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Research Paper

Safe and Effective Permeation Enhancers for Oral Drug Delivery

Kathryn Whitehead,[1] Natalie Karr,[1] and Samir Mitragotri[1,2]

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Purpose. The use of intestinal permeation enhancers to overcome the absorption challenges associated with oral drug delivery has been hampered by the notion that enhancer efficacy is directly linked to toxicity. This study attempts to gain insight into the principles governing the potency and toxicity behavior of enhancers.
Methods. Fifty-one enhancers were selected from 11 chemical categories and their potency and toxicity were analyzed in Caco-2 monolayers at concentrations spanning three orders of magnitude.
Results. A small but significant fraction of the 153 enhancer formulations studied demonstrated unexpected but desired behavior, that is, substantial efficacy without marked toxicity. Our results revealed that both chemical category and concentration proved critical in determining the usefulness of many enhancers, and the concept of an enhancer's 'therapeutic window' is discussed. Several of the most promising enhancers identified by the study were tested for their effect on the transport of the marker molecules mannitol and 70 kDa dextran across Caco-2 cells and were capable of increasing permeability more than 10-fold.
Conclusions. The results presented here underscore the potential of chemical permeation enhancers while providing valuable direction as to what classes and concentrations of compounds are of interest when searching for safe and effective additions to oral formulations.

KEY WORDS: Caco-2; oral delivery; permeation enhancers; potency; toxicity.

INTRODUCTION

Oral delivery is a highly sought-after means of drug administration due to its convenience and positive effect on patient compliance. However, the oral route cannot be utilized for the delivery of proteins and other macromolecules due to enzymatic degradation in the gastrointestinal tract and limited transport across the intestinal epithelium (1,2). While the former issue is being tackled by innovative encapsulation strategies and enzyme inhibitors (3,4), the latter can potentially be addressed by using chemicals to promote drug uptake across the epithelium (5).

Chemical permeation enhancers (CPEs) aid oral drug absorption by altering the structure of the cellular membrane (transcellular route) and/or the tight junctions between cells (paracellular route) of the intestinal epithelium (6,7). Unfortunately, many reports indicate that enhancer efficacy is often linked to toxicity (8,9), and as a result, permeation enhancers are not widely used in oral formulations. However, the full potential of CPEs for oral delivery remains unclear since there is no fundamental understanding of the principles that govern enhancer behavior. Specifically, it is unclear whether the experimentally observed correlation between the potency and toxicity of CPEs is intrinsic in nature or whether it is a consequence of the limited conditions of previous studies. Additionally, little awareness exists as to how chemical category and concentration can influence the interplay between potency and toxicity. Due to the narrow scope of the existing data on CPE potency and toxicity and the irreconcilable differences in experimental models and test conditions, these critical questions previously have gone unanswered. Herein, we bring resolution to these important issues through the generation of a large dataset on CPE potency and toxicity and its subsequent analysis.

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[1] Department of Chemical Engineering, University of California, Santa Barbara, California 93106-5080, USA.
[2] To whom correspondence should be addressed. (e-mail: samir@engineering.ucsb.edu)

ABBREVIATIONS: AS, anionic surfactant; BS, bile salt; CPE, chemical permeation enhancer; CS, cationic surfactant; DMEM, Dulbecco's Modified Eagles Medium; EP, enhancement potential; FA, fatty acid; FE, fatty ester; FM, fatty amine; MTT, methyl thiazole tetrazolium; NR, nitrogen-containing ring; NS, nonionic surfactant; OP, overall potential; OT, other; PPZ, phenyl piperazine; SDC, sodium deoxycholate; SLA, sodium laureth sulfate; SOA, sodium salt of oleic acid; SS, sodium salt of fatty acid; TEER, transepithelial electrical resistance; TP, toxicity potential; ZS, zwitterionic surfactant.

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 (AS), cationic surfactants (CS), zwitterionic surfactants (ZS), nonionic surfactants (NS), bile salts (BS), fatty acids (FA), fatty esters (FE), fatty amines (FM), sodium salts

of fatty acids (SS), nitrogen-containing rings (NR), and others (OT). 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 CPEs. All compounds were tested at concentrations of 1, 0.1, and 0.01% w/v, and were completely soluble in Dulbecco's Modified Eagles Medium (DMEM, American Type Culture Collection (ATCC), Rockville, MD).

Cell Culture

Caco-2 cell line HTB-37 (ATCC, Rockville, MD), derived from human colon cells, was used for all experiments. Cells were maintained in DMEM supplemented with 25 IU/ml of penicillin, 25 mg/L of streptomycin, 250 ug/L of amphotericin B and 100 ml/L of fetal bovine serum. Monolayers were grown on BD Biocoat™ collagen filter supports (Discovery Labware,

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

Bedford, MA) according to supplier instructions. 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 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)

All TEER values were normalized by their initial values. EP was calculated as the reduction in TEER of a Caco-2 monolayer after 10 min of exposure to that CPE, normalized to the reduction in TEER after exposure to the positive control, 1% Triton X-100:

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

where $\mathrm{TEER_{CPE}}$ and $\mathrm{TEER_{+}}$ are the resistance values (% of initial) of the enhancer solution and positive control solution, respectively, after 10 min 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. 10 μl 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.

Permeability Experiments

Solutions containing enhancers and 1 μCi/ml of tritium-labeled mannitol or 70 kDa dextran (American Radiolabeled Chemicals, St. Louis, MO) were applied to the apical side of Caco-2 monolayers. Samples were taken from the basolateral compartment every 10 min for 1 h and the radiolabeled contents were analyzed with a scintillation counter (Packard Tri-Carb 2100 TR, Meriden, CT). Permeability was calculated using a standard equation (10):

$$P = \frac{\Delta M}{C_M A_{xs} \Delta t}$$

where ΔM is the amount of solute transported across the barrier in the time Δt, C_M is the concentration of solute in the apical compartment, and A_{xs} is the cross-sectional area of epithelium in contact with the apical solution. Positive control experiments were performed on BD Biocoat™ filter supports in the absence of cells. Exchange of tritium with water was monitored and did not pose an issue for this system.

RESULTS

Enhancement Potential of CPEs

Using TEER as a surrogate marker for solute permeability, the potency of all CPE formulations was assessed. An inverse relationship between the permeability of polar solutes and TEER has previously been established in the literature (11, 12) and was confirmed using the marker molecule, mannitol, which is 180 Da in size. Figure 1 demonstrates a significant correlation between the two parameters for the enhancers of this study (r^2=0.92). The use of TEER as an alternative measurement for permeability has several advantages, including convenience and a lack of dependence on the size of the solute, thereby ensuring the generality of results. EP values of all 153 enhancer formulations exhibited significant variations with respect to concentration. The median EP value of all CPEs was 0.20 at a concentration of 0.01% w/v, increasing to 0.43 at 0.1% w/v, and 0.96 at a concentration of 1% w/v. The full set of EP data can be found in Table SI of the supplementary text. At each concentration, EP values also exhibited systematic variations with respect to chemical category. For example, fatty esters possessed very little potency at all concentrations. Surfactants displayed more variation with concentration. At low concentrations (0.01%), most ionic surfactants demonstrated significantly higher potency values compared to other categories (P<0.05). The difference in potency between ionic surfactants and other categories

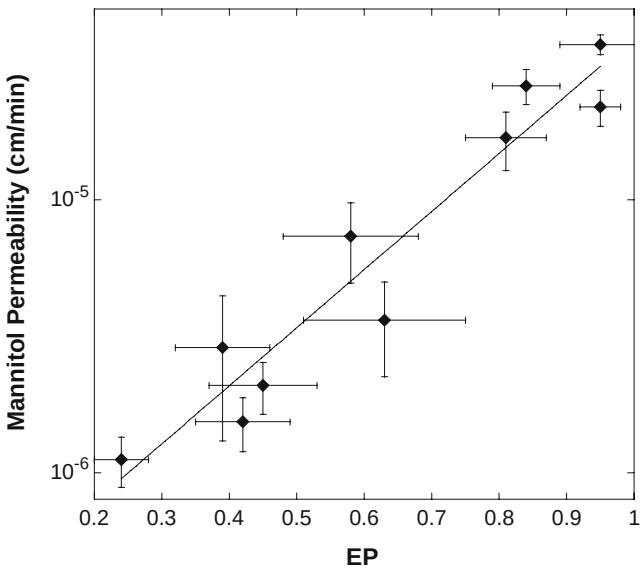

Fig. 1. Enhancement potential is shown to correlate well with mannitol permeability for the enhancers of this study (r^2=0.92). Error bars represent standard deviations (n=3–6).

decreased at intermediate concentrations (0.1% w/v) and nearly disappeared at the highest concentration of 1% w/v. For each chemical category, potency increased with increasing concentration. However, the exact dependence varied significantly for each category.

Toxicity Potential of CPEs based on MTT assay

Toxicity potential of enhancers showed a distribution that was almost bimodal (below 0.2 or above 0.8), regardless of the concentration. At low concentration (0.01%), about 80% of CPEs exhibited TP<0.2, whereas at high concentration (1%), the same percent of CPEs exhibited TP>0.8. The median TP values at low, intermediate and high concentration were 0.07, 0.14, and 0.94, respectively. The full set of TP values is included in Table SI of the supplementary text. TP values demonstrated a strong dependence on enhancer chemistry. For example, cationic surfactants often demonstrated high toxicity values at all concentrations. At high concentration (1%), many CPEs in addition to surfactants exhibited high TP. Interestingly, fatty esters demonstrated extremely low toxicity at all concentrations studied.

Relationships between Enhancement Potential and Toxicity Potential

Having assessed enhancement and toxicity potentials for 51 enhancers (3 concentrations each), the relationship between the two was then evaluated (Fig. 2). This graph has two important features. First, there are clusters of data points in the 'low EP-low TP' and 'high EP-high TP' regions, which supports the commonly perceived notion that oral permeation enhancers are either 'potent and toxic' or 'weak and safe.' However, the second and most striking feature of this graph is that there are many exceptions to this rule. Specifically, 15 out of 153 enhancer formulations recorded high EP (>0.50) and low TP (<0.50), demonstrating the existence of a sizable group of CPEs that are relatively potent and safe.

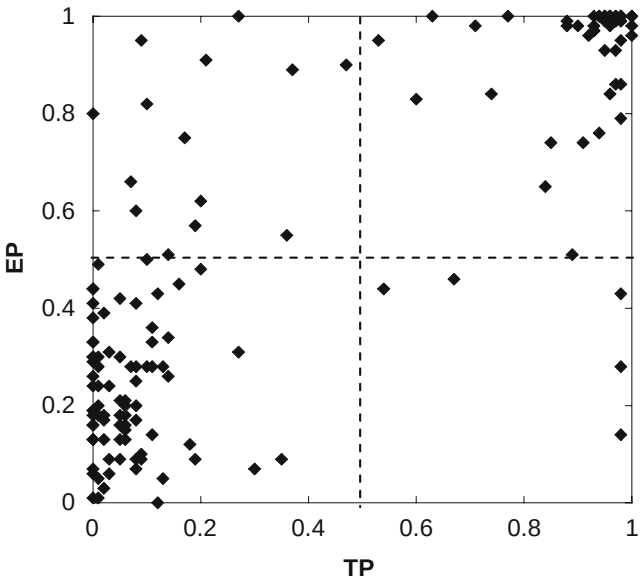

Fig. 2. Enhancement potential (EP) *versus* toxicity potential (TP) data for all 153 enhancer formulations studied. The region of high potency and low toxicity (EP>0.5 and TP<0.5) is dominated by surfactants, nitrogen-containing rings and others. n=3–6. Error bars are not shown in the figure for clarity. Mean standard deviations are 0.07 and 0.09 for EP and TP values, respectively.

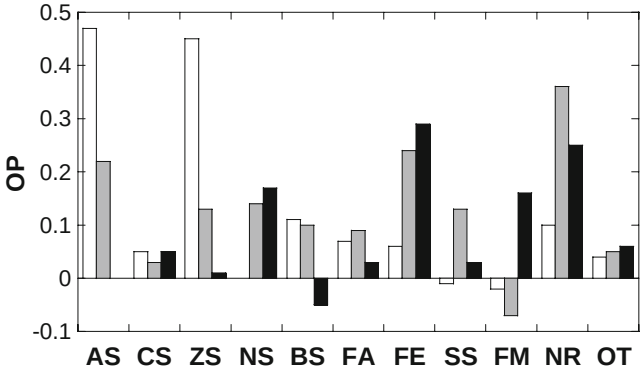

Fig. 3. Distribution of OP values by chemical category (averaged over all enhancers within each category). Higher OP values correspond to more safe and effective enhancers. 0.01, 0.1, and 1% (w/v) concentrations are denoted by *white*, *gray*, and *black* bars, respectively.

To arrive at a parameter that represents the balance of potency and safety of permeation enhancers, we defined a new parameter, overall potential (OP), as the difference between EP and TP (OP=EP−TP, −1<OP<1). Although higher OP values typically indicate increased potential for use, it is important to always consider EP and TP values in conjunction with OP values when assessing an enhancer. The mean OP values for each of the chemical categories can be found in Fig. 3 (averaged separately for each concentration). As a group, anionic surfactants at 0.01% concentration displayed the largest OP, followed by zwitterionic surfactants at 0.01%. A list of the top ten CPEs, ranked by their OP value, can be found in Table II. The list is dominated by nitrogen-containing rings, zwitterionic surfactants, and anionic surfactants, indicating that chemical category has important implications for potent and safe behavior. Interestingly, surfactants at 0.01% concentration appear frequently on this list of best enhancers despite their general reputation as toxic agents (13). This phenomenon underscores the importance of including concentration when commenting on the overall worth of a permeation enhancer.

Table II. The Most Safe and Effective CPEs

CPE	Category	Conc. (%)	OP	Rank
PPZ	NR	0.1	0.86	1
PPS	ZS	0.01	0.80	2
MPZ	NR	1	0.73	3
MPS	ZS	0.01	0.72	4
SLS	AS	0.01	0.70	5
SLA	AS	0.01	0.59	6
PCC	ZS	0.01	0.57	7
MTH	OT	1	0.52	8
NLS	AS	0.01	0.51	9
CL	NR	1	0.48	10

Therapeutic Concentration Windows for CPEs

Based on the results mentioned above, the impact of concentration on potency and toxicity behaviors was explored more deeply by analyzing select enhancers at 14 discrete concentrations spanning four orders of magnitude. One CPE from each of the 11 chemical categories was chosen for further investigation. Of the group studied, three different potency and toxicity profiles stood out as being the most typical. The first profile is shown in Fig. 4A and represents data for sodium dioxycholate (SDC), a bile salt. In this instance, the EP curve (circles) fell nearly on top of the TP curve (squares), and at all concentrations the utility of SDC in enhancing permeation is accompanied by comparable toxicity. This profile was fairly uncommon, with Triton-X100 serving as the only other example of this behavior among the 11 CPEs studied.

Figure 4B, on the other hand, demonstrates a more frequently occurring profile. In the case of the sodium salt of oleic acid (SOA), the drop-off for toxicity occurred at a slightly higher concentration than the drop-off for potency. Therefore, a narrow concentration region existed for SOA in which EP values were still quite high while TP values were low. This region can be thought of as a 'therapeutic concentration win-

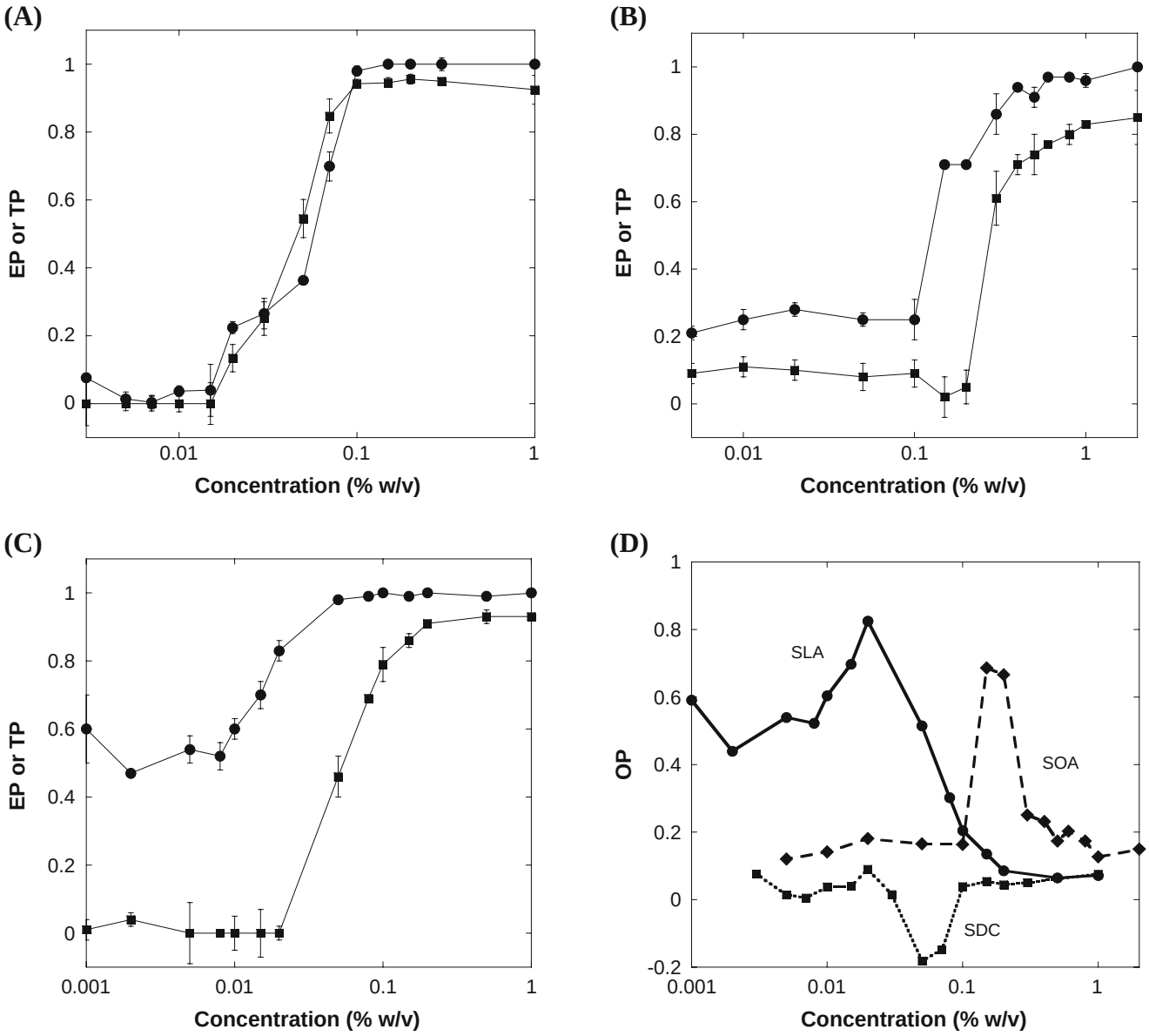

Fig. 4. Common trends for relationships between EP and TP as a function of concentration. In each example, error bars represent standard deviation, $n=6$. **(A)** For sodium deoxycholate, a bile salt, EP (*circles*) and TP (*squares*) curves lie on top of one another, indicating that there is no concentration at which this enhancer is both effective and non-toxic. **(B)** The sodium salt of oleic acid demonstrates a trend where EP (*circles*) and TP (*squares*) curves drop off at different concentrations, yielding a narrow therapeutic concentration window between 0.15 and 0.2% in which the enhancer is both safe and potent. **(C)** In the case of sodium laureth sulfate, an anionic surfactant, EP (*circles*) and TP (*squares*) curves diverge as concentration decreases, indicating the enhancer's usefulness at lower concentrations. **(D)** Overall potential (OP) *versus* concentration data for the three enhancers above. The width of the maximum OP peak corresponds to the size of the therapeutic concentration window of the enhancer.

dow' for an enhancer. Several other enhancers demonstrated similar trends, including phenyl piperazine and pinene oxide. The last type of common profile was exemplified by the anionic surfactant, sodium laureth sulfate (SLA), in Fig. 4C. In this situation, the distance between EP and TP curves was small at higher concentration but grew larger as concentration decreased until it reached a plateau at low concentration. Thus, the therapeutic concentration window was larger than in Fig. 4B. This behavior was typical for other charged surfactants, including the cationic surfactant, decyltrimethyl ammonium bromide, and the zwitterionic surfactant, palmityldimethyl ammonio propane sulfonate. Figure 4D displays overall potential (OP) data for each of the three previously mentioned examples in Fig. 4A–C. In the case of SDC (squares, small dashed line), OP never ventured appreciably above zero, indicating that there is no therapeutic concentration for this particular enhancer. On the other hand, SOA (diamonds, large dashed line) and SLA (circles, solid line) exhibited pronounced maxima in OP at 0.15 and 0.02%, respectively. The width of the peak in OP corresponds to the size of an enhancer's therapeutic concentration window.

Applications of CPEs

Once potency and toxicity information was obtained for each CPE, enhancers with potential for further application were identified. Phenyl piperazine (PPZ), the most safe and effective enhancer identified as judged by methods used in this study, is a member of the piperazine family. Piperazines have a long history of use for therapeutic applications, including the treatment of intestinal parasites (14), impotence (15), and depression (16). Our results indicated that 0.1% PPZ increased the permeability of hydrophilic marker molecules mannitol and 70 kDa dextran more than 14- and 11-fold, respectively (Table III). These values were close to the maximum attainable permeability increases achieved by a positive control. Recovery of cell monolayers after PPZ-induced permeabilization was also assessed. Upon removal of 0.1% PPZ from the cell monolayer, TEER values recovered to 100% of their original value within 24 h. This serves as an excellent example of the ability of a CPE to increase transport of drug-like molecules across epithelial cells without inducing toxicity.

DISCUSSION

Chemical permeation enhancers show significant promise as a solution to the low permeability issues of the intestinal epithelium. In general, enhancers offer the most potential when incorporated into a localized drug delivery system, such as intestinal patches (17) or hydrogels (3), in order to avoid non-specific permeation increases. Although epithelial permeability enhancers have long been studied, their use has been limited due to a large body of data suggesting a correspondence between enhancer efficacy and toxicity. Despite this preexisting evidence, a larger dataset of enhancer behavior was needed to understand the true potential of chemical permeation enhancers. The study reported here offers the much needed data and subsequently uncovers previously unexplored trends that highlight the potential of enhancers for future applications in oral delivery.

Of the top ten CPEs in terms of highest OP values (Table II), only a small number, including the anionic surfactant, sodium lauryl sulfate (SLS), and the zwitterionic surfactant, palmitoyl carnitine chloride (PCC), have been previously analyzed for oral delivery (18,19). This suggests that many safe and effective enhancer formulations exist that simply have not been discovered in the appropriate context. Most importantly, information gained from this work strongly indicates that the potency of CPEs is not inextricably linked to their toxicity. For many enhancers, EP and TP seem to be linked (i.e. OP is close to zero). Many commonly studied enhancers, including sodium glycocholate (20), capric acid (21), EDTA (22), and glyccyrhetinic acid (23), fall into concentration and chemical categories possessing an OP close to zero. Thus, it is understandable that researchers have had a difficult time identifying safe and potent CPEs.

However, this broad set of data reveals a variety of enhancers at specific concentrations that display unrelated efficacy and toxicity behaviors (Table II). For example, anionic surfactants, although generally regarded as unsafe and unusable (13), function very well at low concentration (0.01%) and offer the best performance as a group, as judged by OP. Similarly, zwitterionic surfactants are appreciably safe at 0.01% concentration while retaining most of their enhancing function. Nitrogen-containing rings and fatty esters are excellent enhancer candidates at higher concentrations (1 and 0.1%), but are highly ineffective at the lowest concentration. Furthermore, Fig. 4A–D demonstrate the varied interplay between potency and toxicity effects as a function of concentration for several enhancers. These relationships, particularly those of Fig. 4B and C, provide more thorough evidence of the unrelated behaviors of toxicity and potency. Figure 4 also introduces the concept of a therapeutic concentration window for enhancer use and emphasizes the need to consider concentration when analyzing potency-cytotoxicity relationships of CPEs. No enhancer can be accurately judged on chemical structure alone. This realization may be of particular importance if a specific type or chemical category of enhancer must be employed in combination with a certain drug of interest. When enhancer options are limited, a thorough

Table III. Permeability Data for 0.1% PPZ

	Mannitol (180 Da)			Dextran (70 kDa)		
	$P_{app} \times 10^6$ (cm/s)	StDev $\times 10^6$ (cm/s)	Ratio	$P_{app} \times 10^6$ (cm/s)	StDev $\times 10^6$ (cm/s)	Ratio
Negative control	0.3	0.16	1	0.9	0.2	1
0.1% PPZ	4.6	0.27	14.0	9.8	1.2	11.4
Positive control	5.5	1.3	16.8	10.0	0.75	11.7

examination of potency and toxicity behaviors across a wide range of concentrations may yield the most promising results.

While relying on the conclusions reached in this study, it is important to note that this study does not attempt to define a threshold above which an enhancer should be considered toxic. Acceptable values of TP for safe use *in vivo* would depend on many factors, including the test model and conditions as well as the frequency and duration of use. In the absence of this information, it is difficult to define a threshold value of TP. Instead of establishing definitive guidelines for enhancer safety, this study attempts to provide a comparative analysis of toxicity based on results in Caco-2 cells. Additionally, it must be realized that no screening of systemic toxicity is reported here. The method used to evaluate toxicity, the MTT assay, measured the effect of enhancers directly on epithelial cells. Hence, no assumption should be made about the systemic safety of these CPEs, and further studies are needed to shed light on this topic.

CONCLUSIONS

Results presented here reveal that chemical permeation enhancers can offer a viable means of enhancing permeability without inducing significant toxicity. These data clearly indicate that potency does not necessarily compromise safety and thus encourage the continued study of permeation enhancers. The results presented here demonstrate that concentration and chemical structure have strong influences on enhancer behavior and that both of these factors must be taken into consideration when drawing conclusions regarding safety and potency. Additionally, the study highlights an effective and non-toxic enhancer, 0.1% PPZ, and demonstrates its abilities for transepithelial drug delivery applications. These results provide support for the continued study of permeation enhancers and offer valuable direction in the hunt for safe and effective additives to oral drug delivery formulations.

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