Effects of Permeation Enhancers on the Transport of a Peptidomimetic Thrombin Inhibitor (CRC 220) in a Human Intestinal Cell Line (Caco-2)

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Purpose. The effects of five different permeation enhancer systems on the transport properties of a peptidomimetic thrombin inhibitor, CRC 220, were investigated in monolayers of a human intestinal cell line (Caco-2).

Methods. The transepithelial transport rates and additionally the cytotoxic properties of these enhancers were characterized using the following tests: measurement of the transepithelial electrical resistance (TEER), the MTT-transformation, the protein content and the release of cytosolic lactate dehydrogenase (LDH), as well as FITC-phalloidin and propidium iodide staining.

Results. All permeation enhancer systems showed concentration-dependent effects on cell permeability and toxicity. The most prominent effects on peptide transport were seen at the highest concentration (40 mM), yielding the rank order, NaTC > NaTC/Cholesterol > Solulan C24 > NaTC/Oleic acid > NaTC/PC18. Using the TEER after 120 min exposure as the most sensitive parameter describing cytotoxicity, the following order was obtained: Solulan C24 > NaTC > NaTC/PC18 = NaTC/Cholesterol > NaTC/Oleic acid > NaTC/PC. Generally, efficient enhancement of peptide transport was associated with a noticeable influence on cell viability under in-vitro conditions.

Conclusions. Taking into account permeation and cytotoxicity as a function of concentration, both NaTC at 15 mM and the mixed micellar system NaTC/oleic acid at 0.75 mM offer interesting enhancement properties, showing an 18-fold increase in CRC 220 transport rates. The effects on cell viability and cytotoxicity were comparatively low and of reversible nature.

KEY WORDS: peptide transport; permeation enhancers; micellar systems; cell monolayers; cytotoxicity.

INTRODUCTION

Peroral administration of peptides and proteins would offer significant advantages over the parenteral application, especially in the longterm treatment of chronic diseases (1). Biologically active peptides have become available in larger amounts recently. The limiting factors for the peroral delivery remain the low rate and extend of gastrointestinal absorption, the metabolic lability and hence a low and highly variable oral bioavailability (2).

To achieve therapeutically relevant plasma levels, absorption or permeation enhancing systems are necessary. Although many absorption enhancers were studied under in vivo and in vitro conditions, the enhancer mechanisms are not yet fully understood. As absorption promoters, surfactants, bile salts, fatty acids, derivates of carnitines or of fusidic acid, mixed micelles containing various substances and cyclodextrines (3) have been proposed for further investigations.

Recently new low-molecular-weight thrombin inhibitors with peptidomimetic properties have been synthesized (4). Their oral bioavailability in rats was low (5) and an increase of the absorption would be desirable for further investigations. The transport rate of the most promising candidate, CRC 220 (MG 637), was selected to investigate different "permeation" enhancers in an in-vitro cell culture system. These studies were conducted using the Caco-2 cell culture model.

In this study we investigated the effect of sodium taurocholate (NaTC) and NaTC mixed micelles containing oleic acid, cholesterol or phosphatidylcholines on the in-vitro transport of the thrombin inhibitor CRC 220. All components of the mixed micellar system are of physiological origin and pose little toxicological concerns (3). In addition, a synthetic non-ionic surfactant with a steroid structure similar to bile acids, polyoxyethylene-24-cholesterol-ether (Solulan C-24) was selected. We examined the effects on the monolayer, to ascertain whether surfactant-induced absorption enhancement and cell toxicity are correlated. The reversibility of the cell damage was evaluated and the optimal effective concentration of each absorption enhancer system was determined. From the results obtained in transport and toxicity studies it was hoped to gain insight into the mechanism of absorption enhancement.

MATERIALS AND METHODS

Materials

The thrombin inhibitor CRC 220 (4-methoxy-2,3,5-trimethylphenylsulfonyl-L-aspartyl-4-amidino-D-phenylalanylpiperide hydrochloride) was synthesized by Behringwerke AG (Marburg, Germany). Sulforhodamine (SR 101), Cholesterol, Phalloidin, MTT and the LDH-kit (DG-1340-K) were obtained from Sigma Chemical Co., St.Louis, MO. All phosphatidylcholines were purchased from Avanti Polar Lipids, Inc., Alabaster, Alabama. Solulan was kindly provided by Amerchol, Hamburg, Germany. All other chemicals were obtained from E. Merck (Darmstadt, Germany) in analytical quality. Tissue culture reagents were bought from Gibco (Eggenstein, Germany) except for fetal calf serum (FCS) which was from Biozol (Eching, Germany). Tissue culture articles were purchased from Nunc (Wiesbaden, Gemany). Polycarbonate membrane cell culture inserts were from Tecnomara, Fernwald, Germany.

Preparation of Test Solutions

NaTC (Calbiochem, Bad Soden, Germany) and Solulan were dissolved at the required concentrations in Hank's balanced salt solution (HBSS, with bicarbonate), pH 6.9.

Mixed Micelles

11.4 mL (=0.3 mmol Egg-PC) of the Egg-PC solution (20mg/mL hexan), was evaporated under a nitrogen stream

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at room temperature. The dry lipid film was subsequently solubilized using 10 mL of a 40 mM NaTC solution and sonicated (System 582, KLN Ultraschall GmbH, Germany) for 2 min at room temperature to obtain a clear solution. This stock solution was diluted to the required concentrations (table I).

Specified amounts (11.25, 15, 22.5 or 30 mM, respectively) of the synthetic phospholipids 1,2-dimyristoyl-sn-glycero-3-phosphocholin (PC-14), 1,2-dipalmitoyl-sn-glycero-3-phosphocholin (PC-16) or 1,2-distearoyl-sn-glycero-3-phosphocholin (PC-18) were added to NaTC solutions (15, 20, 30 or 40 mM, respectively) and sonicated for 2 min at room temperature to yield transparent solutions.

A stock solution of 10 mg/mL cholesterol was prepared in chloroform. 77.32 μL (=2 μmol cholesterol) of this solution was dried under a nitrogen stream at room temperature and solubilized in 10 mL of a 40 mM NaTC solution after 5 min sonication. All other concentrations were prepared by the same method.

NaTC/oleic acid mixed micelles (1 mM) were prepared in a ratio of 1:1 as follows: 3.18 μL oleic acid (99% purity, ICN, Meckenheim, Germany) was neutralized by 110 μL 1N NaOH and added to 5.4 mg NaTC. Afterwards the mixture was supplemented with 0.05 M phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} to a final volume of 10 mL. At a pH of 7.75 a clear solution was obtained (modified from 6).

The formulations used in this study are summarized in table I. The code is based on the NaTC concentrations.

Characterization of the Micellar Systems

The formation of mixed micellar systems were verified by NMR and QLS studies as previously described (7).

Cell Cultures

Caco-2 cells from Dr. Löhrke (DKFZ, Heidelberg, Germany) were routinely cultivated in our laboratory and grown to monolayers as reported (5).

Table 1. Composition and Declaration of the Mixed Micelle Systems Used

Composition	Code
15 mM NaTC + 11.25 mM PC	NaTC/PC 15 mM
20 mM NaTC + 15 mM PC	NaTC/PC 20 mM
30 mM NaTC + 22.5 mM PC	NaTC/PC 30 mM
40 mM NaTC + 30 mM PC	NaTC/PC 40 mM
15 mM NaTC + 0.075 mM Cholesterol	NaTC/CH 15 mM
20 mM NaTC + 0.1 mM Cholesterol	NaTC/CH 20 mM
30 mM NaTC + 0.15 mM Cholesterol	NaTC/CH 30 mM
40 mM NaTC + 0.2 mM Cholesterol	NaTC/CH 40 mM
0.25 mM NaTC + 0.25 mM oleic acid	NaTC/OA 0.25 mM
0.5 mM NaTC + 0.5 mM oleic acid	NaTC/OA 0.5 mM
0.75 mM NaTC + 0.75 mM oleic acid	NaTC/OA 0.75 mM
1 mM NaTC + 1 mM oleic acid	NaTC/OA 1 mM

Transport Studies

Transport studies were performed directly on polycarbonate filters as previously described (5). 1.5 mL of the drug solution, consisting of 500 μg/mL CRC 220 and 10 μg/mL SR 101 dissolved in the appropriate enhancer solution, was placed on the apical and 2.5 mL transport buffer on the basolateral side of the monolayers. After different time intervals 1 mL samples were withdrawn from the basolateral chamber and replaced by fresh buffer.

Sample Analysis

The CRC 220 amount was determined by reversed phase HPLC described recently (5). SR 101 was analyzed in parallel using a fluorescence detector (Hitachi F1000, Merck, Germany). Detection wavelength was 586 nm for excitation and 620 nm for emission. Detection limits were 50 ng/mL for CRC 220 and 0.2 ng/mL for SR 101.

Transepithelial Electrical Resistance (TEER)

The integrity of each monolayer was checked at the beginning and the end of the experiment by determination of the TEER with the Endohm^R apparatus (WPI, Germany) in HBSS at room temperature. The initial value (450 ohm * cm^2) was set to 100%. For reversibility studies the monolayers were rinsed with HBSS directly after the transport experiment (2h duration) and supplemented with fresh culture medium on both, the apical and the basolateral side. The cells were further cultivated under normal conditions. At different time points the filters were washed with HBSS, TEER was determined in HBSS and the monolayers were returned to the cell culture medium again.

Actin Staining

For actin staining, the cells were grown for 21 days on glass cover slides and treated for 2 h with the enhancer solution. Afterwards they were rinsed three times with PBS and the remaining actin was stained using FITC-labelled phalloidin (8). The sample was examined under a Zeiss fluorescence microscope (Germany) with a ×500 magnification. Microscopic photographs were taken with a Contac RTS II camera (Germany) and Fuji 100 ASA films.

Lactate Dehydrogenase (LDH)

LDH is a cytosolic enzyme and its presence in the apical compartment is generally regarded as evidence for cell membrane damage (9). For LDH studies, cells were seeded at densities equal to transport studies in 24—well dishes and used after 21 days. 1 mL of the enhancer solution was placed on the monolayer and 50 μL samples were withdrawn after 20, 60 and 90 min of exposure. The LDH content in the samples was assayed utilizing a LDH-kit (DG 1340-K, Sigma), which allows the spectrophotometric determination (λ = 340 nm, Spectra Physics Photometer) of the reduction of NAD in the presence of lactate and LDH. Control experiments performed with 0.1% Triton-X 100 resulted in a LDH release, set as 70% toxicity (9). The LDH amounts found in the apical solution of monolayers treated by the enhancer systems were

related to the Triton-X 100 value and expressed as percent release. The remaining amount of LDH within the cells (rem. LDH) was the difference between the theoretical 100% LDH within the cells and the LDH amount found in the apical compartment. The only tested enhancer which interfered with the determination of LDH activity was NaTC/PC-18 causing a turbidity.

Protein Determination

Subsequent to the LDH experiment the cells were rinsed with HBSS and treated with 0.5 mL 0.1 N NaOH for 24 h at 4°C to cause cell lysis. The protein content was determined according to Lowry et al. (10) and the amount of buffer exposed monolayers was set as 100%.

MTT-transformation

The effects of enhancers on the mitochondrial activity was determined using a colorimetric assay (MTT) based on the cleavage of a yellow tetrazolium salt to purple formazan crystals by mitochondrial enzymes of viable cells (11). Cells were seeded in 96-well culture dishes in densities of $6.5 * 10^5$ cells/cm^2 and cultivated under normal conditions for 21 days. Afterwards the cells were incubated for 2 h with each test solution (Solulan was additionally tested after 1 h exposure), rinsed with HBSS, added with 200μL medium containing 0.5 mg/mL MTT and incubated for another 4 h. The MTT solution was removed, developed formazan crystals were dissolved with 200 μL DMSO and the colour intensity was determined with a multiwell scanning spectrophotometer (Titerek, ICN, Germany) at a wavelength of 570 nm. The MTT transformation of the buffer treated cells were set as 100% viability.

Propidium Iodide Staining

Propidium iodide (PI) enters cells with damaged membranes and binds to nucleic acids, thereby producing a red fluorescence in dead cells. PI is excluded by the intact plasma membrane of living cells (12).

Cells grown on glass slides for 21 days were treated with the appropriate enhancer solution for 2 h. Subsequently they were rinsed carefully with HBSS and incubated for 5 min with 7.5 μM propidium iodide (Molecular Probes, Inc., Eugene, OR) in HBSS at room temperature. The number of dead cells was determined under a Zeiss fluorescent microscope (Germany) with a ×500 magnification. Microscopic photographs were taken with a Contac RTS II camera (Germany) and Fuji 100 ASA films.

Data Treatment

Effective permeability coefficients were calculated from the receiver compartment concentrations and the following relationship:

$$P_{eff} = \frac{V_R}{AC_0}\frac{dc}{dt}$$

where V_R is the volume of the receiver compartment, A is the membrane surface area (4.71 cm^2), C_0 is the initial donor concentration of solute and dc/dt is the slope of the regression line describing the cumulative receiver concentration versus time. All results are expressed as the mean of at least 3 experiments ± standard deviations and statistical analysis was performed using two-sided independent t-test.

RESULTS AND DISCUSSION

Recently a series of L-Asp-D-Phe derivatives with antithrombotic activity was synthesized (13). One of the inhibitors, CRC 220 showed a significantly higher transport rate through Caco-2 monolayers than the other inhibitors yielding an oral bioavailability of 4% in rats (5), due to an active transport component. A further increase of the absorption and bioavailability is desirable, therefore, the selection of an untoxic absorption enhancer becomes a major concern.

The transport of CRC 220 and sulforhodamine (SR 101), a hydrophilic marker molecule mainly transported by the paracellular route (9), were studied in Caco-2 monolayers to characterize the different absorption enhancing systems. The opening of tight junctions was demonstrated by TEER measurements as well as actin staining of the perijunctional ring.

The cytotoxicity or viability of cell monolayers was tested using several cell membrane damage markers, such as LDH release from cytoplasma, MTT turnover in mitochondria, residual cell protein and propidium iodide staining of dead cells.

Transport and Toxicity Studies Without an Enhancer

As baseline values for the effective permeability coefficients P_{eff} of CRC 220 in Caco-2 cell monolayers without permeation enhancers were found to be $1.09 \pm 0.04 * 10^{-7}$ cm/s performed in HBSS or PBS respectively. The hydrophilic paracellular marker substance SR 101 was transported with a P_{eff} of $0.21 \pm 0.05 * 10^{-7}$ cm/s. The TEER of the Caco-2 cell line was in the range of 452 ± 77 ohm * cm^2. These results obtained in HBSS medium were set to 100% viability as presented in Table II. To compare toxicity data from several enhancer principals as a function of concentration, different biochemical markers were studied and a deviation of ±20% from the control value was generally regarded as innocuous.

Sodium Taurocholate (NaTC)

NaTC is a physiologically occurring bile acid, frequently used as permeation enhancer for peptides (13). In agreement with Anderberg et al. (14), we observed a dose-dependent absorption enhancement, but also a dose-dependent toxicity. The P_{eff} and toxicity values arising from coadminsitration of NaTC at different concentrations to Caco-2 monolayers are shown in figure 1. Up to 15 mM of NaTC, a moderate increase of CRC 220 and SR 101 transport were observed. Concentrations up to 40 mM resulted in a 280-fold increase in transport rates for CRC 220 and a 530-fold increase for SR 101.

Corresponding to the absorption enhancement, the cell viability decreased. TEER values, measured at the end of the transport experiments, diminished already at concentrations >5 mM NaTC, indicating the opening of tight junctions. This assumption was verified by histological staining of the Caco-2 monolayers, using FITC-labelled phalloidin. Figure 2 shows

Table II. Transport Enhancing Capacity and Toxicity Values of NaTC and NaTC/PC Mixed Micelles. Values Are Expressed as Mean of at Least 3 Determinations ± Standard Deviation

	CRC P_{eff} [a]10^{-7}[cm/s]	SR P_{eff} [a]10^{-7}[cm/s]	TEER 120 min [%]	LDH 90 min [%]	rem.LDH 90 min [%]	MTT 120 min [%]	Protein 90 min [%]
HBSS	1.1 ± 0.04	0.21 ± 0.05	114.2 ± 6.9	2.3 ± 0.3	97.7 ± 0.3	100.0 ± 4.0	100.5 ± 2.7
NaTC							
2.5 mM	3.1 ± 0.65	1.0 ± 0.43	103.9 ± 5.6	2.4 ± 0.95	97.6 ± 0.95	100.5 ± 44.2	106.8 ± 2.4
5 mM	1.7 ± 0.2	0.3 ± 0.06	103.4 ± 2.9	6.0 ± 3.6	94.0 ± 3.6	103.1 ± 4.8	89.7 ± 5.2
10 mM	12.1 ± 2.5	1.75 ± 0.3	75.5 ± 3.9	6.75 ± 2.3	93.25 ± 2.3	105.8 ± 3.1	88.6 ± 4.21
15 mM	18.3 ± 1.3	4.7 ± 0.4	48.2 ± 3.3	8.4 ± 0.5	91.6 ± 0.5	96.4 ± 3.8	96.4 ± 6.7
20 mM	15.6 ± 2.4	3.6 ± 0.3	30.0 ± 1.9	23.0 ± 1.7	77.0 ± 1.7	75.3 ± 2.4	64.6 ± 4.3
30 mM	103 ± 18.5	18.8 ± 1.8	11.3 ± 1.0	33.8 ± 1.4	66.2 ± 1.4	35.9 ± 4.2	33.2 ± 12.6
40 mM	284 ± 10	111.7 ± 6.0	4.5 ± 0.1	43.2 ± 2.9	56.8 ± 2.9	13.8 ± 1.9	11.6 ± 0.8
NaTC/Egg-PC							
15 mM	1.0 ± 0.04	0.7 ± 0.5	101.8 ± 5,7	0.4 ± 0.1	99.6 ± 0.1	91.8 ± 2.4	93.8 ± 3.3
20 mM	1.3 ± 0.12	0.4 ± 0.2	92.5 ± 4.6	1.9 ± 0.3	98.1 ± 0.3	95.2 ± 4.1	104.1 ± 6.0
30 mM	0.7 ± 0.01	0.4 ± 0.2	95.2 ± 3.7	1.7 ± 0.4	98.3 ± 0.4	93.0 ± 3.4	103.3 ± 8.0
40 mM	1.0 ± 0.03	0.4 ± 0.06	104.7 ± 5.4	1.9 ± 0.1	98.1 ± 0.1	91.1 ± 3.4	108.6 ± 15
NaTC/PC-14							
15 mM	1.3 ± 0.3	0.4 ± 0.07	94.3 ± 13.2	1.1 ± 0.3	98.9 ± 0.3	99.9 ± 6.8	100 ± 10
20 mM	1.3 ± 0.1	0.7 ± 0.08	106.8 ± 15.3	1.6 ± 0.03	98.4 ± 0.03	70.2 ± 11.5	100.8 ± 5.0
30 mM	0.9 ± 0.1	0.5 ± 0.01	107.1 ± 4.8	1.7 ± 0.2	98.3 ± 0.2	62.0 ± 6.1	101.6 ± 2.8
40 mM	1.2 ± 0.2	0.4 ± 1.16	100.8 ± 4.8	1.8 ± 0.1	98.2 ± 0.1	64.1 ± 7.7	112.8 ± 2.0
NaTC/PC-16							
15 mM	1.1 ± 0.04	0.4 ± 0.03	129.4 ± 2.9	1.2 ± 0.1	98.8 ± 0.1	77.8 ± 10.8	103.7 ± 7.9
20 mM	0.8 ± 0.2	0.3 ± 0.06	112.8 ± 0.8	1.6 ± 0.1	98.4 ± 0.1	65.8 ± 16.7	113.7 ± 7.5
30 mM	0.9 ± 0.2	0.4 ± 0.1	110.4 ± 4.2	1.7 ± 0.1	98.3 ± 0.1	74.8 ± 13.81	110.7 ± 3.1
40 mM	0.6 ± 0.04	0.2 ± 0.04	105.1 ± 3.2	2.0 ± 0.1	98.0 ± 0.1	71.3 ± 7.6	109.0 ± 8.0
NaTC/PC-18							
15 mM	3.2 ± 0.5	0.9 ± 0.07	72.8 ± 7.4	nd	nd	94.2 ± 3.8	102.7 ± 5.7
20 mM	4.5 ± 0.5	2.4 ± 0.5	37.9 ± 11.8	nd	nd	114.2 ± 10.3	97.5 ± 3.5
30 mM	4.5 ± 0.4	2.0 ± 0.2	46.2 ± 4.5	nd	nd	72.7 ± 6.5	71.4 ± 4.3
40 mM	12.0 ± 0.7	6.7 ± 0.6	18.9 ± 2.9	nd	nd	21.1 ± 4.8	44.4 ± 5.8

[a] nd : Not determined.

the photomicrographs of actin staining after 2 h exposure of 0, 10, 20, 30 and 40 mM NaTC. At the lower concentrations (<20 mM) the staining became fuzzy and not all cells were surrounded by a fluorescent belt, but the cells seemed to be healthy. In contrast, at high concentrations, i.e. 30 and 40 mM NaTC, defined actin structures disappeared and the cells were obviously damaged. These findings suggest, that the increased cell monolayer permeability at concentrations of 20 mM NaTC and higher is caused by cytotoxic effects of NaTC. The cell membranes became leaky as demonstrated by the release of cytosolic LDH and protein release. In addition the activity of mitochondrial enzymes, determined by MTT transformation, decreased. To visualize dead cells, we stained the NaTC treated monolayers with propidium iodide, which only penetrates the cytoplasmatic membrane of damaged cells, demonstrating an increasing number of dead cells at NaTC concentrations >20 mM (data not shown).

NaTC/PC Mixed Micelles

Phosphatidylcholines, the main components of cell membranes, are known to protect cells from the toxic effects of bile salts (15) by formating mixed micelles. To estimate the absorption enhancing and cytotoxic effects of different NaTC/PC-mixed micellular systems, containing various phospholipids at a molar ratio of 1:0.75, were investigated as shown in table II.

Egg-PC

Egg-PC decreased the cytotoxicity but lowered the absorption promoting effect of NaTC. Since Egg-PC is a mixture of phosphatidylcholine esterified with different medium and long chain length fatty acids, we tried to evaluate which lipid residues were responsible for the observed result. Therefore we select three saturated fatty acid conjugates, PC-14, PC-16 and PC-18.

PC-14, PC-16, and PC-18

Similar to NaTC/Egg-PC mixed micelles, PC-14 and PC-16 NaTC/phosphatidylcholine compositions were well tolerated by the Caco-2 monolayers, showing a membrane protecting effect. Unfortunately, transport of CRC 220 and the marker SR 101 were not significantly different from control values, suggesting that this permeation enhancer is not effective for the peptide under investigation.

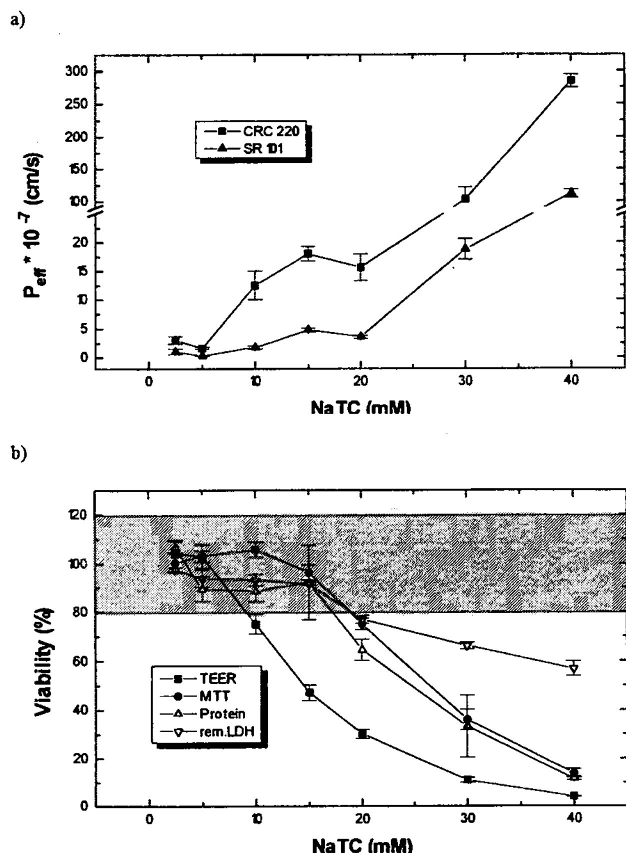


Fig. 1. Concentration dependence of a) the transepithelial transport of CRC 220 and SR 101 (note the y-axis-break) and b) the toxicity values of Caco-2 monolayers measured after incubation with NaTC. Data are expressed as mean of at least 3 determination ± standard deviation.

Surprisingly, mixed micellar systems containing PC-18 did not neutralize the membrane perturbing effects of NaTC as PC-14 or PC-16. Consequently, the absorption promoting capacities of NaTC remained intact, but were less pronounced. For example, the exposure of 40 mM NaTC/PC-18 resulted only in a 12-fold increased transport of CRC 220 in contrast to the 280-fold increase caused by 40 mM NaTC.

Membrane damage, evaluated by protein and MTT data and propidium iodide staining (not shown) was not noticable <30 mM NaTC/PC-18, indicating a slight protection of PC-18 compared to NaTC. On the other hand, TEER values decreased already at 15 mM NaTC/PC-18, demonstrating the opening of tight junctions before cell damage occured. These observations were confirmed by histological actin staining, which was similar to figure 2c.

These results are in agreement with literature data (15), because we obtained no tissue damage as well as no absorption enhancement with the tested NaTC-phosphatidylcholin systems, except in the case of NaTC/PC-18 mixed micelles.

The different behaviour of PC-18 may be caused by its lower solubility in NaTC due to the longer side chains. In contact with plasma membranes NaTC seemed to have a higher affinity to membrane bound phosphatidylcholines than to PC-18 and could solubilize these phosphatidylcholines from the bilayer.

NaTC/Cholesterol

Cholesterol, a rigid molecule, stabilizes the biomembranes by insertion into the bilayer. Since cholesterol is not water-soluble, bile salts are required to obtain clear solutions, e.g. 1 mM NaTC and 5 μM cholesterol. This small amount of cholesterol did not totally inhibit the toxic effect of NaTC, but a small protection was noticed (table III). Similar to NaTC/PC-18, NaTC/cholesterol concentrations <30 mM caused no membrane perturbation, but again the opening of the tight junctions occured before cell damage. Accordingly, the P_{eff} values increased with higher enhancer concentrations. A critical toxicity level was observed >20mM.

NaTC/oleic Acid

Fusogenic fatty acids like oleic acid interact with membrane components and induce permeability changes (16). Since the effects of NaTC and oleic acid accumulate, very low concentrations were used. We found 1 mM NaTC/oleic acid to cause a slight increased of LDH release. In this study, we estimated the absorption effects at concentrations of 0.25, 0.5, 0.75 and 1 mM NaTC/oleic acid (1:1) mixed micelles (table III).

As viewed by light microscopy, Caco-2 cell monolayers treated with 1 mM NaTC/oleic acid, significantly changed their appearance, showing rounded cells, similar to trypsin treatment (not shown). Nevertheless these cells showed no propidium iodide influx, indicating their viability.

A significant increase in P_{eff}-values was obtained with 0.5 mM NaTC/oleic acid although all toxicity studies were negative at this concentration. For the intracellular enzyme markers this was true even at higher concentrations. Only the TEER values decreased, resulting in higher transport rates.

Bile salt-fatty acid mixed micellar systems are supposed to be very potent absorption enhancers but harmless to the tissue (3). These observations correlated with the results found in our study. We observed an opening of tight junctions and a 18-fold increase of paracellular transport by oleic acid whereas the plasma membrane was not damaged.

Solulan C-24

The absorption enhancing properties of Solulan C-24, a synthetic, non-ionic cholesterol POE ether were recently evaluated by Drewe et al. (17). Solulan C-24 was described to be effective at low concentrations. Therefore, we tested a concentration range of 0.01 to 0.1% (w/v). In contrast to the previously described enhancers, Solulan showed a non-linear time course at all tested concentrations. The absorption promoting effect of solulan required at least 60 min pretreatment. Accordingly, the permeability coefficients P_{eff} were calculated for the 60–120 min interval (table III). LDH-release and mitochondrial activity also reflect a similar time dependence. TEER and MTT values measured after 120 min decreased even at concentrations, which did not show an increased transport rate, e.g. 0.02%, while protein and LDH release results remained in the untoxic range. It should be noted, that the latter ones were measured already after 90 min exposure, suggesting that the values at 120 min might be lower and in agreement with MTT results. This is of importance, since

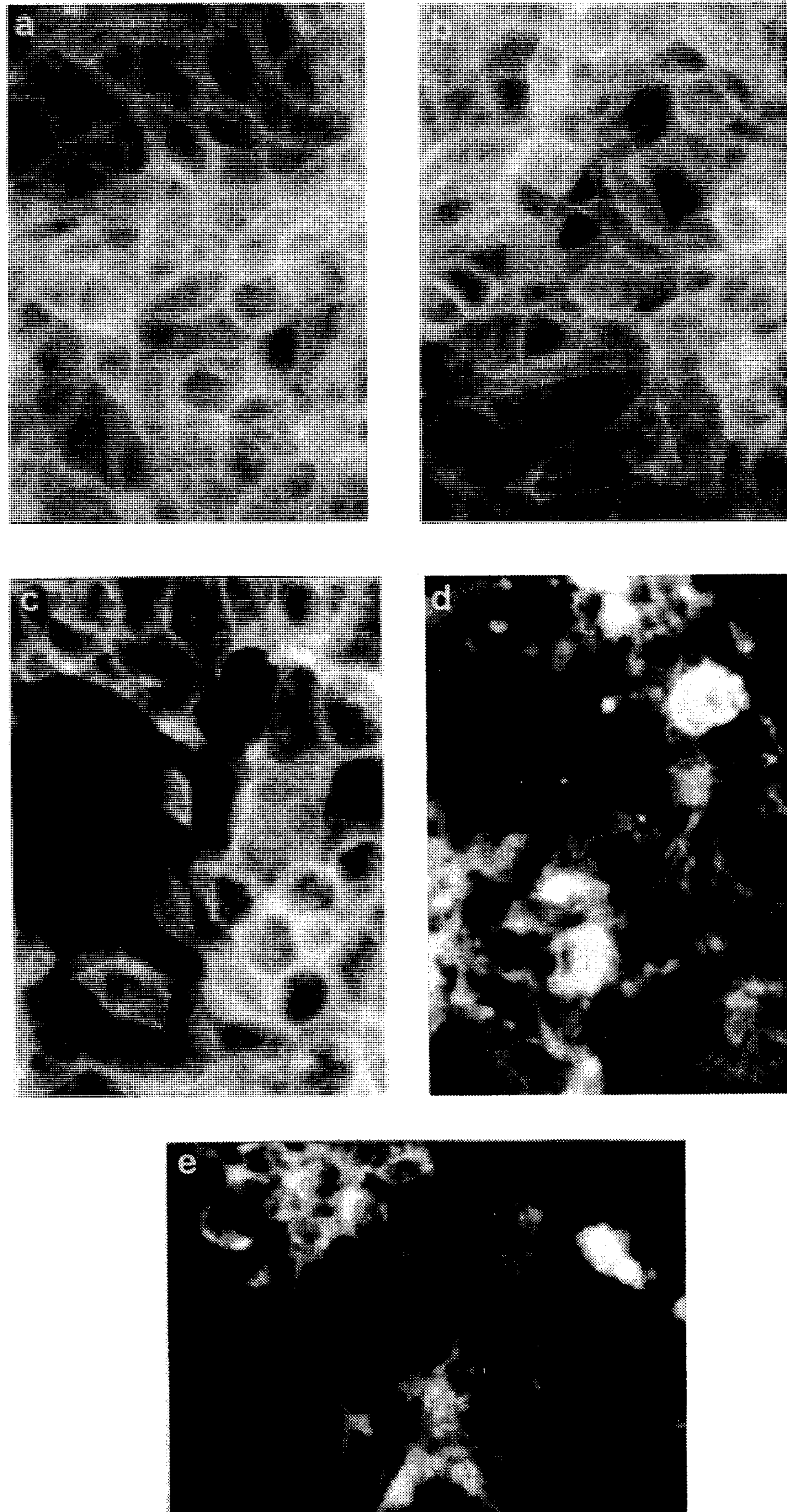


Fig. 2. Staining of tight junctions by phalloidin of a Caco-2 monolayer after 2h exposure of a) without NaTC b) 10mM, c) 20mM, d) 30mM, and e) 40mM NaTC (312×).

Table III. Transport Enhancing Capacity and Toxicity Values of NaTC/Cholesterol, NaTC/oleic Acid Mixed Micelles, and Solulan C-24. Values Are Expressed as Mean of at Least 3 Determinations ± Standard Deviation

	CRC P_{eff}[a] 10^{-7}[cm/s]	SR P_{eff} [a]10^{-7}[cm/s]	TEER 120 min [%]	LDH 90 min [%]	rem.LDH 90 min [%]	MTT 120 min [%]	Protein 90 min [%]
NaTC/CH							
15 mM	5.8 ± 0.6	2.3 ± 0.2	67.8 ± 2.9	9.2 ± 0.8	91.8 ± 0.8	98.2 ± 4.4	79.9 ± 4.6
20 mM	7.6 ± 1.2	4.7 ± 0.9	53.2 ± 3.2	13.5 ± 2.0	86.5 ± 2.0	80.9 ± 3.7	80.5 ± 1.7
30 mM	23 ± 6.9	16.9 ± 7.5	36.5 ± 6.2	25.8 ± 3.9	74.2 ± 3.9	44.0 ± 1.2	63.6 ± 2.5
40 mM	198 ± 32.4	40.9 ± 4.4	23.3 ± 4.0	37.3 ± 1.6	62.7 ± 1.6	14.6 ± 1.0	13.7 ± 2.9
NaTC/OA							
0.25 mM	1.6 ± 0.5	0.7 ± 0.4	109.7 ± 2.0	1.5 ± 0.1	98.5 ± 0.1	89.2 ± 4.3	117.4 ± 5.5
0.5 mM	3.6 ± 0.3	1.8 ± 0.3	104.0 ± 4.7	5.9 ± 1.7	94.1 ± 1.7	83.5 ± 4.8	108.2 ± 2.5
0.75 mM	17.8 ± 2.6	18.6 ± 3.4	36.3 ± 0.5	14.9 ± 1.7	85.1 ± 1.7	81.5 ± 7.0	87.3 ± 6.6
1 mM	29.4 ± 4.1	nd	27.8 ± 3.3	40.4 ± 4.4	59.6 ± 4.4	84.9 ± 6.0	53.4 ± 6.1
Solulan							
0.01 %	3.8 ± 0.5	1.4 ± 0.2	76.9 ± 5.8	1.8 ± 0.1	98.2 ± 0.1	92.3 ± 3.3	100.8 ± 3.2
0.02 %	7.2 ± 0.8	3.3 ± 0.1	40.9 ± 4.3	4.6 ± 0.3	95.4 ± 0.3	76.3 ± 1.3	94.0 ± 3.8
0.03 %	11.4 ± 0.9	6.4 ± 0.8	24.2 ± 3.4	7.4 ± 0.7	92.6 ± 0.7	55.4 ± 5.0	98.1 ± 4.5
0.05 %	82.3 ± 17	13.3 ± 0.4	7.2 ± 0.6	13.0 ± 0.1	87 ± 0.1	46.6 ± 2.8	80.7 ± 3.2
0.1 %	165 ± 5.8	18.6 ± 0.9	2.6 ± 0.7	38.2 ± 6.6	61.8 ± 6.6	33.2 ± 2.7	56.5 ± 1.1
Triton-X 100							
0.1 %	nd	nd	nd	73.3 ± 8.4	26.7 ± 8.4	4.5 ± 0.1	32.8 ± 3.5
1 %	nd	nd	nd	53.7 ± 1.2	46.3 ± 1.2	nd	32.4 ± 5.3

[a] nd : Not determined.

both, the transport enhancing and the cell damaging effect of Solulan seemed to be clearly time dependent.

A sharp decrease in TEER values could be noticed with increasing concentrations, but actin staining showed at all concentrations an intact monolayer (Fig. 3a) in contrast to NaTC, where at similar TEER values the monolayer appeared leaky. Nevertheless, the cells were obviously damaged, as confirmed by propidium iodide staining (Fig. 3b).

Recently, Drewe et al. (17) observed an increased intracellular fluorescence of NBD-labelled octreotide and an enhanced transport of [^{3}H]-PEG 4000 accompanyed by a decrease in TEER values, suggesting an opening of both, the transcellular and the paracellular pathways.

We could not confirm this hypothesis, since in our study the tight junctions, responsible for the paracellular pathway, were not affected. Furthermore we explain these results by a perforation of the apical and the basolateral plasma membranes affecting the TEER values, since they reflect the extent of ion fluxes, without the opening of tight junctions. In the same way PEG 4000 was able to diffuse through the dead cells, i.e. transcellulary and was found in the basolateral chamber despite intact junctions.

Reversibility of Enhancer Induced Cytotoxical Effects

One major function of the intestinal epithelia is its barrier function for most substances in the gut lumen. To study the reversibility of our enhancer systems, we replaced the test solution after 2 h incubation. TEER measurements as function of time (Fig. 4a) were used to demonstrate the reversibility enhancer effect. With the exception of Solulan, which affected the TEER in an irreversible manner, the barrier function of the Caco-2 monolayer was restored to initial values after 3 to 5h. The restoration of tight junction organization could also be demonstrated by actin staining experiments. As shown in figure

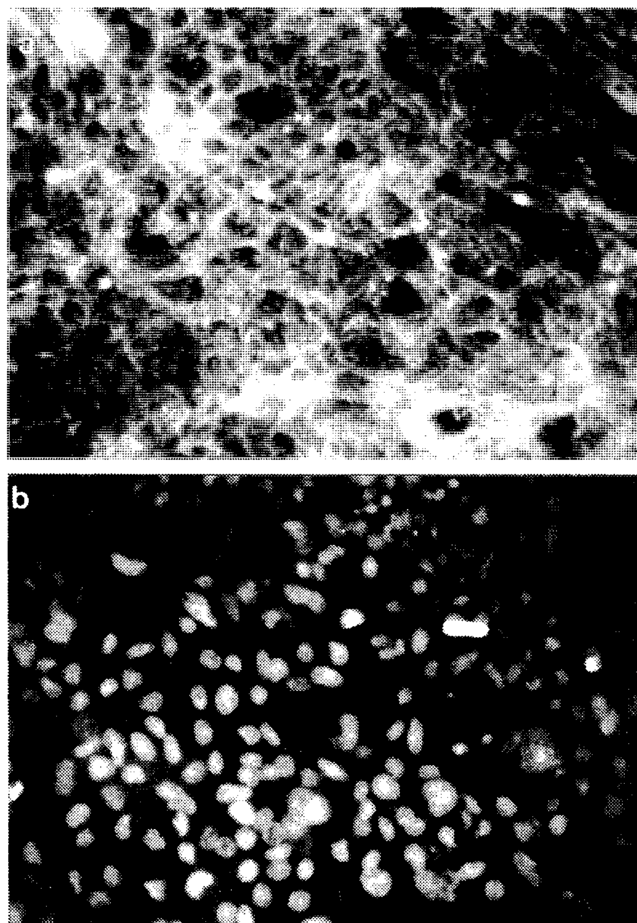
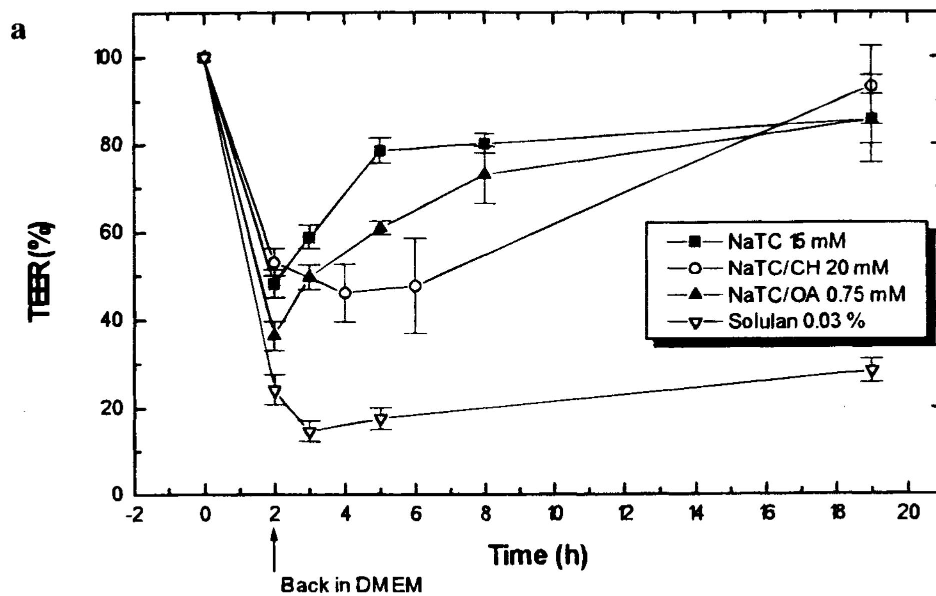


Fig. 3. a) Phalloidin staining of a Caco-2 monolayer after the incubation with 0.1% Solulan C-24 revealed complex actin structures, b) but all cells were dead, as demonstrated by propidium iodide influx (312×).



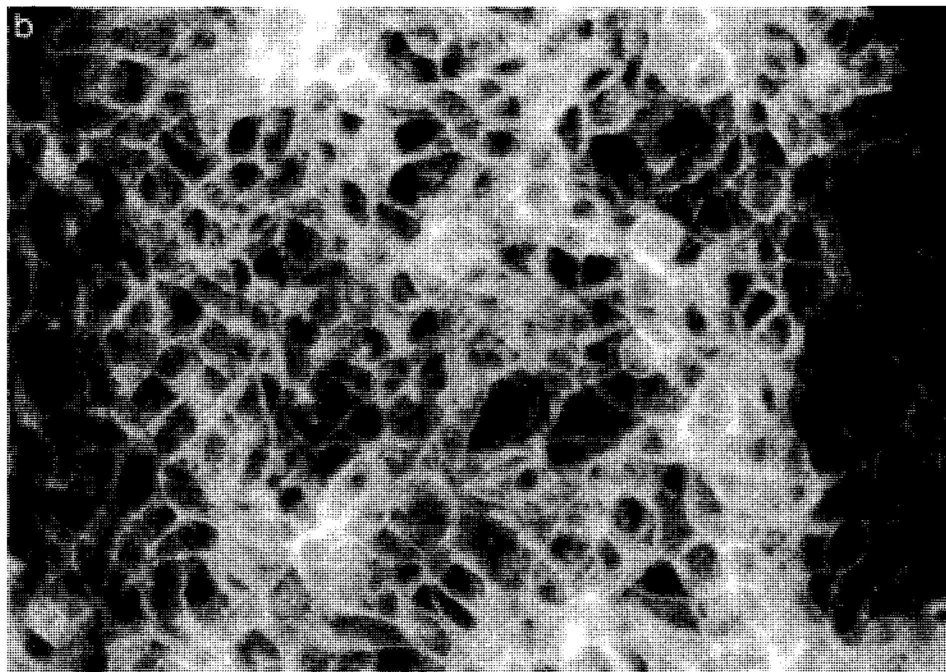


Fig. 4. a) Reversibility of the TEER decrease caused by four enhancer systems at intermediate concentrations. Data are expressed as mean of at least 3 determinations ± standard deviation. b) Recovery of the junctional complex of the monolayer after 2h exposure of 20 mM NaTC and subsequent replacement in normal culture medium for another 12h (312×).

4b, tight junctions return to their normal appearance after exposure to 20 mM NaTC, when the cells are returned to normal culture conditions.

CONCLUSIONS

We evaluated in this study the absorption enhancing and cell damaging effects of various enhancer systems on Caco-2 cells. At lower concentrations NaTC caused a reversible opening of tight junctions and allowed drug permeation through expanded paracellular spaces, whereas at higher concentrations cell membranes were irreversibly damaged leading to an enhanced permeation of drug at sites of cell damage.

Phosphatidycholines generally minimized the permeation enhancing and toxical properties of NaTC, except of PC-18, which showed the same toxicity as NaTC but a lower transport rate of drugs. Cholesterol had no effect on the NaTC results. The addition

of oleic acid resulted in an enhanced drug transport at very low concentrations. The last evaluated enhancer, Solulan C-24 showed a different behaviour. An absorption enhancement occured only through cell damage, while the cytoskeleton remained coherent. The cell destruction was shown to be irreversible.

To conclude, for further in-vivo studies of the thrombin inhibitor CRC 220, NaTC 15mM and NaTC/oleic acid 0.75 mM might be interesting.

REFERENCES

1. P. L. Smith, D. A. Wall, C. H. Gochoco and G. Wilson. (D) Routes of delivery: Case studies. (5) Oral absorption of peptides and proteins. *Adv. Drug Delivery Rev.* **8**:253–290 (1992).
2. V. H. L. Lee and A. Yamamoto. Penetration and enzymatic barriers to peptide and protein absorption. *Adv. Drug Delivery Rev.* **4**:171–207 (1990).
3. S. Muranishi. Absorption enhancers. *Critical Reviews in Therapeutic Drug Carrier Systems* **7**:1–33 (1990).
4. W. Stüber, R. Koschinsky, G. Dickneite and C. Kolar. Amidinophenylalanin-Derivate, Verfahren zu deren Herstellung, deren Verwendung und diese enthaltende Mittel als Antikoagulantien. *EP. 0513543* (1992).
5. E. Walter, T. Kissel, M. Reers, G. Dickneite, D. Hoffmann and W. Stüber. Transepithelial transport properties of peptidomimetic thrombin inhibitors in monolayers of a human intestinal cell line (Caco-2) and their correlation to in vivo data. *Pharm. Res.* **12**:360–365 (1995).
6. T. Ohtani, M. Murakami, A. Yamamoto, K. Takada and S. Muranishi. Effect of absorption enhancers on pulmonary absorption of fluorescein isothiocyanate dextrans with various molecular weights. *Int. J. Pharm.* **77**:141–150 (1991).
7. U. Werner. Modelle der nasalen und intestinalen Mukosa für Transport-und Metabolismusuntersuchungen. *PhD-thesis, Cuvillier Verlag Göttingen ISBN: 3-89588-403-0* (1995).
8. U. Werner and T. Kissel. Development of a human nasal epithelial cell culture model and its suitability for transport and metabolism studies under in-vitro conditions. *Pharm. Res.* **12**:565–571 (1995).
9. C. S. Schasteen, M. G. Donovan and J. N. Cogburn. A novel in vitro screen to discover agents which increase the absorption of molecules across the intestinal epithelium. *J. Control. Rel.* **21**:49–62 (1992).
10. O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
11. T. Mosmann. Rapid colorimetric assay for cellular growth and survial: Application to proliferation and cytotoxcity assays. *J. Immunol. Methods* **65**:55–63 (1983).
12. B. J. Kroesen, G. Mesander, J. G. ter Haar, T. H. The and L. de Leij. Direct visualisation and quantification of cellular cytotoxicity using two colour fluorescence. *J. Immunol. Meth.* **156**:47–52 (1992).
13. F. G. J. Poelma, J. J. Tukker and D. J. A. Crommelin. The role of bile salts in the intestinal absorption of drugs. *Acta Pharm. Technol.* **36**:43–52 (1990).
14. E. K. Anderberg, C. Nyström and P. Artursson. Epithelial transport of drugs in cell culture. VII: Effects of pharmaceutical surfactant excipients and bile acids on transepithelial permeability in monolayers of human intestinal epithelial (Caco-2) cells. *J. Pharm. Sci.* **81**:879–887 (1992).
15. A. L. M. Velardi, A. K. Groen, R. P. J. O. Elferink, R. van der Meer, G. Palasciano and G. N. J. Tytgat. Cell type-dependent effect of phospholipid and cholesterol on bile salt cytotoxicity. *Gastroenterology* **101**:457–464 (1991).
16. M. Murakami, K. Takada and S. Muranishi. Further mechanistic study on intestinal absorption enhanced by unsaturated fatty acids: reversible effect by sulfhydryl modification. *Biochim. Biophys. Acta* **1117**:83–89 (1992).
17. J. Drewe, G. Fricker, J. Vonderscher and C. Beglinger. Enteral absorption of octreotide: absorption enhancement by polyoxyethylene-24-cholesterol ether. *Br. J. Pharmacol.* **108**:298–303 (1993).