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Physiological barriers to the oral delivery of curcumin

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Curcumin, a principal component from *Curcuma longa*, with antioxidant and anti-inflammatory activities was proposed as a potential candidate for the preventation and/or treatment of cancer and chronic diseases. However, curcumin could not achieve its expected therapeutic outcome in clinical trials due to its low solubility and poor bioavailability. The actual intestinal physiological barriers limiting curcumin absorption after oral administration have not been fully investigated. To identify the main barriers curtailing its absorption, *in vitro* permeability of curcumin and flux of its glucuronide were monitored in rat jejunum and Transwell grown Caco-2 cells. Curcumin was more permeable under acidic conditions, but the permeability was substantially below the permeability of highly permeable standards. Its efflux could not be inhibited by specific Pgp and MRP inhibitors. BCRP was found to participate in curcumin transport, but the Organic Anion Transporting Polypeptide (OATP) did not. The permeability of curcumin significantly increased when the structure of mucus was compromised. The inhibitor of curcumin metabolism, piperin, failed to act as a permeability enhancer. Piperin inhibited Pgp and MRP transporters and decreased the amount of glucuronide transported back into the intestine. Inclusion of piperin in curcumin-containing formulations is highly recommended as to inhibit curcumin glucuronidation and to increase the transport of formed glucuronides into the plasma, therefore increasing the probability of glucuronide distribution into target tissue and inter-convertion to curcumin. It would also be beneficial, if curcumin delivery systems could reversibly compromise the mucous integrity to minimize the non-specific binding of curcumin to its constituents.

1. Introduction

The rhizome of *Curcuma longa* represents the main source of turmeric, a yellow spice with a long history of use for dietary and medicinal purposes in Indian and Chinese cultures (Hatcher et al. 2008). The main constituent of turmeric extract, curcumin, a hydrophobic polyphenole, is poorly absorbed after oral application. Low systemic availability thus mitigates against using curcumin in the prevention of conditions distal from the gastrointestinal tract (GIT) (Hatcher et al. 2008; Basnet et al. 2011). Namely, therapeutic plasma concentrations required to elicit biochemical changes germane to chemoprevention, antioxidant, antiviral and anti-inflammatory effects determined in *in vitro* experiments are in the micromolar range (Zhang and Lim 2008; Wortelboer et al. 2003; Ireson et al. 2001), while clinical studies in cancer patients, receiving 4–12 g of pure curcumin per day determined low, nanogram steady-state levels (22–41 ng/ml after 12 g p.o. dose (Dhillon et al. 2008), 4 ng/mL after 3.6 g p.o. dose (Sharma 2004)) of circulating curcumin. The predominant species of curcumin in plasma are represented by its conjugated metabolites (glucuronides and sulfates) and products of reduction reactions (tetrahydro- and hexahydrocurcumin and hexahydrocurcuminol) (Basnet et al. 2011). Although less effective than the parent compound, these metabolites are believed to be responsible for systemic activity (i.e. lowering plasma levels of glucose, cholesterol, triglycerides, anti-obesity effect etc.) after curcumin consumption (Hatcher et al. 2008; Basnet et al. 2011), a promising feature rendering this polyphenolic compound worthy of further research.

Poor curcumin absorption from intestine is a feature pertinent to this phytochemical, observed across several animal species (Basnet et al. 2011). Low water solubility, decomposition at neutral or alkaline pH, photosensitivity and a coordinately regulated alliance between metabolizing enzymes and transporters, all act in tandem with the net result of low curcumin absorption (Hatcher et al. 2008; Wahlang et al. 2011). *In vitro* studies with Caco-2 (Hatcher et al. 2008; Wahlang et al. 2011), MDCKII (Wortelboer et al. 2003) and LLC-PK1 (Zhang et al. 2008) cells, experiments with vesicles isolated from Multidrug Resistance Associated Proteins 1 and 2 (MRP-1 and MRP-2) transfected Sf8 cells (Wortelboer et al. 2003) and CYP3A4 studies (Zhang et al. 2008) identified P-glycoprotein (Pgp), MRP-1, MRP-2, Cytochrome P450 isoenzyme 3A4 (CYP3A4), sulfotransferase 1A1 and 1A3 (SULT1A1 and 1A3) (Ireson et al. 2002), UDP-glucuronyl transferases (Ireson et al. 2001; Liu et al. 2006) and nonspecific oxydoreductases (Ireson 2001) as the key step of entero-enteric and hepatic pre-systemic metabolism.

However, no proper *in vitro* permeability studies providing deeper knowledge about barriers to its absorption have been performed by now. A detailed review of *in vitro* curcumin permeability studies through cell cultures reveals that the determination of curcumin permeability was either not the purpose of these studies (curcumin was only used to inhibit transport of Pgp substrates digoxin, rhodamine (Zhang et al. 2008); MRP2

substrate calcein (Wortelboer 2003)) or, that the experimental design of these studies was improper considering stability and solubility data on curcumin (curcumin concentrations tested were as high as 170 μM, the solutions for testing were prepared at pH 6.5 or 7.4) (Zhang et al. 2008; Wortelboer et al. 2003; Wahlang et al. 2011). Namely, it is known that curcumin has extremely low water solubility (0.03 μM) and decomposes in water at pH values above 7.2 (Hatcher et al. 2008; Kamiga et al. 2003). This could significantly influence the measurement of curcumin's permeability. Therefore the aim of this study was to evaluate curcumin's intestinal permeability through Caco-2 cell monolayers as well as through rat jejunum and to investigate the influence of physiological barriers on its permeability (i.e. mucus, pH along intestinal tract, participation of intestinal transporters). The potential of piperin, a known inhibitor of curcumin pre-systemic glucuronidation, to act as permeability enhancer for curcumin was also investigated. In all experiments albumin was used in both donor and acceptor solutions to increase curcumin stability and solubility. In addition, the transport of curcumin glucuronides was also investigated.

2. Investigations and results

2.1. Curcumin stability in Ringer buffer pH 6.5 and 7.4

To perform curcumin permeability experiments, the stability of curcumin in donor solutions were first evaluated. 50 μM curcumin in Ringer buffer of pH 6.5 and 7.4 was prepared from curcumin DMSO stock solution (final DMSO concentration 0.1%), similarly to the procedure described in published papers (Wahlang et al. 2011; Zhang et al. 2008). The concentration of curcumin in the heated (37 °C) and oxidized donor solutions was analyzed 25 min after their preparation and after 3 h. The LC-MS/MS measurements indicated a significant decline in curcumin concentrations in the timeframe of 25 min (less than 15% of initial curcumin concentration was measured). After 3 h, curcumin concentration was below the detection limit. Because the stability of curcumin can be improved with the addition of plasma proteins (Hatcher et al. 2008; Kaminaga et al. 2003) 2 vol/vol % of human albumin was added to donor solutions, used for permeability experiments. With albumin in the solutions, the concentrations of curcumin remaining in the donor solutions after 3 h incubation remained unchanged (97.2 ± 3.2% of the initial curcumin concentration). The solutions with 2 vol/vol % of albumin were thus used for further permeability experiments. The mass balance and the recovery of curcumin during permeability experiments remained practically unchanged and above 90%. The use of other surface acting agents was intentionally avoided, because they are known to modify membrane fluidity, transporter-enzyme interplay and could thus lead to false permeability measurements.

2.2. Curcumin permeability through Caco-2 monolayers

The permeability of 100 μM curcumin was determined through Transwell grown Caco-2 cell monolayers with 2% of albumin added to both sides of the monolayer. Due to the presence of albumin, the concentration of the unbound curcumin in donor solutions was unknown, most probably below curcumin water solubility (0.03 μM), but constant throughout the experiment. The permeability was determined in the absorptive (AP-BL) and the opposite (BL-AP) direction at acidic and neutral pH (i.e. 6.5 and 7.4) on the apical side of the cells, while the basolateral pH was kept constant at pH 7.4. The participation of efflux (Pgp, MRPs and Breast Cancer Resistance Protein (BCRP)) and absorptive (Organic Anion Transporting Polypeptide OATP) transporters was assessed by using appropriate inhibitors and pH conditions on the apical side of the cells.

Table 1: Permeability of curcumin through Caco-2 cell monolayers

pH conditions (AP/BL)	Curcumin Caco-2 permeability P_{app} ($\times 10^{-6}$ cm/s) Experimental design	AP-BL	BL-AP
6.5/7.4	Ref	13.9 ± 0.5	19.8 ± 1.0*
	E3S (0.5 mM)	13.5 ± 1.5	
7.4/7.4	Ref	6.1 ± 1.9	10.2 ± 0.5*
	PSC833 (5 mM)		10.8 ± 0.6
	MK571 (50 mM)		10.0 ± 0.4
	FumC (5 mM)		39.6 ± 3.8

The permeability (P_{app}) in absorptive (AP-BL) and secretory (BL-AP) directions were evaluated for curcumin alone (Ref). The influence of OATP transporter was evaluated with estrone-3-sulfate (E3S) in the apical donor solution, while the contribution of efflux transporters was determined with Pgp inhibitor PSC833, MRPs inhibitor MK571 and fumitromorgin C (FumC) as BCRP inhibitor. The concentrations of inhibitors in the apical acceptor solutions are given in brackets

* the difference between AP-BL and corresponding BL-AP P_{app} values are statistically significant ($p < 0.05$)

The results, presented in Table 1, indicated asymmetrical transport properties of curcumin regardless of the pH applied to the apical side. In both cases, the BL-AP permeability was significantly higher than the absorptive one ($p < 0.05$). However, under slightly acidic conditions (pH 6.5) on the apical side, the absorptive permeability of curcumin significantly surpassed the one, determined at iso-pH conditions (apical and basolateral pH 7.4). Although higher, AP-BL permeability value measured at pH 6.5 was not in the range of AP-BL permeabilities determined in our laboratory for highly permeable standards through Transwell grown Caco-2 monolayers (Yu et al. 2002), therefore curcumin could not be classified as high but as low permeable compound.
To identify transporters involved, specific inhibitors were used (PSC833 for Pgp, MK571 for MRPs and fumitromorgin C for BCRP) at appropriate concentrations and iso-pH conditions, as indicated in Table 1. We did not observe any permeability decrease in BL-AP directions when specific Pgp and MRP inhibitors were added. For the first time the participation of BCRP to curcumin efflux from Caco-2 cells was also assessed. We used fumitromorgin C as a specific BCRP inhibitor, which increased the BL-AP permeability of curcumin significantly. The permeability increase observed in the presence of 5 mM fumitromorgin C was not due to Caco-2 cell monolayer injuries, because TEER values and the permeability of paracellular marker fluorescein (the marker of tight junction integrity) did not change (data not shown).
According to curcumin structure and its acidic pKa values ($pKa_1 = 8.38 \pm 0.04$; $pKa_2 = 9.88 \pm 0.02$; $pKa_3 = 10.51 \pm 0.01$) (Wahlang et al. 2011), the main form of curcumin present at pH 6.5 and 7.4 is predominately unionized (Tønnesen et al. 2002). Because polyphenolic compounds have been identified as OATP substrates/inhibitors (Fuchikami et al. 2006), we also anticipated that this absorptive transporter could be involved in the absorption of curcumin. Since OATPs exert their maximal transporter efficiency in acidic environment, the impact of this transporter was monitored at apical pH 6.5 and estron-3-sulfate (E3S) was used for its inhibition (Nozawa et al. 2004). E3S did not affect curcumin AP-BL permeability, which remained practically identical to the reference AP-BL curcumin permeability values.

2.3. Curcumin permeability through rat jejunum

To address the participation of intestine in limiting oral absorption of curcumin, Sprague-Dawley rats were used. The

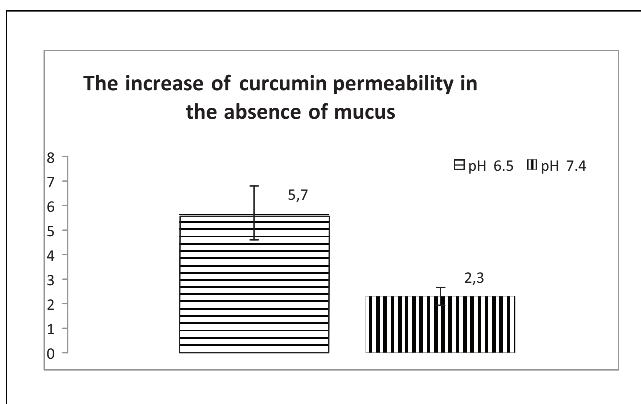

Fig. 1: Impact of mucus on the permeability of curcumin through rat jejunum at apical pH 6.5 and 7.4

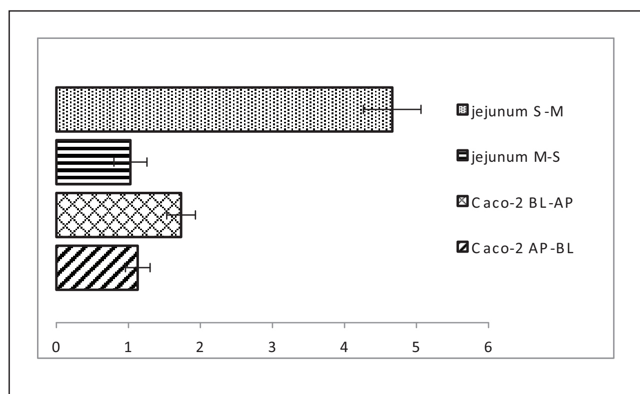

Fig. 2: Impact of 100 μM piperin on the permeability of curcumin through Caco-2 cell monolayers and rat jejunum in the absorptive (AP-BL/M-S) and secretory (BL-AP/S-M) directions

metabolism of curcumin to glucuronides and reduced curcumin species was negligible, because the quantitiy of metabolites in the acceptor/donor solutions or in the tissue extract, prepared after the permeability experiments, were below LC-MS/MS detection limit (data not shown). At apical pH 6.5 and basolateral pH 7.4 the M-S permeability in the absorptive direction was $(14.3 \pm 2.5) \times 10^{-7}$ cm/s, while at apical pH 7.4 permeability was significantly lower $(3.0 \pm 0.2) \times 10^{-7}$ cm/s, similarly to the results obtained with Caco-2 model. The S-M permeability was not significantly different from the corresponding M-S permeabilities (the S-M permeability at acidic apical pH was $(10.4 \pm 3.1) \times 10^{-7}$, while at apical pH 7.4 the S-M values were $(2.5 \pm 0.6) \times 10^{-7}$.

2.4. *The impact of mucus on curcumin permeability*

To disrupt mucus layer in rat jejunum *in vitro*, 2 mM N-acetyl-L-cysteine (NAcCys) was applied in the incubation salines (pH 6.5 and 7.4) on the mucosal side and incubated for 2 h. Afterwards, these buffers were removed and replaced by donor solutions, containing 100 μM curcumin, 2 mM NAcCys and 2% albumin and the permeability of curcumin was monitored for additional 2 h. Simultaneously, reference experiments were conducted, where permeability of curcumin was monitored without NAcCys in the donor solutions. In the absence of mucus, the M-S curcumin permeability at apical pH 7.4 significantly increased (the permeability increased 2.3-times) and the effect was even more pronounced at acidic apical pH of 6.5, where a 5.7-time increase of curcumin absorptive permeability was noted (Fig. 1).

2.5. *Influence of piperin on the permeability of curcumin*

To evaluate whether piperin could be used as permeability enhancer for curcumin, the experiments were performed at apical pH 6.5, and the permeability of 100 μM curcumin was monitored through rat jejunum and Transwell grown Caco-2 cell monolayers in the presence of piperin (100 μM) on the mucosal/apical side. The results are presented in Fig. 2. As shown, piperin failed to increase the absorptive permeability of curcumin in both models used (the AP-BL/M-S permeabilities determined in the presence of piperin did not change significantly compared to reference AP-BL/M-S values).
Instead of increasing the amount of curcumin absorbed in the M-S/AP-BL direction, piperin significantly ($p < 0.01$) enhanced the active efflux of curcumin from the Caco-2 cells and rat enterocytes even more than determined in the reference experiments (the S-M/BL-AP permeabilities, determined in the presence of piperin, were significantly ($p < 0.01$) higher than the corresponding, reference S-M/BL-AP permeabilities - see 2.2 and Table 1). The S-M curcumin permeability increased more than 4-times through rat jejunum, while the effect was not so pronounced in Caco-2 cells (only 1.7-time increase of BL-AP permeability).

2.6. *Curcumin glucuronide transport in Transwell grown Caco-2 cells*

Transport properties of curcumin glucuronide were monitored in Transwell grown Caco-2 cells (Table 2). The results measured under iso-pH conditions (Table 2, 7.4/7.4 REF) indicated that the glucuronides formed inside Caco-2 cells undergo active transport into the apical and basolateral sides, but the secretion of glucuronides (BL-AP flux) back into the apical side significantly ($p < 0.001$) surpassed their absorption (AP-BL flux) into the basolateral compartment. With the use of specific ABC transporter inhibitors (Table 2; PSC833, MK571, FumC) the efflux of curcumin glucuronides significantly declined, but so did its flux in the absorptive direction. However, the ratio between AP-BL and BL-AP fluxes increased (from 0.50 for reference experiments to 0.62 for PSC and MK571) for all inhibitors, except for FumC. The ratio between AP-BL and BL-AP fluxes in the presence of FumC decreased significantly.
At apical pH 6.5, which resembles physiological conditions in the proximal GIT better, fluxes of glucuronides in boh directions were significantly ($p < 0.01$) lower than at iso-pH conditions (Table 2). However, the flux of glucuronide in the absorptive (AP-BL) direction exceeded that in the opposite – BL-AP direc-

Table 2: Fluxes of curcumin glucuronide through Transwell grown Caco-2 cells

Curcumin glucuronide fluxes [nmol/h × cm^2]			
pH conditions	Inhibitors	AP-BL	BL-AP
7.4/7.4	REF	0.065 ± 0.000	0.131 ± 0.026[*]
	PSC833	0.056 ± 0.000[a]	0.091 ± 0.001[*a]
	MK571	0.056 ± 0.000[a]	0.090 ± 0.001[*a]
	FumC	0.042 ± 0.001[a]	0.096 ± 0.003[*a]
6.5/7.4	REF	0.046 ± 0.002	0.032 ± 0.002[*]
	Piperin	0.048 ± 0.001	0.025 ± 0.006[*a]

The fluxes of curcumin glucuronides were measured in basolateral (AP-BL) and apical (BL-AP) solutions at apical pH 6.5 and 7.4 in the absence (reference – REF) or in the presence of various transporter inhibitors. The results are presented as mean ± SD of four measurements.
[*] the fluxes of curcumin glucuronides into the basolateral compartment are significantly ($p < 0.05$) higher (at apical pH 7.4) or lower (at apical pH 6.5) than the fluxes into the apical compartment.
[a] the addition of an inhibitor (PSC833, MK571, FumC, piperin) significantly ($p < 0.05$) lowered curcumin glucuronides flux compared to the corresponding reference (REF) values.

tion (Table 2, 6.5/7.4 REF). In the presence of piperin (100 μM) BL-AP flux of glucuronide declined significantly (Table 2).

3. Discussion

The contribution of intestinal physiological barriers to the absorption of curcumin after oral delivery has not yet been evaluated. Namely, the use of curcumin in the *in vitro* permeability studies through cell cultures has been limited only to the use of curcumin as Pgp/MRP inhibitor (Zhang et al. 2008; Wortelboer 2003). There is only one paper published by Wahlang et al. (2011), where the permeability and the impact of efflux transporter Pgp to curcumin permeability were determined. However, very high donor concentrations (170 μM) of curcumin in HBBS pH 6.5 were applied to the cells. This group also reported that the recovery of curcumin after permeability experiments was very low (only 35–54%) (Wahlang et al. 2008), indicating that the experimental design used was most probably improper considering stability and solubility data on curcumin. Namely, curcumin is very unstable in aqueous acidic or neutral solutions, because more than 90% of initial quantity of curcumin prepared in water buffer pH 7.2 decomposes in 30 min at 37 °C (Hatcher et al. 2008). Furthermore, water solubility of curcumin is less than 0.03 μM at room temperature (Hatcher et al. 2008). The instability/poor water solubility of curcumin was also demonstrated in our preliminary experiments, where the recovery of curcumin in Ringer buffer at pH 6.5 and 7.4 was below the LC-MS/MS detection limit. The decline in curcumin's concentrations in our solutions was thus most probably caused by the non-specific binding of curcumin to the plastic, accompanied by precipitation and decomposition. Based on this data, we could not perform experiments with curcumin solutions in Ringer buffers. However, the stability of curcumin can be greatly improved in the presence of plasma proteins (Hatcher et al. 2011; Kaminaga 2003). Namely, in the presence of 10% of FBS, only 50% of curcumin decomposed at 37 °C during 8 h incubation. Thus, for the purpose of this study, human albumin (2 vol/vol %) was added into donor and acceptor Ringer solutions, used for permeability experiments, which significantly improved curcumin stability. With albumin in the solutions, the mass balance and the recovery of curcumin during permeability experiments remained practically unchanged (> 90%), which is significantly greater than the recovery (< 50%) determined for *in vitro* permeability experiments with Caco-2 cell monolayers published by Wahlang (Wahlang et al. 2011).

Poor physio-chemical properties, instability in GIT, metabolism and active efflux of curcumin could all contribute simultaneously to curcumin low *in vitro* permeability through rat jejunum and Transwell grown Caco-2 monolayers, determined in this study (Hatcher et al. 2011; Basnet et al. 2011). Curcumin has been used to inhibit secretion of Pgp and MRP2 substrates (Zhang et al. 2008; Wortelboer et al. 2003). However, the identification of transporters involved in curcumin transport through the intestine has not yet been investigated. Wahlang et al. (2008) monitored curcumin permeability through Caco-2 monolayers in both directions but found no differences between AP-BL and BL-AP permeabilities. Verapamil, a nonspecific Pgp inhibitor, had no impact on the permeability either (Wahlang et al. 2011). Contrary to their results, we showed that curcumin efflux from intestinal cells could represent an important barrier to curcumin absorption, since BL-AP permeability significantly exceeded the one in AP-BL direction at both pH. However, the efflux could not be inhibited by specific Pgp (PSC883) or MRP (MK-571) inhibitors, indicating that the binding affinity of curcumin towards all Pgp and MRP binding sites can be much higher than the one of inhibitors or on the other hand, the binding of curcumin to these proteins was irreversible, making curcumin more potent Pgp and MRP inhibitor than those currently on the market. BCRP inhibitor fumitromorgin C significantly increased curcumin efflux from Caco-2 cells. Although monolayer vitality and integrity were not compromised by fumitromorgin C, the permeability increase was most probably induced by positive-cooperation between fumitromorgin C and curcumin in BCRP. There are multiple binding sites in the structure of efflux transporters (Pgp has four, MRP-2 and BCRP have two) (Huang et al. 2006; Berginc et al. 2010; Sharom 2008). The substrate can thus occupy only one or more binding sites, depending on its affinity and concentration. It is possible that the binding affinity of curcumin towards BCRP was not as high as in the case of Pgp and MRPs, where specific transporter inhibitors failed to displace curcumin from protein and decrease its efflux. It is also possible, that the donor curcumin concentration was too low to occupy both BCRP binding sites simultaneously. This would leave one BCRP site free for the inhibitor or the inhibitor could successfully displace curcumin from at least one of the binding sites. Because simultaneous binding of two different substrates to their individual binding site, otherwise known as positive-cooperation between binding sites (Berginc et al. 2010), results in allosteric changes and increased transporter binding affinity towards both of its substrates, their transport significantly increases (Berginc et al. 2010; Sharom 2008). Positive-cooperation between both binding sites in BCRP was also most probably the reason, why we failed to inhibit BCRP mediated curcumin transport in the BL-AP direction. We also confirmed that OATP transporters do not participate in facilitated curcumin diffusion into enterocytes, although these transporters have been shown to increase the amount of polyphenolic-type of compounds (Fuchikami et al. 2006).

The role of the intestine in contributing to sub-therapeutic curcumin plasma concentrations after per-oral application was also evaluated *in vitro* with rat jejunum, due to the dissimilarities in physiological characteristics between cancerous (i.e. Caco-2) and non-cancerous (i.e. rat jejunum) models (Yu et al. 2002). It is known that curcumin bioavailability after oral administration is low regardless of the animal model (Hatcher et al. 2008) and that its metabolism in F344 rats gives the same profile of metabolites qualitatively as in humans, but overall, the metabolism of curcumin in rats highly underestimates human situation (Ireson et al. 2001). However, our rat model was unsuitable for metabolism studies, because no metabolites could be detected in the incubation solutions or in tissue extract. Since metabolism could not affect curcumin donor concentration in our permeability studies, this model can be readily used to determine intestinal curcumin permeability. Surprisingly, the permeability of curcumin through rat intestine was even lower than through Caco-2 cell monolayers. There were also no significant differences between permeabilities in M-S and S-M direction, indicating that efflux transporters did not participate in the intestinal absorption from rat jejunum. The compound's permeability can be assessed with models of various degrees of complexity according to the FDA guidelines (Yu et al. 2002). Usually, a low permeable compound, whose permeability has been determined with excised intestinal tissue of animals, shows even lower permeability values through Caco-2 cell monolayers, because there are less tight junctions with narrower openings in Caco-2 cells than in animal intestinal tissue and because the expression patterns of transporters and membrane fluidity significantly differ among models used (Artursson et al. 2001). However, in the case of curcumin, the opposite was observed; the permeability of low permeable compound was higher in Caco-2 model than in rat jejunum. Considering the differences between both models, we anticipated that mucus could represent an additional barrier to curcumin absorp-

tion. Namely, mucus is actively secreted to the mucosal side of enterocytes, but it is not present in the apical side of Caco-2 cells. Although mucus thickness varies depending on the bowl movements and content mixing along the GIT (Atuma et al. 2001), it is basically water filled layer of glycoproteins, lipids and water, sustaining acidic microclimate pH (Legen and Kristl 2003), which could bind free curcumin, lower its concentration gradient and thus decrease its permeability through enterocytes. Disruption of mucus above rat jejunum by the mucolytic agent NAcCys significantly increased curcumin absorptive permeability, especially under acidic conditions. Guided by these results it is obvious that mucus and its constituents might represent an additional barrier, which non-specifically binds curcumin and prevents it from sufficient absorption after oral application. There are contradictory reports about mucus influencing the absorption and permeability of drugs. The *in vitro* permeability of testosterone has been shown to be greatly (up to 80%) influenced by the mucus (Karlsson et al. 1993). Permeability increase was observed also with dextropropoxyphene through Caco-2-Ht29GlucH co-cultures in the presence of mucolytic agent NAcCys (Meaney and O'Driscoll 1999). In another study with pig mucus the diffusion of lipophilic drugs (hydrocortisone, propranolol, metoprolol and testosterone) was shown to be profoundly influenced by mucus, especially by the lipids in it (Wikman Larhed and Björk 1998). Although numerous reports can be found claiming that mucus is not a diffusion barrier to smaller molecules (molecular weight below 700 g/mol) (Legen and Kristl 2001), it seem that generalization of this statement is not possible. It most probably depends on the compound and its characteristics, whether the mucus can hinder its diffusion from the lumen to the absorptive surface. In the case of curcumin, also a very lipophilic compound (log $P > 3$), mucus seems to participate in its non-specific binding either to the proteins or to the lipids (Legen and Kristl 2001).

Because reversible mucus removal from the surface of intestine *in vivo* presents safety issues for the patient, the attempts to increase curcumin per-oral bioavailability have turned to formulation strategies. Thus nanoparticles, cyclodextrines, natural viscous polymers, liposomes and mixed micelles have been used as novel drug delivery systems which appear to assure better permeability and resistance to metabolism (Hatcher et al. 2008; Huang et al. 2006; Tønnesen 2006). Besides formulation strategies, curcumin oral absorption can also be enhanced by the concomitant consumption of piperin. The *in vivo* study in humans has shown a 2000% increase in curcumin bioavailability after oral application of 2 g curcumin together with 20 mg of piperin, which inhibited intestinal and hepatic curcumin glucuronidation, leading to a higher extent of curcumin absorption without posing safety issues for the volunteers (Shoba et al. 1998). The attempt of this study was thus also to evaluate whether piperin could be used as permeability enhancer to improve curcumin absorption and not just its metabolism. Because the absorptive curcumin permeability did not change, piperin cannot be used as a permeability enhancer for curcumin, although some studies have demonstrated its capability of modulating membrane dynamics by modifying the fluidity of the apical membrane (Khajuria et al. 1998). Instead of improving curcumin absorption, piperin significantly enhanced curcumin efflux in both tested models. As shown for FumC and BCRP transporter, the increase of curcumin S-M/BL-AP permeability with piperin in the donor solution most probably resulted from positive-cooperation triggered by piperin on one of the ABC transporters (most probably BCRP), involved in curcumin efflux. Based on the *in vivo* data, concomitant consumption of curcumin and piperin results in higher curcumin per-oral bioavailability. Therefore, the *in vivo* inhibitory effect of piperin on the intestinal glucuronidation most probably exceeds the positive-cooperation and increased the efflux of curcumin from enterocytes, because this latter process tends to lower the amount of curcumin absorbed.

In spite of curcumin pharmacokinetic shortcomings emanating from its low solubility, poor absorption, rapid metabolism and elimination (Hatcher et al. 2008), which render the use of curcumin as remedy for per-oral application quite ineffective, recent studies have suggested that curcumin metabolites could be responsible for some of its systemic effects (Hatcher et al. 2008; Basnet et al. 2011). There are no available curcumin metabolite standards on the market, therefore pharmacological activity of curcumin metabolites has not been sufficiently communicated yet. However, data for tetrahydrocurcumin suggests that this metabolite exerts more potent antidiabetic and antioxidant activities than its parent compound and that it actively inhibits the assembly of microtubule proteins, an activity devoid from curcumin (Hatcher et al. 2008). Furthermore, studies with radiolabelled curcumin in rats have shown distribution of curcumin and its metabolites into the lungs, muscles, spleen, liver, kidney, intestine, blood and to a lesser extent into the brain after intra peritoneal applications (Hatcher et al. 2008). These organs are thus all potential sites exposed to pharmacological activities of curcumin and/or its metabolites. Namely, although metabolites are the only species of curcumin detected in measurable levels in the plasma, after their distribution into target sites they might inter-converse back into the curcumin by enzymes (such as β-glucuronidases and sulfatases) residing in these tissues (Hatcher et al. 2008). Therefore it might be beneficial not to monitor only curcumin plasma levels, but also the amount of curcumin metabolites absorbed from the intestine. Higher amounts of curcumin metabolites absorbed could thus increase the probability of metabolites distributing into these target sites. Our studies with Caco-2 cells indicated that the glucuronide formed inside the cells was actively transported to both sides of the cells. By using specific transporter inhibitors (PSC883, MK-571, Fum-C), we have shown that the transport of glucuronide to the apical side was most probably mediated by Pgp and MRP2, while MRP1 and MRP3 were probably responsible for the flux of metabolite to the basolateral side. Since the ratio between AP-BL and BL-AP fluxes decreased in the case of FumC, therefore penalizing the amount of glucuronide absorbed, BCRP was most probably not involved in intestinal distribution of curcumin glucuronide. However, the lower glucuronide formation rate in the presence of FumC might also be a consequence of the reduced parent curcumine intracellular concentration caused by greater BCRP mediated curcumin apical excretion rate due to the already discussed activating effect of FumC on curcumine BL-AP transport (Table 1). The flux of glucuronide in the secretory direction could additionally be inhibited by piperin, which most probably inhibited ABC transportes (i.e. Pgp, MRP2) in the apical side of Caco-2 cells. In this manner, piperin could contribute to higher amounts of glucuronides absorbed, therefore increasing the probability of a higher systemic pharmacological activity.

Owing to stability and solubility issues, the *in vitro* curcumin permeability assessment could only be performed with albumin addition to the incubations salines, used for the permeability experiments. In this manner, decomposition, precipitation and non-specific plastic binding of curcumin were avoided, enabling correct permeability measurements. The permeability of curcumin was pH dependent with higher values obtained under slightly acidic conditions. Still, the permeability of curcumin was lower than the permeabilities of highly permeable FDA standards, classifying curcumin as BCS IV compound. At iso-pH values curcumin was subjected to a profound efflux back into the intestinal lumen by BCRP and to some extent probably by Pgp and MRP-2, but the binding affinity of curcumin towards

Pgp and MRP-2 was higher than that of specific Pgp/MRP2 inhibitors. Thus, curcumin could be an even more effective Pgp/MRP inhibitor. Used *in vivo*, it could increase the absorption of drugs, transported by those two ABC proteins, but these questions need further *in vivo/in vitro* conformation. Mucus was found to significantly hinder the absorption of curcumin because its main constituents, glycoproteins/lipids, most probably bind free curcumin, thus preventing adequate diffusion to the enterocytes. Piperin, a permeability enhancer and inhibitor of curcumin glucuronidation, failed to increase the absorption of curcumin, but it increased curcumin active extrusion from cells with ABC proteins. This effect is probably of no importance *in vivo*, since clinical studies report increased curcumin per-oral bioavailability when it was consumed with piperin. However, piperin significantly increased the amount of curcumin metabolite transported to the basolateral compartment, therefore making this adjuvans a useful tool for increasing the circulating levels of glucuronides, therefore increasing the probability of glucuronide distribution into tissues, distal from the GIT, and consequently their inter-conversion to curcumin. Curcumin delivery systems should therefore pursue the option of delivering curcumin to the upper, acidic part of the GIT (i.e. immediate release formulations), where curcumin permeability is higher. The per-oral curcumin bioavailability could further be increased, if these formulations would contain piperin and could reversibly loosen/compromise the mucus structure.

4. Experimental

4.1. Materials

Salts for incubation salines, curcumin, fluorescein, estrone-3-sulfate (E3S), MK571, fumitromorgin C (FumC), *N*-acetyl-L-cysteine (NAcCys) and all the necessary ingredients for cell cultivation were from Sigma Aldrich Chemie (Deisenhofen, Germany). PSC was from Tocris. All chemicals used in this study were of the highest grade available.

4.2. Methods

4.2.1. *In vitro transport studies across Caco-2 cell monolayers and rat small intestine*

Caco-2 cells were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) ACC 169, lot 12 and were grown on Transwell Costar culture inserts with a polycarbonate membrane (diameter 12 mm and pore size 0.4 μm). 65,000 cells/filter membranes were used for seeding and the medium was changed every two days. At day 15, transepithelial electrical resistance (TEER) was measured for each filter with Caco-2 cell monolayers. If the TEER values were in the range of 300–400 Ωcm^2, the Caco-2 cell monolayers were used for the subsequent testing of permeability at day 21.

The Caco-2 cells grown on Transwell Costar culture inserts were carefully rinsed with Ringer buffer. 1.5 mL and 0.5 ml of bathing solution (Ringer buffer) on basolateral and apical sides of the Caco-2 cell monolayer, respectively, was maintained at 37 °C and continuously oxygenated with carbogen (95% O_2, 5% CO_2) and lightly shaken during experiment. 625 mM glucose and 625 mM mannitol were always added to the basolateral (BL) and apical (AP) sides, respectively, to give final 10 mM concentrations. Albumin (2 vol/vol %) was added to transport buffer on both sides to increase curcumin stability and to avoid precipitation and decomposition. Curcumin was added to the AP side (if studying apical-to-basolateral (AP-BL) transport) or the BL side (if studying basolateral-to-apical (BL-AP) transport). Samples (150 μL) were withdrawn from the acceptor side every 20 min up to 120 min and replaced by fresh Ringer buffer containing all necessary ingredients at appropriate concentrations. The fluxes of curcumin glucuronide, which was synthesized inside cells from curcumin, were also monitored by sampling donor and acceptor compartments at the same time-points. Only those Caco-2 cell monolayers with constant TEER values during the whole experiment were used. The experiments conducted with rat intestinal tissue conform to the Law for the Protection of Animals (Republic of Slovenia) and were registered at the Veterinary Administration of the Republic of Slovenia. Rat small intestine was obtained from male Sprague-Dawley rats (250–320 g) fasted 18 h prior to the experiments. After euthanasia and laparotomy, the intestine was rinsed with ice-cold 10 mM glucose Ringer solution. Jejunum, located 25–60 cm distally from the pyloric sphincter was used for the experiments. The intestinal tissue was cut into 3 cm long segments, excluding visible Peyer's patches. These intestinal segments were opened along the mesenteric border, stretched onto inserts with an exposed tissue area of 1 cm^2 and then placed between two compartments of EasyMount side-by-side diffusion chambers (Physiologic Instruments, San Diego, USA). The experimental procedure continued in the same manner as described for Caco-2 cell monolayers.

4.2.2. *Electrical measurements*

The diffusion chambers were equipped with two pairs of Ag/AgCl electrodes for measuring transepithelial potential difference (PD) and short circuit current (I_{sc}) with a multi channel voltage-current clamp (model VCC MC6, Physiologic Instruments). The viability and integrity of tissue was checked by monitoring PD, I_{sc} and TEER every 20 min during the experiments. In viable rat tissues TEER did not change statistically significantly during the whole experiment. The average TEER from 20 min to 120 min was calculated and also used for evaluating the tissue/cell integrity and viability. The viability of rat small intestine was additionally checked by recording the increase of I_{sc} and PD after the addition of stock glucose solution to the mucosal compartment at the end of experiment (final glucose concentration was 25 mM). Tissue segments were considered viable if the PD value after the addition of glucose was lower than –1.0 mV and if the average TEER values recorded during the experiment were between 20 and 40 Ωcm^2.

4.2.3. *LC-MS/MS analysis of curcumin and curcumin metabolites*

Samples obtained during the permeability experiments were precipitated with ice cold MeOH (1:3 vol/vol), vigorously vortexed and left at −20 °C for 48 h. Afterwards, the samples were centrifuged (15 min at 4 °C and 15000 × *g*), the supernatant was transferred into autosampler 96–well plate tray for subsequent analysis by LC-MS/MS. LC/MS/MS apparatus consisted of an Agilent 6460 triple quadrupole mass spectrometer equipped with a JetStream interface and connected to an Agilent 1290 Infinity UPLC (Agilent Techologies, Santa Clara, USA). For chromatographic separation, a Phenomenex Kinetex 50 × 2.0 mm C-18 column with 2.6-mm particles was used (Phenomenex, Torrance, USA). The injection volume was 2 μL and the column temperature was 50 °C. The mobile phase consisted of a linear gradient of water (A) and acetonitrile (B), both containing 0.1% formic acid. The gradient started with 10% B which increased linearly to 80% B over 2 min and then returned to original conditions in 5 s. The flow rate was 0.65 mL/min and the total time of analysis was 2.7 min. The mass spectrometer was operated in positive ionization mode with the following parameters: drying gas temperature: 275 °C, drying gas flow: 5 L/min, nebulizer: 45 PSI (3.1×10^5 Pa), sheath gas temperature: 320 °C, sheath gas flow: 11 L/min, capillary entrance voltage: 4000 V, nozzle voltage: 1000 V, delta EMV: 200 V. The MRM's used for the quantification of curcumine and curcumine-glucuronide were as follows: The multiple reaction monitoring *m/z* transitions and collision energies used for the quantification of curcumine and curcumine glucuronide were 369→177 at 16 eV and 545→369 at 25 eV, respectively.

4.2.4. *Data analysis*

The apparent permeability coefficient (P_{app}) of curcumine and fluxes (J) of curcumine metabolites were calculated according to Eq. (1) and Eq. (2)

$$P_{app} = \frac{dc}{dt}\frac{V}{c_0 A} \tag{1}$$

$$J = \frac{dM}{A \times dt} \tag{2}$$

where *dc*/*dt* and *dM/dt* represent changes in concentration/mass of the examined substance/metabolites in the acceptor/donor compartment per unit time under steady state conditions, *V* is the volume of the acceptor compartment, *A* the exposed surface area (1 cm^2 for rat jejunum and 1.13 cm^2 for Caco-2 cell monolayers) and c_0 the initial concentration of curcumin. Curcumin permeabilities were determined in AP-BL/M-S and BL-AP/S-M directions. Results in Tables and Figures are presented as means ± SD of at least 3 measurements. Data were evaluated statistically using SPSS 16.0 for Windows. Where appropriate, F-test for testing the equality of variances and, afterwards, 2-tailed student t-test ($p = 0.05$), were used. Otherwise, one way ANOVA, followed by Bonferroni post-hoc test were applied.

References

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

Atuma C, Strugala V, Allen A, Holm L (2001) The adherent gastrointestinal mucus gel layer: thickness and physical state *in vivo*. Gastrointest Liver Physiol 280: G922–G929.

Basnet P, Skalko-Basnet N (2011) Curcumin: An anti-inflammatory molecule from a curry spice on the path to cancer treatment. Molecules 16: 4567–4598.

Berginc K, Milisav I, Kristl A (2010) Garlic flavonoids and organosulfur compounds: impact on the hepatic pharmacokinetics of saquinavir and darunavir. Drug Metab Pharmacokinet 25: 521–530.

Dhillon N, Aggarwal BB, Newman R, Wolff RA, Kunnumakkara AB, Abbruzzese JL, Ng CS, Badmaev V, Kurzrock R (2008) Phase II trial of curcumin in patients with advanced pancreatic cancer. Clin Cancer Res 14: 4491–4499.

Fuchikami H, Satoh H, Tsujimoto M, Ohdo S, Ohtani H, Sawada Y (2006) Effects of herbal extracts on the function of human organic-anion transporting polypeptide OATP-B. Drug Metab Dispos 34: 577–582.

Hatcher H, Planalp R, Cho J, Torti FM, Torti SV (2008) Curcumin: from ancient medicine to current clinical trials. Cell Mol Life Sci 65: 1631–1652.

Huang L, Wang Y, Grimm S (2006) ATP-dependent transport of rosuvastatin in membrane vesicles expressing Breast Cancer Resistance Protein. Drug Metab Dispos 34: 738–742.

Ireson C, Orr S, Jones DJL, Verschoyle R, Lim CK, Luo JL, Howells L, Plummer S, Jukes R, Williams M, Steward WP, Gescher A (2001) Characterization of metabolites of the chemopreventive agent curcumin in human and rat hepatocytes and in the rat *in vivo*, and evaluation of their ability to inhibit phorbol ester-induced prostaglandin E_2 production. Cancer Res 61: 1058–1078.

Ireson CR, Jones DJL, Orr S, Coughtrie MWH, Boocock DJ, Williams ML, Farmer PB, Steward WP, Gescher AJ (2002) Metabolism of the cancer chemopreventive agent curcumin in human and rat intestine. Cancer Epidemiol Biomarkers Prev 11: 105–111.

Kaminaga Y, Nagatsu A, Akiyama T, Sugimoto N, Yamazaki T, Maitani T, Mizukami H (2003) Production of unnatural glucosides of curcumin with drastically enhanced water solubility by cell suspension cultures of Catharantus roseus. FEBS Letters 555: 311–316.

Karlsson J, Wikman A, Artursson P (1993) The mucus layer as a barrier to drug absorption in monolayers of human intestinal epithelial HT29-H goblet cells. Int J Pharm 93: 209–218.

Khajuria A, Zutshi U, Bedi KL (1998) Permeability characteristics of piperin on oral absorption – an active alkaloid from peppers and a bioavailability enhancer. Indian J Exp Biol 36: 46–50.

Legen I, Kristl A (2001) Comparative permeability of some acyclovir derivatives through native mucus and crude mucin dispersions. Drug Dev Ind Pharm 27: 669–674.

Legen I, Kristl A (2003) Factors affecting the microclimate pH of the rat jejunum in Ringer bicarbonate buffer. Biol Pharm Bull 26: 886–889.

Liu A, Lou H, Zhao L, Fan P (2006) Validated LC/MS/MS assay for curcumin and tetrahydrocurcumin in rat plasma and application to pharmacokinetic study of phospholipid complex of curcumin. J Pharmaceut Biomed 40: 720–727.

Meaney C, O'Driscoll C (1999) Mucus as a barrier to the permeability of hydrophilic and lipophilic compounds in the absence and presence of sodium taurocholate micellar systems using cell culture models. Eur J Pharm Sci 8: 167–175.

Nozawa T, Irnai K, Nezu JI, Tsuji A, Tamai I (2004) Functional characterization of pH-sensitive organic anion transporting polypeptide OATP-B in human. J Pharmacol 308: 438–445.

Sharma RA, Euden SA, Platton SL, Cooke DN, Shafayat A, Hewitt HR, Marczylo TH, Morgan B, Hemingway D, Plummer SM, Pirmohamed M, Gescher AJ, Steward WP (2004) Phase I clinical trial of oral curcumin: biomarkers of systemic activity and compliance. Clin Cancer Res 10: 6847–6854.

Sharom FJ (2008) ABC multidrug transporters: structure, function and role in chemoresistance. Pharm 9: 105–127.

Shoba G, Joy D, Joseph T, Majeel M, Rajendran R, Sirinivas PSSR (1998) Influence of piperin on the pharmacokinetics of curcumin in animals and human volunteers. Planta Med 64: 353–356.

Tønnesen HH, Masson M, Loftsson T (2002) Studies of curcumin and curcuminoids. XXVII. Cyclodextrin complexation: solubility, chemical and photochemical stability. Int J Pharm 244: 127–135.

Tønnesen HH (2006) Solubility and stability of curcumin in solutions containing alginate and other viscosity modifying macromolecules. Pharmazie 61: 696–700.

Wahlang B, Pawar YB, Bansal AK (2011) Identification of permeability-related hurdles in oral delivery of curcumin using Caco-2 cell model. Eur J Pharm Biopharm 77: 275–282.

Wikman Larhed A, Björk E (1998) The influence of intestinal mucus components on the diffusion of drugs. Pharm Res 15: 66–71.

Wortelboer HM, Usta M, van der Velde AE, Boersma MG, Spenkelink B, van Zenden JJ, Rietjens IMCM, van Bladeren PJ, Cnubben NHP (2003) Interplay between MRP inhibition and metabolism of MRP inhibitors: the case of curcumin. Chem Res Toxicol 16: 1642–1651.

Yu LX, Amidon GL, Polli JE, Zhao H, Mehta MV, Conner DP, Shah VP, Lesko LJ, Chen ML, Lee VHL (2002) Biopharmaceutical classification system: the scientific basis for biowaiver extensions. Pharm Res 19: 921–925.

Zhang W, Lim LY (2008) Effects of spice constituents on P-glycoprotein-mediated transport and CYP3A4-mediated metabolism *in vitro*. Drug Metab Dispos 36: 1283–1290.