Structure–Activity Relationships for Enhancement of Paracellular Permeability by 2-Alkoxy-3-alkylamidopropylphosphocholines across Caco-2 Cell Monolayers

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Abstract □ The oral route is the preferred route of delivery for a large number of drug molecules. However, the intestinal epithelium presents a formidable barrier for delivery of drugs into systemic circulation. Phospholipids are among compounds that enhance the absorption of drugs across the intestinal epithelium. In this paper, we describe structure–activity relationships for phospholipid derivatives as enhancers of paracellular permeability across Caco-2 cell monolayers. In a series of 2-alkoxy-3-alkylamidopropylphosphocholine derivatives, compounds with a long chain at C-3 (R_3) and short chain at C-2 (R_2) were potent in causing a decrease in transepithelial electrical resistance (TEER) and an increase in mannitol transport, but also showed significant cytotoxicity. Compounds with 9–11 carbons at C-3 and 6–10 carbons at C-2 provided good separation (up to 2.7-fold) between activity and cytotoxicity. Notably, a good correlation ($r^2 = 0.93$) was observed between EC_{50} (TEER) [concentration that caused a drop in TEER to 50% of its control (untreated) value] and $EC_{10\times}$ (mannitol) [concentration that caused 10-fold increase in mannitol transport over the control (untreated) value], confirming that a decrease in TEER is associated with enhanced permeability of the hydrophilic compounds across Caco-2 cell monolayers. Compounds with *medium to long carbon chains* at C-2 and C-3, *and* the total carbons in the alkyl chains > 20, showed poor activity and no cytotoxicity.

Introduction

The oral route is the preferred route of delivery for a large number of drug molecules. However, the intestinal epithelium presents a formidable barrier for delivery of many drugs into systemic circulation. Lipophilic drug molecules can cross this barrier by diffusion through the cell membranes, whereas small hydrophilic compounds diffuse through the intercellular space via the paracellular route. Some drugs can act as substrates for the carrier proteins present in the intestinal epithelium (e.g., amino acid, di/tripeptide, glucose, etc.), and are absorbed by a carrier-mediated active transport process. For those hydrophilic drugs that are likely to traverse the intestinal epithelium via the paracellular route, the intestinal barrier is particularly formidable because of the presence of the tight junctions that severely restrict the free passage of solutes through the paracellular space.[1,2] Hence, development of absorption enhancers that can improve the absorption of hydrophilic molecules via the paracellular route is an active area of research in many laboratories. To date, a large number of absorption enhancers with diverse chemical structures have been identified. These include bile salts, medium chain fatty acids, phospholipids, acyl carnitines, Ca^{2+} chelators, surfactants, and detergents.[3−23]

Among many compounds that can enhance the intestinal absorption of hydrophilic drug molecules, phospholipid-like agents with medium length fatty acid chains are of special interest, because they are ubiquitous in living organisms and are found in the membranes and membranous organelles of all living matter.[7] In two separate studies, formulations containing dodecanoylphoshatidylcholine achieved ~8−13% bioavailability (with respect to the iv bolus dose) of insulin after intranasal administration to healthy volunteers.[8,9] Lysophosphatidylcholine, containing a mixture of palmitoyl and stearoyl side chains, significantly increased the nasal absorption of human growth hormone in rats, rabbits, and sheep.[10] Palmitoyllysophosphatidylcholine[11] as well as its analogues[12] increased the permeability of poorly permeable vasopressin analogues across Caco-2 cell monolayers by almost 2 orders of magnitude. The increase in the permeability was achieved at concentrations of the enhancer that did not perturb the membrane significantly (trypan blue exclusion).[12] Lysophoshatidic acid caused a decrease in transepithelial electrical resistance (TEER) and an increase in sucrose flux across cultured brain capillary endothelial cells.[13] Our recent studies have shown that dodecylphosphocholine (DPC) can cause an increase in the permeability of hydrophilic compounds and a decrease in TEER across Caco-2 cell monolayers by modulation of the tight junctions.[14]

Very little information is available regarding the mechanism by which the phospholipid derivatives are able to enhance the absorption of drug molecules or the structural requirements for their activity. In vitro studies[11−14] suggest that these agents increase the paracellular permeability of compounds by modulation of the tight junctions. Studies with the brain endothelial capillary cells[13] suggest that such agents may alter the permeability of the tight junctions by intracellular mechanisms such as tyrosine phosphorylation of proteins at focal contact, recruitment of focal adhesion components, and activation of yet undefined signaling pathways. Our studies have shown that DPC displaces some of the tight junction (zonula occludens)-associated protein ZO-1 at a concentration that causes a decrease in TEER and an increase in paracellular perme-

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ability across Caco-2 cells.[14] Our studies with DPC[14] have further suggested that the presence of a medium (or long) alkyl chain and a zwitterionic headgroup are essential for the activity of phospholipid-like agents as enhancers of paracellular permeability across Caco-2 cell monolayers. Similar requirements (i.e., alkyl chain and zwitterionic headgroup) for enhancement of paracellular permeability by structurally unrelated acyl carnitines[14,15] suggest the possibility of an emerging structure−activity relationship for enhancement of paracellular permeability across intestinal epithelium by modulation of tight junctions.

In this paper, we describe a more definitive and detailed study to delineate the structure−activity relationship for phospholipid-like agents as enhancers of paracellular permeability across Caco-2 cell monolayers. An excellent group of compounds for such a structure−activity relationship study is a series of 2-alkoxy-3-amidopropylphosphocholine derivatives[22] with varying lengths of alkyl chains at C-2 and C-3 positions. As proposed in our previous study,[14,23] we have applied the concept of potency index in developing the structure−activity relationship. Embedded in the potency index are two parameters—one reflecting the potency of the compounds as modulators of the tight junctions, i.e., transepithelial electrical resistance (TEER), and the other reflecting their potency in causing cytotoxicity by indiscriminate damage to the cell membrane (MTT test). We report here one of the first attempts to delineate the relationship between phospholipid structure and enhancement of paracellular permeability in relation to the cytotoxicity of this class of compounds.

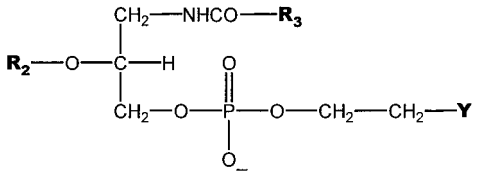
Materials and Methods

Materials—Caco-2 cells were purchased from American Type Culture Collection (Rockville, MD). Eagle's Minimum Essential Medium (EMEM), 0.25% trypsin/0.02% ethylenediaminetetraacetic acid−tetrasodium salt (EDTA-4Na), and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY). Nonessential amino acids (NEAA), Hank's balanced salt solution (HBSS), antibiotic antimycotic solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), palmitoyl-DL-carnitine chloride, sodium dodecyl sulfate (SDS), DPC, 1-myristoyl-sn-glycero-3-phosphate sodium (MGP), 1-lauroyl-sn-glycero-3-phosphocholine (LGPC), 3-amino-1,2-propanediol, acyl chlorides, triphenylmethyl chloride, 60% sodium hydride (in oil), alkyl halides, *p*-toluenesulfonic acid monohydrate, and aqueous (40%) trimethylamine were purchased from either Sigma (St. Louis, MO) or Aldrich (Milwaukee, WI). *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) was obtained through Tissue Culture Facilities (UNC at Chapel Hill, NC). Transwell plates and inserts (12 wells/plate, 3.0 μm pore and 1.0 cm^2 area, polycarbonate) were purchased from Corning-Costar (Cambridge, MA). [^{3}H]Mannitol was obtained from DuPont NEN (Boston, MA).

Caco-2 Cell Culture—Caco-2 cells were grown in EMEM containing 10% FBS, 1% l-glutamine, 1% NEAA and antibiotics (100 U/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B) in 75 cm^2 culture flasks. The cultures were kept at 37 °C in an atmosphere of 5% CO_2, 95% air, and 90% relative humidity. Cells were passaged after 95% confluency and were seeded with a density of 1.0×10^5 cells/mL on to porous polycarbonate filter membranes with a pore size of 3.0 μm and a surface area of 1.0 cm^2. Cells of passage number 45−55 were used in all the studies. Media were changed every 2 days after seeding until late confluence (20−22 days). Just before the experiments, the culture medium was replaced with HBSS transport buffer (HTB) that contained 1x HBSS, 25 mM HEPES, and 25 mM glucose at pH 7.4 and incubated for 1 h at 37 °C. The cell monolayers with TEER values in the range of 600−800 Ω·cm^2 were used for the experiments.

General Chemical Methods—Compounds **1**−**12** (Table 1) were synthesized as described previously.[24,25] Briefly, 3-amino-1,2-propanediol was reacted with various acyl chlorides to give the corresponding (alkylamido)propanediols. The primary hydroxyl group was selectively protected as the trityl ether with triphenylmethyl chloride in pyridine. Reaction of the secondary hydroxyl with sodium hydride and various alkyl halides gave the C2 alkyl ether. Deprotection of the primary hydroxyl group with *p*-toluenesulfonic acid in CH_2Cl_2/MeOH gave the 3-alkylamido-2-alkoxy-1-propanol. The phosphocholine was formed in two steps: (1) reaction with 2-bromoethyl dichlorophosphate and (2) amination with aqueous trimethylamine in $CHCl_3$/*i*-PrOH/DMF. Confirmation of chemical structure and determination of purity of the compounds have been reported.[26,27]

Table 1—Structures of 2-Alkoxy-3-alkylamidopropylphosphocholines



group	enhancers	R_3	R_2	Y
I	**1**	$C_{17}H_{35}$	CH_3	$N(CH_3)_3$
	2	$C_{16}H_{33}$	C_2H_5	$N(CH_3)_3$
	3	$C_{17}H_{35}$	C_2H_5	$N(CH_3)_3$
	4	$C_{19}H_{39}$	C_2H_5	$N(CH_3)_3$
II	**5**	C_9H_{19}	C_6H_{13}	$N(CH_3)_3$
	6	$C_{11}H_{23}$	C_8H_{17}	$N(CH_3)_3$
	7	$C_{11}H_{23}$	C_8H_{17}	$CH_2N(CH_3)_3$
	8	C_9H_{19}	$C_{10}H_{21}$	$N(CH_3)_3$
III	**9**	$C_{17}H_{35}$	C_8H_{17}	$N(CH_3)_3$
	10	$C_{11}H_{23}$	$C_{10}H_{21}$	$N(CH_3)_3$
	11	C_9H_{19}	$C_{12}H_{25}$	$N(CH_3)_3$
	12	$C_{11}H_{23}$	$C_{12}H_{25}$	$N(CH_3)_3$

The stock solutions of phospholipids (80 mM) were obtained by dissolving them in HBSS/ethanol (20:80, v/v) and were stored at −20 °C. Just before the experiments, the phospholipid solutions were thawed and diluted to the appropriate concentrations with HTB (final ethanol concentrated up to 2% v/v) and sonicated for 5 min in an ice bath.

Cytotoxicity Assay—The cell viability was measured by the MTT assay.[26,27] Approximately 3.0×10^3 Caco-2 cells (in 100 μL of cell culture medium) were seeded into each of the wells in a 96-well tissue culture plate (Corning-Costar, Cambridge, MA). The cells were then cultured under the same condition (see Caco-2 Cell Culture) for 96 h, and the culture medium was changed once. Just prior to the start of each experiment, the medium was removed from the wells, and 100 μL of the compound solution in HTB was added to each well. After 20 min, 20 μL of a 5 mg/mL MTT solution was added to each well, and the cells were incubated for another 90 min. Then 100 μL of 10% SDS in 0.02 M HCl/isobutanol (1:1, v/v) solution was added to stop the reaction. The cells without the treatment of any compound were harvested as above and were used as controls. Absorbance was measured at 590 nm (indicative of the formation of the formazan product by mitochondrial dehydrogenase of the viable cells) using a multiwell scanning spectrophotometer (Bio-Rad, Hercules, CA). IC_{50} represented the concentration of the phospholipid derivatives that caused 50% cell death as measured by the mitochondrial dehydrogenase activity.

EC_{50} (TEER) Determination—To measure the effect of the phospholipids on the TEER values of Caco-2 cell monolayers, the test compounds, dissolved in 0.5 mL of HTB at various concentrations, were applied to the apical side. The monolayers were treated for 20 min at room temperature and the TEER measured using an Epithelial Tissue Voltohmmeter (EVOM, World Precision Instruments, Sarasota, FL) and calculated as Ω·cm^2 by multiplying it with the surface area of the monolayer (1.0 cm^2). The resistance due to the cell monolayers was determined after subtracting the contribution of the blank filter and HTB. From the data, EC_{50} for TEER, the concentration at which the phospholipid decreases the TEER of cell monolayer by 50% of the control (untreated) value, was calculated.

Transport Experiments—All transport studies were performed at room temperature on filter-grown Caco-2 monolayers. The transport experiments were carried out under sink conditions such that the concentration of the transported compound in the

receiver compartment was less than 10% of its initial concentration in the donor compartment. Permeability coefficients (P_{app}) were calculated using the following equation:

$$P_{app} = (dQ/dt)\ (1/A)\ (1/C_o)$$

where dQ/dt (mol transported/s) is the flux of the marker compound across the Caco-2 cell monolayer, A (cm^2) represents the diffusional area of the inserts, and C_o (M) denotes the initial concentration of marker compound in the donor compartment. All measurements were in triplicate and expressed as mean ± SD values.

Transport without the Tight Junction Barrier—To evaluate the maximum transport rate of mannitol without the functional barrier of epithelial tight junctions, EDTA was used to open up the tight junction completely.[28] A solution of EDTA at concentration range of 0.001 mM to 15 mM was prepared using HTB without Ca^{2+}/Mg^{2+}. The solution was applied to the apical and basolateral sides of cell monolayer for 30 min. Then the solution was removed, and the cells were washed once with HTB without Ca^{2+}/Mg^{2+}. [^{3}H]-Mannitol (0.5 mL of 0.1 mM, 0.25 μCi) was added to the apical compartment, and the transport rate was determined by measuring the radioactivity (PACKARD 1600 TR liquid scintillation counter, Downers Grove, IL) appearing in the basolateral compartment as a function of time.

Absorption Enhancement—To calculate the compound concentration at which the permeability of mannitol is increased by 10-fold [$EC_{10\times}$ (mannitol)], the compound solution at concentration range of 0.05 to 5.0 mM in HTB (0.5 mL) was added to the apical side of the cells. HTB (1.5 mL) was added to the basolateral side. After treatment for 20 min, the lipid solution was removed and the monolayer was washed once with fresh HTB. Following the measurement of the TEER, 0.5 mL of HTB solution containing 0.1 mM [^{3}H]mannitol (0.25 μCi) was applied to the apical side. Samples from the basolateral side were taken at 10, 20, 40, and 60 min. Transport rates were determined by measuring the radioactivity in the basolateral side by liquid scintillation counter.

Results

Previous studies with two structurally distinct compounds, i.e., DPC and PC, have shown that the minimum structural requirements to cause a decrease in TEER and a corresponding increase in paracellular permeability across Caco-2 cells are (i) the presence of a medium to long alkyl chain and (ii) a zwitterionic group.[14,15] The same structural features may also confer a similar activity to phospholipid derivatives.[7−12,14] Here we report the effect of varying the length of alkyl chains at C-2 and C-3 positions of phospholipid derivatives on TEER, paracellular permeability across Caco-2 cell monolayers, and cytotoxicity to these cells.

Effect of Synthetic Phospholipid Derivatives on TEER and Paracellular (mannitol) Permeability across Caco-2 Cells—The chemical structures of the 2-alkoxy-3-alkylamidopropylphosphocholines studied are shown in Table 1. The effect of these compounds on TEER across Caco-2 cell monolayers was determined by exposing the cells to various concentrations of these agents on the apical side for 20 min prior to the measurement of TEER. The time of exposure was determined based on our studies with DPC which showed that maximal effect on TEER was achieved in 20 min.[14] Under these conditions, the mannitol permeability across Caco-2 cell monolayers was also determined. The typical TEER−concentration profiles as well as mannitol permeability−concentration profiles observed are shown in Figure 1. Based on the TEER/mannitol permeability−concentration profiles, the synthetic ether phospholipids were classified into three groups. Compounds in group I (**1**−**4**, Table 1) caused a precipitous drop in TEER and a steep rise in mannitol permeability with concentration (e.g., compound **2**, Figure 1A). These compounds are

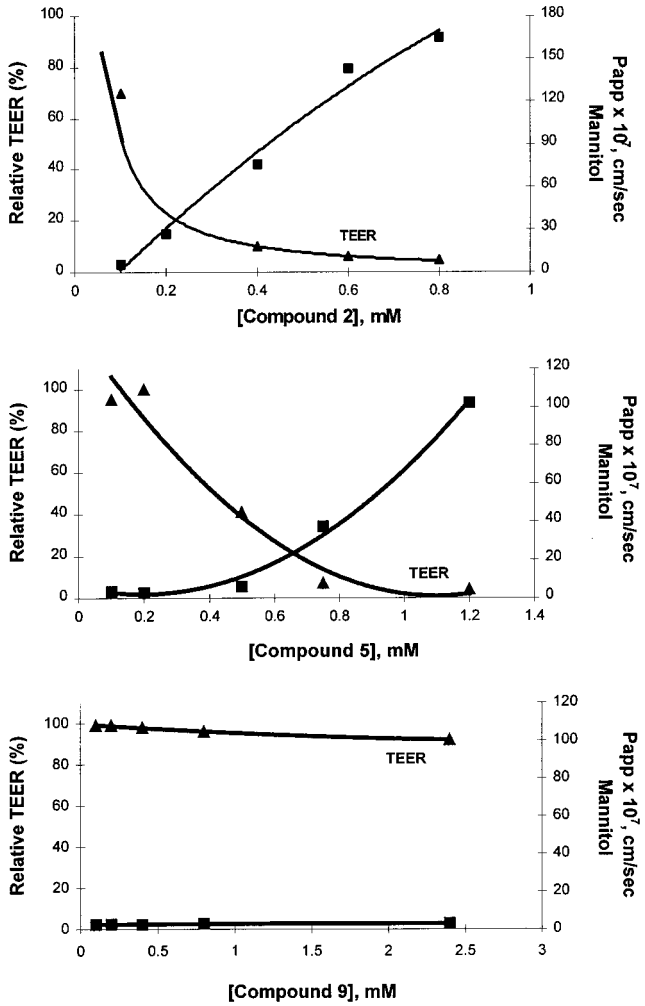


Figure 1—Effect of concentration on TEER and mannitol permeability for 2-alkoxy-3-alkylamidopropylphosphocholines. A, compound **2**; B, compound **5**; C, compound **9**. ■, mannitol permeability; ◆, TEER. Compounds were applied to the apical side of the monolayers for 20 min at room temperature.

characterized by a short alkyl chain (1−2 carbons) as the R_2 substituent and a long alkyl chain (16−19 carbons) as the R_3 substituent. In contrast, compounds in group II (**5**−**8**, Table 1) caused a more gradual decrease in TEER and increase in mannitol permeability with concentration (e.g., compound **5**, Figure 1B). These compounds contain medium length alkyl chains as the R_2 (6−10 carbons) and R_3 (9−11 carbons) substituents, with the total carbons in both the alkyl chains < 20. Compounds in group III (**9**−**12**, Table 1) had little or no effect on TEER or mannitol permeability over the concentration range examined (e.g., compound **9**, Figure 1C). These compounds contain a medium length alkyl chain as the R_2 substituent (8−12 carbons) and a medium to large alkyl chain as the R_3 substituent (9−17 carbons) with the total number of carbons in both alkyl chains > 20.

For comparison, the effect of EDTA, a known modulator of paracellular permeability via tight junction modulation,[28] on TEER and mannitol permeability is shown in Figure 2. The mannitol permeability reached a plateau at high concentrations of EDTA; no such plateau was reached with 2-alkoxy-3-alkylamidopropylphosphocholines (Figures 1 and 2). The compounds in groups I and II appear to be more potent than EDTA in causing a drop in TEER and an increase in the mannitol permeability across Caco-2 cell monolayers.

Comparison of the Effect of Synthetic Phospholipids on TEER and Mannitol Permeability across

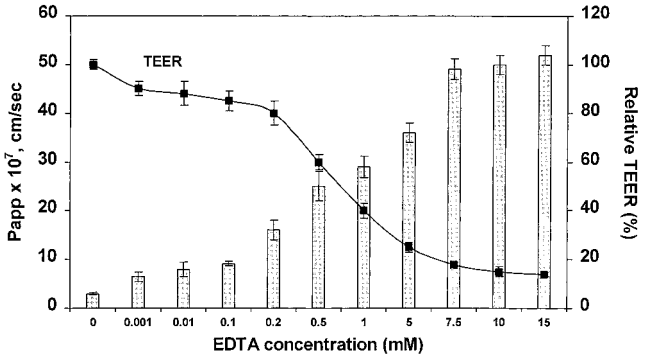


Figure 2—Effect of EDTA concentration on TEER and mannitol permeability. EDTA was applied on both apical and basolateral side of the monolayers for 30 min at room temperature. All measurements were in triplicate and expressed as mean ± SD.

Table 2. Values of EC_{50}, $EC_{10\times}$, IC_{50}, and PI of the Synthetic Phospholipids

group	enhancers	EC_{50} (TEER)[a]	$EC_{10}\times$ (mannitol)[a]	IC_{50} (MTT)[a]	PI[b]
I	1	0.14 (0.02)	0.21 (0.02)	0.16 (0.02)	1.14
	2	0.12 (0.01)	0.20 (0.02)	0.11 (0.01)	0.83
	3	0.16 (0.01)	0.29 (0.02)	0.16 (0.02)	1.02
	4	0.28 (0.02)	0.42 (0.01)	0.25 (0.02)	0.89
II	5	0.42 (0.03)	0.73 (0.05)	1.10 (0.05)	2.62
	6	0.25 (0.02)	0.70 (0.03)	0.68 (0.02)	2.72
	7	0.42 (0.03)	0.73 (0.05)	1.10 (0.10)	2.62
	8	0.80 (0.02)	1.35 (0.12)	1.40 (0.25)	1.75
III	9	no effect	ND[c]	>2.0	–
	10	1.45 (0.02)	ND	1.50 (0.05)	1.03
	11	1.6 (0.1)	ND	2.0 (0.2)	1.25
	12	no effect	ND	no effect	–

[a] Values of EC_{50} (TEER), $EC_{10}\times$ (mannitol) and IC_{50} (MTT) are expressed as mM; the numbers in parentheses indicate SD. [b] PI: the ratio of IC_{50}/EC_{50} based on the data from Caco-2 cells. [c] ND: not detectable because of the insolubility of the lipids at higher concentrations.

Caco-2 Cells—To express the effect of these compounds on TEER and paracellular permeability quantitatively, we have defined the terms EC_{50} (TEER)[14] and $EC_{10\times}$ (mannitol).[22] EC_{50} (TEER) is the concentration of an enhancer at which TEER drops to 50% of its control value (untreated cells), and $EC_{10\times}$ (mannitol) is the concentration at which mannitol permeability is increased by 10-fold over the control value (untreated cells). In case of mannitol permeability, the term $EC_{10\times}$ was defined instead of EC_{50} because the plateau in the mannitol permeability was not always easy to obtain (see Figure 1); hence, a percentage of maximal effect could not always be determined. Furthermore, 10-fold increase in mannitol permeability over the control value ($\sim 2.5 \times 10^{-7}$ cm/s) represented 50% of the maximal mannitol permeability (25×10^{-7} cm/s) obtained by opening up the tight junctions with EDTA (Figure 2). The EC_{50} (TEER) and $EC_{10\times}$ (mannitol) for the synthetic phospholipids are shown in Table 2. By both these parameters, the compounds in group I were the most potent phospholipid derivatives tested, the compounds in group II showed moderate potency, and those in group III were weakly active or inactive.

The relationship between the EC_{50} (TEER) and the EC_{10x} (mannitol) is shown in Figure 3. Clearly, an excellent correlation exists between the EC_{10x} (mannitol) values and the EC_{50} (TEER) values. Hence, EC_{50} (TEER) can serve as a reliable index of the potency of the compounds as enhancers of paracellular permeability if the data on mannitol permeability are not available.

Interestingly, at their EC_{50} concentrations, the changes in TEER and mannitol transport caused by group II compounds were reversible. After removal of the compounds and recovery of the cells in complete cell culture medium (10% fetal calf serum, 1% NEAA in EMEM) for 2 h, the decreased TEER and the enhanced mannitol transport returned to their initial values (approximately 85–95% TEER recovery and 90–95% recovery of mannitol permeability). However, on similar treatment of the cell monolayers with group I compounds, the TEER and mannitol transport values did not recover, indicating that tight junction integrity cannot be restored after exposure of the cells to group I compounds.

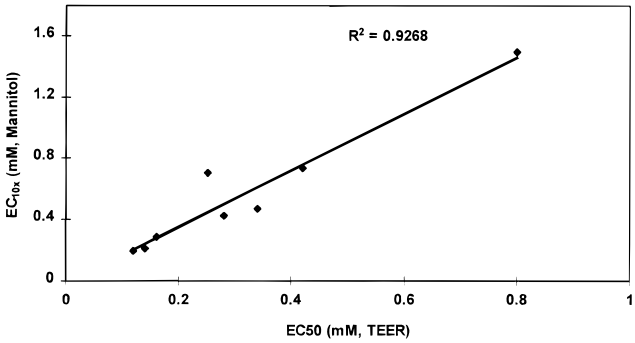


Figure 3—Relationship between EC_{50} (TEER) and EC_{10x} (mannitol) for 2-alkoxy-3-alkylamidopropylphosphocholines. EC_{50} (TEER) and EC_{10x} (mannitol) have been defined in Materials and Methods.

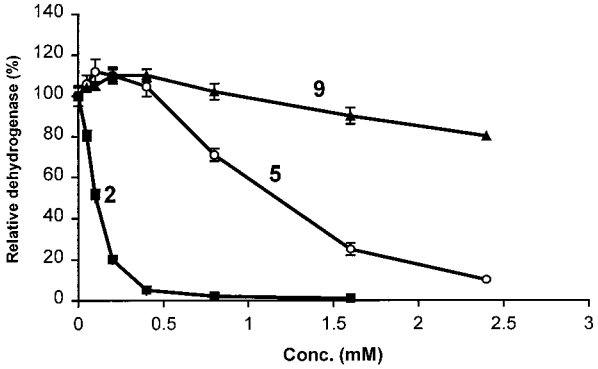


Figure 4—Relationship between concentration and mitochondrial dehydrogenase activity (MTT) for 2-alkoxy-3-alkylamidopropylphosphocholines that are typical of three groups of compounds within this series. All measurements were in triplicate and expressed as mean ± SD.

Cytotoxicity of the Synthetic Phospholipids—To evaluate cytotoxicity of the synthetic phospholipids toward Caco-2 cells, the viability of the cells was evaluated using the MTT method.[26,27] MTT is a tetrazolium salt that is cleaved by mitochondrial dehydrogenase in living cells to give a dark blue formazan product. Damaged or dead cells show reduced or no mitochondrial dehydrogenase activity. Cytotoxicity of compounds **2**, **5**, and **9**, representing the compounds in groups I, II, and III, respectively, as a function of their concentration is shown in Figure 4. The toxicity of these compounds is expressed as IC_{50}, which is defined as the concentration at which the compounds caused 50% decrease in mitochondrial dehydrogenase activity in the MTT test. The IC_{50} values of all the compounds tested are given in Table 2. Among the three compounds shown in Figure 4, compound **2** was most cytotoxic ($IC_{50} = 0.11$ mM), compound **5** showed moderate cytotoxicity (IC50 = 1.1 mM), and compound **9** did not show any toxicity even at the highest concentration (2.5 mM) tested. The cytotoxicity of compounds **2**, **5**, and **9** was representative of the cytotoxicity exhibited by compounds in their respective groups.

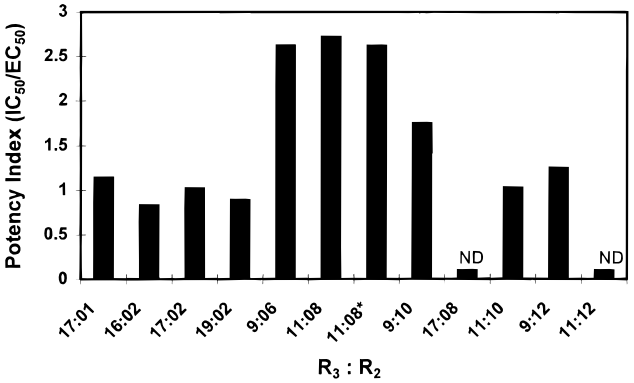


Figure 5—Relationship between R2 and R3 substituents in 2-alkoxy-3-alkylamidopropylphosphocholines and the potency index, PI [IC_{50}/EC_{50}(TEER)]. The compounds are in numerical order (**1** to **12**) from left to right. *The trimethylammonium group is separated from the phosphate group by a three-carbon chain instead of a two-carbon chain

Discussion

A decrease in TEER and an increase in mannitol permeability across Caco-2 cell monolayers caused by 2-alkoxy-3-alkylamidopropylphosphocholines suggest that this class of compounds has the potential to act as absorption enhancers. Their effect on TEER and mannitol permeability suggests that they are acting via modulation of the tight junctions; however, the evidence is entirely circumstantial. In a previous study,[14] we had presented evidence that a long alkyl chain and a zwitterionic functionality are essential for the activity of such compounds as modulators of paracellular permeability. In the present study, we have examined a limited structure−activity relationship (2-alkoxy-3-alkylamidopropylphosphocholines) that provides more insights on the effect of branching of the alkyl chain on the activity (as enhancers of paracellular permeability) and toxicity of phospholipid derivatives. The branching is not achieved with branched alkyl chains, but rather by introducing an ether functionality at C-2 and an amido functionality at C-3 of a propylphosphocholine.

Surprisingly, introduction of a small alkyl group at the C-2 position (R_2) significantly enhanced the potency of phospholipids in causing a decrease in TEER and an increase in mannitol permeability across Caco-2 cell monolayers. For example, the EC_{50} (TEER) value of compound **2** (0.12 mM) was 3.5- to 5-fold lower than the previously reported values for PC (0.42 mM), DPC (0.65 mM), or LGPC (0.65 mM). Unfortunately, this structural feature also caused the phosphocholine derivatives to be more potent cytotoxic agents. Thus the IC_{50} value for compounds **2** (0.11 mM) is much lower than that for PC (0.45 mM), DPC (0.92 mM), and LGPC (0.84 mM).[14] Increasing the length of C-2 alkyl chain to 6−8 carbon atoms while simultaneously decreasing the length of C-3 alkyl chain caused a decrease in the potency of the compounds as modulators of the paracellular permeability with a concurrent decrease in cytotoxicity. Finally, further increase in the length of the C-2 or C-3 alkyl chains rendered the compound inactive.

In developing a structure−activity relationship for paracellular permeation enhancers, the potency of these agents must be addressed in relation to their cytotoxicity. Hence, we have defined the term potency index (PI), which is a ratio of the IC_{50} (MTT) value of a compound to its EC_{50} (TEER) value.[14,22] For the synthetic phospholipid derivatives, the PI values are listed in Table 2 and plotted in Figure 5. Clearly, for all compounds having a short alkyl chain at C-2 (group I), the PI value is ∼1; i.e., the concentration at which they can exhibit enhancement in paracellular permeability is very similar to the concentration at which they are cytotoxic. In contrast, the compounds in group II, with medium length alkyl chains at C-2 and C-3 (total carbons < 20), have PI values in the range of 1.75−2.72 and achieve the best separation of activity as paracellular permeation enhancer and cytotoxicity among all the compounds tested including those tested in our previous study.[14] This PI range is significantly greater than that for the extensively investigated enhancer PC (PI ≅ 1). Compounds **6** and **7** exhibit comparable PI values despite the fact that their cationic and anionic centers are separated by two and three carbons, respectively. Thus a small change in the distance between the cationic and the anionic centers does not lead to any change in activity or toxicity. Some compounds in group III (medium to long alkyl chains at C-2 and C-3 *and* total carbons > 20) have PI values of approximately 1, whereas others are too inactive to determine either the EC_{50} or the IC_{50} value.

In conclusion, with this limited series of compounds, we have been able to demonstrate that the structure−activity (modulation of paracellular permeability) relationship for synthetic phospholipid derivatives is distinct from the structure−toxicity relationship. Interestingly, the separation of activity and toxicity is most pronounced (>2.5-fold) for moderately active compounds, while the activity and the toxicity appear to converge among the more potent compounds. Hence, further assessment of the structure−activity relationships should focus on phospholipid derivatives with medium-size alkyl substituents at C-2 and C-3. The relationship between relative position and distance of the anionic and cationic centers and the activity/toxicity should be further explored.

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