Drug Development and Industrial Pharmacy, 34:1343–1349, 2008

ISSN: 0363-9045 print / 1520-5762 online
DOI: 10.1080/03639040802098185

informa
healthcare

Intestinal Drug Absorption Enhancers: Synergistic Effects of Combinations

Gill M. Enslin and Josias H. Hamman
Department of Pharmaceutical Sciences, Tshwane University of Technology, Pretoria, South Africa

Awie F. Kotzé
School of Pharmacy, Department of Pharmaceutics, North-West University, Potchefstroom, South Africa

Although many absorption enhancers have been investigated, very few are used clinically. A need exists therefore for more effective absorption enhancers. The drug-absorption-enhancing effects of combinations of *N*-trimethyl chitosan chloride (TMC) with degrees of quaternization of 48 and 64%, dicarboxymethyl chitosan oligosaccharide, and chitosan lactate oligomer with monocaprin and melittin were compared to their individual performances using the in vitro Caco-2 cell model. Combining the absorption enhancers showed synergism in both the reduction of the transepithelial electrical resistance (TEER) and the enhancement of the transport of a macromolecular model compound across this intestinal epithelial cell layer. Lower concentrations of the absorption enhancers in the combination groups exhibited greater effects on the epithelial cells compared with the individual absorption enhancers.

Keywords absorption enhancement; chitosan; monocaprin; melittin; Caco-2 cells; transepithelial electrical resistance

INTRODUCTION

The route of choice for drug administration is often the oral route due to its many advantages such as convenience, safety, and acceptability (Cleland, Daugherty, & Mrsny, 2001). However, poor bioavailability of certain types of drug compounds precludes their administration via the oral route. These drugs must therefore be administered via an alternative route, usually by parenteral injection. An oral drug delivery system for these drugs would be desirable, particularly for drugs intended for chronic use. The likeliness of producing drug molecules with poor biopharmaceutical properties has increased immensely since the initiation of biotechnology and advanced drug design technologies (Sharma, Varma, Chawla, & Panchagnula, 2005; Van Hoogdalem, De Boer,& Breimer, 1989; Zhou, 1994), which intensifies the challenges related to their effective delivery (Gomez-Orellana, 2005). Therefore, the situation has arisen where the progress in developing new drug entities is far ahead of the development of effective delivery systems for these new drugs (Kotzé et al., 1999; Shen, 2003; Soltero & Ekwuribe, 2001).

Strategies that have been proposed to maximize the bioavailability of poorly absorbable drugs include chemical modification, special drug delivery systems, targeted delivery, and co-administration of enzyme inhibitors and absorption enhancers (Hamman, Enslin, & Kotzé, 2005). Absorption-enhancing agents that facilitate the absorption of hydrophilic macromolecules by increasing their transcellular and/or paracellular transport across the intestinal epithelium have been studied extensively (Lecluyse & Sutton, 1997; Ward, Tippin, & Thakker, 2000). Absorption enhancers that have been evaluated in the past represent a group of compounds that differ in their chemical, mechanistic, and toxicity profiles (Junginger & Verhoef, 1998). The mechanism of an absorption enhancer may encompass several processes, and the absorption enhancer chosen for a drug compound depends on the particular barrier to that particular compound's absorption (Lecluyse and Sutton, 1997).

Glycerides of medium chain fatty acids have been studied as permeation enhancers of hydrophilic drugs across epithelial cells. Monocaprin is the monoglyceride of a C-10 fatty acid that has been shown to increase the permeability of Caco-2 cell monolayers by a mechanism thought to be indicative of the modulation of tight junction permeability (Brown, Collett, Attwood, Ley, & Sims, 2002). Melittin is an amphipathic, cationic peptide that consists of 26 amino acids and is a component of honey bee venom. Melittin solutions between 1.2 and 1.5 μM were shown to increase the transport of mannitol across Caco-2 cell monolayers by a factor of 3.5, and at a concentration of less than 2.4 μM, it is expected to exhibit minimal toxicity (Liu, Davis, Liu, & Krishnan 1999). Addition of melittin to the apical side of Caco-2 cell monolayers resulted in the opening of tight junctions as indicated by reductions in transepithelial electrical resistance (TEER) and increased flux of dextrans across the cell monolayers (Maher, Feighery, Brayden,

Address correspondence to Josias H. Hamman (PhD), Department of Pharmaceutical Sciences, Tshwane University of Technology, Private Bag X680, Pretoria, 0001, South Africa. E-mail: Hammanjh@tut.ac.za

& McClean, 2007a). Melittin also had a reversible noncytotoxic concentration-dependant decrease in TEER of E-12 cell monolayers and increased the permeability of rat colonic mucosae to [^{14}C]-mannitol (Maher, Feighery, Brayden, & McClean, 2007b). Chitosan, a cationic polysaccharide, and its derivatives such as methylated and carboxymethylated chitosan, are able to promote the transmucosal absorption of small polar molecules as well as peptide and protein drugs (Dodane, Khan, & Merwin, 1999; Hamman, Schultz, & Kotzé, 2003; Hejazi & Amiji, 2003; Illum, 1998; Junginger & Verhoef, 1998; Kotzé et al., 1997; Lueßen et al., 1997; Paul & Sharma, 2000; Thanou, Verhoef, & Junginger, 2001).

The quest for more effective drug absorption enhancers has stimulated this investigation where combinations of chitosan derivatives, chitosan oligomers, monocaprin, and melittin have been evaluated for their in vitro absorption enhancement effects across the intestinal epithelial cell line, Caco-2. The aim is to determine whether different agents would potentiate their individual absorption-enhancing effects when applied in combinations to the epithelial cells. It was further postulated that a lower concentration of each absorption-enhancing agent is needed in combination to reach a higher absorption enhancement effect compared to their individual performances.

MATERIALS AND METHODS

Materials

Chitosan oligomers used in this study included chitosan lactate oligomer (Chitosan Oligo Lactate MW ˜4,000 Da, Promex Ingredients, Avaldness, Norway) and a chitosan derivative oligomer, dicarboxymethyl chitosan oligomer (synthesized from COS-Y chitosan oligomer degree of deacetylation, 70%, 13,500 g/mol). *N*-trimethyl chitosan chloride (TMC), a quaternized derivative of chitosan with degrees of quaternization of 48% (166,000 g/mol, prepared from Seacure 244, degree of deacetylation 93%, Pronova Biopolymer, Drammen, Norway) and 64% (143,000 g/mol, prepared from Seacure 244, degree of deacetylation 93%, Pronova Biopolymer), was used. Monocaprin, melittin, and fluorescein isothiocyanate (FITC)–dextran were purchased from Sigma-Aldrich (Schnelldorf, Germany), and all other chemicals were of analytical grade or high-performance liquid chromatography (HPLC) grade.

Study Group Composition

The following absorption enhancer solutions and combinations thereof were tested on the Caco-2 cell model for their effects on TEER and permeation of a macromolecular model compound, FITC–dextran (MW 4,400), across Caco-2 cell monolayers cultured on transwell filters.

Individual absorption enhancers used were as follows: TMC-48 (*N*-trimethyl chitosan chloride with a degree of quaternization of 48% in concentrations of 0.25 and 0.5%, wt/vol); TMC-64 (*N*-trimethyl chitosan chloride with a degree of quaternization of 64, 0.25, and 0.5%, wt/vol); CLO (chitosan lactate oligomer, 0.25 and 0.5%, wt/vol); DCMCO (dicarboxymethyl chitosan oligomer, 0.25 and 0.5%, wt/vol); monocaprin (1.3 and 2 mM); and melittin (1 and 1.5 μM).

Absorption enhancer combinations used were as follows: TMC-48 (0.25%, wt/vol) + melittin (1 μM); TMC-64 (0.25%, wt/vol) + melittin (1 μM); CLO (0.25%, wt/vol) + melittin (1 μM); DCMCO (0.25%, wt/vol) + melittin (1 μM); monocaprin (1.3 mM) + melittin (1 μM); TMC-48 (0.25%, wt/vol) + monocaprin (1.3 mM); TMC-64 (0.25%, wt/vol) + monocaprin (1.3 mM); CLO (0.25%, wt/vol) + monocaprin (1.3 mM); and DCMCO (0.25%, wt/vol) + monocaprin (1.3 mM).

Seeding and Culturing of Caco-2 Cell Monolayers

Caco-2 cells (American Type Culture Collection, Manassas, VA, USA) were cultured and seeded onto Transwell filters (12-well plates for transepithelial electrical resistance studies and 6-well plates for transport studies) according to a previously described method (Hamman et al., 2003).

Measurement of the Effect on the Transepithelial Electrical Resistance

The effect of the absorption enhancers alone and in different combinations on the TEER of Caco-2 cell monolayers was measured as an indication of the "tightness" of the tight junctions between adjacent epithelial cells. The absorption enhancer solutions were prepared in serum-free Dulbecco's Modified Eagles Medium (DMEM) and the pH was adjusted to 6.2 or 7.4 with 0.1 M HCl or 0.1 M NaOH as required. Test solutions or suspensions were freshly prepared on the day the TEER experiments were performed.

The effect of the selected absorption enhancers on the TEER of the Caco-2 cell monolayers was measured with a Millicell ERS meter (Millipore Corporation, Billerica, MA, USA) connected to a pair of "chopstick" electrodes. Prior to the start of each TEER experiment, the medium was removed and replaced with DMEM buffered at pH 7.4 with 25 mM *n*-(2-hydroxymethyl) piperazine-*N*-(2-ethanesulfonic acid), (HEPES) (Sigma). Measurements were started 1 h prior to incubation with test compound solutions on the apical side of the cell monolayers. TEER was measured at 20 min intervals at pH 6.2 and pH 7.4 during incubation with the absorption enhancer solutions at 37°C in an atmosphere of 95% air and 5% carbon dioxide up to 2 h. A control group was included with every TEER experiment under the same conditions as described above, except that the absorption enhancer solutions were replaced with serum-free DMEM. All TEER experiments were carried out in triplicate.

Measurement of the Effect on the Transport of a Macromolecular Compound

The effect of the individual absorption enhancers and combinations thereof on the permeability of a model compound,

fluorescence isothiocyanate dextran (FITC–dextran, MW 4,400), was determined across Caco-2 cell monolayers. The permeability studies were conducted between days 21 and 28 after seeding onto the six-well filter plates. The Caco-2 cell monolayers were incubated at 37°C under 95% air and 5% carbon dioxide with the absorption enhancer solutions containing 1 mg/mL FITC–dextran applied to the apical side of the cell monolayers at time zero. Samples (200 μL) were withdrawn from the basolateral chamber at the following time intervals after administration at 20, 40, 60, 80, 100, 120, 150, 180, and 240 min. The medium in the basolateral chamber, which consisted of DMEM buffered at pH 7.4 with HEPES, was replenished with an equal volume of HEPES-buffered DMEM after each sample was withdrawn. All control experiments were performed as above with 1 mg/mL FITC–dextran in serum-free DMEM without the test compounds. All transport experiments were carried out in triplicate. The FITC–dextran was determined using size exclusion HPLC with fluorescence detection.

High-Performance Liquid Chromatography Analysis of FITC–Dextran

Quantification of FITC–dextran in the samples was carried out using HPLC with size exclusion chromatographic separation and fluorescence detection. The chromatographic system and conditions were as follows: spectraphysics liquid chromatographic system—pump (model P1000); autosampler (model AS3000); fluorescence detector (model FL2000), excitation wavelength 494 nm and emission wavelength 518 nm; PolySep-GFC-P Linear size exclusion column, 300 × 7.80 mm; and PolySep-GFC-P guard column, 35 × 7.80 mm (Phenomenex, USA distributed by Separations, Johannesburg, South Africa). The mobile phase was acetonitrile: 0.05 M phosphate buffer (12:88), delivered at a flow rate of 1.5 mL/min. The buffer component of the mobile phase was prepared with deionized water and the pH adjusted to 7.0. The prepared mobile phase was filtered through a 0.45-μm nylon filter and degassed under vacuum.

Data Analysis

The results obtained from the transport studies were expressed as cumulative transport (% of initial dose) and the apparent permeability coefficients (P_{app}) were calculated using the following equation:

$$P_{app} = \frac{dQ}{dt}\left(\frac{1}{A_{60}C_0}\right) \quad (1)$$

where P_{app} is the apparent permeability coefficient (cm/s), dQ/dt is the permeability rate (amount permeated per minute), A is the diffusion area of the cell monolayer, and C_0 is the initial concentration of FITC–dextran.

Transport enhancement ratios (R) were calculated from P_{app} values using the following equation:

$$R = \frac{P_{app}(\text{sample})}{P_{app}(\text{control})} \quad (2)$$

Statistical Analysis

The reduction in TEER and transport values of the different experimental groups were compared by means of a one-way ANOVA to determine if differences were significant ($p < .05$).

RESULTS AND DISCUSSION

Measurement of the Effect on the TEER

The TEER (% of initial value) plotted as a function of time for the highest concentrations of the individual absorption enhancers at pH 6.2 are shown in Figure 1A and that for the individual absorption enhancers at pH 7.4 in Figure 1B.

The results obtained from the TEER experiments showed that TMC-48 and TMC-64 both reduced TEER in an apparently

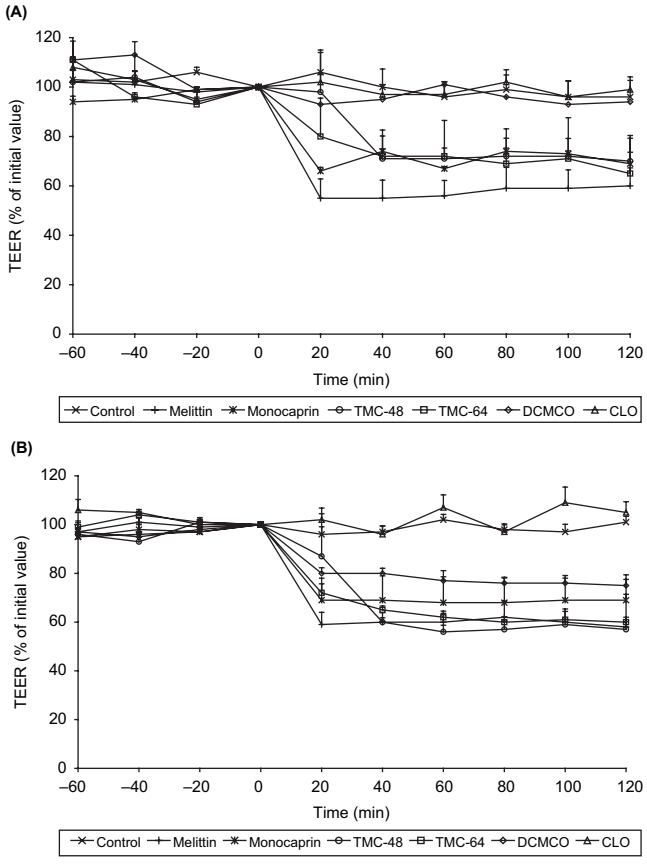

FIGURE 1. (A) Reduction in TEER (% of initial value) of Caco-2 cell monolayers as a function of time for the individual absorption enhancers (high concentrations) at pH 6.2 and (B) at pH 7.4.

concentration dependent manner, but this was less marked for TMC-48 at pH 7.4. This is in agreement with previous in vitro research findings (Hamman & Kotzé, 2003; Kotzé et al., 1997). Melittin exhibited similar TEER reduction for both pH values at the higher concentration (1.5 μM) used, but not at the lower concentration (1.0 μM). At pH 6.2 both CLO, DCMCO and melittin at 1.0 μM did not result in a statistically significant decrease in TEER compared with the control group ($p > .05$). Melittin has several actions on membranes which are complex in nature and are dependent on many factors, including pH, concentration, membrane composition, and hydration level and ionic strength of the medium which influence the conformation and aggregation state of the peptide itself. Permeabilization of membranes occurs at low concentrations of melittin and is indicative of an alteration in the organizational properties of the membrane lipids (Dempsey, 1990). It was shown that melittin reduces TEER of Caco-2 cell monolayers in a concentration-dependent way with a threshold concentration of 0.7 μM. The mechanism for reduction in TEER was found to involve alterations in specific tight junction proteins (Maher, Feighery, Brayden, & McClean, 2007a). This study confirmed a threshold value for melittin in its TEER reduction effect when applied alone to the Caco-2 cell monolayers as it was only effective at the higher concentration tested.

Monocaprin showed similar effectiveness and reduced TEER significantly ($p \leq .05$) at both pH values, with the higher concentration leading to a greater reduction in TEER. The independence of the effect from pH of the medium is to be expected from an uncharged molecule such as monocaprin. The Caco-2 monolayers exhibited no recovery toward pretest TEER values 1 h after removal of the monocaprin solutions for either concentration. Brown, Collett, Attwood, Ley, & Sims (2002) have, however, reported a recovery to 80% of pretest values for monolayer treated with 1.3 mM monocaprin solutions after 30 h of incubation in DMEM, whereas no such recovery was noted for 2.0 mM monocaprin-solution-treated monolayers.

DCMCO reduced TEER statistically significantly in a concentration-dependent manner at pH 7.4 ($p \leq .05$), but the reduction in TEER at pH 6.2 was not statistically significant ($p > .05$). Chitosan and its derivatives are reported to exhibit a molecular weight threshold for opening tight junctions (Schipper, Vårum, & Artursson, 1996). The findings of this study are consistent with this in that CLO exhibited negligible TEER reduction, whereas DCMCO exhibited only some reduction in TEER at 0.5% (wt/vol) concentration. A possible explanation for this poor performance of chitosan oligomers has been provided by Jonker-Venter et al. (2006), who propose that it is possible that oligomers interact with the cell membrane to open tight junctions, but because they are themselves smaller molecules than polymers, they enter the paracellular space between cells and thereby block the intercellular space for the passage of other molecules.

The TEER (% of initial value) plotted as a function of time for the combinations of absorption enhancers (lower concentrations were used in the combinations as described under materials and methods) at pH 6.2 are depicted in Figure 2A and that for the combinations of absorption enhancers at pH 7.4 in Figure 2B.

The most effective absorption enhancer combinations at pH 6.2 were the TMC polymers in combination with monocaprin and melittin where a synergistic reduction in TEER was observed with an increase in the effect on the TEER from 15 ± 7.0% for TMC-48 (0.25%, wt/vol) alone to 29 ± 1.1% when combined with monocaprin and to 56 ± 18.4% when combined with melittin. The effect of TMC-64 (0.25%, wt/vol) alone on the TEER was increased from 26 ± 6.6 to 59 ± 6.9% when combined with melittin, whereas no increase was observed when combined with monocaprin.

Combining the different chitosan derivatives and oligomers with melittin and monocaprin showed increased effects on the TEER of the Caco-2 monolayers at pH 7.4. The most prominent synergistic effect was obtained with TMC-48 (0.25%, wt/vol) where the reduction in TEER was significantly increased from 43 ± 7.2% for TMC-48 alone to 59 ± 2.3% in combination with

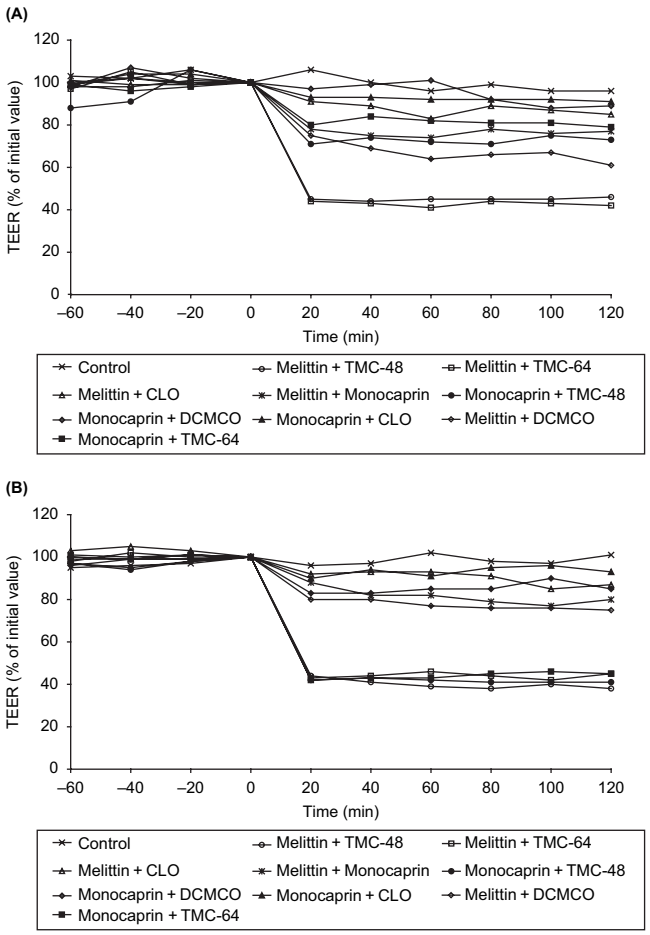

FIGURE 2. (A) Reduction in TEER (% of initial value) of Caco-2 cell monolayers as a function of time for the combinations of absorption enhancers (low concentrations) at pH 6.2 and (B) at pH 7.4. (Error bars omitted for reasons of clarity).

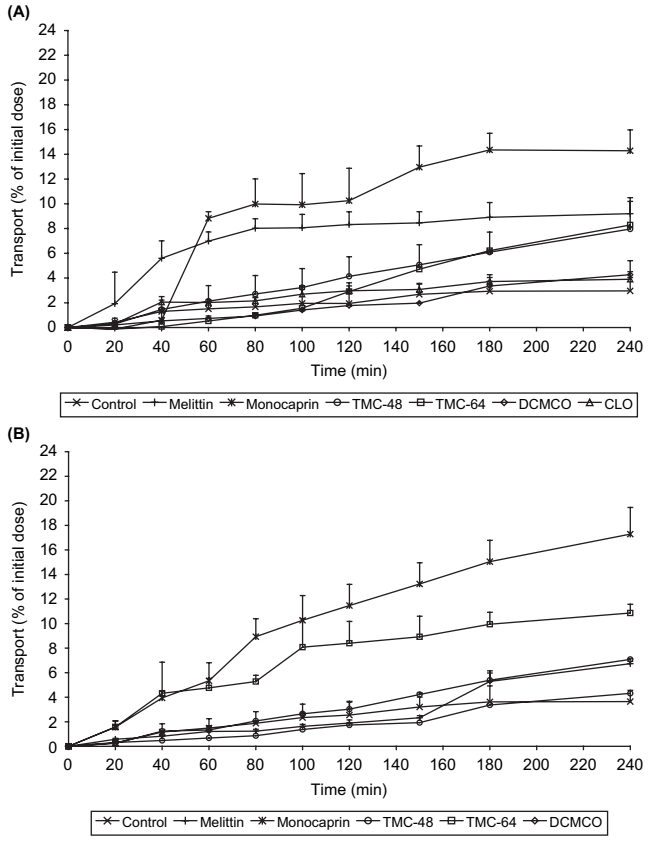

FIGURE 3. (A) Cumulative transport (% of initial dose) of FITC–dextran as a function of time for the individual absorption enhancers at pH 6.2 and (B) at pH 7.4.

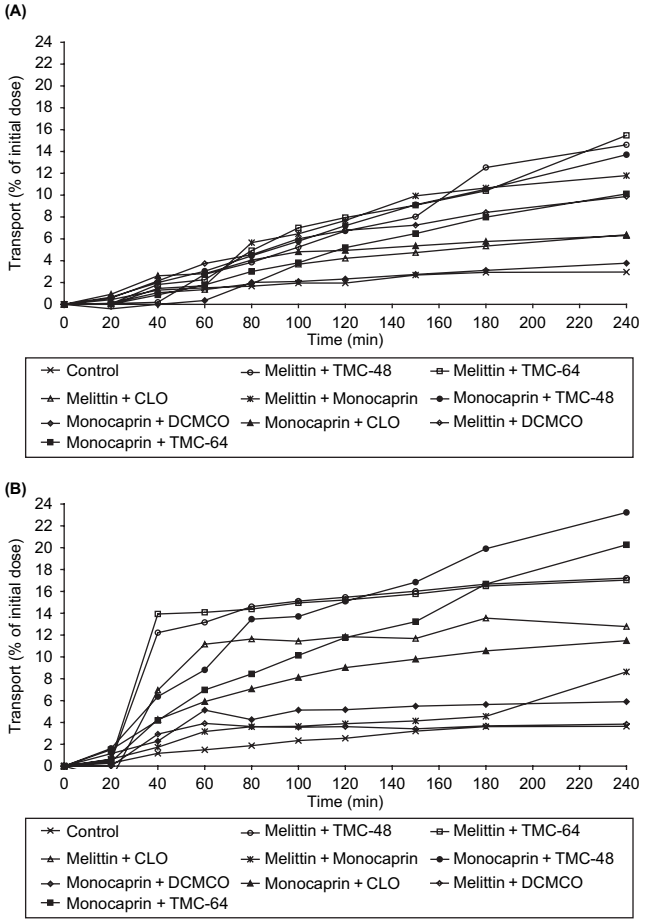

FIGURE 4. (A) Cumulative transport (% of initial dose) of FITC–dextran as a function of time for the combinations of absorption enhancers at pH 6.2 and (B) at pH 7.4. (Error bars omitted for reasons of clarity).

monocaprin and to 62 ± 7.1% in combination with melittin. The reduction in TEER for TMC-64 (0.25%, wt/vol) increased from 35 ± 4.7% when applied alone to 58 ± 2.7% in combination with monocaprin and to 58 ± 4.6% in combination with melittin. The effect on TEER was also significantly increased for DCMCO (0.25%, wt/vol) from 12 ± 1.5% when applied alone to 17 ± 1.8% with monocaprin and to 31 ± 3.1% with melittin. A similar trend was observed for CLO (0.25%, wt/vol) where the TEER reduction increased from 2 ± 1.6% when applied alone to 9 ± 2.9% when combined with monocaprin and to 15 ± 2.2% when combined with melittin.

The synergistic effect on the TEER of the epithelial cell monolayers obtained by combining the different absorption enhancers can possibly be attributed to the different mechanisms of opening tight junctions by the different absorption enhancers with probable interactions between these mechanisms and/or some overlap.

Measurement of the Effect on the Transport of a Macromolecular Compound

The cumulative transport (% of initial dose) of FITC–dextran plotted as a function of time for the individual absorption enhancers at pH 6.2 is shown in Figure 3A, whereas that for the individual absorption enhancers at pH 7.4 in Figure 3B. The transport plots for the combinations of absorption enhancers at pH 6.2 are depicted in Figure 4A and those at pH 7.4 are shown in Figure 4B. The calculated P_{app} values as well as enhancement ratios (R) are shown in Table 1 for the individual absorption enhancers, and the values for the combinations are shown in Table 2.

Of the individual absorption enhancers, monocaprin (2.0 mM) showed the highest absorption enhancement effect with a P_{app} value of $24.4 \pm 2.4 \times 10^{-7}$ cm/s at pH 6.2 and $26.1 \pm 3.8 \times 10^{-7}$ at pH 7.4. This can possibly be explained by transport enhancement of the model compound via the paracellular and transcellular pathways, and toxic effects may be caused to some extent by this relatively high concentration of monocaprin even though previous studies suggested no toxic effects at this concentration (Brown Collett, Attwood, Ley, & Sims, 2002; Liu et al., 1999). The transport enhancement effects of all the absorption enhancers tested in this study were clearly concentration dependent, whereas in general a less pronounced dependency was observed for pH. As expected, DCMCO was

TABLE 1
The Apparent Permeability Coefficients (P_{app}) and Transport Ratios (R) of FITC–Dextran (Molecular Weight 4,400) Across Caco-2 Cell Monolayers Co-Applied with the Individual Absorption Enhancers

Individual Absorption Enhancers	pH 6.2		pH 7.4	
	$P_{app} \times 10^{-7}$(cm/s)*	R	$P_{app} \times 10^{-7}$(cm/s)*	R
DCMCO 0.5% (wt/vol)	6.40 ± 0.2^{A}	1.45	6.49 ± 0.2^{B}	1.10
DCMCO 0.25% (wt/vol)	4.14 ± 3.6	0.94	4.54 ± 1.8	0.77
TMC-48 0.5% (wt/vol)	12.00 ± 2.9^{A}	2.71	10.41 ± 0.4^{B}	1.77
TMC-48 0.25% (wt/vol)	6.60 ± 0.5^{A}	1.49	9.18 ± 0.6^{B}	1.56
TMC-64 0.5% (wt/vol)	13.56 ± 2.6^{A}	3.06	15.44 ± 0.5^{B}	2.63
TMC-64 0.25% (wt/vol)	7.46 ± 2.3^{A}	1.69	10.25 ± 1.7^{B}	1.74
Monocaprin 2.0 mM	24.42 ± 2.4^{A}	5.52	26.08 ± 3.8^{B}	4.44
Monocaprin 1.3 mM	7.32 ± 5.2	1.66	10.53 ± 2.3^{B}	1.79
Melittin 1.5 μM	14.67 ± 1.9^{A}	3.32	10.06 ± 0.5^{B}	1.71
Melittin 1 μM	10.16 ± 1.6^{A}	2.30	5.69 ± 0.3	0.97
Control	4.42 ± 1.8	–	5.88 ± 1.4	–

*Values marked with an A are statistically significantly different from control at pH 6.2 ($p \leq .05$) and B are statistically significantly different from control at pH 7.4 ($p \leq .05$), $n = 3$, mean ± *SD*.

TABLE 2
Effect of the Combinations of Absorption Enhancers on the Apparent Permeability Coefficients (P_{app}) and Transport Ratios (R) of FITC–Dextran (Molecular Weight 4,400) Across Caco-2 Cell Monolayers

Absorption Enhancer Combinations	pH 6.2		pH 7.4	
	$P_{app} \times 10^{-7}$(cm/s)*	R	$P_{app} \times 10^{-7}$(cm/s)*	R
Melittin 1 μM + Monocaprin 1.3 mM	20.44 ± 1.6^{A}	4.62	8.56 ± 0.48^{B}	1.46
Monocaprin 1.3 mM + DCMCO 0.25% (wt/vol)	6.94 ± 1.9	1.57	8.79 ± 3.2	1.49
Monocaprin 1.3 mM + TMC-48 0.25% (wt/vol)	$21.15 \pm 1.7^{A,C}$	4.78	$35.95 \pm 2.8^{B,D}$	6.11
Monocaprin 1.3mM + TMC-64 0.25% (wt/vol)	$16.24 \pm 2.5^{A,C}$	3.67	$30.43 \pm 0.7^{B,D}$	5.18
Melittin 1 μM + DCMCO 0.25% (wt/vol)	$15.25 \pm 1.8^{A,C}$	3.45	4.88 ± 2.1	0.83
Melittin 1 μM + TMC-48 0.25% (wt/vol)	$23.94 \pm 1.30^{A,C}$	5.42	$25.32 \pm 1.6^{B,D}$	4.31
Melittin 1 μM + TMC-64 0.25% (wt/vol)	$22.91 \pm 1.7^{A,C}$	5.18	$20.54 \pm 2.8^{B,D}$	3.49

*Values marked with an A are statistically significantly different from control at pH 6.2 ($p \leq .05$), B are statistically significantly different from control at pH 7.4, C are statistically significantly different from single component chitosan derivative at pH 6.2 ($p \leq .05$), and D are statistically significantly different from single component chitosan derivative at pH 7.4 ($p \leq .05$), $n = 3$, mean ± *SD*.

less effective than the other absorption enhancers at both pH values for reasons already mentioned in the discussion of the TEER results.

In accordance with the TEER results, synergism in terms of absorption enhancement was observed for the combinations of the TMC polymers with melittin as well as with monocaprin. Furthermore, melittin and monocaprin in combination with each other and with DCMCO also exhibited synergistic effects. The transport enhancement ratio of 5.42 obtained with the combination of melittin (1 μM) and TMC-48 (0.25%, wt/vol) is of special importance if taken into consideration that this effect was achieved with relatively low concentrations of these absorption enhancers.

As mentioned before, synergism may result from combinations of absorption enhancers that act on the same cellular mechanism that is not yet saturated or may be due to different mechanisms of action, such as tight junction permeability modulation and cell membrane phase transition.

CONCLUSIONS

This study has shown that combinations of absorption enhancers are more effective in reducing the TEER of Caco-2 cell monolayers and more effective in enhancing the transport of a model compound, FITC–dextran, across these cell monolayers at both pH 6.2 and 7.4 than any of the selected absorption

enhancers alone. Synergistic effects were observed, which most likely result from the different mechanisms by which the constituent absorption enhancers act. This synergism resulted in lower concentrations of absorption enhancers to cause higher drug absorption enhancing effects on the intestinal epithelium compared with the individual absorption enhancing agents.

REFERENCES

Brown, J. R., Collett, J. H., Attwood, D., Ley, R. W., & Sims, E. E. (2002). Influence of monocaprin on the permeability of a diacidic drug BTA-243 across Caco-2 cell monolayers and everted gut sacs. *Int. J. Pharm., 245*, 133–142.

Cleland, J. L., Daugherty, A., & Mrsny, R. (2001). Emerging protein delivery methods. *Curr. Opin. Biotechnol., 12*, 212–219.

Dempsey, C. E. (1990). The actions of melittin on membranes. *Biochim. Biophys. Acta, 1031*, 143–161.

Dodane, V., Khan, M. A., & Merwin, J. R. (1999). Effect of chitosan on epithelial permeability and structure. *Int. J. Pharm., 182*, 21–32.

Gomez-Orellana, I. (2005). Strategies to improve oral drug bioavailability. *Expert Opin. Drug. Deliv., 2*, 419–433.

Hamman, J. H., Enslin, G. M., & Kotzé, A. F. (2005). Oral delivery of peptide drugs: Barriers and developments. *BioDrugs, 19*, 165–177.

Hamman, J. H., Schultz, C. M., & Kotzé, A. F. (2003). *N*-trimethyl chitosan chloride: Optimum degree of quaternization for drug absorption enhancement across epithelial cells. *Drug Dev. Ind. Pharm., 29*, 161–172.

Hejazi, R., & Amiji, M. (2003). Chitosan-based gastrointestinal delivery systems. *J. Control. Release, 89*, 151–165.

Illum, L. (1998). Chitosan and its use as a pharmaceutical excipient. *Pharm. Res., 15*, 1326–1331.

Jonker-Venter, C., Snyman, D., Janse van Rensburg, C., Jordaan, E., Schultz, C., Steenekamp, J. H., Hamman, J. H., & Kotzé, A. F. (2006). Low molecular weight quaternised chitosan (II): *In vitro* assessment of absorption enhancing properties. *Pharmazie, 61*, 301–305.

Junginger, H. E., & Verhoef, J. C. (1998). Macromolecules as safe penetration enhancers for hydrophilic drugs—a fiction. *Pharm. Sci. Technol. Today, 1*, 370–376.

Kotzé, A. F., De Leeuw, B. J., Lueßen, H. L., De Boer, A. G., Verhoef, J. C., & Junginger, H. E. (1997). Chitosans for enhanced delivery of therapeutic peptides across intestinal epithelia: In vitro evaluation in Caco-2 cell monolayers. *Int. J. Pharm., 159*, 243–253.

Kotzé, A. F., Thanou, M. M., Lueßen, H. L., De Boer, A. G., Verhoef, J. C., & Junginger, H. E. (1999). Effect of the degree of quaternization of *N* -trimethyl chitosan chloride on the permeability of intestinal epithelial cells (Caco-2). *Eur. J. Pharm. Biopharm., 47*, 269–274.

Lecluyse, E. L., & Sutton, S. C. (1997). *In vitro* models for selection of development candidates. Permeability studies to define mechanisms of absorption enhancement. *Adv. Drug Deliv. Rev., 23*, 163–183.

Liu, P., Davis, P., Liu, H., & Krishnan, T. R. (1999). Evaluation of cytotoxicity and absorption enhancing effects of melittin—a novel absorption enhancer. *Eur. J. Pharm. Biopharm., 48*, 85–87.

Lueßen, H. L., De Leeuw, B. J., Langemeÿer, W. E., De Boer, A. B. G., Verhoef, J. C., & Junginger, H. E. (1997). Mucoadhesive polymers in peroral peptide drug delivery. VI. Carbomer and chitosan improve the intestinal absorption of the peptide drug buserelin *in vivo*. *Pharm. Res., 13*, 1668–1672.

Maher, S., Feighery, L., Brayden, D. J., & McClean, S. (2007a). Melittin as an epithelial permeability enhancer I: Investigation of its mechanism of action in caco-2 monolayers. *Pharm. Res., 24*, 1336–1345.

Maher, S., Feighery, L., Brayden, D. J., & McClean, S. (2007b). Melittin as an epithelial permeability enhancer II: In vivo investigations in human mucus secreting monolayers and rat colonic mucosae. *Pharm. Res., 24*, 1346–1356.

Paul, W., & Sharma, C. P. (2000). Chitosan, a drug carrier for the 21st century: A review. *S.T.P. Pharma Sci., 10*, 5–22.

Schipper, N. G. M., Vårum, K. M., & Artursson, P. (1996). Chitosans as absorption enhancers for poorly absorbable drugs. 1: Influence of molecular weight and degree of acetylation on drug transport across human intestinal epithelial (Caco-2) cells. *Pharm. Res., 13*, 1686–1691.

Sharma, P., Varma, M. V. S., Chawla, H. P. S., & Panchagnula, R. (2005). Absorption enhancement, mechanistic and toxicity studies of medium chain fatty acids, cyclodextrins and bile salts as peroral absorption enhancers. *Farmaco, 60*, 884–893.

Shen, W. (2003). Oral peptide and protein delivery: Unfulfilled promises. *Drug Discov. Today, 8*, 607–608.

Soltero, R., & Ekwuribe, N. (2001). The oral delivery of protein and peptide drugs. *Innovations Pharm. Technol.*, 106–110.

Thanou, M., Verhoef, J. C., & Junginger, H. E. (2001). Oral drug absorption enhancement by chitosan and its derivatives. *Adv. Drug Deliv. Rev., 52*, 117–126.

Van Hoogdalem, E. J., De Boer, A. G., & Breimer, D. D. (1989). Intestinal drug absorption enhancement: An overview. *Pharm. Ther., 44*, 407–443.

Ward, P. D., Tippin, T. K., & Thakker, D. K. (2000). Enhancing paracellular permeability by modulating epithelial tight junctions. *Pharm. Sci. Technol. Today, 3*, 346–358.

Zhou, X. H. (1994). Overcoming enzymatic and absorption barriers to non-parenterally administered protein and peptide drugs. *J. Control. Release, 29*, 239–252.