentrations: 0.25; 5.0; 15.0 and 40 mmol/l.

Table 6: *Results of transport experiments No. 10-13. All experiments were performed in a-b direction with iso-pH conditions (apical pH 6.5; basolateral 6.5); conc. means planned salicylic acid concentration (mmol/l), real conc. means start sample concentration (mmol/l), avg. slope means average slope (flux), Papp means apparent permeability coefficient (cm · s^{-1}) x 10^{-6}, rel. SD means standard deviation in % (n=3).*

No.	conc.	real conc.	passage	Avg. Slope	rel. SD	Papp	rel. SD
10	0.25	0.23	40	0.79	4.36	52.32	4.23
11	5.0	4.44	40	9.38	7.47	32.00	9.58
12	15.0	13.26	40	18.48	8.67	21.11	8.85
13	40.0	35.57	40	22.84	1.02	9.73	0.86

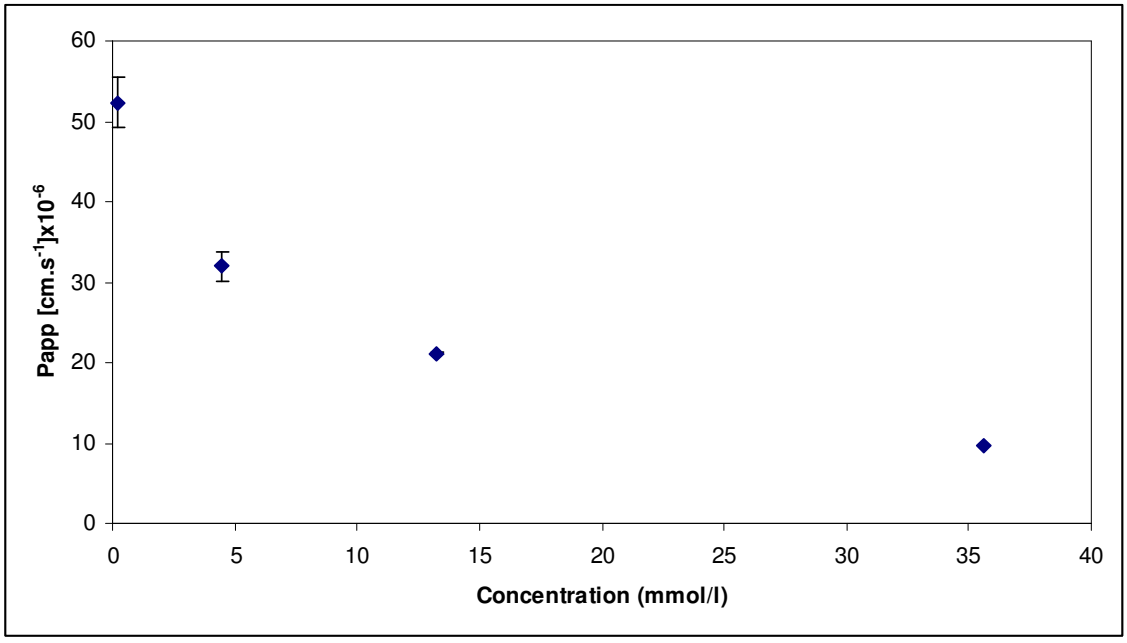


Figure 8*: Papp values dependency on concentration of salicylic acid in a-b direction, apical pH 6.5; basolateral pH 6.5. The apparent permeability coefficients (Papp) across Caco-2 cell monolayers of salicylic acid are decreasing with increasing concentration of salicylic acid. Each point indicates mean ± SD.*

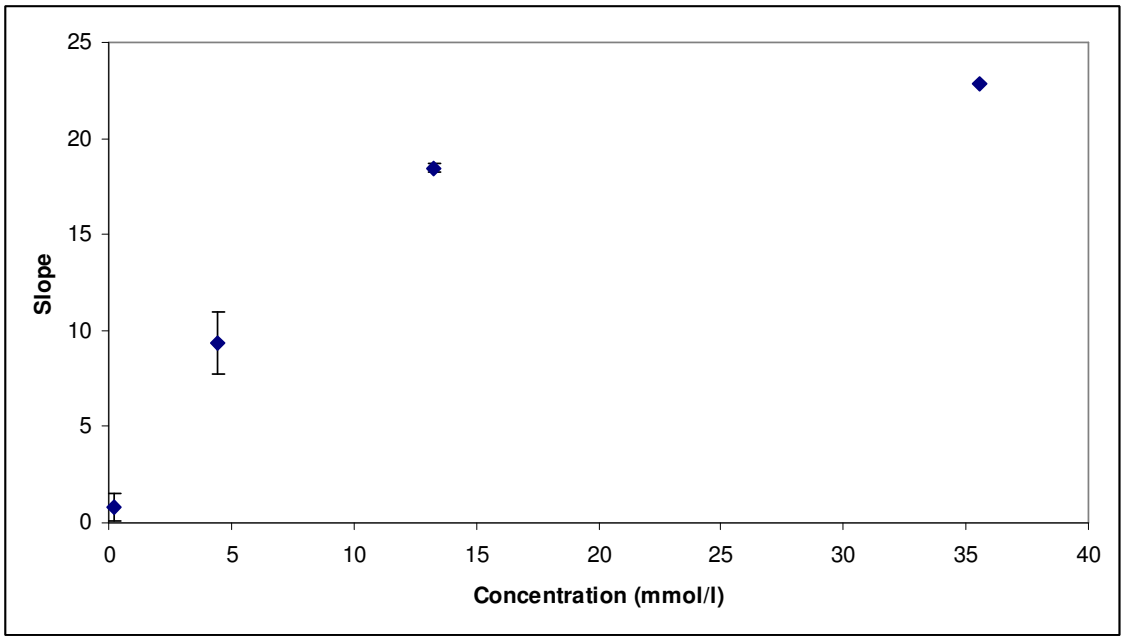


***Figure 9**: Slope values dependency on concentration of salicylic acid in a-b direction, apical pH 6.5; basolateral pH 6.5. Slope values are growing non-linearly with increasing salicylic acid concentration. Each point indicates mean ± SD.*

With iso-pH 6.5 condition it is possible to observe the same tendency as in previous experiments. With increasing concentration of salicylic acid decreases Papp values and increases slope values. Even though pH is even on both sides of Caco-2 cell monolayer, there are still two partial pH gradient systems, apical side pH 6.5/monolayer pH 7.4 and monolayer pH 7.4/basolateral side pH 6.5. This is probably the reason of this course of transport as opposite to apical pH 7.4/basolateral pH 7.4 conditions where was not noticed any dependence of Papp on concentration levels (Neuhoff *et al.* 2005).

Anyway, both slope and Papp values are in general lower in these conditions than with pH gradient conditions what is probably caused by higher ionization of salicylic acid molecule at pH 6.5 and by decrease of H^+ dependent carrier mediated co-transport of salicylic acid.

7.2. Gentisic Acid Transport

This set of experiments was made for investigate properties of transport across Caco-2 cell monolayers of derivates of salicylic acid and prove that it has similar properties as the transport of salicylic acid. Experiments were made with eight concentrations (mmol/l) 2.5; 5.0; 8.0; 10.0; 15.0; 20.0; 30.0 and 40.0. All samples from gentisic acid experiment were analyzed immediately after the run, because of it's relatively instability. During experiment, samples were protected from light by smoked glass walls of the incubator. All experiments were done in pH gradient conditions (apical pH 5.5; basolateral pH 7.4) in a-b direction, sampling interval was 30 minutes.

Table 7: *Results of transport experiments with gentisic acid. All experiments were performed in a-b direction with pH gradient (apical pH 5.5; basolateral 7.4), sampling interval used to be 30 minutes; conc. means planned gentisic acid concentration (mmol/l), real conc. means start sample concentration (mmol/l), avg. slope means average slope (flux), Papp means apparent permeability coefficient (cm · s^{-1}) x 10^{-6}, rel. SD means standard deviation in % (n=3).*

conc.	real conc.	passage	Avg. Slope	rel. SD	Papp	rel. SD
2.5	2.17	36	0.43	3.51	2.97	4.02
5.0	4.28	36	0.86	2.29	3.04	1.01
8.0	6.68	39	1.90	2.09	4.30	2.11
10.0	8.63	37	2.16	2.31	3.80	6.84
15.0	13.06	36	2.04	4.52	2.37	4.12
20.0	16.71	39	3.98	2.10	3.61	4.56
30.0	25.66	36	3.00	3.62	1.77	3.86
40.0	32.48	37	4.97	7.66	2.32	6.00

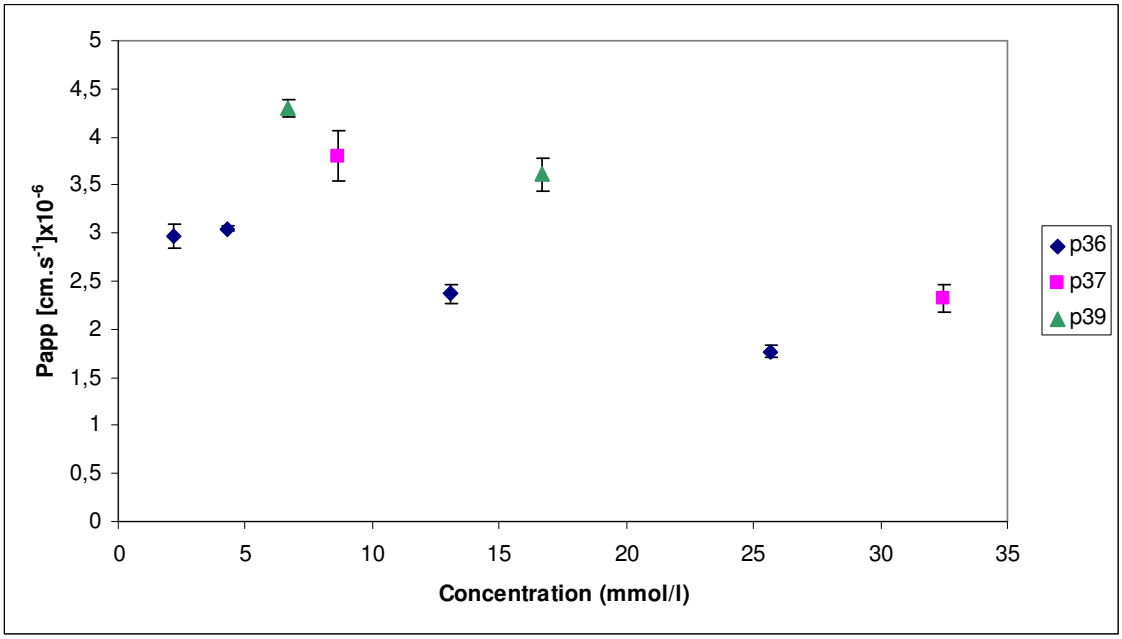


***Figure 10**: Papp values dependency on concentration of gentisic acid in a-b direction, apical pH 5.5; basolateral pH 7.4. The apparent permeability coefficients (Papp) across Caco-2 cell monolayers of gentisic acid do not show any significant trend with increasing concentration of gentisic acid. Each point indicates mean ± SD.*

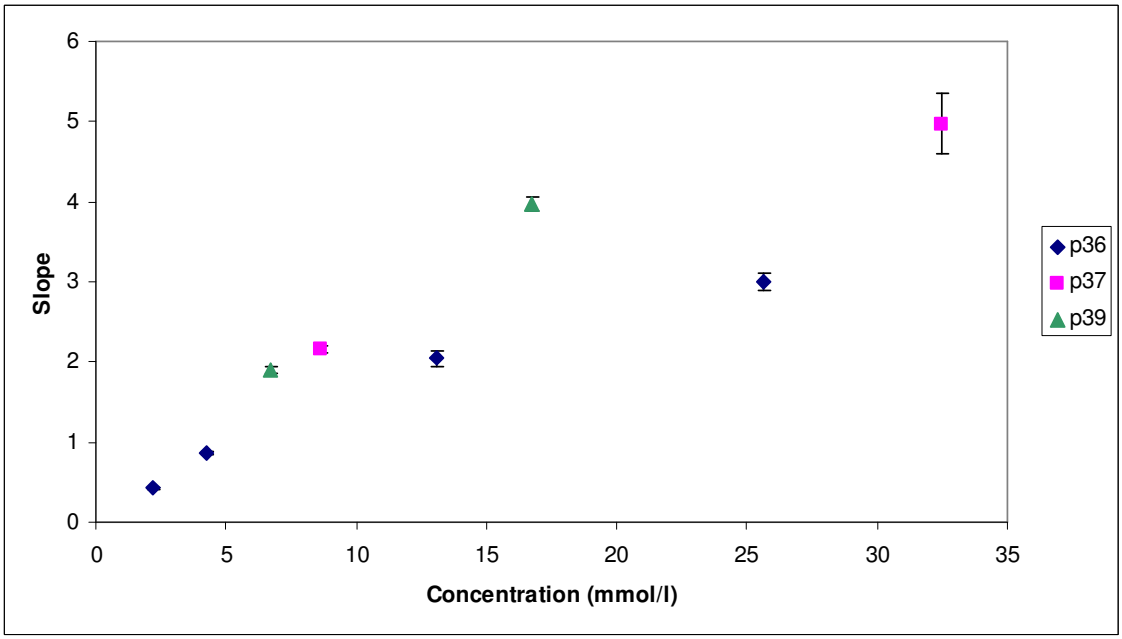


***Figure 11**: Slope values dependency on concentration of gentisic acid in a-b direction, apical pH 5.5; basolateral pH 7.4. Slope values are growing non-linearly with increasing gentisic acid concentration. The tendency is not regular; it's possible to observe dependency on passage number. Each point indicates mean ± SD.*

With gentisic acid the tendency of transport rate is not as clear as it was with salicylic acid. It is not possible to see any common trend neither from slope and Papp graph. What is certain is that both slopes and Papp values are significantly lower. The reason is probably lower LogP of gentisic acid compare to salicylic acid; it means that passive transport of gentisic acid is not so fast.

From the results obtained it is not possible to see clearly, whether gentisic acid is the substrate for active transport across Caco-2 cell monolayers. The slope of this experiment is definitely not linear, but I didn't measure enough of samples to consider it. With the high concentration of gentisic acid it is possible to observe significant increase of Papp values, which is probably caused by toxic influence to cells.

7.3. 5-hydroxyisophthalic Acid Transport

5-hydroxysophthalic acid is transported even slower than gentisic acid. The goal of this part was to extend general notion about transport rate of salicylic acid derivates. Experiments were performed in a-b direction, in pH gradient conditions (apical pH 5.5; basolateral pH 7.4). Because of doubts about results of first part of this experiment set, in the second part two experiments were repeated; experiments were performed with concentrations (mmol/l) 2.5; 5.0; 10.0; 15.0; 20.0 and 30.0. Sampling interval was 30 minutes.

Table 8: *Results of transport experiments with 5-hydroxyisophthalic acid. All experiments were performed in a-b direction with pH gradient (apical pH 5.5; basolateral 7.4), sampling interval used to be 30 minutes; conc. means planned gentisic acid concentration (mmol/l), real conc. means start sample concentration (mmol/l), avg. slope means average slope (flux), Papp means apparent permeability coefficient (cm · s^{-1}) x 10^{-6}, rel. SD means standard deviation in % (n=3).*

conc.	real conc.	passage	Avg. Slope	rel. SD	Papp	rel. SD
2.5	2.14	36	0.028	16.35	0.201	15.91
5.0	4.25	36	0.068	6.87	0.242	6.31
10.0 (n = 2)	9.70	39	0.087	6.04	0.136	7.42
15.0	13.24	36	0.110	4.57	0.126	4.32
15.0	14.49	37	0.104	11.66	0.108	12.14
20.0	19.91	39	0.268	2.88	0.204	5.59
30.0	25.14	36	0.434	23.48	0.261	22.81
30.0	28.78	37	0.400	3.76	0.211	5.58

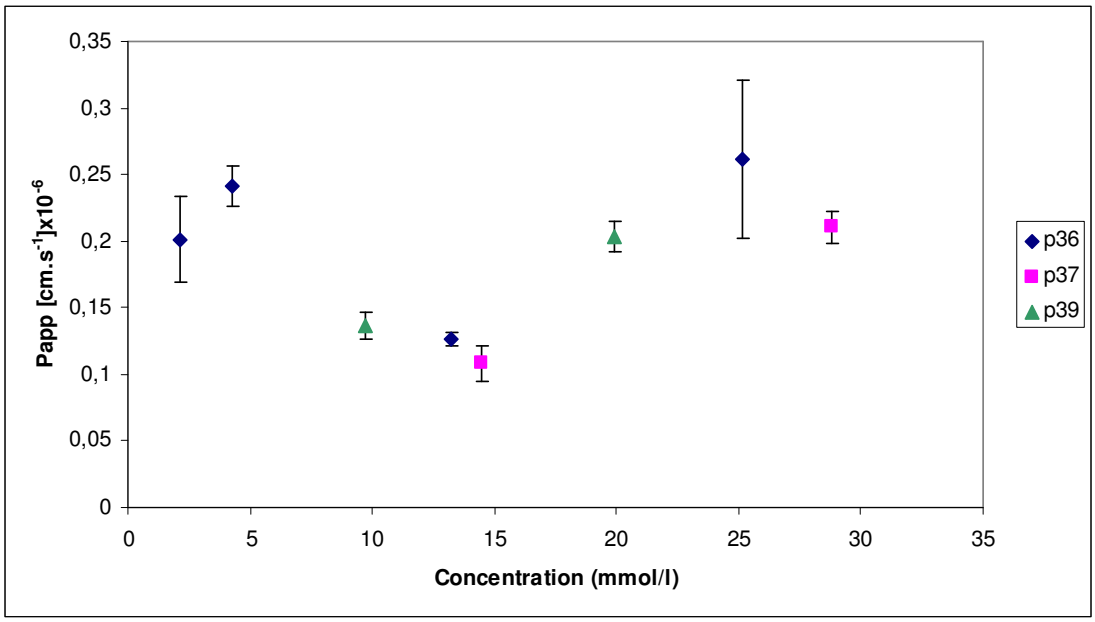


***Figure 12**: Papp values dependency on concentration of 5-hydroxyisophthalic acid in a-b direction, apical pH 5.5; basolateral pH 7.4. Papp across Caco-2 cell monolayers of 5-hydroxyisophthalic acid do not show any significant trend with increasing concentration of 5-hydroxyisophthalic acid. Each point indicates mean ± SD.*

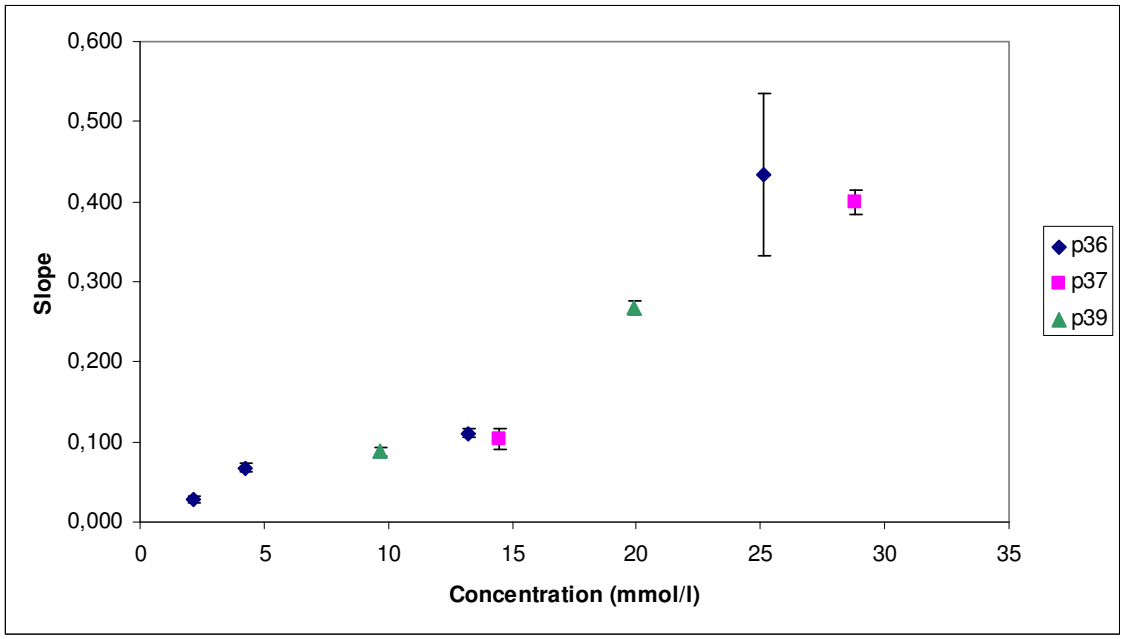


***Figure 13**: Slope values dependency on concentration of 5-hydroxyisophthalic acid in a-b direction, apical pH 5.5; basolateral pH 7.4. Slope values are growing non-linearly with increasing 5-hydroxyisophthalic acid concentration. The tendency is not regular; it is possible to observe dependency on passage number. Each point indicates mean ± SD.*

5-hydroxyisophthalic has the lowest transport rate compared to other examined drugs. The reason might be in lower LogP of 5-hydroxyisophthalic compared to salicylic acid and gentisic acid; 5-hydroxyisophthalic acid is not good compound for passive transport across Caco-2 cell monolayers. The other reason is 5-hydroxyisophthalic is not substrate for monocarboxylic acid transporters, because it is dicarboxylic acid. The non-linear shape of the slope curve implies that 5-hydroxyisophthalic acid is a substrate for active transport; however, the concentration range is not so wide. With higher concentration of the substrate it is possible to observe significant increase of Papp values, which is probably caused by toxic influence of higher concentrations of 5-hydroxyisophthalic acid to Caco-2 cells.

7.4. The Influence of Absorption Enhancers

Absorption enhancer is a substance which opens tight junctions of Caco-2 cell monolayers. This was used to investigate how significant is the effect of enhancing paracellular pathway on salicylic acid transport. To analyze this, some other compound were used. Antipyrin was used as a compound transported passively transcellulary and ^{14}C-mannitol was used as a paracellular marker.

In the role of absorption enhancer was used ethylenediaminetetraacetic acid (EDTA), in the first part just on apical side of monolayer, in the second part on both sides. The concentration of EDTA in both cases was 2.0 mmol/l. All monolayers were pre-incubated for 30 minutes with EDTA (2.0 mmol/l) solution.

Even thought some damage of the integrity of some monolayers during the b-a direction experiment was observed, the results have been published, because the trend of enhancing effect is evident.

Table 9: *Review of Papp values of antipyrin across Caco-2 cell monolayer. The sampling interval was 15 minutes; Papp means apparent permeability coefficient ($cm \cdot s^{-1}$) x 10^{-6}, rel. SD means standard deviation in % (n=3), w/o means without*
Iso-pH conditions (apical pH 7.4; basolateral pH 7.4), without EDTA or EDTA only on apical side

	a-b w/o EDTA	a-b with EDTA	b-a w/o EDTA	b-a with EDTA
Papp	52.70	46.90	56.10	56.60
rel. SD	11.54	10.54	1.71	3.78

Iso-pH or pH gradient (apical pH 5.5; basolateral pH 7.4), EDTA on both sides

	a-b pH-grad	a-b pH 7.4	b-a pH-grad	b-a pH 7.4
Papp	86.80	66.10	69.20	68.50
rel. SD	n=2	5.36	8.13	14.84

Table 10: *Expression of EDTA influence on antipyrin transport; juxtaposition of Papp values with EDTA on both sides and without EDTA (Papp with EDTA / Papp w/o EDTA). Iso-pH conditions (apical pH 7.4; basolateral pH 7.4); Papp means apparent permeability coefficient (cm · s^{-1}) x 10^{-6}*

	Papp with EDTA	**Papp w/o EDTA**	**quotient**
apical-to-basolateral	66.1	52.7	1.25
basolateral-to-apical	68.5	56.1	1.22

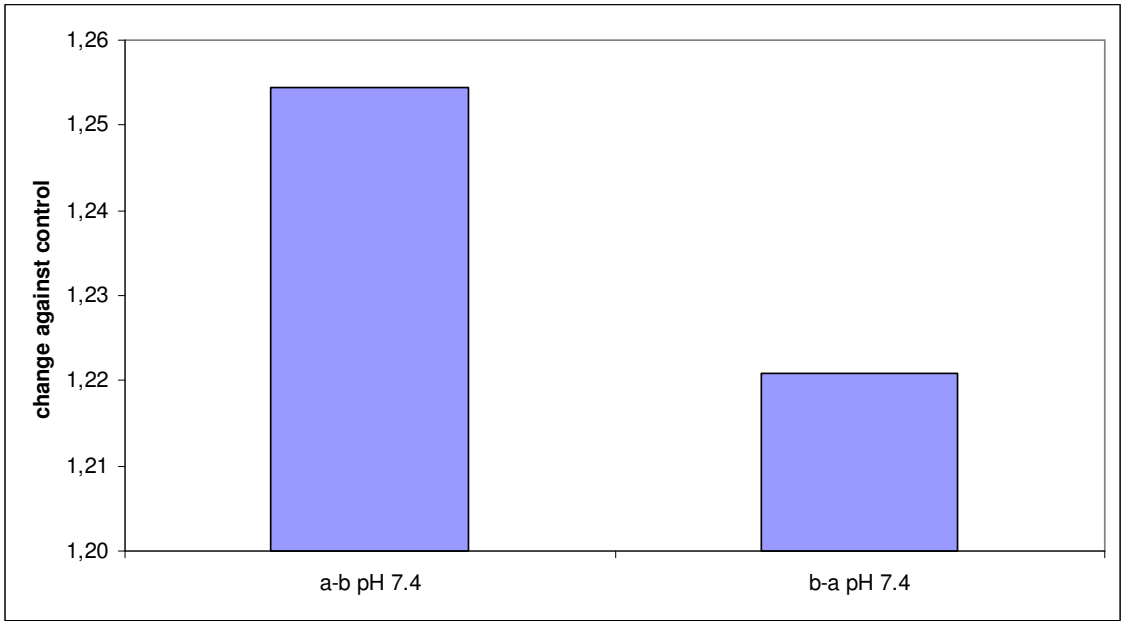


Figure 14: *Influence of EDTA (both sides) on antipyrin transport across Caco-2 cell monolayer. Change against the control is expressed by quotient of Papp values with and without EDTA*

Table 11: *Review of Papp values of ^{14}C-Mannitol across Caco-2 cell monolayer. The sampling interval was 15 minutes; Papp means apparent permeability coefficient (cm · s^{-1}) x 10^{-6}, rel. SD means standard deviation in % (n=3), w/o means without*
Iso-pH conditions (apical pH 7.4; basolateral pH 7.4), without EDTA or EDTA only on apical side

	a-b w/o EDTA	**a-b with EDTA**	**b-a w/o EDTA**	**b-a with EDTA**
Papp	0.543	0.538	0.457	0.520
rel. SD	4.07	5.79	2.80	4.92

Iso-pH or pH gradient (apical pH 5.5; basolateral pH 7.4), EDTA on both sides

	a-b pH-grad	a-b pH 7.4	b-a pH-grad	b-a pH 7.4
Papp	8.59	42.2	11.4	34.4
rel. SD	n=2	8.53	6.12	15.30

Table 12: *Expression of EDTA influence on ^{14}C-Mannitol transport; juxtaposition of Papp values with EDTA on both sides and without EDTA (Papp with EDTA / Papp w/o EDTA). Iso-pH conditions (apical pH 7.4; basolateral pH 7.4); Papp means apparent permeability coefficient (cm · s^{-1}) x 10^{-6}*

	Papp with EDTA	Papp w/o EDTA	quotient
apical-to-basolateral	42.2	0.54	77.7
basolateral-to-apical	34.4	0.46	75.4

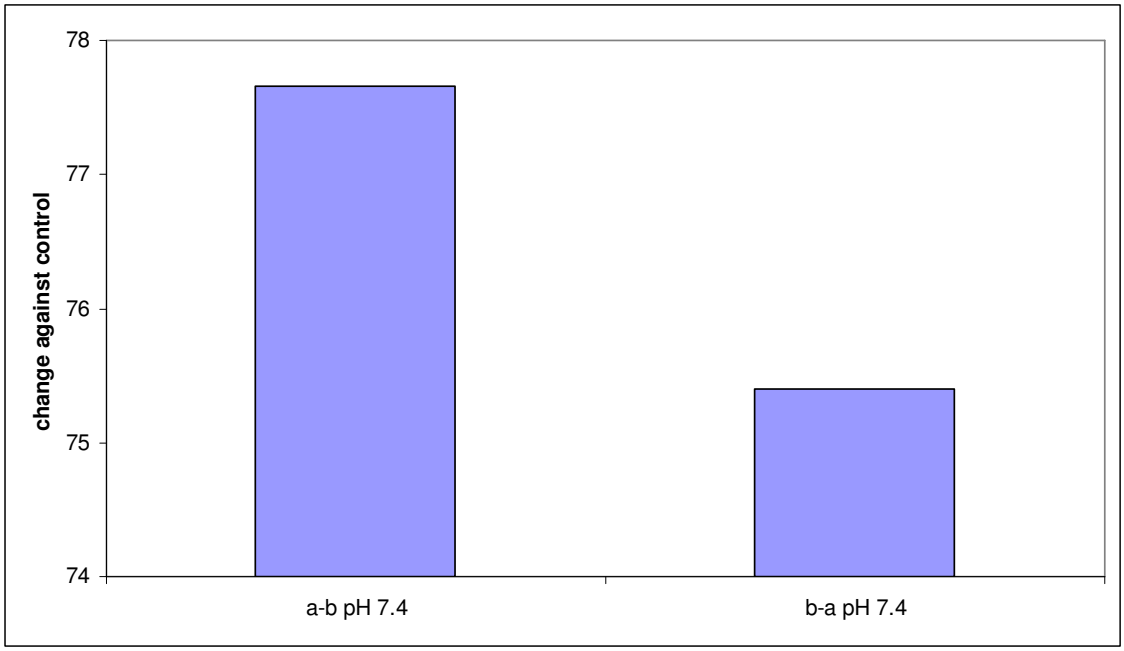


Figure 15: *Influence of EDTA (both sides) on ^{14}C-Mannitol transport across Caco-2 cell monolayer. Change against the control is expressed by quotient of Papp values with and without EDTA*

It is obvious from both experiments that the influence of absorption enhancers (EDTA in this case) is not equal and it differs depending on type of molecule.

Firstly, Papp values of antipyrin were measured. Antipyrin is a compound, which is transported across Caco-2 cell monolayers transcellulary and strictly passively what means that no protein transporters are involved.

It showed up that Papp values of antipyrin are the same both in apical-to-basolateral and basolateral-to-apical direction. EDTA just on apical side does not have significant influence on antipyrin Papp values. Using EDTA on both sides changed measured values and the transport rate was increased. It seems that EDTA effect is larger in apical-to-basolateral direction. However, these changes are not considerable.

Secondly, Papp values of ^{14}C-Mannitol were measured. Labeled mannitol is used as a indicator of Caco-2 cell monolayers integrity and its Papp values should not exceed 0.5%. As the results prove, measured values are around this value. Again, effect of EDTA on apical side only was irrelevant.

Using EDTA on both sides of monolayer it is possible to see considerable increase of Papp values, with EDTA these are about seventy times higher then in the control sample. This has been expected, since ^{14}C-Mannitol is transported by spaces in between cells and any change of permeability or composition of tight junctions should have huge effect on the paracellular transport.

With salicylic acid was examined influence of EDTA (2.0 mmol/l) again on apical side only and on both sides of monolayer, all monolayers were pre-incubated with EDTA solution, concentration 2.0 mmol/l. Results were matched with control Papp values of samples without EDTA. The salicylic acid concentration was 0.25 mmol/l.

Table 13: *Review of Papp values of salicylic acid across Caco-2 cell monolayer. The sampling interval was 2 minutes; Papp means apparent permeability coefficient* $(cm \cdot s^{-1}) \times 10^{-6}$*, rel. SD means standard deviation in % (n=3).*

	a-b pH grad	**a-b iso-pH**	**b-a pH grad**	**b-a iso-pH**
Papp	174.76	12.18	4.54	19.57
rel. SD	12.82	3.41	1.95	3.16

Review of Papp values of salicylic acid across Caco-2 cell monolayer, influence of EDTA on apical side of monolayer only. Sampling interval was 2 minutes for pH gradient (apical pH 5.5; basolateral pH 7.4) and 5 minutes for iso-pH condition (apical pH 7.4; basolateral pH 7.4);

	a-b pH grad	a-b iso-pH	b-a pH grad	b-a iso-pH
Papp	197.80	16.32	7.10	22.09
rel. SD	4.62	12.40	4.73	5.95

Iso-pH or pH gradient (apical pH 5.5; basolateral pH 7.4), EDTA on both sides. Sampling interval was 2 minutes for pH gradient and 5 minutes for iso-pH.

	a-b pH-grad	a-b pH 7.4	b-a pH-grad	b-a pH 7.4
Papp	157.01	52.27	9.24	51.57
rel. SD	n=2	3.61	16.08	9.08

Table 14: *Expression of EDTA influence on salicylic acid transport; juxtaposition of Papp values with EDTA on both sides and without EDTA (Papp with EDTA / Papp w/o EDTA). Iso-pH and pH gradient conditions; Papp means apparent permeability coefficient ($cm \cdot s^{-1}$) x 10^{-6}*

	Papp with EDTA	Papp w/o EDTA	quotient
a-b pH grad	157.01	174.76	0.90
a-b iso-pH	52.27	12.18	4.29
b-a pH grad	9.24	4.54	2.04
b-a iso pH	51.57	19.57	2.64

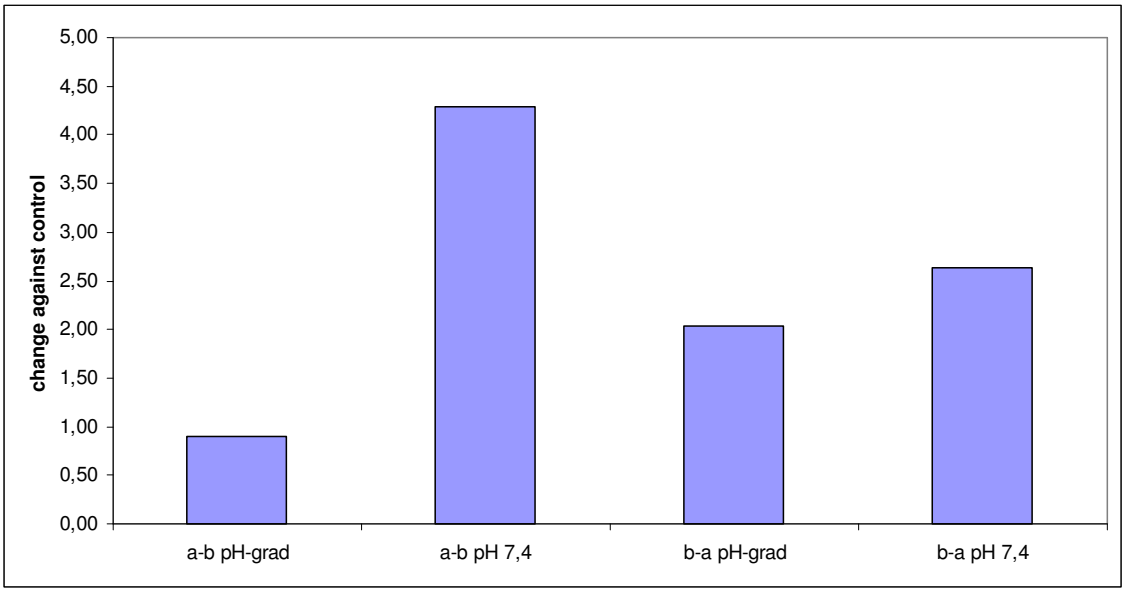


Figure 16: *Influence of EDTA (both sides) on salicylic acid transport across Caco-2 cell monolayer. Change against the control is expressed by quotient of Papp values with and without EDTA*

From data obtained from salicylic acid experiment follows that using EDTA just on apical side slightly raises Papp values. It is unexpected and might be caused by irritation of cells by effect of EDTA. Due to this effect tight junctions are not so sensitive to the enhancing effect of EDTA. However, this increase is not significant.

Using EDTA on both sides brings mixed results. Papp values of a-b transport under pH gradient are lower than with control sample. On the other hand, the rest of values show increase.

Decrease of Papp values in a-b direction under pH gradient might be caused by buffering ability of EDTA, increasing pH and inhibition of H^+ dependent co-transport. Slight increase in the rest of performed experiments is probably caused by opening tight junctions.

In general it is possible to observe that EDTA effect on salicylic acid transport is not remarkable what suggests high representation of transcellular transport (whether passive or active) compared to paracellular transport.

7.5. Inhibition Studies

The goal of this part of the experiment was to examine the influence of compounds with structure similar to salicylic acid. The possibility of inhibiting transporter proteins directly was also examined. All inhibition experiments were performed with cell passage p37 under pH gradient (apical pH 5.5; basolateral pH 7.4). The sampling interval was 2 minutes. In one case dimethylsulfoxide (DMSO) was used and one control sample containing salicylic acid and DMSO was measured.

Table 15: *Review of inhibition transport experiments. Concentration of salicylic acid is 0.048 mmol/l; SA is salicylic acid, DMSO is dimethylsulfoxide, CHC is α-Cyano-4-hydroxycinnamic acid, 3-combination means 2 mmol/l probenecid + 5 mmol/l pravastatin + 1 mmol/l CHC. Papp means apparent permeability coefficient (cm · s^{-1}) x 10^{-6}, rel. SD means standard deviation in % (n=3).*

	Papp	**rel. SD**	**% of control**
SA+DMSO (0.16%)	219.81	25.22	116.26
0.048 mmol/l SA	**189.08**	**9.44**	**100.00**
SA + 10 mmol/l 5-hydroxyisophthalic acid	159.07	9.09	84.13
SA + 1 mmol/l CHC	146.04	21.02	77.24
SA + 2 mmol/l probenecid	143.63	3.95	75.96
SA + 2 mmol/l probenecid on both sides	127.22	12.48	67.28
SA + 3-combination	102.57	6.90	55.36
SA + 10 mmol/l 2-hydroxy-3-isopropylbenzoic acid + DMSO 0.16%	78.69	12.40	41.62

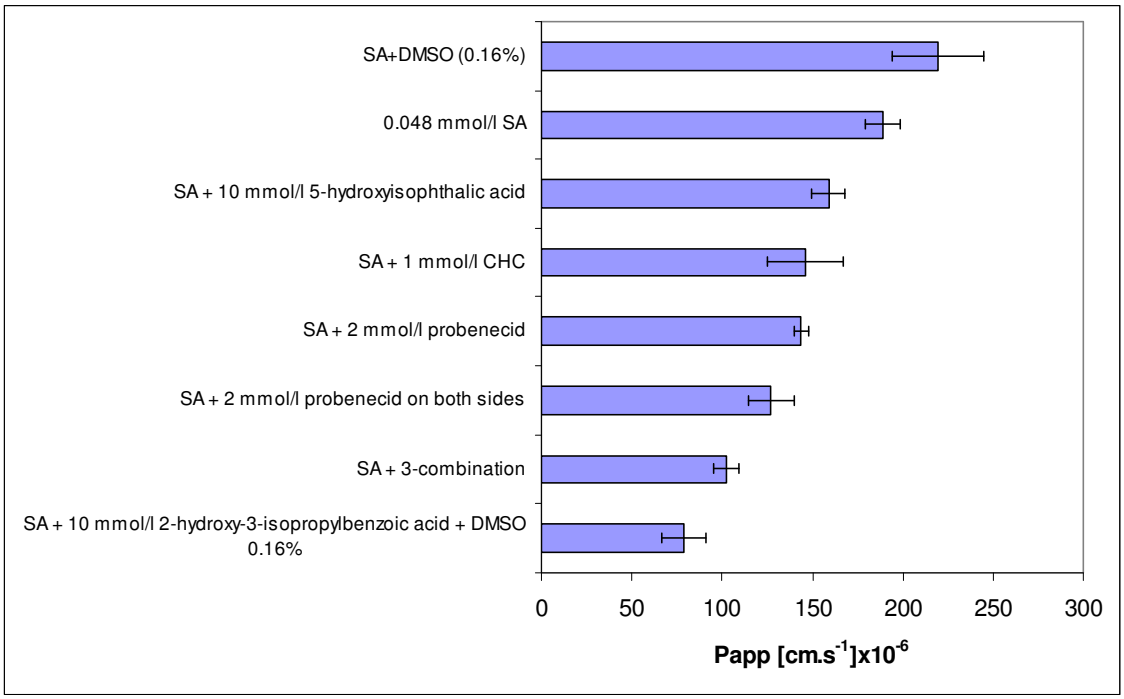


Figure 17: *The influence of inhibitors on transport of salicylic acid across Caco-2 cell monolayers. SA is salicylic acid. Each bar indicates mean ± SD (n=3).*

Benzoic acid, pravastatin and 2-hydroxy-3-isopropylbenzoic acid are substrates for active transport across Caco-2 cell monolayers (benzoic acid both for OATP and MCT (Tsuji *et al.* 1994), 2-hydroxy-3-isopropylbenzoic acid as well and pravastatin is substrate for OATP (Nozawa *et al.* 2004). CHC is MCT inhibitor (Deuticke 1982) and probenecid is OATP inhibitor (Yasui-Furukori *et al.* 2005). The participation of 5-hydroxyisophthalic acid on active transport is discussed in chapter 7.3. With combination of OATP inhibitor, MCT inhibitor and OATP substrate we obtained additive inhibitive effect compared to single studies, what implies that both OATP and MCT are involved in salicylic acid transport.

The difference between inhibition influences of each compound is connected to their physico-chemical properties and affinity to OATP and MCT. It is possible to see that the most visible inhibitive effect was caused by 2-hydroxy-3-isopropylbenzoic acid. It is also possible to observe the difference between effects of probenecid present only on apical side and on both sides.

8. Conclusions

This thesis investigated transport properties of salicylic acid and its derivates across Caco-2 cells monolayers. It has been obtained same results as published previously; salicylic acid is transported both by passive transcellular and active transcellular transport. The K_m of salicylic acid transport was calculated under pH gradient conditions (apical pH 5.5; basolateral pH 7.4), K_m = 3.15 mmol/l. Salicylic acid transport was not affected by opening tight-junctions by EDTA; however, this opening was clearly visible on radio-labeled mannitol. It was possible to see inhibitive effect of substrates of OATPs and MCTs on salicylic acid transport rate. The biggest inhibitive effect from compounds used shown 2-hydroxy-3-isopropylbenzoic acid.

Gentisic acid and 5-hydroxyisophthalic acid are both transported similarly to salicylic acid – by passive and active transport, nevertheless, results are not so clear and further studies are required. Plots obtained from these experiments shown significant increase with higher concentrations of substrates what can be caused by toxic effect on Caco-2 cells.

Abbreviations

ABL – aqueous boundary layer

ATP – adenosine triphosphate

Caco-2 – adenocarcinoma cell line derived from human colonic epithelia

DMEM – Dulbecco‘s Modified Eagle Medium

DMSO – dimethyl sulphoxide

EDTA – ethylenediaminetetraacetic acid

HBSS – Hank’s Balanced Salt Solution

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIFBS – heat-inactivated fetal bovine serum

HPLC – high-performance liquid chromatography

MCT – monocarboxylic transporter

MDCK – Madin-Darby canine kidney cells

MES – 2-(N-Morpholino)ethanesulfonic acid

NEAA – non-essential amino acids

OAT – organic anion transporter

OATP – organic anion transporting polypeptide

Papp – apparent permeability coefficient

PBS – phosphate-buffered saline solution

PEST – penicillin and streptomycin

P-gp – P-glycoprotein

TEER – transepithelial electrical resistence

TFA – trifluoroacetic acid

References

Adibi, S. A. (1997). "The oligopeptide transporter (Pept-1) in human intestine: biology and function." *Gastroenterology* **113**(1): 332-40.

Adson, A., Burton, P. S., Raub, T. J., Barsuhn, C. L., Audus, K. L. and Ho, N. F. (1995). "Passive diffusion of weak organic electrolytes across Caco-2 cell monolayers: uncoupling the contributions of hydrodynamic, transcellular, and paracellular barriers." *J Pharm Sci* **84**(10): 1197-204.

Adson, A., Raub, T. J., Burton, P. S., Barsuhn, C. L., Hilgers, A. R., Audus, K. L. and Ho, N. F. (1994). "Quantitative approaches to delineate paracellular diffusion in cultured epithelial cell monolayers." *J Pharm Sci* **83**(11): 1529-36.

Artursson, P. (1990). "Epithelial transport of drugs in cell culture. I: A model for studying the passive diffusion of drugs over intestinal absorptive (Caco-2) cells." *J Pharm Sci* **79**(6): 476-82.

Artursson, P. and Karlsson, J. (1991). "Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells." *Biochem Biophys Res Commun* **175**(3): 880-5.

Artursson, P., Palm, K. and Luthman, K. (2001). "Caco-2 monolayers in experimental and theoretical predictions of drug transport." *Adv Drug Deliv Rev* **46**(1-3): 27-43.

Artursson, P., Ungell, A. L. and Lofroth, J. E. (1993). "Selective paracellular permeability in two models of intestinal absorption: cultured monolayers of human intestinal epithelial cells and rat intestinal segments." *Pharm Res* **10**(8): 1123-9.

Augeron, C. and Laboisse, C. L. (1984). "Emergence of permanently differentiated cell clones in a human colonic cancer cell line in culture after treatment with sodium butyrate." *Cancer Res* **44**(9): 3961-9.

Balimane, P. V. and Sinko, P. J. (1999). "Involvement of multiple transporters in the oral absorption of nucleoside analogues." *Adv Drug Deliv Rev* **39**(1-3): 183-209.

Bonen, A., Miskovic, D., Tonouchi, M., Lemieux, K., Wilson, M. C., Marette, A. and Halestrap, A. P. (2000). "Abundance and subcellular distribution of MCT1 and MCT4 in heart and fast-twitch skeletal muscles." *Am J Physiol Endocrinol Metab* **278**(6): E1067-77.

Briske-Anderson, M. J., Finley, J. W. and Newman, S. M. (1997). "The influence of culture time and passage number on the morphological and physiological development of Caco-2 cells." *Proc Soc Exp Biol Med* **214**(3): 248-57.

Carlstedt, I., Sheehan, J. K., Corfield, A. P. and Gallagher, J. T. (1985). "Mucous glycoproteins: a gel of a problem." *Essays Biochem* **20**: 40-76.

Collett, A., Sims, E., Walker, D., He, Y. L., Ayrton, J., Rowland, M. and Warhurst, G. (1996). "Comparison of HT29-18-C1 and Caco-2 cell lines as models for studying intestinal paracellular drug absorption." *Pharm Res* **13**(2): 216-21.

de Aizpurua, H. J. and Russell-Jones, G. J. (1988). "Oral vaccination. Identification of classes of proteins that provoke an immune response upon oral feeding." *J Exp Med* **167**(2): 440-51.

Deuticke, B. (1982). "Monocarboxylate transport in erythrocytes." *J Membr Biol* **70**(2): 89-103.

Dharmsathaphorn, K., McRoberts, J. A., Mandel, K. G., Tisdale, L. D. and Masui, H. (1984). "A human colonic tumor cell line that maintains vectorial electrolyte transport." *Am J Physiol* **246**(2 Pt 1): G204-8.

Dix, C. J., Hassan, I. F., Obray, H. Y., Shah, R. and Wilson, G. (1990). "The transport of vitamin B12 through polarized monolayers of Caco-2 cells." *Gastroenterology* **98**(5 Pt 1): 1272-9.

Duizer, P., Penninks, A. H., Stenhuis, W. H. and Groten, J. P. (1997). "Comparison of permeability characteristics of the human colonic Caco-2 and rat small intestinal IEC-18." *J Control Release* **49**(1): 39-49.

Flanagan, S. D., Takahashi, L. H., Liu, X. and Benet, L. Z. (2002). "Contributions of saturable active secretion, passive transcellular, and paracellular diffusion to the overall transport of furosemide across adenocarcinoma (Caco-2) cells." *J Pharm Sci* **91**(4): 1169-77.

Fogh, J., Wright, W. C. and Loveless, J. D. (1977). "Absence of HeLa cell contamination in 169 cell lines derived from human tumors." *J Natl Cancer Inst* **58**(2): 209-14.

Gan, L. S., Yanni, S. and Thakker, D. R. (1998). "Modulation of the tight junctions of the Caco-2 cell monolayers by H2-antagonists." *Pharm Res* **15**(1): 53-7.

Garcia, C. K., Goldstein, J. L., Pathak, R. K., Anderson, R. G. and Brown, M. S. (1994). "Molecular characterization of a membrane transporter for lactate, pyruvate, and other monocarboxylates: implications for the Cori cycle." *Cell* **76**(5): 865-73.

Grasset, E., Pinto, M., Dussaulx, E., Zweibaum, A. and Desjeux, J. F. (1984). "Epithelial properties of human colonic carcinoma cell line Caco-2: electrical parameters." *Am J Physiol* **247**(3 Pt 1): C260-7.

Hagenbuch, B. and Meier, P. J. (2004). "Organic anion transporting polypeptides of the OATP/ SLC21 family: phylogenetic classification as OATP/ SLCO superfamily, new nomenclature and molecular/functional properties." *Pflugers Arch* **447**(5): 653-65.

Halestrap, A. P. and Meredith, D. (2004). "The SLC16 gene family-from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond." *Pflugers Arch* **447**(5): 619-28.

Halestrap, A. P. and Price, N. T. (1999). "The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation." *Biochem J* **343 Pt 2**: 281-99.

Hayton, W. L. (1980). "Rate-limiting barriers to intestinal drug absorption: a review." *J Pharmacokinet Biopharm* **8**(4): 321-34.

Heyman, M., Crain-Denoyelle, A. M., Nath, S. K. and Desjeux, J. F. (1990). "Quantification of protein transcytosis in the human colon carcinoma cell line CaCo-2." *J Cell Physiol* **143**(2): 391-5.

Hidalgo, I. J., Raub, T. J. and Borchardt, R. T. (1989). "Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability." *Gastroenterology* **96**(3): 736-49.

Hillgren, K. M., Kato, A. and Borchardt, R. T. (1995). "In vitro systems for studying intestinal drug absorption." *Med Res Rev* **15**(2): 83-109.

Horio, M., Chin, K. V., Currier, S. J., Goldenberg, S., Williams, C., Pastan, I., Gottesman, M. M. and Handler, J. (1989). "Transepithelial transport of drugs by the multidrug transporter in cultured Madin-Darby canine kidney cell epithelia." *J Biol Chem* **264**(25): 14880-4.

Hu, M. and Borchardt, R. T. (1990). "Mechanism of L-alpha-methyldopa transport through a monolayer of polarized human intestinal epithelial cells (Caco-2)." *Pharm Res* **7**(12): 1313-9.

Cho, M. J., Thompson, D. P., Cramer, C. T., Vidmar, T. J. and Scieszka, J. F. (1989). "The Madin Darby canine kidney (MDCK) epithelial cell monolayer as a model cellular transport barrier." *Pharm Res* **6**(1): 71-7.

Irvine, J. D., Takahashi, L., Lockhart, K., Cheong, J., Tolan, J. W., Selick, H. E. and Grove, J. R. (1999). "MDCK (Madin-Darby canine kidney) cells: A tool for membrane permeability screening." *J Pharm Sci* **88**(1): 28-33.

Kanai, N., Lu, R., Bao, Y., Wolkoff, A. W. and Schuster, V. L. (1996). "Transient expression of oatp organic anion transporter in mammalian cells: identification of candidate substrates." *Am J Physiol* **270**(2 Pt 2): F319-25.

Kobayashi, D., Nozawa, T., Imai, K., Nezu, J., Tsuji, A. and Tamai, I. (2003). "Involvement of human organic anion transporting polypeptide OATP-B (SLC21A9) in pH-dependent transport across intestinal apical membrane." *J Pharmacol Exp Ther* **306**(2): 703-8.

Kusuhara, H. and Sugiyama, Y. (2002). "Role of transporters in the tissue-selective distribution and elimination of drugs: transporters in the liver, small intestine, brain and kidney." *J Control Release* **78**(1-3): 43-54.

Lee, K., Ng, C., Brouwer, K. L. and Thakker, D. R. (2002). "Secretory transport of ranitidine and famotidine across Caco-2 cell monolayers." *J Pharmacol Exp Ther* **303**(2): 574-80.

Lee, K. and Thakker, D. R. (1999). "Saturable transport of H2-antagonists ranitidine and famotidine across Caco-2 cell monolayers." *J Pharm Sci* **88**(7): 680-7.

Lee, V. H. (2000). "Membrane transporters." *Eur J Pharm Sci* **11 Suppl 2**: S41-50.

Leibovitz, A., Stinson, J. C., McCombs, W. B., 3rd, McCoy, C. E., Mazur, K. C. and Mabry, N. D. (1976). "Classification of human colorectal adenocarcinoma cell lines." *Cancer Res* **36**(12): 4562-9.

Lentz, K. A., Hayashi, J., Lucisano, L. J. and Polli, J. E. (2000). "Development of a more rapid, reduced serum culture system for Caco-2 monolayers and application to the biopharmaceutics classification system." *Int J Pharm* **200**(1): 41-51.

Lesuffleur, T., Barbat, A., Dussaulx, E. and Zweibaum, A. (1990). "Growth adaptation to methotrexate of HT-29 human colon carcinoma cells is associated with their ability to differentiate into columnar absorptive and mucus-secreting cells." *Cancer Res* **50**(19): 6334-43.

Lipinski, C. A., Lombardo, F., Dominy, B. W. and Feeney, P. J. (2001). "Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings." *Adv Drug Deliv Rev* **46**(1-3): 3-26.

Mueckler, M., Caruso, C., Baldwin, S. A., Panico, M., Blench, I., Morris, H. R., Allard, W. J., Lienhard, G. E. and Lodish, H. F. (1985). "Sequence and structure of a human glucose transporter." *Science* **229**(4717): 941-5.

Neuhoff, S. (2005). Refined in vitro Models for Prediction of Intestinal Drug Transport: Role of pH and Extracellular Additives in the Caco-2 Cell Model. Uppsala, Acta Universitatis Upsaliensis**:** 83.

Neuhoff, S., Ungell, A. L., Zamora, I. and Artursson, P. (2005). "pH-Dependent passive and active transport of acidic drugs across Caco-2 cell monolayers." *Eur J Pharm Sci* **25**(2-3): 211-20.

Nozawa, T., Imai, K., Nezu, J., Tsuji, A. and Tamai, I. (2004). "Functional characterization of pH-sensitive organic anion transporting polypeptide OATP-B in human." *J Pharmacol Exp Ther* **308**(2): 438-45.

Olson, A. L. and Pessin, J. E. (1996). "Structure, function, and regulation of the mammalian facilitative glucose transporter gene family." *Annu Rev Nutr* **16**: 235-56.

Oxender, D. L. (1972). "Membrane transport." *Annu Rev Biochem* **41**(10): 777-814.

Palacin, M., Estevez, R., Bertran, J. and Zorzano, A. (1998). "Molecular biology of mammalian plasma membrane amino acid transporters." *Physiol Rev* **78**(4): 969-1054.

Pappenheimer, J. R. and Reiss, K. Z. (1987). "Contribution of solvent drag through intercellular junctions to absorption of nutrients by the small intestine of the rat." *J Membr Biol* **100**(2): 123-36.

Prasad, P. D., Wang, H., Kekuda, R., Fujita, T., Fei, Y. J., Devoe, L. D., Leibach, F. H. and Ganapathy, V. (1998). "Cloning and functional expression of a cDNA encoding a mammalian sodium-dependent vitamin transporter mediating the uptake of pantothenate, biotin, and lipoate." *J Biol Chem* **273**(13): 7501-6.

Py, G., Eydoux, N., Perez-Martin, A., Raynaud, E., Brun, J. F., Prefaut, C. and Mercier, J. (2001). "Streptozotocin-induced diabetes decreases rat sarcolemmal lactate transport." *Metabolism* **50**(4): 418-24.

Quan, Y. S., Hattori, K., Lundborg, E., Fujita, T., Murakami, M., Muranishi, S. and Yamamoto, A. (1998). "Effectiveness and toxicity screening of various absorption enhancers using Caco-2 cell monolayers." *Biol Pharm Bull* **21**(6): 615-20.

Shu, Y., Bello, C. L., Mangravite, L. M., Feng, B. and Giacomini, K. M. (2001). "Functional characteristics and steroid hormone-mediated regulation of an organic cation transporter in Madin-Darby canine kidney cells." *J Pharmacol Exp Ther* **299**(1): 392-8.

Taipalensuu, J., Tornblom, H., Lindberg, G., Einarsson, C., Sjoqvist, F., Melhus, H., Garberg, P., Sjostrom, B., Lundgren, B. and Artursson, P. (2001). "Correlation of gene expression of ten drug efflux proteins of the ATP-binding cassette transporter family in normal human jejunum and in human intestinal epithelial Caco-2 cell monolayers." *J Pharmacol Exp Ther* **299**(1): 164-70.

Takanaga, H., Tamai, I. and Tsuji, A. (1994). "pH-dependent and carrier-mediated transport of salicylic acid across Caco-2 cells." *J Pharm Pharmacol* **46**(7): 567-70.

Tamai, I. (1997). "[Molecular characterization of intestinal absorption of drugs by carrier-mediated transport mechanisms]." *Yakugaku Zasshi* **117**(7): 415-34.

Tamai, I., Sai, Y., Ono, A., Kido, Y., Yabuuchi, H., Takanaga, H., Satoh, E., Ogihara, T., Amano, O., Izeki, S. and Tsuji, A. (1999). "Immunohistochemical and functional characterization of pH-dependent intestinal absorption of weak organic acids by the monocarboxylic acid transporter MCT1." *J Pharm Pharmacol* **51**(10): 1113-21.

Tamai, I., Takanaga, H., Maeda, H., Sai, Y., Ogihara, T., Higashida, H. and Tsuji, A. (1995). "Participation of a proton-cotransporter, MCT1, in the intestinal transport of monocarboxylic acids." *Biochem Biophys Res Commun* **214**(2): 482-9.

Tavelin, S. (2003). New Approaches to Studies of Paracellular Drug Transport in Intestinal Epithelial Cell Monolayers. Uppsala, Acta Universitatis Upsaliensis**:** 66.

Tavelin, S., Milovic, V., Ocklind, G., Olsson, S. and Artursson, P. (1999). "A conditionally immortalized epithelial cell line for studies of intestinal drug transport." *J Pharmacol Exp Ther* **290**(3): 1212-21.

Tsuji, A., Takanaga, H., Tamai, I. and Terasaki, T. (1994). "Transcellular transport of benzoic acid across Caco-2 cells by a pH-dependent and carrier-mediated transport mechanism." *Pharm Res* **11**(1): 30-7.

Tsukaguchi, H., Tokui, T., Mackenzie, B., Berger, U. V., Chen, X. Z., Wang, Y., Brubaker, R. F. and Hediger, M. A. (1999). "A family of mammalian Na+-dependent L-ascorbic acid transporters." *Nature* **399**(6731): 70-5.

Wang, J., Schaner, M. E., Thomassen, S., Su, S. F., Piquette-Miller, M. and Giacomini, K. M. (1997). "Functional and molecular characteristics of Na(+)-dependent nucleoside transporters." *Pharm Res* **14**(11): 1524-32.

Ward, P. D., Tippin, T. K. and Thakker, D. R. (2000). "Enhancing paracellular permeability by modulating epithelial tight junctions." *Pharm. Sci. Technol. Today* **3**(10): 346-358.

Wikman, A., Karlsson, J., Carlstedt, I. and Artursson, P. (1993). "A drug absorption model based on the mucus layer producing human intestinal goblet cell line HT29-H." *Pharm Res* **10**(6): 843-52.

Yang, C. Y., Dantzig, A. H. and Pidgeon, C. (1999). "Intestinal peptide transport systems and oral drug availability." *Pharm Res* **16**(9): 1331-43.

Yasui-Furukori, N., Uno, T., Sugawara, K. and Tateishi, T. (2005). "Different effects of three transporting inhibitors, verapamil, cimetidine, and probenecid, on fexofenadine pharmacokinetics." *Clin Pharmacol Ther* **77**(1): 17-23.

Yee, S. (1997). "In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man--fact or myth." *Pharm Res* **14**(6): 763-6.

Zhang, L., Brett, C. M. and Giacomini, K. M. (1998). "Role of organic cation transporters in drug absorption and elimination." *Annu Rev Pharmacol Toxicol* **38**: 431-60.

Zhou, S. Y., Piyapolrungroj, N., Pao, L., Li, C., Liu, G., Zimmermann, E. and Fleisher, D. (1999). "Regulation of paracellular absorption of cimetidine and 5-aminosalicylate in rat intestine." *Pharm Res* **16**(11): 1781-5.

Zweibaum, A., Pinto, M., Chevalier, G., Dussaulx, E., Triadou, N., Lacroix, B., Haffen, K., Brun, J. L. and Rousset, M. (1985). "Enterocytic differentiation of a subpopulation of the human colon tumor cell line HT-29 selected for growth in sugar-free medium and its inhibition by glucose." *J Cell Physiol* **122**(1): 21-9.