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Research paper

Anthranoid laxatives influence the absorption of poorly permeable drugs in human intestinal cell culture model (Caco-2)

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Abstract

Interactions between widely used anthranoid laxatives and other simultaneously administered drugs are not known. In this paper, the influence of rhein, danthron, sennidins A/B, sennosides A/B, and senna leaf infusion was investigated on the permeability of furosemide, ketoprofen, paracetamol, propranolol, verapamil, digoxin, and Rhodamine 123 across Caco-2 monolayers. The effects on monolayer integrity ([^{14}C]mannitol permeability, TEER) were also determined.

The *in vitro* absorption of highly permeable drugs was not strongly affected during co-administration of the laxatives. Furosemide permeability was enhanced by rhein and danthron (3.6 and 3.0-fold), which may partly be due to opening of the paracellular spaces and/or effects on active efflux. However, the secretory permeability of digoxin and Rho 123 was not strongly affected by rhein and danthron, suggesting that inhibition of MDR1 was not responsible for the increased permeation of furosemide. The absorptive permeability of digoxin was decreased by rhein and danthron, offering evidence for effects on apical membranes. The effects on monolayer integrity were detectable, but reversible. According to presented experiments, daily use of laxatives with well-absorbing drugs would seem unlikely to affect drug permeability, but the effects on the absorption of poorly permeable drugs cannot be excluded.

Keywords: Anthranoid laxatives; Sennosides; Caco-2 permeability; Absorption of drugs

Abbreviations: AP-BL, apical-to-basolateral; BL-AP, basolateral-to-apical; C_0, initial concentration (μM, nM); DMSO, dimethyl sulfoxide; dpm, disintegrations per minute; FBS, foetal bovine serum; HBSS, Hank's balanced salt solution; DMEM, Dulbecco's modified Eagle's medium; Hepes, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; HPLC, high-performance liquid chromatography; log *P*, octanol–water partitioning coefficient; MDR1, multidrug resistance protein 1; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; MRP, multidrug resistance associated protein; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolum bromide; NEAA, non-essential amino acids; P_{app}, apparent permeability coefficient (cm/s); pK_a, ionisation constant; Rho 123, rhodamine 123; TEER, transepithelial electrical resistance.

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1. Introduction

Anthranoid laxatives are widely used laxatives of natural origin. Sennosides, the most known anthranoid compounds, are extracted from the dried leaves and pods of senna plants *Cassia senna* L. (*Cassia acutifolia* Delile) and *Cassia angustifolia* Vahl. Other anthranoids, such as aloe-emodin, chrysophanol, and rhein, are obtained from *Rhamnus purshiana* DC and *Rheum palmatum* [1]. The basic structure of anthranoid laxatives is the anthracene ring (Fig. 1a), where C-9 is substituted with a hydroxyl or a carbonyl group. For the laxative function, C-8 is substituted with hydroxyl groups [2]. Based on the moiety present at C-10, anthranoids are called anthrones (H_2), anthraquinones (=O) or dianthrones (Fig. 1b).

In plants, the anthranoids are mostly present as sugar derivatives (=glycosides). Glucose or rhamnose molecules are bound by β-glycosidic linkages to the OH groups at C-8 or at C-1 (*O*-glycosides) (Fig. 1b). The β-glycosidic linkage between the sugar moiety and the anthranoid backbone stabilizes the molecule and protects it against hydrolysis in the stomach and α-glucosidase activity in the small intestine [3]. The glycosidic anthranoid laxatives are not absorbed from the small intestine. Bacterial enzymes, β-glycosidase and reductase, present in the large intestine transform the glycosidic sennosides first to sennidin monoglucosides and sennidin, and further to corresponding active aglycone anthrones, i.e., rhein anthrone, and rhein [2,4]. Unlike the glycones, which are absorbed after bacterial transformation to aglycones mainly in the large intestine, the orally ingested aglycones are absorbed in the upper parts of the gastrointestinal tract [5].

Direct interactions between rhein anthrone or anthraquinone (rhein, danthron) and the intestinal epithelium cause epithelial cell damage, which lead to motility changes and accelerated intestinal transit both in the small and large intestine [6,7]. Alterations in intestinal water absorption and secretion, which lead to fluid accumulation in the intestine [8,9], lead to further acceleration of intestinal transit. The effects on secretion and absorption are induced by a direct interaction between the laxative and the epithelial cells [10]. Additionally, anthranoids may cause decreased production of ATP by uncoupling mitochondrial oxidative phosphorylation [11]. Low ATP concentration and inhibition of the Na^+/K^+ ATPase lead to the breakdown of the ion gradient across the epithelial cell mem-

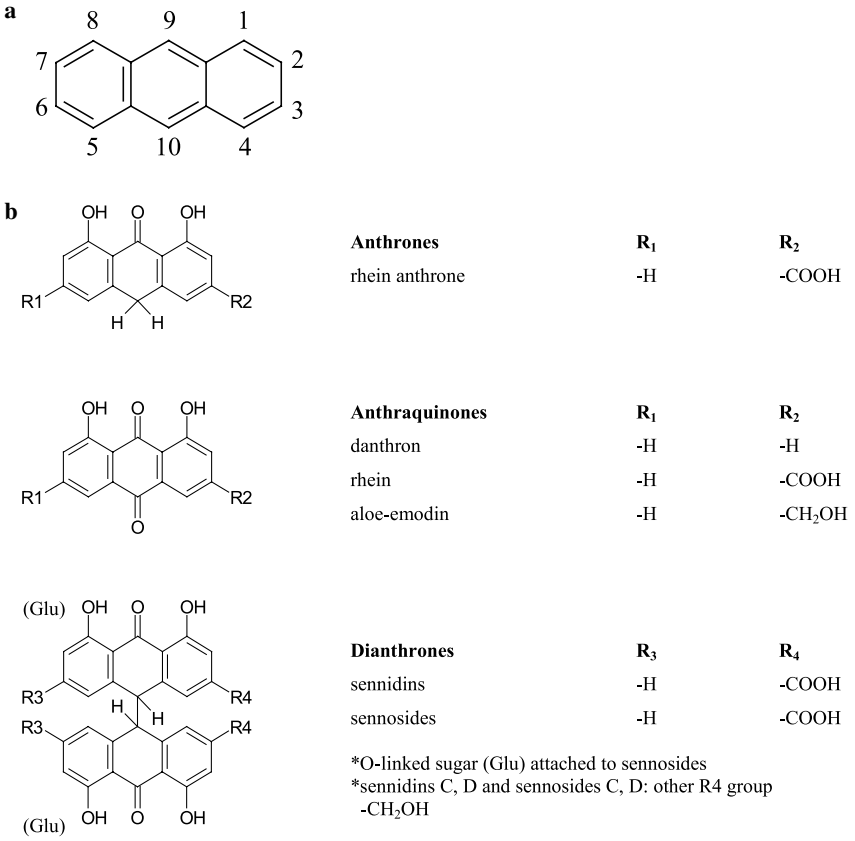


Fig. 1. (a) Anthracene ring, the basic structure of all anthranoid laxatives. (b) Three groups of anthranoid laxatives: anthrones with two hydrogen atoms in C-10, anthraquinones with carbonyl at C-10. Dianthrones consist of two linked anthrone molecules with or without O-linked sugar (=sennosides or sennidines, respectively). Two optic isomers can be distinguished depending on the configuration of the C-10; sennoside A is a dextrorotary *trans*-form and sennoside B is a *meso*-form.

brane. This prevents the absorption of sodium and water from the intestinal lumen to the blood, and may affect the permeability of co-administered drugs. Disruption of tight junctions between intestinal epithelial cells induces increased net secretion of water and electrolytes into the intestinal lumen [12]. This might inhibit the absorptive permeability of hydrophilic poorly absorbing drugs from the intestinal lumen or enhance the secretory permeability, even though a change of fluid flux from net secretion to net absorption has been shown not to increase the permeability of antipyrine, a highly permeable drug [13]. Although changes in water and electrolyte absorption and efflux in the small intestine caused by laxatives may be compensated for in the colon, they may affect the bioavailability of co-administered drugs at the main site of their absorption, the small intestine.

The aim of this paper was to probe whether anthranoid laxatives rhein, danthron, sennidin A/B, sennoside A/B (Fig. 1b), or a senna leaf infusion have the ability to affect the absorptive permeability of well-permeating drugs ketoprofen (active transport by monocarboxylic acid transporter), paracetamol (passive permeability), propranolol (passive permeability; interacts with MDR1), verapamil (substrate/inhibitor of MDR1), poorly permeating furosemide (active transport/efflux) and digoxin (active efflux by MDR1) across Caco-2 cell monolayers. Caco-2 cell cultures are widely used as *in vitro* model for intestinal absorption and secretion of nutritional and drug molecules [14–16]. These cell cultures are used as a model to investigate drug–drug (for example [17]), or drug–food interactions during the absorption phase [18,19]. Additionally, the effects of the anthranoid laxatives on the absorptive and secretory permeabilities of [^{14}C]mannitol (a paracellular marker molecule) and Rhodamine 123 (Rho 123) (paracellular absorption and MDR1-mediated active efflux) were determined.

2. Materials and methods

2.1. Materials

The anthranoid laxatives rhein, sennidin A/B, and sennoside A/B, purity 98%, with small amounts of sennosides C and D, were donated by Extracta Ltd. (Helsinki, Finland). Danthron was bought from ICN Biomedicals Inc. (Aurora, OH, USA) and senna leaves intended for laxative use were from Helsinki University Pharmacy (Helsinki, Finland).

Ketoprofen, propranolol, and verapamil were bought from ICN Biomedicals Inc. (Aurora, OH, USA). Furosemide and paracetamol were donated by Orion Pharma (Espoo, Finland). [^{14}C]mannitol (specific activity 58.0 mCi/ml) was bought from Amersham Pharmacia Biotech UK Ltd. (Amersham, England). Rhodamine 123 (Rho 123) was purchased from Fluka Chemie GmbH (Buchs, Switzerland) and [^{3}H]digoxin (specific activity 21.8 Ci/mmol) was from Perkin-Elmer Life Sciences (Boston, MA, USA). All chemicals for cell culturing were purchased from Gibco Invitrogen Corp. (Life Technologies Ltd., Paisley, Scotland) or from Sigma Chemical Co. (St. Louis, MO, USA). All of the organic solvents and other chemicals used in the analyses were of analytical or chromatographic grade and were bought from Riedel-de Haën (Seelze, Germany), Rathburn (Walkerburn, Scotland), Merck (Darmstadt, Germany), or ICN Biomedicals Inc. (Aurora, OH, USA).

2.2. Cell culture

The Caco-2 cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultivated as described earlier [17,19] and seeded at a density of 6.8×10^4 cells/cm^2 onto tissue culture inserts (Transwell 3401, 12 mm diameter, 0.4 μm pore size, 1.1 cm^2 growth area polycarbonate filters, Corning Costar Corp., Cambridge, MA). Cells from passage numbers 31 to 42 at ages ranging from 21 to 28 days were used for the transport experiments.

2.3. Preparation of the solutions

The studied compounds ketoprofen, paracetamol, propranolol, verapamil, and furosemide were dissolved in HBSS (pH 5.8) to a concentration of 200 μM (100 μM Rho 123). After dissolution, the pH of the solution was re-adjusted, if needed. 1.6 μCi/ml [^{3}H]digoxin and 1.2 μCi/ml [^{14}C]mannitol in HBSS were prepared.

For drug-permeability experiments, the anthranoid laxatives (rhein, danthron, sennidin and sennoside) were first dissolved in DMSO. These solutions were added to HBSS, resulting in 200 μM laxative concentrations and 2% DMSO content. The senna leaf infusion (senna tea) was prepared according to package instructions: the senna leaves were infused for 15 min in boiling water and filtered after cooling to room temperature. HBSS 10× concentrate was added to final senna leaf concentration 20 mg/ml. All test solutions contained 10 mM Mes.

Prior to starting the experiment, the anthranoid and drug solutions were combined (1:1), and the pH of the final test solutions was measured and corrected, if needed.

2.4. MTT toxicity test

MTT test (a colorimetric assay) can be used to determine cell viability (mitochondrial activity) by measuring the extent of formazan formation after lysis of the living material and solubilisation of formazan crystals [20]. The seeding of Caco-2 cells and test procedure is explained in [19]. The cells were exposed over 60 min to ethanol (0.25–30%, v/v), 90 min to DMSO (0.2–35%, v/v), the drugs (1.0 and 10.0 μM, digoxin; 100 and 500 μM, other drugs), or laxatives (different concentrations, from 50 to 500 μM; 2.50, 5.00, and 10.00 mg/ml, senna infusion), at 37°C. Results ($n = 6$–8) were expressed as percentages of the control value (cells treated with HBSS only).

2.5. Permeability experiments

All of the permeability experiments were performed under "sink conditions", meaning that amounts of compound transported to the acceptor compartment during individual sampling intervals did not exceed 10% of the amounts in the donor compartment.

Before the permeability experiments, the cell monolayers were washed twice with HBSS containing 10 mM Hepes, pH 7.4. After equilibration in the experimental conditions, the transepithelial electrical resistance (TEER) was measured using a Millicell® ERS Voltohmmeter (Millipore Corp., Bedford, MA, USA). Cell monolayers with TEER values below 250 $\Omega\,cm^2$ were not used.

The abilities of 100 μM ketoprofen, paracetamol, propranolol, verapamil, and furosemide to permeate across the Caco-2 monolayers without or with co-administration of 100 μM laxatives (senna infusion 10 mg/ml) were studied in an absorptive (apical-to-basolateral, AP-BL) direction at an apical pH of 5.8 and a basolateral pH of 7.4. For Rho 123, 50 and 5 μM donor concentrations were used in AP-BL and secretory (basolateral-to-apical, BL-AP) directions, respectively. For [^{3}H]digoxin, solutions with 0.8 μCi/ml activity were used in both directions.

After equilibration, the apical solutions were changed to HBSS containing the compounds without or with the laxatives. Samples were collected after 15, 30, 45, 60, and 90 min (ketoprofen, paracetamol, propranolol, and verapamil), or after 30, 60, 90, and 120 min (furosemide and digoxin) by moving the cell monolayers to a new well containing fresh HBSS. In the case of digoxin, BL-AP samples were collected by removing the whole acceptor (apical) volume and replacing it with fresh HBSS solution. All of the transport experiments were conducted in triplicate. Samples were kept at −22°C until analysed (for no longer than 35 days).

In Rho 123 experiments, a 60-min pre-incubation was performed with the anthranoids before the actual permeability test. After the pre-incubation, the laxatives were removed from the apical compartment, the cell monolayers were washed once with fresh HBSS (apical + basolateral compartments), and Rho 123 in HBSS was added in donor compartments. Samples were collected after 15, 30, 45, 60, 90, and 120 min by moving the monolayers to a new well with fresh pre-warmed HBSS (AP-BL), or by removing the whole acceptor (apical) volume and replacing it with fresh HBSS solution (BL-AP).

To determine monolayer integrity after each experiment, the cell cultures were washed once with HBSS, pH 7.4, and TEER values were measured. If values were below 220 $\Omega\,cm^2$, the cell monolayers were further incubated with HBSS, and electrical resistance was measured again after 60 min. Monolayer integrity was further assessed after the drug-permeability tests with [^{14}C]mannitol. The apical washing solution was changed to test solution with [^{14}C]mannitol solution (0.6 μCi/ml, pH 5.8). After 60 min, 100 μl samples were withdrawn from the basolateral compartments for the activity measurements. Diffusion rates $\leqslant$0.5%/h were considered as "normal".

2.6. Long-term effects on monolayer integrity

In order to study long-term effects of the anthranoid laxatives on the paracellular permeability of the monolayers, an 8-h experiment with [^{14}C]mannitol was performed. The AP-BL and BL-AP permeability of [^{14}C]mannitol was monitored after 60-min pre-exposure to 100 μM laxative concentrations (senna infusion at 10 mg/ml). TEER of the monolayers was measured at $t = -5$, 30, and 55 min from the start of the pre-exposure. After 60 min, the laxative solutions were removed from the donor compartments and mannitol in DMEM (without additives used in growth medium) buffered to pH 5.8 (10 mM Mes) was added to the donor compartment, and DMEM buffered to 7.4 (10 mM Hepes) was added to the acceptor compartments. Mannitol samples were collected after 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 360, 420, and 480 min and TEER was measured at time points 60, 120, 180, 240, 300, 360, 420, and 480 min. Cumulative sample concentrations (dpm, ^{14}C-disintegrations per minute) between time points 90 and 270–300 min were used for permeability calculations.

2.7. Analytical methods

Drug concentrations in the acceptor compartments were determined using HPLC (Waters Millennium, Milford, USA). The determination conditions are explained in [19]. Propranolol was determined using same method with verapamil. Rho 123 concentrations in samples were determined by Wallac Victor^2 1420 Multilabel HTS Counter (Wallac, Turku, Finland), [^{14}C]mannitol and [^{3}H]digoxin with liquid scintillation counting, using a WinSpectral 1414 Liquid Scintillation Counter (Wallac, Turku, Finland), described elsewhere [19].

2.8. Data analysis

The AP-BL and BL-AP permeability (apparent permeability coefficient, P_{app}) of each compound was calculated according to the following equation where

$$P_{app}\ (cm/s) = \frac{dQ}{dt}\left(\frac{1}{C_0 A}\right), \quad (1)$$

where dQ/dt is the cumulative rate of appearance of drugs on the acceptor side (μmol/s or nmol/s), C_0 is the initial drug concentration on the donor side (μmol/ml or nmol/ml), and A is the surface area (1.1 cm^2) of the monolayers.

The results were tested statistically using unpaired t-test combined with Dunn–Sidak Adjusted Probability and Bonferroni Adjusted Probability test using SYSTAT® version 10.2 for Windows® (SYSTAT Software Inc., Richmond, CA, USA). Significance level of 5% was used.

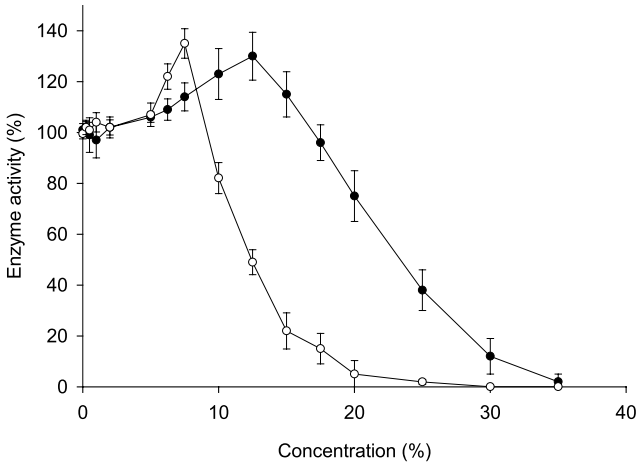


Fig. 2. Effects of DMSO (●) and ethanol (○) on the intracellular dehydrogenase activity of Caco-2 cells (means ± SD, $n = 4$). The cells were exposed to DMSO for 90 min and to ethanol for 60 min.

3. Results and discussion

3.1. MTT test

The effects of ethanol, DMSO, the drugs studied, and of the anthranoid laxatives on the mitochondrial dehydrogenase activity were studied on Caco-2 cell monolayers prior to the transport experiments. Slightly increased enzyme activities were observed at ethanol and DMSO concentrations (v/v) 5.0–10% and 5.0–15.0%, respectively, indicating sub-toxic effects on the cell line (Fig. 2). In order to ensure cell viability during the permeability experiments, the concentration of DMSO was restricted to 1.0% in the laxative solutions containing rhein, danthron, sennidin, and sennoside. Ethanol was not used as a solubility enhancing excipient in the experiments.

No toxic effects in relation to mitochondrial enzyme activity were seen with any of the studied drugs even at 500 μM concentration (Table 1), whereas laxatives caused a clear reduction in mitochondrial enzyme activity at higher concentrations (⩾250 μM). In order to ensure the solubility of the laxatives at higher concentrations, the concentration of DMSO in the MTT test was 2.5% (v/v). This should, however, not compromise the results as DMSO effects at this concentration are still minor. The laxative concentrations for the permeability experiments were chosen according to the above results: rhein, danthron, sennidin A/B, and sennoside A/B were used at 100 μM (including 1.0% DMSO). The senna infusion exhibited no toxicity at the concentrations tested (2.5, 5.0 or 10.0 mg/ml) (Table 1). In the subsequent permeability experiments, the concentration of the senna infusion was 10.0 mg/ml.

Table 1
The MTT test

Compound	Concentration (μM)	Enzyme activity (%)
Digoxin	0.1	105 ± 8.0
	1.0	97 ± 2.9
Furosemide	100	101 ± 11
	500	102 ± 12
Ketoprofen	100	101 ± 3.1
	500	106 ± 3.1
Paracetamol	100	107 ± 2.0
	500	101 ± 2.2
Propranolol	100	97 ± 3.7
	500	101 ± 3.2
Verapamil	100	105 ± 2.6
	500	99 ± 9.0
Rho 123	100	98 ± 5.3
	500	96 ± 6.3
Rhein[a]	50	91 ± 5.9
	100	89 ± 9.2
	250	88 ± 8.9
	500	45 ± 9.9
Danthron[a]	50	95 ± 7.8
	100	89 ± 25
	250	32 ± 5.4
	500	28 ± 11
Sennidin A/B[a]	50	97 ± 19
	100	85 ± 22
	250	54 ± 27
Sennoside A/B[a]	100	102 ± 13
	250	111 ± 15
	500	45 ± 11
Senna infusion	2.50[b]	106 ± 23
	5.00[b]	102 ± 15
	10.00[b]	92 ± 12

Caco-2 cells (16×10^4 cells/cm^2) were exposed to the compounds studied and the anthranoid laxatives for 90 min. Results are expressed as percentage of control value (100%) obtained after exposure to HBSS only (means ± SD, $n = 8$).
[a] With 25 mg/ml DMSO.
[b] Concentration mg/ml.

3.2. Drug permeability

The abilities of the highly permeable drugs ketoprofen, paracetamol, propranolol, and verapamil to permeate across Caco-2 cell monolayers were to some extent affected by the anthranoid laxatives (Fig. 3). The permeability of paracetamol (pK_a 9.63, acid), a compound permeating mainly by passive transcellular diffusion, was one of the least affected besides propranolol and verapamil. As a very weak acid, which is practically 100% unionised at the apical pH 5.8, it should also be fairly insensitive to any small changes in the pH potentially caused by the co-administered compounds, especially since a further acidification is more likely.

The permeability of ketoprofen, which is, at least in part, actively transported by a pH-dependent carrier-mediated H^+ co-transporter in the AP-BL direction [21,22], was most affected by danthron (27% reduction) and the senna infusion (48% increment) (Fig. 3). Ketoprofen, a lipophilic acid, has a calculated log $D_{5.8}$ of 1.34 (based on pK_a 3.98,

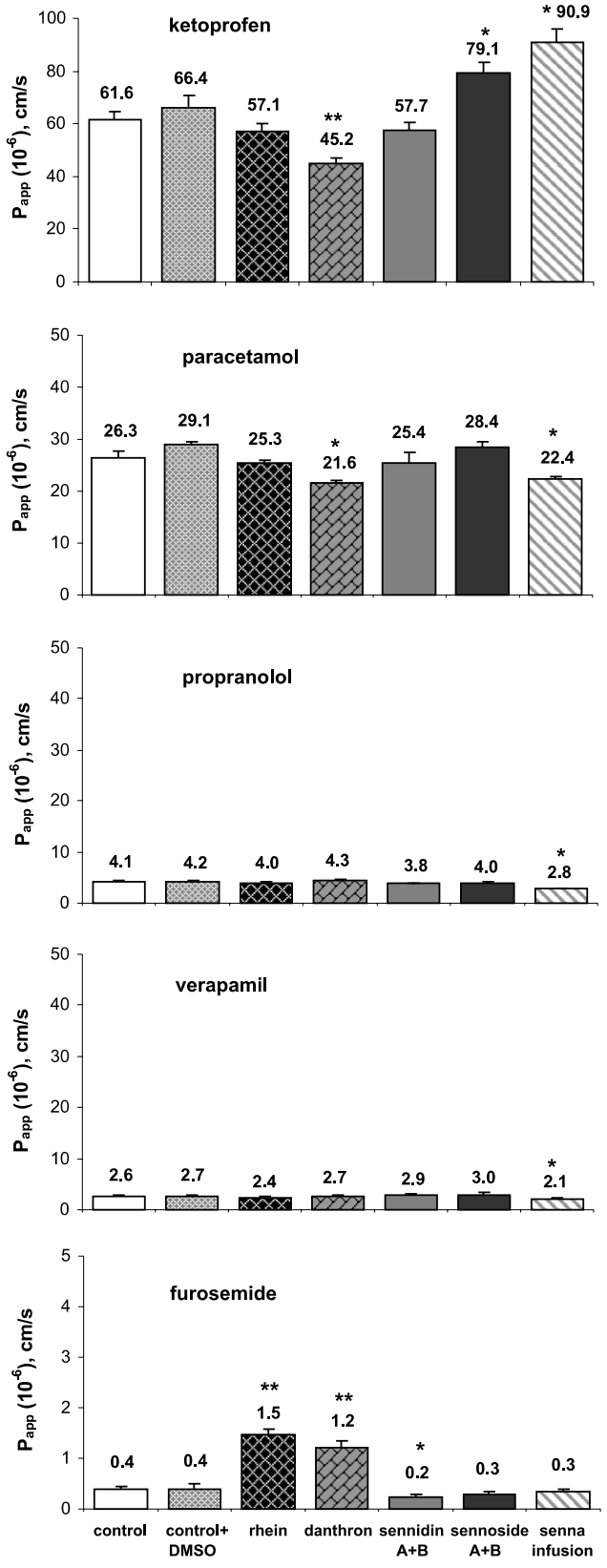


Fig. 3. P_{app} values for 100 μM ketoprofen, paracetamol, propranolol, verapamil and furosemide with co-administration of HBSS only (control), DMSO as a solvent in HBSS, rhein, danthron, sennidin, sennoside and senna infusion across Caco-2 cell monolayers (means ± SD, $n = 3$–4). $^{*}p < 0.05$; $^{**}p < 0.01$.

log P 3.16 [23]), which as such would suggest fair passive absorptive permeability despite a high extent of ionisation. Anthranoids, such as danthron, are supposed to have the ability to uncouple mitochondrial oxidative phosphorylation, leading potentially to a decreased ATP production [24,11]. The pH-dependent carrier-mediated H^+ co-transporter might suffer from low ATP concentrations leading to diminished active transport. The reduced permeability values in the presence of danthron, 45.2 ± 1.7 (10^{-6}) cm/s, compared with the control values, 61.6 ± 2.8 (10^{-6}) cm/s, could therefore be explained by the reduction in ATP concentrations caused by danthron in Caco-2 cells.

The senna infusion contains mainly sennosides A and B, small amounts of sennosides C and D, aloe-emodin, rhein 8-glucosides, mucilage, flavonoids (kaemferol, its respective glycosides kaemferin and isorhamnetin), and naphthalene precursors, but also salicylic acid and oxalate [1,25] and resin, saponins, and polysaccharide hydrocolloids [26,1]. This infusion had the ability to enhance ketoprofen permeability almost 1.5-fold. Since senna leaves contain acids, which are stronger than ketoprofen (salicylic acid, oxalate), but also many active compounds capable of interacting with co-administered actively transported drugs, it is not easy to explain what could happen between drugs and these compounds. Small regional changes in the pH of the transport buffer at the site of absorption might cause changes in the permeabilities of co-administered drugs by strengthening the H^+ gradient across the Caco-2 cell membranes.

Propranolol and verapamil, which are considered MDR1 efflux transport substrates [27,28], were affected by co-administered senna infusion (Fig. 3). Some anthracyclines, which also belong to anthraquinones and are used in cancer treatment, are determined as MDR1 and/or MRP1 substrates [29]. The permeability of not only anthracyclines, but also other "natural" drugs (such as rhein and danthron), might be affected by the efflux proteins. No considerable effects of rhein or danthron were, however, detectable on propranolol and verapamil permeability (Fig. 3). For many substrates of MDR1, passive permeability dominates at high drug concentrations, if both the affinity to the MDR1 protein and the passive permeability are high as in the case of verapamil [30]. However, more recent studies do not confirm propranolol as a substrate for MDR1; it is considered a potential modulator (activator) of MDR1-associated ATPase activity [31,32]. Thus, the slight permeability reducing effect of the senna infusion might be due to small regional acidification of the transport buffer at the site of absorption, leading to increased degree of ionisation of the basic compounds.

The absorption of furosemide, a poorly permeable drug, is a mixture of trans- and paracellular diffusion and saturable active transport mediated potentially by several efflux proteins [33–35]. The actual efflux proteins involved in the poor absorption of furosemide have not been positively identified, but MDR1, MRP1, and/or MRP2 have been suggested. Rhein and danthron enhanced furosemide per-

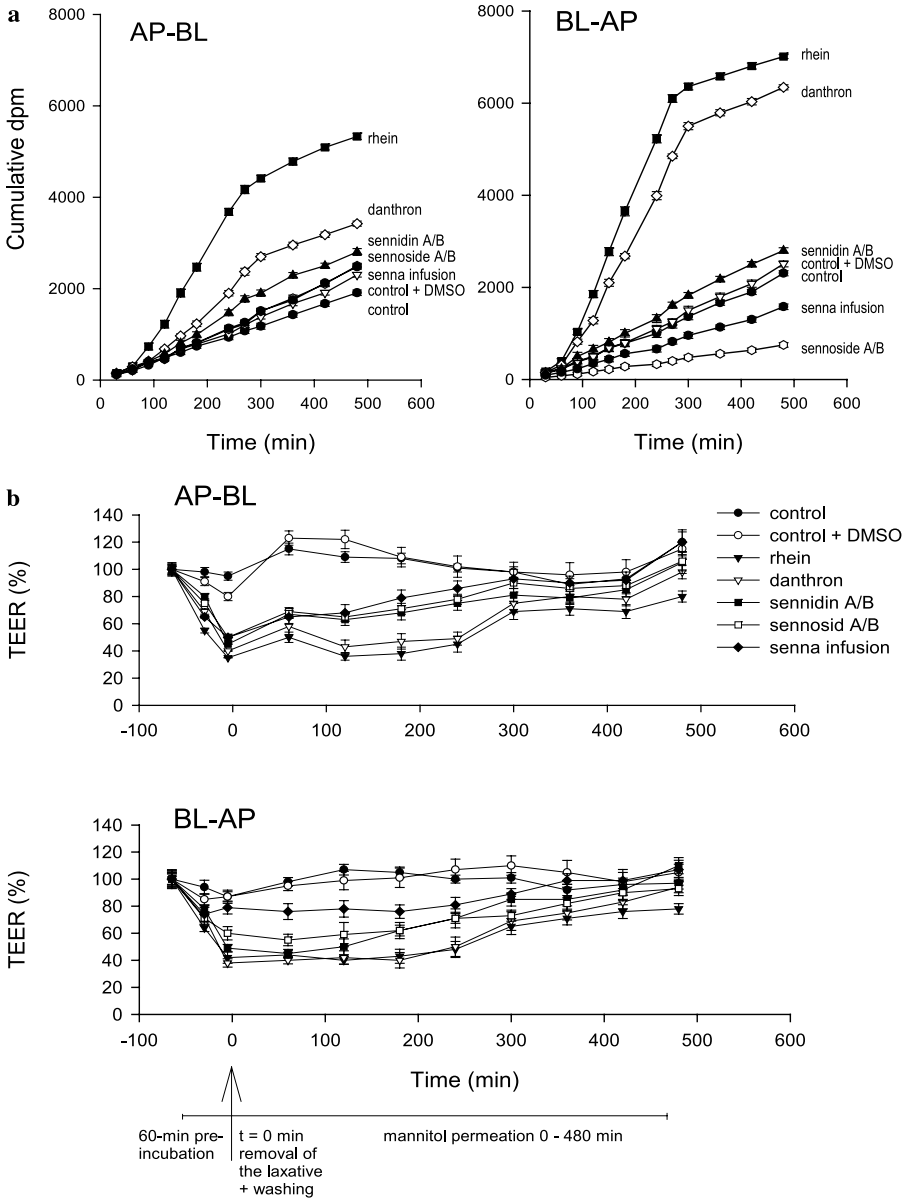


Fig. 4. Long-term effects of laxatives on monolayer integrity. The cell monolayers were pre-treated (60 min) with 100 μM rhein, danthron, sennidin A/B, sennoside A/B, or 10 mg/ml senna leaf infusion. At time point 0, the laxatives were removed and the monolayers washed with buffered DMEM, pH 7.4. The donor transport medium was changed to DMEM + [^{14}C]mannitol buffered to pH 5.8 (apical) or pH 7.4 (basolateral), and the acceptor medium to buffered DMEM, respectively. (a) Mannitol permeability. The cumulative paracellular diffusion of [^{14}C]mannitol (measured as disintegrations/min), dpm, after pre-treatment with HBSS only (control), 1% DMSO in HBSS (control + 1.0% DMSO), 100 μM rhein, danthron, sennidin, sennoside and 10 mg/ml senna infusion (means ± SD, $n = 3$). (b) % changes in monolayer electrical resistance (TEER; means ± SD, $n = 3$) after 60 min pre-treatment with HBSS only (control), 1% DMSO in HBSS, rhein, danthron, sennidin, sennosides and senna infusion, and during the 8-h mannitol permeability experiment. Initial TEER values prior to pre-incubation were used as 100%.

meability 3.6- and 3.0-fold, respectively (Fig. 3). This might be caused by interactions with the efflux protein(s), if the anthranoids had higher affinity to the secretory protein than furosemide. If the paracellular permeability were enhanced, opening of the paracellular spaces between Caco-2 cells would have been observed as decreased TEER values and enhanced mannitol permeability. Indeed, mannitol permeability was enhanced to 0.6 and 0.55%/h (control 0.2%/h) during the furosemide assay when co-administered with rhein and danthron, respectively (data not shown). TEER values decreased reversibly to about 40% as indicated also in Fig. 4b. However, due to the high acid strength of furosemide (determined pK_{a1} 3.70, pK_{a2} 9.93) and the presence of a pH gradient across the cell monolayer (pH 5.8 vs 7.4), a significant fraction is absorbed transcellularly at the used pH [33]. A partial opening of the paracellular spaces would, therefore, not lead to a very strong enhancement of permeability, since the paracellular route represents a relatively small fraction of the accessible absorptive area. Hence, inhibition of efflux would be likely

to contribute to the enhanced furosemide permeability in the presence of rhein and danthron.

Furosemide permeability was decreased by sennidin to about 60% and by sennoside to 70% of the control value (Fig. 3). Tight junctions between Caco-2 cells were to some extent affected, as indicated by TEER measurements (about 60% of the control values, Fig. 4b) and 60-min mannitol diffusion experiment after the permeability assay (0.32 and 0.30%/h, data not shown), but these changes would be more indicative of increased permeability. However, studies probing the transporters involved in furosemide secretion show decreased permeation of furosemide in both absorptive and secretive direction in the presence of sulfinpyrazone, an inhibitor of the efflux proteins MRP1 and 2 [34,35].

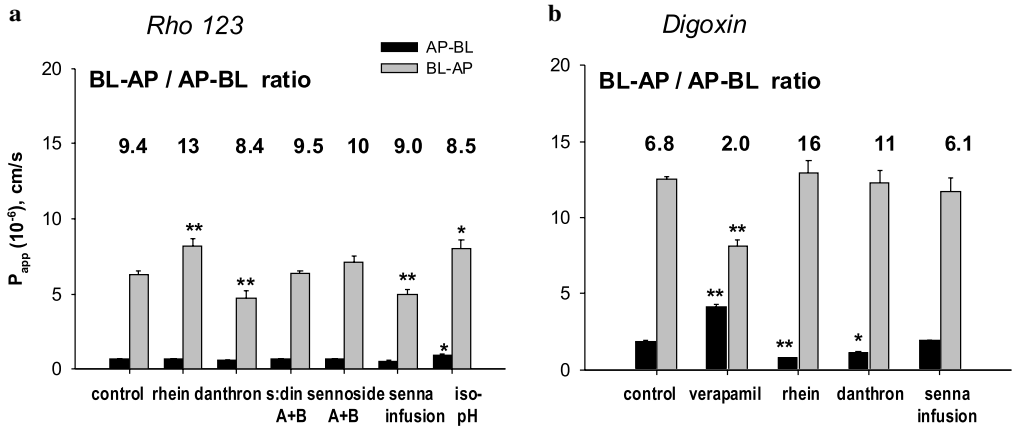
Rho 123 is a substrate of MDR1 efflux and its absorptive permeability is quite low. According to Troutman and Thakker [36], the AP-BL permeability is passive and mainly paracellular, with little contribution from transcellular diffusion. The permeability of Rho 123 without laxative pre-incubation was 0.67 ± 0.06, and 6.25 ± 0.52 (10^{-6}) cm/s (pH gradient) in AP-BL and BL-AP direction, respectively, which indicates the presence of active efflux (Fig. 5a). At equal pH conditions (iso-pH 7.4), the AP-BL permeability was 0.94 ± 0.06 and BL-AP permeability 8.0 ± 0.7 (10^{-6}) cm/s (Fig. 5a), which results in 8.5-fold directional difference. This offers evidence for the fact that the pH conditions are not responsible for the differences in absorption and excretion of Rho 123.

The AP-BL permeability of Rho 123 was not affected during the experiment time by any of the laxatives, the P_{app} values were very close to each other (Fig. 5a). If enhancement of paracellular absorptive permeability were noticed, low TEER values would indicate for opening of the paracellular spaces. This did indeed partly happen; TEER values measured after the pre-incubation with the anthranoids were about 50% of the initial value (Fig. 4b), but after the Rho 123 permeability assay TEER values were almost 90% and 80% in AP-BL and BL-AP experiments, respectively (Fig. 5c). According to Troutman and Thakker [36], the paracellular absorptive permeability of Rho 123 is not affected by MDR1-mediated efflux activity.

Rhein enhanced significantly (30%) the BL-AP permeability of Rho 123, whereas danthron and senna infusion inhibited it (25% and 20%, respectively). This was noticed as a slightly reduced ratio between BL-AP and AP-BL permeabilities ($P_{app[BL\text{-}AP]}$ vs $P_{app[AP\text{-}BL]}$) for danthron (8.4) and senna leaf infusion (9.0). Other laxatives had no effect on the secretory permeability of Rho 123 (Fig. 5a).

Digoxin is a well-known substrate of MDR1 [37]. MDR1 inhibitors, such as verapamil and GW918, have been shown to increase absorptive and decrease secretory permeability of digoxin [36]. Additionally, there is some evidence to suggest that other efflux proteins may partly contribute to digoxin secretory permeability [38].

The permeability of [^{3}H]digoxin was not affected by sennidines or sennosides (data not shown). Two hundred micromolar of verapamil enhanced AP-BL and decreased



c TEER, % of the control value (S.D.)

	control	rhein	danthron	sennidin A+B	sennoside A+B	senna infusion
AP-BL	95 (2.6)	62 (2.7)*	86 (3.9)*	86 (6.7)*	89 (9.2)	85 (4.3)
BL-AP	98 (5.2)	69 (3.8)*	105 (5)*	81 (6)	77 (6.3)*	89 (7.3)

Fig. 5. Apparent permeabilities (P_{app}; means ± SD, $n = 3$–4) of Rho 123 and [^{3}H]digoxin across Caco-2 cell monolayers at apical pH 5.8 and basolateral pH 7.4. The permeability ratios $P_{app[BL\text{-}AP]}$ vs $P_{app[AP\text{-}BL]}$ are also indicated. (a) After 60-min pre-incubation with HBSS only (control), 100 μM rhein, danthron, sennidin, sennoside, and 10 mg/ml senna leaf infusion and at iso-pH conditions (with HBSS, when both apical and basolateral pH was 7.4). (b) Without (control) or with 200 μM verapamil, 100 μM rhein, danthron and 10 mg/ml senna infusion. (c) % changes in monolayer electrical resistance (TEER) after Rho 123 AP-BL and BL-AP permeability experiments. Initial TEER value at the start of the experiment (prior to pre-incubation) is 100% (means ± SD, $n = 3$).

BL-AP permeability of [^{3}H]digoxin (Fig. 5b), as expected. Rhein and danthron were able to decrease the AP-BL permeability of digoxin, but the BL-AP permeability was not affected (Fig. 5b). This leads to high BL-AP/AP-BL ratio (6.8, 16, and 11 for control, rhein, and danthron, respectively). Rhein and danthron are relatively small (m.w. < 300 g/mol) planar molecules (Fig. 1b), and are therefore possibly able to intercalate with cell membranes. This leads to changes in membrane fluidity (possibly to increased membrane rigidity) and effects capability of drugs to interfere with MDR1 in the apical membranes. This would not affect the secretory permeability, because in this case drugs enter apical membranes (and the efflux protein) from the basolateral side.

The mechanism behind the interactions during absorption between the senna infusion and drugs is not easily detected because of several active compounds present in the infusion. Besides several compounds of laxative effect, the infusion also contains flavonoids, such as kaemferol and respective glycosides kaemferin and isorhamnetin [25], which are able to interact with active transport proteins and cell membranes [39,40].

3.3. Long-term effects on monolayer integrity

The apparent permeabilities for mannitol without laxative pre-incubation were 0.48 ± 0.02 (AP-BL) and 0.52 ± 0.01 (BL-AP) (10^{-6}) cm/s during the 480 min assay, calculated between time points 90 and 270 min (Fig. 4a). Inclusion of 1% DMSO in the 60-min pre-treatment (apical compartment) solution (control + 1% DMSO) did not significantly enhance mannitol permeability. Pre-treatment with 100 μM rhein with 1% DMSO in HBSS enhanced long-term mannitol permeability; P_{app} values were 2.30 ± 0.23 and 3.38 ± 0.13 (10^{-6}) cm/s for AP-BL and BL-AP permeabilities, respectively. Corresponding P_{app} values after danthron pre-treatment were 1.19 ± 0.09 and 2.69 ± 0.11 (10^{-6}) cm/s. Characteristic for all enhanced mannitol permeabilities was that after the first 60 min of almost control-like permeability, a clear step for higher permeability was detected (Fig. 4a). Same phenomenon was observed also during cumulative AP-BL permeability of Rho 123 (paracellular route) after pre-incubation with rhein (data not shown). This phenomenon could be explained by the possibility of rhein and danthron to intercalate with apical cell membranes, leading to additional effects on paracellular spaces when increased rigidity of the membranes might inhibit normal flexibility of the paracellular spaces. However, again after 300 min, mannitol permeability evened out and the final slope (300–480 min) of the cumulative permeability curve was similar to that of the control (Fig. 4a). This happens because laxative molecules slowly leave the cell membranes. Sennidin pre-treatment caused only a slight enhancement in the P_{app} values (1.9- and 1.4-fold enhancements in AP-BL and BL-AP permeabilities) during the 8-h experiment. Sennoside A/B or senna infusion pre-treatment did not affect the absorptive permeability of mannitol (1.2-fold enhancement), but the BL-AP permeability was decreased to 35% (sennoside) and 71% (senna infusion) compared to the control value.

Transepithelial electrical resistance (TEER) of the monolayers was followed over the 8-h mannitol permeability experiment (Fig. 4b). As expected, high mannitol permeability is connected with low TEER values, indicating opening of the paracellular spaces between the Caco-2 cells. Rhein and danthron caused a strong decrement in the TEER values. However, all cell monolayers recovered during the 8-h experiment showing almost control-like values at the end of the experiment. This is indeed consistent with the results in mannitol permeability. Sennidin, sennoside and senna infusion caused also a strong decrement in the TEER, to about 55% of the control value, but the recovery of the monolayers was fast (at $t = 180$ min up to 75%), and therefore only slight effects on mannitol permeability were observed.

Anthraquinone laxatives have the ability to enhance fluid secretion to the small and large intestine [8,9]. This is caused by the disruption of tight junctions between colonic epithelial cells [12]. Mannitol, being a small paracellularly diffusing molecule (m.w. 182 g/mol), is a good marker molecule for tight junctional integrity of cell monolayers [15].

It has been observed that secretion of water, electrolytes, and [^{14}C]erythritol (a paracellular permeability marker molecule) into the rat colon happens 6 h after ingestion of sennosides [41]. In those experiments, it took several hours for sennosides to enter the colon, where bacterial β-glucosidase breaks down the β-glycosidic linkages between the glucose and the anthranoid molecules. In our experiments, sennosides did not cause prominent elevation of [^{14}C]mannitol permeability, but pre-treatment with sennidin (sennoside molecule without glucose moiety) caused a slight increment in AP-BL permeability. Because β-glucosidase was not present in our experiments, sennidin should act on Caco-2 monolayers as sennosides do when the glucose molecule is cleaved from the main dianthrone molecule in the colon. In fact, the TEER values had decreased by 40–45% after the sennidin and sennoside pre-treatment (Fig. 4b), but the cells recovered almost completely within 180 min, and the final TEER values were very close to the original values.

4. Conclusions

The abilities of highly permeable drugs paracetamol, propranolol, and verapamil to diffuse across Caco-2 cell monolayers were not strongly affected by any of the anthranoid laxatives. The effects of danthron and rhein on active transport of drugs may be due to reduced ATP production in Caco-2 cells, or by their affinity to intercalate with cell membranes, leading to changes in membrane fluidity. The effects of senna infusion on drug permeability are

variable, depending on several active compounds present in the infusion. The enhancement of paracellular permeability of drugs was evidenced by mannitol long-term assay and TEER measurements. Laxatives rhein and danthron were able to reversibly open the paracellular spaces between Caco-2 cells, leading to enhanced mannitol permeability and decreased TEER.

Our results indicate that anthranoid laxatives and drugs with high passive permeability can be contemporaneously ingested. However, if poorly permeable drugs are administered with anthranoid laxatives, effects on drug permeabilities cannot be predicted. *In vitro* experiments offer valuable information about possible interactions during absorption.

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