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Research Paper

Mechanistic Analysis of Chemical Permeation Enhancers for Oral Drug Delivery

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Purpose. Traditionally, the oral route cannot be employed for the delivery of macromolecular drugs such as proteins and peptides due, in large part, to limited transport across the epithelial membrane. This particular challenge can potentially be addressed through the use of chemical permeation enhancers, which affect transcellular and/or paracellular transport routes. Although certain permeation enhancers have been proposed for use in oral delivery, potential for application is often unclear when the route of enhancer action is unknown.
Methods. A combination of theory and experiments was developed for determining mechanism of enhancer action. The effect of 51 enhancers on Caco-2 cells was studied using TEER, MTT, and LDH assays.
Results. The mechanistic details of intestinal permeability enhancement were uncovered for a broad set of enhancers *in vitro*. Understanding gained from enhancer mechanisms enabled the deduction of structure–function relationships for hydrophilic and hydrophobic permeation enhancers as well as the identification of a transcellular enhancer, 0.01% (*w*/*v*) palmityldimethyl ammonio propane sulfonate, which enabled the non-cytotoxic intracellular delivery of a model drug.
Conclusions. The results presented here emphasize the importance of understanding enhancer mechanism and uncover a zwitterionic surfactant capable of safely and effectively achieving intra-epithelial drug delivery *in vitro*.

KEY WORDS: Caco-2; mechanism; oral drug delivery; permeation enhancer; transcellular.

INTRODUCTION

Although needles are the primary mode of administering macromolecular drugs such as proteins and peptides, their limitations have motivated researchers to explore alternative routes. One of the most popular alternatives to parenteral drug administration is oral delivery (1,2), which requires transport across the intestinal epithelial membrane. Because the epithelium protects against the entry of xenogenic substances into the body, it also acts as a barrier to the delivery of macromolecules (3). This issue of limited transport can be potentially addressed through the use of chemicals to promote drug absorption across the epithelium (4).

Chemical permeation enhancers aid drug uptake through two distinct mechanisms, both of which involve the mediation of a physical cellular barrier. The passive transcellular route involves the alteration of the structure of the cell membrane, whereas an enhancement of the paracellular route entails an opening of the tight junctions between epithelial cells (5,6). Many studies have focused their efforts on ascertaining the mechanism of action of a variety of enhancers, such as melittin (7), sodium cholate (8), palmitoyl carnitine (9), and chitosan (10). Numerous methods have been used to make mechanistic assessments, including fluorescence microscopy (11), immunostaining (12,13), voltage clamping (14,15), and permeability studies (7,16). Unfortunately, these techniques are often used inconsistently across laboratories, and mechanistic analysis tends to be incomplete. Specifically, enhancer mechanism is typically considered to be solely transcellular or paracellular, and the ability of an enhancer to affect both routes remains largely unexplored. For example, sodium caprate, which has been shown to be effective *in vivo* (17,18), has been classified as a paracellular enhancer by several studies (19,20) and as a transcellular enhancer by others (21). Few studies have performed the combination of experiments required to conclude that sodium caprate, in fact, acts through both routes (22,23). This example underscores the need to establish a uniform method for determining the route of action of an enhancer. With such a goal in mind, we have created a simple way of quantitatively determining the mechanistic nature of an enhancer through experiments and theoretical analysis. Once this was achieved, we set out to use the understanding gained from such an investigation to elucidate permeation enhancer structure–function relationships and to identify enhancers with new therapeutic properties.

Electronic supplementary material The online version of this article (doi:10.1007/s11095-008-9542-2) contains supplementary materials, which is available to authorized users.

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NOTATION: EP, Enhancement potential; K, Mechanistic parameter; LDH, Lactate dehydrogenase; Log P, Water-octanol partion coefficient; LP, LDH potential; MTT, Methyl thiazole tetrazolium; PPS, Palmityldimethyl ammonio propane sulfonate; TEER, Transepithelial electrical resistance; TP, Toxicity potential.

MATERIALS AND METHODS

Selection of Chemical Permeation Enhancers. Fifty-one enhancers from 11 distinct chemical categories were chosen for this study. These categories include anionic surfactants, cationic surfactants, zwitterionic surfactants, nonionic surfactants, bile salts, fatty acids, fatty esters, fatty amines, sodium salts of fatty acids, nitrogen-containing rings, and others. A complete list of enhancers examined in this study can be found in Table I. Compounds were selected to reflect a diverse library of enhancers and to include several commonly-studied absorption enhancers. All compounds were tested at concentrations of 1, 0.1, and 0.01% w/v in Dulbecco's Modified Eagles Medium (DMEM, American Type Culture Collection, Rockville, MD). All enhancers were soluble in DMEM.

Table I. List of Chemical Permeation Enhancers

Abbreviation	Chemical Name	Category	CAS Number
SLS	Sodium lauryl sulfate	AS	151-21-3
SDS	Sodium decyl sulfate	AS	142-87-0
SOS	Sodium octyl sulfate	AS	142-31-4
SLA	Sodium laureth sulfate	AS	68585-34-2
NLS	*N*-Lauryl sarcosinate	AS	137-16-6
CTAB	Cetyltrimethyl ammonium bromide	CS	57-09-0
DTAB	Decyltrimethyl ammonium bromide	CS	2082-84-0
BDAC	Benzyldimethyl dodecyl ammonium chloride	CS	139-07-1
TTAC	Myristyltrimethyl ammonium chloride	CS	4574-04-3
DPC	Dodecyl pyridinium chloride	CS	104-74-5
DPS	Decyldimethyl ammonio propane sulfonate	ZS	15163-36-7
MPS	Myristyldimethyl ammonio propane sulfonate	ZS	14933-09-6
PPS	Palmityldimethyl ammonio propane sulfonate	ZS	2281-11-0
CBC	ChemBetaine CAS	ZS	N/A (mixture)
CBO	ChemBetaine Oleyl	ZS	N/A (mixture)
PCC	Palmitoyl carnitine chloride	ZS	6865-14-1
IP	Nonylphenoxypolyoxyethylene	NS	68412-54-4
T20	Polyoxyethylene sorbitan monolaurate	NS	9005-64-5
T40	Polyoxyethylene sorbitan monopalmitate	NS	9005-66-7
SP80	Sorbitan monooleate	NS	1338-43-8
TX100	Triton-X 100	NS	9002-93-1
SDC	Sodium deoxycholate	BS	302-95-4
SGC	Sodium glycocholate	BS	863-57-0
CA	Cholic acid	FA	73163-53-8
HA	Hexanoic acid	FA	142-91-6
HPA	Heptanoic acid	FA	111-14-8
LME	Methyl laurate	FE	111-82-0
MIE	Isopropyl myristate	FE	110-27-0
IPP	Isopropyl palmitate	FE	142-91-6
MPT	Methyl palmitate	FE	112-39-0
SDE	Diethyl sebaccate	FE	110-40-7
SOA	Sodium oleate	SS	143-19-1
UR	Urea	FM	57-13-6
LAM	Lauryl amine	FM	124-22-1
CL	Caprolactam	NR	105-60-2
MP	Methyl pyrrolidone	NR	872-50-4
OP	Octyl pyrrolidone	NR	2687-94-7
MPZ	Methyl piperazine	NR	109-01-3
PPZ	Phenyl piperazine	NR	92-54-6
EDTA	Ethylenediaminetetraacetic acid	OT	10378-23-1
SS	Sodium salicylate	OT	54-21-7
CP	Carbopol 934P	OT	9003-04-7
GA	Glyccyrhetinic acid	OT	471-53-4
BL	Bromelain	OT	9001-00-7
PO	Pinene oxide	OT	1686-14-2
LM	Limonene	OT	5989-27-5
CN	Cineole	OT	470-82-6
ODD	Octyl dodecanol	OT	5333-42-6
FCH	Fenchone	OT	7787-20-4
MTH	Menthone	OT	14073-97-3
TPMB	Trimethoxy propylene methyl benzene	OT	2883-98-9

AS Anionic surfactants, *CS* cationic surfactants, *ZS* zwitterionic surfactants, *NS* nonionic surfactants, *BS* bile salts, *FA* fatty acids, *FE* fatty esters, *FM* fatty amines, *SS* sodium salts of fatty acids, *NR* nitrogen-containing rings, *OT* others

Cell Culture. Caco-2 cell line HTB-37 (American Type Culture Collection, Rockville, MD), derived from human colon cells, was used for all experiments. Cells were maintained at 37°C in DMEM supplemented with 25 IU/ml of penicillin, 25 mg/L of streptomycin, 250 μg/L of amphotericin B and 100 ml/L of fetal bovine serum. Monolayers were grown on BD Biocoat™ collagen filter supports (Discovery Labware, Bedford, MA) for 3 days according to supplier instructions. Feeding schedules remained the same for all experiments to ensure comparable monolayer growth. At the end of the growth period, the integrity of the cell monolayer was confirmed by transepithelial electrical resistance (TEER) measurements (Millicell-ERS voltohmmeter, Millipore, Billerica, MA). Only monolayers with TEER values over 700 Ω cm^2 were used for further experimentation. TEER values of unseeded filter supports averaged 100 Ω cm^2.

TEER Experiments. Upper filter supports containing viable Caco-2 monolayers were transferred into a 24-well BD Falcon plate and 1 ml of media was dispensed into each basolateral compartment. Enhancer solutions were applied to the apical compartment and TEER readings were taken at 10 min. TEER recovery was assessed by removing enhancer solutions after 30 min, applying fresh media, and measuring TEER values at 24 h.

Calculation of Enhancement Potential (EP). When determining enhancer potency, TEER was used as a surrogate marker for permeability. The literature has indicated an inverse relationship between TEER and potency (24,25), which was confirmed for the enhancers of this study using the radiolabeled marker molecule mannitol. This inverse relationship has been confirmed previously for our system (26). Although variability in baseline TEER values did occur, all TEER values were normalized by their initial values to allow appropriate comparison between experiments. A quantitative measure of potency, enhancement potential (EP), was calculated as the reduction in TEER of a Caco-2 monolayer after 10 min of exposure to that enhancer, normalized to the reduction in TEER after exposure to the positive control, 1% Triton X-100:

$$EP = \frac{100\% - TEER_E}{100\% - TEER_+}$$

where $TEER_E$ and $TEER_+$ are the resistance values (% of initial) of the enhancer solution and positive control solution, respectively, after 10 minutes of exposure. EP lies on a scale of 0 to 1, with 1 representing maximum enhancement as compared to the positive control.

Methyl Thiazole Tetrazolium (MTT) Experiments. Caco-2 cells were seeded at 10^5 cells/well onto a 96-well plate. Enhancer solutions (100 μl) were applied for 30 min. Ten microliters of reagent from an MTT kit (American Type Culture Collection, Rockville, MD) was applied to each well for 5 h, after which 100 μl of detergent was applied to each well and allowed to incubate in the dark at room temperature for about 40 h. Absorbance was read at 570 nm (MTT dye) and 650 nm (detergent). Toxicity potential (TP) values are reported as the fraction of nonviable cells, as compared to the negative control, DMEM. TP values range from 0 to 1, with 0 indicating no mitrochondrial toxicity, and 1 representing maximum toxicity.

Lactate Dehydrogenase (LDH) Experiments. Caco-2 cells were seeded at 10^4 cells/well onto a 96-well plate. Enhancer solutions (100 μl) were applied for 30 min. Twenty five microliters of the solution was then transferred to a fresh 96-well plate and mixed with 25 μl of LDH reagent from the CytoTox 96® assay (Promega, Madison, WI) and allowed to react for 30 min in the dark at room temperature. Stop solution (25 μl) was then added to each well, and the absorbance was read at 490 nm. LDH potential (LP) values are reported as the fraction of maximal LDH release, as determined by the positive control lysis solution provided with the assay kit (~1% Triton-X100). LP values lie on a scale of 0 to 1, with 0 representing no LDH release, and 1 indicating maximum LDH release.

Calculation of Molecular Parameters. Chemical permeation enhancer structures were drawn using the program Molecular Modeling Pro (ChemSW) and were relaxed to their lowest energy conformation. All parameters were estimated as described in the software. The octanol–water partition coefficient was taken as the average of the three closest of four independent methods: atom-based Log *P*, fragment addition Log *P*, Q Log *P*, and Morigucchi's method.

Fluorescence Microscopy. A solution containing a permeation enhancer and 0.01% (*w*/*v*) calcein dissolved in phosphate buffered saline was applied to Caco-2 cells. After 30 min, solutions were removed and replaced with a solution containing only calcein. After 1 h, samples were washed 3× with phosphate buffered saline and viewed with a Zeiss fluorescence microscope.

RESULTS

Comparison of the MTT and LDH Assays. Although many types of toxicity assays are used to assess the damage caused by an enhancer to epithelium, two of the most common include the LDH and the MTT assays (27,28). The LDH assay measures the amount of lactate dehydrogenase enzyme, present in the cytosol, that leaks out of the cell and into the extracellular fluid. In essence, this assay measures the permeability of the cellular membrane to a 144 kDa enzyme. The MTT assay measures the ability of the cell mitochondria to cleave the MTT salt into a formazan product, which accumulates inside of the cell. Therefore, the MTT assay is a good measure of the overall health of the cell, as it indicates the viability of the cell's primary energy-generating organelle. Additionally, it has been shown to be the more sensitive of the two assays (29). Based on these differences, it was decided that the MTT assay would be used to calculate the quantitative parameter, toxicity potential (TP), of the enhancers analyzed in this study.

Such a choice did not have significant implications for most enhancers, given that the results of the MTT and LDH assays usually correlated very well (Fig. 1, r^2=0.73 for a linear

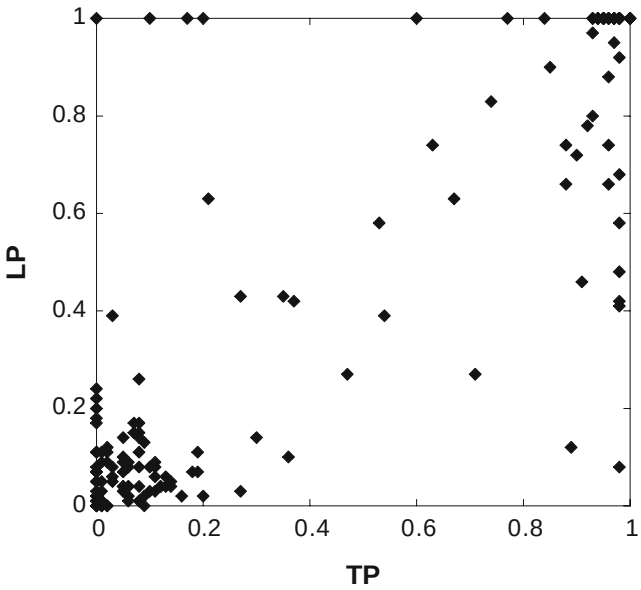

Fig. 1. *LP vs. TP* graph for all 153 enhancer formulations. *LP* generally correlated with *TP* (r^2=0.73 for a linear fit over all points). Outliers include most notably zwitterionic surfactants (high LP, low TP). n=3–6. Error bars are not shown in the figure for clarity. Mean standard deviations are 0.09 and 0.12 for *TP* and *LP* values, respectively.

fit). Only 14% of permeation enhancers did not fall along the 45° line, within error. Most prominently, zwitterionic surfactants tended to display high LP values but low TP values. This observation fundamentally suggests that while zwitterionic surfactants are very effective in perturbing the membrane of epithelial cells, they do not induce toxicity to the mitochondria. While deviating from the norm, the behavior of this group of enhancers was significant enough to provoke further exploration. Subsequently, it was found that discrepancies in the toxicity information gathered via MTT and LDH assays could be exploited in a way that revealed the mechanistic nature of the absorption enhancers.

Mechanisms of Enhancer Action—Transcellular and Paracellular Contributions. Equation 1 has been derived using the ionic permeability theory developed in the Theory section of the Supplementary Text:

$$\mathrm{EP} = \mathrm{LP} + \frac{E_\mathrm{p}}{E_\mathrm{o}^{\max}} \tag{1}$$

where EP is enhancement potential, LP is LDH potential, and $\frac{E_p}{E_o^{max}}$ is a term representing paracellular contributions to permeability. This equation states that the overall potency of an enhancer is equal to a transcellular effect plus a paracellular effect. Equation 1 was used to assess the relative contribution of transcellular and paracellular pathways to permeability of the intestinal epithelium. Figure 2 shows a plot of EP *vs.* LP for all enhancers at the various concentrations used in this study. According to Eq. (1), the line EP = LP corresponds to enhancers that act predominantly by the transcellular route (paracellular contributions are negligible). Enhancers lying on the vertical EP axis primarily utilize the paracellular pathway, since there is no relationship between EP and LP when transcellular contributions are negligible. The relative contribution of the paracellular pathway is higher for enhancers falling closer to the EP axis than to the EP = LP line.

Based on the departure of points from EP = LP, it is possible to quantify the extent of contribution of the paracellular pathway to overall enhancement. For this purpose, we calculated the parameter $\mathrm{K} = \frac{(\mathrm{EP}-\mathrm{LP})}{\mathrm{EP}}$ which represents the relative contribution of the paracellular pathway. *K* values were determined for all enhancers, with theoretical values ranging from 0 (predominantly transcellular) to 1 (predominantly paracellular). For example, 1% EDTA (EP=0.98, LP=0.27) yields K=0.72, indicating that it enhances *in vitro* transport primarily due to contributions from the paracellular pathway, a conclusion that is consistent with the literature (14). The complete set of raw mechanistic data for all enhancers can be found in Table SI of the supplementary text. Analysis of enhancer categories based on *K* is shown in Fig. 3. Although *K* values can vary significantly within the same category, these data provide a general idea of the mechanistic behavior of each chemical group. As a whole, fatty esters (FE) displayed by far the most paracellular behavior, followed by nitrogen-containing rings (NR). Cationic (CS) and zwitterionic (ZS) surfactants demonstrated the most transcellular behavior, which is not surprising given their known ability to disrupt membrane structure (30).

In general, the route of enhancement (transcellular *vs.* paracellular) was not dramatically altered by a change in concentration. Figure 4 demonstrates the likelihood of changes in *K* values upon change in enhancer concentration from 0.01% to 0.1% *w/v* or 0.1% to 1% *w/v*. About half of the time, the change in *K* values was less than 0.1 and in 83% cases, the

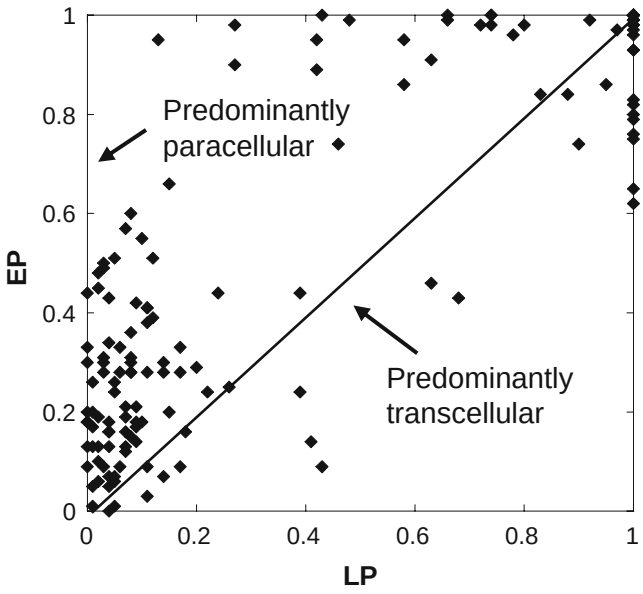

Fig. 2. *EP vs. LP* values for all 153 enhancer formulations (51 enhancers at 3 concentrations each). *Points* falling along the 45° line represent enhancer formulations with strong contributions from transcellular transport. *Points* close to the vertical EP axis represent enhancer formulations with strong contributions from paracellular transport. n=3–6. Error bars are not shown in the figure for clarity. Mean standard deviations are 0.07 and 0.12 for *EP* and *LP* values, respectively.

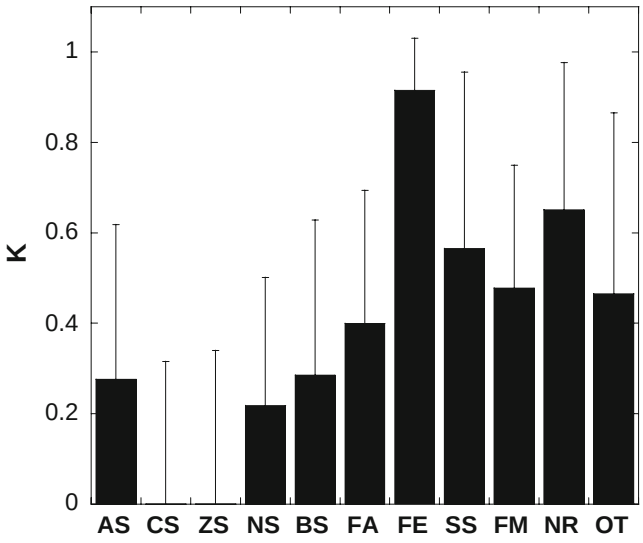

Fig. 3. Distribution of K values by chemical category (averaged over all enhancers and concentrations within each category). Category abbreviations are the same as those used in Table I. Higher K values correspond to a higher contribution from the paracellular route. *Error bars* indicate standard deviation (i.e. the extent to which enhancers within the same category affect the same route). It is not necessarily expected for standard deviations to be small.

change in K was less than 0.5. Larger changes in K were less prominent. Notable exceptions include all five of the anionic surfactants examined in this study, which become increasingly paracellular as concentration was decreased.

Molecular Origins of Mechanism of Action. In order to gain insight into the molecular features of a chemical permeation enhancer that affect potency, 22 molecular descriptors, including the octanol–water partition coefficient (Log P), components of solubility parameters (dispersive, polar and hydrogen bonding), and polar surface area were calculated for each enhancer. These parameters were reduced to a set of eight independent variables by assessing their correlation coefficients. These eight parameters were then analyzed for correlations with potency (EP). The data set at 0.01% concentration was chosen for analysis because it had the greatest distribution of EP values and thus the greatest potential to reveal trends.

Of all of the molecular descriptors that had been calculated, the Log P of the enhancers showed most notable correlations with EP (Fig. 5). Specifically, two distinct trends were observed when EP was plotted *versus* Log *P*. The first trend (dark gray), demonstrates a direct correlation between the two (r^2=0.9). Interestingly, 83% of permeation enhancers in this box are transcellular in nature (K<0.5). The other trend (light gray), shows an inverse trend between EP and Log P (r^2=0.77). 96% of enhancers in this box are paracellular (K>0.5). The analysis of Fig. 5 thus reveals two separate trends for enhancers acting through transcellular or paracellular routes. Specifically, the analysis shows that the potency of transcellular enhancers scales directly with enhancer hydrophobicity whereas that of paracellular enhancers scales inversely with hydrophobicity.

Applications of Chemical Permeation Enhancers. In addition to enhancing transepithelial transport, absorption promoters can also conceivably be used for intraepithelial delivery. This could be an important application in the case of diseases of the epithelia, including pre-cancerous cervical neoplasia (31) and chronic obstructive pulmonary disease (32). With the help of mechanistic data, the zwitterionic

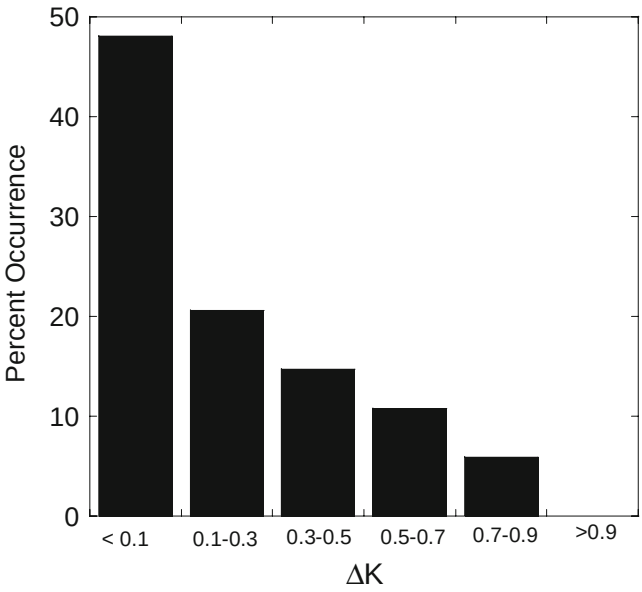

Fig. 4. Likelihood of changes in K values as enhancer concentration changes by one order of magnitude. K values did not change substantially for most enhancers as a function of concentration (higher *percent occurrence* at low ΔK).

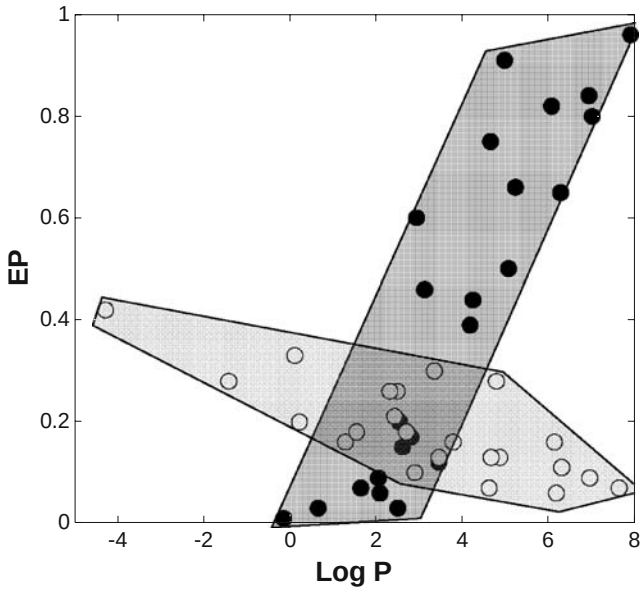

Fig. 5. Correlation between the experimental parameter, *EP*, and the molecular descriptor, *Log P*, for enhancer formulations at 0.01% (*w*/*v*) concentration. *EP* is directly proportional to *Log P* for transcellular enhancers (*closed circles*) and inversely proportional to *Log P* for paracellular enhancers (*open circles*); n=3–6.

surfactant 0.01% (*w*/*v*) palmityldimethyl ammonio propane sulfonate (PPS) was chosen for intraepithelial studies, as it was shown to be safe and effective while utilizing the transcellular route *in vitro* (EP=0.8, TP=0, K=0). It was expected that a transcellular enhancer would be most capable of allowing drug transport into epithelial cells.

Figure 6 demonstrates the ability of 0.01% PPS to permeabilize epithelial cells and allow the entry of the marker molecule calcein. While the negative control was only able to deliver calcein in between the cells, 0.01% PPS enabled the transport of calcein into more than 75% of epithelial cells. In order to confirm that this permeabilization was due to a potent transcellular mechanism, the experiment was also performed with 0.1% phenylpiperazine, a safe and effective paracellular enhancer (EP=0.95, TP=0.09, K=0.86). Use of phenylpiperazine resulted in a situation similar to the negative control, indicating that intraepithelial delivery can be achieved only through transcellular means. It was also confirmed that 0.01% PPS did not damage cell monolayer structure through TEER recovery experiments. These results indicate the feasibility of a new and interesting way to safely and effectively deliver therapeutics into epithelial cells.

DISCUSSION

Permeation enhancers offer significant potential in increasing drug absorption across the intestinal epithelium.

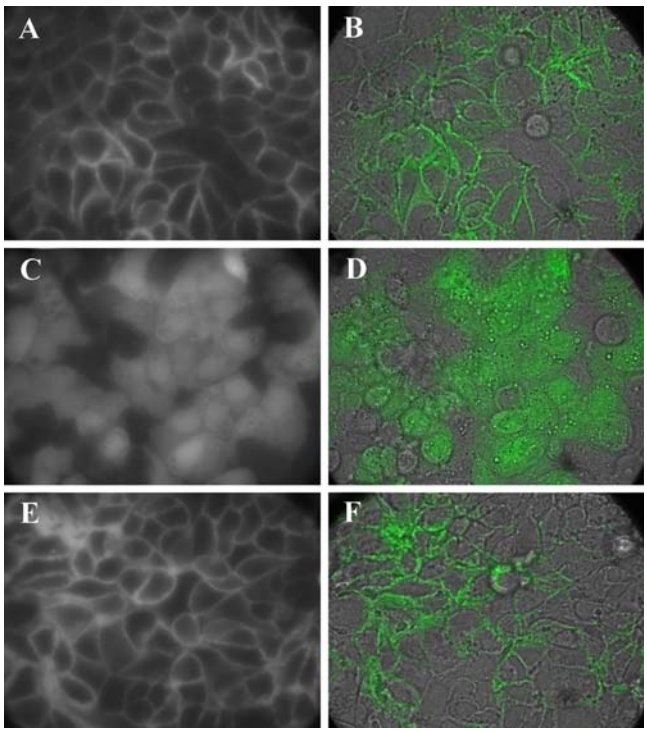

Fig. 6. Fluorescent images of calcein permeation into Caco-2 cells in the presence of a negative control (**A**, **B**), the transcellular enhancer 0.01% PPS (**C**, **D**), and the paracellular enhancer 0.1% phenylpiperazine (**E**, **F**). **A**, **C**, and **E** are pure fluorescent images while **B**, **D**, and **F** show fluorescence overlaid with corresponding brightfield images.

Although oral permeability enhancers have long been studied, understanding of their true potential and mechanism of action still remains unclear. The studies reported here directly address this issue and offer insight into the use of chemical absorption enhancers for applications in Caco-2 delivery. Through a combination of theory and experiments, the degree to which the enhancers acted via the transcellular and paracellular routes was evaluated *in vitro*. This mechanistic analysis was performed in the context of the passive transport of polar and ionic solutes. The transcellular pathway involves transport directly through an epithelial cell (33), whereas the paracellular pathway makes use of the tight junctions that are present between epithelial cells (34). While many permeability enhancers have been previously shown to employ paracellular mechanisms (24,35), it is also possible for an enhancer to engage the transcellular route, or even both routes (22).

Our study revealed significant chemistry-dependent contributions of transcellular and paracellular routes to overall passive permeation. Although several exceptions to the trends existed, most chemical groups behaved in a consistent mechanistic fashion. For example, all fatty esters acted through paracellular mechanisms. On the other hand, cationic and zwitterionic surfactants, in general, acted predominantly through the transcellular route. It is interesting to note the important difference between these two surfactant groups. While the cationic surfactants possessed the highest MTT-associated toxicity levels of any of the chemical categories and offered minimal opportunity for further application, zwitterionic surfactants demonstrated little toxicity to the mitochondria.

This study highlights several ways in which the understanding of enhancer mechanism has lead to other significant information regarding enhancer performance *in vitro*. For example, the deduction of a structure–function relationship for permeation enhancers was possible only once a distinction had been made between enhancers acting predominantly through the paracellular or transcellular routes. Subsequently, we were able to relate an experimentally-determined parameter (enhancement potential) to a molecular descriptor (Log P) in a mechanism-dependent manner. Molecular modeling showed that transcellular contributions to potency correlate with hydrophobicity, suggesting that ability to partition into the epithelial membrane may determine the potency of such enhancers. On the other hand, for paracellular enhancers, potency correlated inversely with Log P, suggesting that paracellular efficacy is the result of a favorable interaction between the absorption promoter and the hydrophilic constituents of the tight junctions. These observations offer insight into the relationship between molecular structure and enhancer potency.

Results presented here also revealed the ability of a transcellular enhancer, 0.01% PPS, to achieve effective intraepithelial delivery *in vitro*. Typically, this type of macromolecular delivery is only achieved through physical means such as ultrasound (36,37) or electroporation (38). Again, such an interesting result would not have surfaced without understanding of enhancer mechanisms. The key feature of 0.01% PPS that enables its use as an intraepithelial permeation enhancer is its low toxicity to the cell mitochondria but high ability to alter the cellular membrane. Although this study

demonstrated the use of only 0.01% PPS for intraepithelial applications, it is speculated that other zwitterionic surfactants would behave in a similar manner. Other zwitterionic surfactants at low concentration may have similar potential, owing to their high EP values but low TP and K values (see Supplementary Text).

It is important to note that the equations developed in the Theory section of the Supplementary Text suggest that all data points in Fig. 2 should lie above the line corresponding to EP = LP. Indeed, about 85% of points lie on or above this line and another 9% are close to the line within error. The remaining 6% of points exist well below the line, indicating disagreement between the theory and experiments. The theory assumes that absorption promoters enhance the permeability of both sides of epithelial cells equally. It is conceivable that certain enhancers produce more pronounced permeabilization of the apical membrane compared to basolateral membrane. In such situations, certain data points are expected to occur below the diagonal. It should be noted that the analysis of enhancer mechanism is presented using ionic permeability as a basis and that experiments have been performed solely *in vitro*. It is expected that these conclusions can be extended to polar solutes, since polar solutes are likely to follow the same pathway as ions across the epithelium. However, extrapolation of these conclusions to other solutes and to *in vivo* settings should be done with caution.

While relying on the conclusions reached in this study, it must be realized that no screening of systemic toxicity is reported here. Both methods of evaluating toxicity, MTT and LDH, measure the effect of enhancers on epithelial cells. Hence, no assumption should be made about the systemic safety of these permeation enhancers, and further studies are needed to shed light on this topic.

CONCLUSIONS

Results presented here reveal a simple and reliable method of quantitatively determining the contributions of paracellular and transcellular transport routes to overall enhancer mechanism *in vitro*. It has been shown that chemical structure has a strong influence on enhancer mechanism and that certain chemical categories have better potential for success in drug delivery formulations. Through understanding gained via mechanistic analysis, the study has identified separate structure–function relationships for hydrophilic and hydrophobic permeation enhancers in relation to potency. Finally, the study highlights an effective and non-cytotoxic transcellular enhancer, 0.01% PPS, which demonstrates a significant ability to deliver therapeutic compounds into epithelial cells and warrants further research for intraepithelial applications.

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