Enhanced Permeability of Molecular Weight Markers and Poorly Bioavailable Compounds across Caco-2 Cell Monolayers Using the Absorption Enhancer, Zonula Occludens Toxin

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Purpose. Zonula occludens toxin (Zot), a protein elaborated from *Vibrio cholerae*, has been shown to be capable of reversibly opening tight junctions. The objective of this work was to determine the stability of Zot and to examine the permeability of a series of molecular weight hydrophilic markers and therapeutic agents in the presence of Zot.

Methods. The transport of molecular weight markers (i.e., PEG 4000, FITC-dextran 10,000 and inulin) and therapeutic agents (i.e., acyclovir, cyclopsorin, paclitaxel, doxorubicin) was evaluated with Zot (0, 1, 2, and 4 μg/mL) using Caco-2 cell monolayers.

Results. Zot was found to be stable over a 10-day period. Significantly higher ($p < 0.05$) permeability of the molecular weight markers, inulin, and PEG4000 were observed with Zot (4 μg/mL). The transport of each therapeutic marker was significantly increased with paclitaxel displaying a >3-fold enhancement in Papp values with Zot (4 μg/mL). A 30% decrease in transepithelial electrical resistance values was observed, which returned to baseline 30 min after Zot was removed.

Conclusions. Considering the problems of poor oral bioavailability, it is concluded that Zot is a promising drug delivery technology to be used to enhance drug transport across the intestinal mucosa. Future applications are targeted at assessing its usefulness in oral drug delivery using *in vivo* systems.

KEY WORDS: Zot; permeability; FITC-10,000; PEG4000; paclitaxel; absorption enhancer.

INTRODUCTION

It is becoming increasingly important to incorporate favorable absorption properties in the drug design process because oral bioavailability may be a significant barrier to effective drug response. An orally administered drug must not only possess intrinsic activity but also favorable pharmacokinetic properties that allow drug molecules to permeate the intestinal mucosa, enter the site of action, and maintain residence at the site of action for a time period sufficient to illicit the intended pharmacodynamic effect. One of the major limitations to systemic bioavailability is the barriers that comprise the intestinal mucosa, the tight junctions that control paracellular transport (1–3). Tight junctions are generally reported to be impermeable to molecules with radii larger than 11 to 15 angstroms and represent the major barrier toward the paracellular transport of compounds (1). The paracellular route is the dominant pathway for passive solute flow across the intestinal epithelial barriers. Tight junctions control the passive diffusion of ions and small water-soluble solutes through the paracellular pathway, thereby often counter-regulating any gradients generated by transcellular pathways. Because tight junctions are major barriers to the paracellular transport of certain agents, studies have been performed to examine the ability of endogenous and exogenous factors to modify the junctions (4–11). These agents, referred to as absorption enhancers, have been reported to modulate intestinal tight junctions, but their development has been slow because of the unwanted side effects.

Absorption enhancers include compounds such as chitosan analogs (5–7,9,10) and acylcarnitines (8). Chitosan analogs, the second most abundant natural-occurring organic material after cellulose, are obtained from chitin, a waste material from the sea (6). Chitosan has been demonstrated to promote the nasal absorption of insulin in rats and sheep (9) and enhance the paracellular transport of peptides *in vitro* and *in vivo* by opening of tight junctions (5,11). However, either unacceptable side effects or poor solubility at physiologic pH values has hampered agents falling within these two general classes of absorption enhancers (i.e., calcium chelators and surfactants). Calcium chelators, which cause calcium depletion, induce global changes in the cells, including the disruption of actin filaments, adherent junctions, and diminished cell adhesion (12). Surfactants may cause exfoliation of the intestinal epithelium irreversibly compromising its important barrier functions (13). Promising chitosan analogs aggregate at pH values above 6. Therefore, the polymer is only soluble in acidic solutions where most of the amino groups necessary to modulate tight junctions are protonated (6).

Recently, a new and novel protein technology has demonstrated the ability to function as an absorption enhancer in a variety of *in vivo* systems. Fasano *et al.* has identified zonula occludens toxin (Zot), a toxin produced by the bacterial strain *Vibrio cholerae*, which activates a complex intracellular cascade of events that regulate tight junction permeability (14). Zot, a 45-kDa protein, is capable of binding to a specific receptor on the luminal surface of the intestine and reversibly opening the tight junctions between intestinal epithelial cells (15). Studies conducted by Fasano and coworkers have shown that the tight junctions can be perturbed enough to allow for the transport of agents across the intestinal mucosa to achieve higher concentrations in the systemic circulation (16,17). Fasano and colleagues have established that Zot is not only tissue specific (intestinal mucosa and blood-brain barrier) but that it produces a dose-dependent and reversible effect on tissue resistance and permeability (16,17). Additionally, the mechanism of Zot is thought to occur by the rearrangement of the cytoskeleton related to changes in protein kinase C (15). This cytoskeletal rearrangement affects the relationship of the proteins constructing the tight junction, thus leading to the opening of this important structure. One of the most persuasive studies conducted with Zot *in vivo* assessed the co-administration of oral insulin with Zot in BB/Wor diabetic male rats (16). Results suggested that the co-administration of oral insulin with Zot resulted in a reduction of blood glucose

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comparable with that seen with a conventional dose of subcutaneous insulin, resulting in an overall increase in bioavailability of approximately 20%. Thus, the relevance of using Zot to enhance the permeability and thus increase oral bioavailability of compounds is apparent.

Our preliminary results have shown that Zot enhances the transport of hydrophilic and small lipophilic (MW ~300 Da) compounds across Caco-2 cell monolayers (18). Hence, the objective of this work was to provide in greater detail a systematic evaluation of Zot as an enhancer of paracellular transport in an intestinal cell culture system, the Caco-2 cell monolayers. Included in this evaluation are the following: (1) an assessment of Zot stability over time, (2) determination of optimal incubation times in the cell culture system, (3) an assessment of the changes in transepithelial electrical resistance with Zot, and (4) an examination of the Caco-2 cell permeability of a series of molecular weight hydrophilic markers in the presence and absence of Zot. The molecular weights of evaluated marker compounds ranged from 0.18–10 kDa (e.g., mannitol, FITC-dextran 10,000, inulin, and polyethylene glycol (PEG) 4000). In addition, the permeability of a series of therapeutic agents in the presence of Zot was also examined. The therapeutic agents selected included acyclovir, cyclosporin, doxorubicin, and paclitaxel. In general these agents have the following characteristics: low bioavailability, hydrophobic, low permeability, and P-glycoprotein substrates. The flux of a transcellular marker, propranolol, in the absence and presence of Zot was also studied.

MATERIAL AND METHODS

The Caco-2 cell line was obtained from American Type Culture Collection (Rockville, MD, USA) at passage 19. Cells used in this study were between passage numbers 25 and 50. Dulbecco's modified Eagle medium (DMEM), Dulbecco's modified phosphate-buffered saline with and without Ca^{2+} and Mg^{2+} (PBS), non-essential aminoacids, fetal bovine serum, L-glutamate, trypsin (0.25%)-EDTA (1 mM), and penicillin G-streptomycin sulfate antibiotic mixture, were purchased from Gibco Laboratories (Lenexa, KS, USA). T- 75 flasks were obtained from Becton Dickinson Labware (Franklin Lakes, NJ, USA). Transwell clusters, 24 mm in diameter (surface area 4.71 cm^2) and 0.4-μm pores were from Corning Costar (Cambridge, MA, USA). [^{14}C]Mannitol (46.6 mCi/mmol), [^{14}C] inulin (14.5 mCi/g), [^{14}C]polyethylene glycol 4000 (15.7 mCi/g), [^{3}H]propranolol, [^{14}C]Paclitaxel (45.3 mCi/mmol), [^{3}H]cyclosporin A (7.00 Ci/mmol), [^{3}H]Acyclovir (1.2 mCi/mL), and [^{14}C]/55 mCi/mmol/doxorubicin, and FITC-dextran 10,000 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dr. Alessio Fasano supplied the Zot. All other chemicals were of Analytical grade from Sigma Chemical Co. and Fisher Scientific (Fair Lawn, NJ, USA).

Cell Culture

Caco-2 Cell Monolayers

The growth, maturation, and seeding of Caco-2 cells have been previously described (18). Caco-2 cells with a passage number between 25 and 50 were used in an effort to derive Caco-2 monolayers with consistent morphologic and biochemical properties. Caco-2 cells were grown in T-150 flasks at 37°C in an atmosphere of 5% CO_2 using DMEM supplemented with 2% L- glutamine, 1% nonessential amino acids, 1% penicillin-streptomycin and 10% fetal bovine serum. The cells were seeded onto polycarbonate filters (mean pore diameter = 0.4 μm, surface area = 4.71 cm^2) in the transwell tissue culture inserts, at a density of 80,000 cells/cm^2 (3.8×10^5 cells/mL). The medium (1.5 mL in the apical chamber and 2.6 ml in the basolateral chamber) was changed every other day after seeding. Cells were used for transport studies between days 21–28 after seeding. The integrity of the monolayers was determined by measuring the transepithelial electrical resistance (TEER) with a Millicell-ERS (Millipore Corp., Bedford, MA, USA) at different times in culture during 22 days. Transepithelial transport of radiolabeled [^{14}C]-mannitol served as an additional control for monolayer integrity. Before the experiments, TEER values were assessed for Caco-2 cell monolayers and were in the order of 250–350 Ω·cm^2.

Zot Stability and Pre-Incubation Time Evaluation

Studies assessing the stability of Zot dissolved in PBS (4 μg/mL) over a period of 10 days were performed. During this period, Zot was stored at 4°C. To determine the consistent activity (stability) of Zot, the transport of mannitol was assessed on days 1, 4, 7, and 10. To evaluate the enhancement properties of Zot (4 μg/ml) on the flux of a variety of compounds, our laboratory has established that Zot should be allowed to pre-incubate with the cell monolayers for a specific time period before the addition of drug to allow for the effective opening of the tight junctions. To evaluate the most effective length of pre-incubation time, Zot was allowed to incubate with Caco-2 monolayers for the following length of times: 5, 15, 30, 45, and 60 min. The transport of mannitol was then evaluated up to 2 h.

Caco-2 Transport Studies

Dose-Response Effect of Zot and Marker Transport

The transport of radiolabeled and unlabeled paracellular markers was examined in the presence or absence of Zot. The molecular weights of evaluated compounds ranged from 0.18–10 kDa (e.g., mannitol 182 Da, inulin 5000 Da, FITC-dextran 10,000 Da and polyethylene glycol [PEG] 4000 Da). Caco-2 cells were grown to confluency and transport studies were conducted between days 21 and 28. To assess the dose response effect of Zot on paracellular transport, cell monolayers (n = 6) were pre-incubated for 30 min with Zot (0, 1, 2, and 4 μg/mL). Markers were added to inserts at time 0. Inserts were moved at times 5, 10, 15, 20, 30, 45, 60, 75, 90, and 120 min and transferred to fresh solutions (2.6 mL) of phospate-buffered saline in the basolateral chamber. All incubations with Zot were maintained at 37°C and monolayers were agitated orbitally at 50–60 rpm during the course of the permeability study. Samples were collected over 120 min from the basolateral chamber and analysis was performed by radiographic methods or high-performance liquid chromatography methods.

Caco-2 Transport of Therapeutic Agents

The transport of the therapeutic agents {[^{14}C]mannitol, [^{14}C]paclitaxel, [^{3}H]cyclosporin A, [^{3}H] acyclovir (1.2 mCi/

ml), and [^{14}C]doxorubicin (55 mCi/mmol)} were examined in the presence or absence of Zot. Caco-2 cells were grown to confluency and transport studies were conducted between days 21 and 28. To examine any possible influence of Zot on the transcellular pathway in Caco-2 cells, the transport of propranolol, a transcellular marker was evaluated. To assess the dose response effect of Zot on drug transport, cell monolayers (n = 6) were pre-incubated for 30 min with Zot (0, 1, 2, and 4 μg/mL), therapeutic agents were added to inserts and transport studies were performed as previously described.

TEER Measurements

To assess the dose response effect of Zot on TEER, cell monolayers (n = 6) were incubated over a 3-h period at the following concentrations of Zot: 0, 1, 2, and 4 μg/mL. Incubations were maintained at 37°C, monolayers were agitated orbitally at 50-60 rpm and TEER values were monitored over a 3-h period with Millicell ERS meter. Two hours after the initiation of the experiment, the monolayers were washed and the TEER was measured until recovery to baseline.

Viability of Caco-2 Cell Monolayers

Cell viability was evaluated using the lactate dehydrogenase release assay. A set of cell monolayers were incubated with Zot as stated in the transport studies above. At the end of the incubation period, the apical and basolateral media from individual cell wells were pooled and extracellular LDH activity was measured. Cell monolayers were treated with 0.1%Triton-X100 in PBS for 5 min at 40°C to determine the intracellular LDH activity. All samples were processed according to the manufacturer's instructions.

FITC High-Performance Liquid Chromatography Analysis

A selective and specific high-performance liquid chromatography method was developed to quantitate FITC-dextrans in transport media, which included size-exclusion chromatography with fluorescence detection (20). The chromatographic system for FITC analysis included the following: 1) model 515 liquid chromatograph (Waters-Millipore, Milford, MA, USA); 2) 717 Waters autosampler; 3) LC 20 fluorescence detector (Perkin Elmer, PA, USA) set at an excitation and emission wavelength of 494 and 518 nm, respectively; 4) 3390A Hewlett Packard Integrator (Avondale, PA, USA); and 5) Polysep-GFC-P linear size exclusion column (300 × 7.80 mm I.D., Phenomenex, Torrance, CA, USA) was used as the analytical column. The buffer component of the mobile phase (0.05 M phosphate buffer) was prepared with deionized water and the pH was adjusted to 7.0. The mobile phase was filtered through a 0.45-μm nylon filter and degassed under ultra-sound and vacuum for 15 min. The mobile phase was delivered at a flow rate of 1 mL/min. The mobile phase consisted of acetonitrile: 0.05 M phosphate buffer (12:88, v/v). The assay was linear ($r \geq 0.999$) and inter-day precision ranged from 0.5–5.5%.

Data Analysis

Permeability Coefficient

Apparent permeability coefficients (P_{app}) were calculated according to the following equation:

$$\text{Papp} = \frac{\Delta Q}{\Delta t} \cdot \frac{1}{A \cdot C_{(0)}} \quad (1)$$

where $\Delta Q/\Delta t$ is equal to the linear appearance rate of mass in the receiver solution, A to the cross sectional area, and $c(0)$ to the initial test agent concentration in the donor compartment. All values are represented as mean and standard deviation of the values from six monolayer transwell inserts prepared under identical conditions and from the same preparation of cells. The percent flux of transport of the tested agents was determined as follows:

$$\%Flux_{0-2hr} = \frac{Amount\ transported_{0-2hr}}{Initial\ amount} \cdot 100 \quad (2)$$

The percent flux was used as a measure of the extent of transport, the initial amount reflects the amount of marker compound placed in the transwell at $t = 0$. Transport enhancement ratios R were calculated from Papp values:

$$R = \frac{\text{Papp}_{Zot}}{\text{Papp}_{Control}} \quad (3)$$

Statistical Analysis

Correlation coefficients (r^2) obtained by linear regression analysis were in the range of 0.98–1.00. Permeability coefficients generated from the transport experiments were statistically compared by multivariate analysis of variance using post-hoc analysis. Statistical significance was set at $p < 0.05$.

RESULTS

Stability of Zot and Pre-Incubation Times with Zot

The stability of Zot (4 μg/mL) was evaluated over a 10-day period by conducting [^{14}C]mannitol transport studies across Caco-2 cell monolayers in the apical to basolateral direction. Figure 1A displays the % [^{14}C]mannitol flux after 2 h for control and Zot treatment experiments on days 1, 4, 7, and 10. As seen in Fig. 1A, the percentage of [^{14}C]mannitol flux of Zot-treated monolayers at 2 h for days 1, 4, 7, and 10 ranged from 4.98–5.30% whereas the control ranged from 0.13–0.17%. There were no significant differences found in the [^{14}C]mannitol percent flux across the Caco-2 cell monolayers, suggesting that Zot is stable over a 10-day period when stored at 4°C.

To determine the optimal time for Zot (4 μg/mL) to effectively open the tight junctions in Caco-2 cell monolayers, a series of pre-incubation times were evaluated followed by [^{14}C]mannitol transport studies. Fig. 1B illustrates the percent flux of [^{14}C]mannitol over a range of pre-incubation times (i.e., 5, 15, 30, 45, 60 min). As seen in Fig. 1B, the pre-incubation of Zot at both 5 and 15 min before the addition of [^{14}C]mannitol did not significantly change the percent [^{14}C]manntiol flux as compared with the control (i.e., mannitol alone). At 30 min, the pre-incubation of Zot significantly increased ($p < 0.05$) the [^{14}C]mannitol flux as compared with control at 2 h (0.06 to 5.12%). There was also a significant increase of [^{14}C]mannitol percent flux at 45 and 60 min vs. control; however, this increase was not significantly higher than the 30 min pre-incubation period. At 30 min, there was a mean 99.6% increase in percent [^{14}C]mannitol flux at 2 h vs. control. Thus, in subsequent studies assessing the transport of

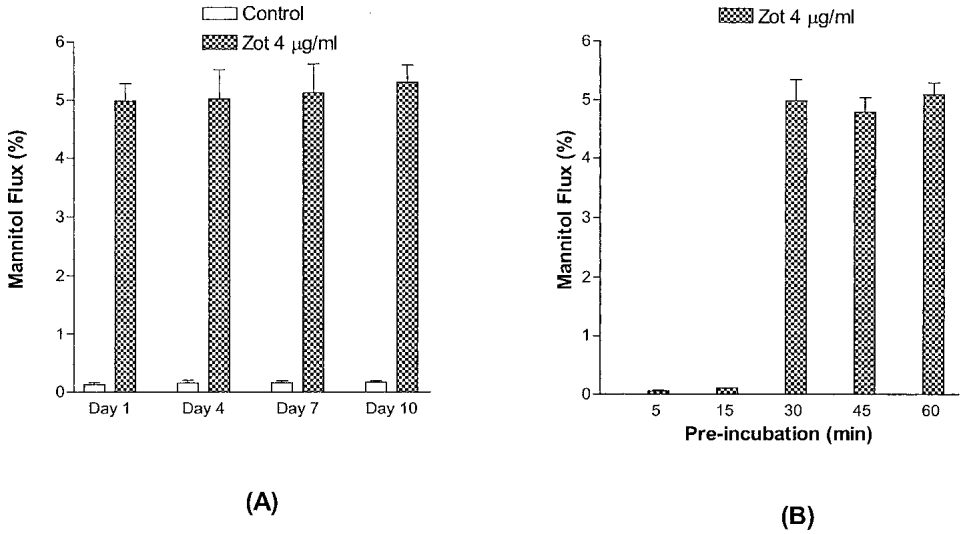

Fig. 1. Percent [^{14}C]mannitol flux at 2 h observed in stability and pre-incubation studies with Zot. (A) Stability of Zot (4 μg/mL) on days 1, 4, 7, and 10 as indicated by [^{14}C]mannitol transport studies across Caco-2 cell monolayers (n = 6) in the apical to basolateral direction. During the stability studies, Zot was stored at 4°C. (B) Pre-incubation times (5, 15, 30, 45, and 60 min) with Zot (4 μg/mL) using [^{14}C]mannitol transport studies across Caco-2 cell monolayers (n = 6) in the apical to basolateral direction. P_{app} was 7.0×10^{-7} cm/s and 90×10^{-7} cm/s for mannitol control and Zot 4 μg/mL, respectively.

various marker compounds in the presence of Zot, Caco-2 monolayers were pre-incubated with Zot for 30 min.

Transport of Paracellular and Transcellular Markers

To assess monolayer integrity and the tightness of the junctions in between cells, the permeability of various paracellular markers was evaluated in the presence and absence of Zot. Table I summarizes the permeability coefficients for the marker compounds {[^{14}C]PEG-4000, [^{14}C]inulin (5000 Da), and FITC-dextran 10,000} evaluated in this study. In general, Zot-induced treatment at the highest concentration produced a significant increase in transport for those markers with a molecular weight less than 5 kDa. The enhancing effect of Zot on the linear hydrophillic paracellular marker, PEG-4000 when incubated with 0, 1, 2, and 4 μg/mL of Zot is presented in Fig. 2A. Significantly higher ($p < 0.05$) percent flux was observed for the transport of [^{14}C]PEG-4000 at Zot 4 μg/mL vs. control. After pre-incubation with Zot 4 μg/mL the [^{14}C]PEG-4000 P_{app} was 1.68×10^{-6} cm/s (Table I) vs. 1.44×10^{-6} cm/s for the control.

The percent flux of [^{14}C]inulin, a paracellular marker with a molecular weight of 5 kDa, was not significantly enhanced with the 1, 2, or 4 μg/mL Zot treatments at 1 h. However, there was a significant increase ($p < 0.05$) in the percent flux of [^{14}C]inulin at 2 h (Fig. 2B) with the 4 μg/mL Zot treatment. In addition, a significant increase ($p < 0.05$) in [^{14}C]inulin P_{app} (4.37×10^{-6} cm/s) at 4 μg/mL Zot was found vs control (7.0×10^{-7} cm/s) with a fold enhancement of 6.2. Figure 2C presents the flux of FITC-dextran 10,000. No significant increases was observed in the flux of this hydrophilic markers across Zot pretreated Caco-2 cell monolayers.

Table I. Apparent Permeability Coefficient (P_{app}) of Radiolabeled and Unlabeled Compounds alone and in the Presence of Zot (0, 1, 2, and 4 μg/mL) across Caco-2 Monolayers (n = 6)

Compound	P_{app} Control[a]	Papp Zot 1.0 μg/mL	Papp Zot 2.0 μg/mL	Papp Zot 4.0 μg/mL[a]
PEG 4000	1.44 (0.17)	1.45 (0.18)	1.53 (0.13)	1.68* (0.13)
Inulin	0.70 (0.01)	0.71 (0.02)	0.76 (0.01)	4.37* (0.01)
FITC-dextran 10,000	0.24 (0.03)	0.30 (0.05)	0.32 (0.04)	0.31 (0.07)
Acyclovir	1.91 (0.13)	2.85 (0.24)	3.00* (0.47)	3.46* (0.45)
Paclitaxel	9.8 (0.83)	10.3 (0.96)	20.5* (3.4)	30.7* (3.3)
Cyclosporin A	0.67 (0.02)	0.75 (0.024)	0.80 (0.02)	8.10 (0.04)
Doxorubicin	1.52 (0.15)	2.44 (0.17)	3.48* (0.28)	4.01* (0.20)
Propranolol	30.2 (1.2)	34.5 (1.5)	27.1 (1.3)	28.2 (1.3)

[a] P_{app} coefficients listed as 10^{-6} cm/s.
* Significant difference at $p < 0.05$ when compared with control.

Caco-2 Transport of a Series of Therapeutic Agents

Table I also summarizes the permeability coefficients for the therapeutic agents evaluated. In general, Zot-induced treatment at the highest concentration (4 μg/mL) produced a significant increase in transport for each agent. The enhancing effect of Zot on the low permeable, but highly soluble drug, acyclovir, when incubated with 0, 1, 2, and 4 μg/mL of Zot is presented in Fig. 3A. Significantly higher ($p < 0.05$) percent flux was observed for the transport of [^{3}H]acyclovir at 1, 2, and 4 μg/mL of Zot vs. control. Furthermore, the percent increase in percent flux at 2 h vs. control at the 1, 2, and 4 μg/mL of Zot was 28.6, 51.7, and 92.3% vs. control and permeability enhancement ratios at these concentrations were

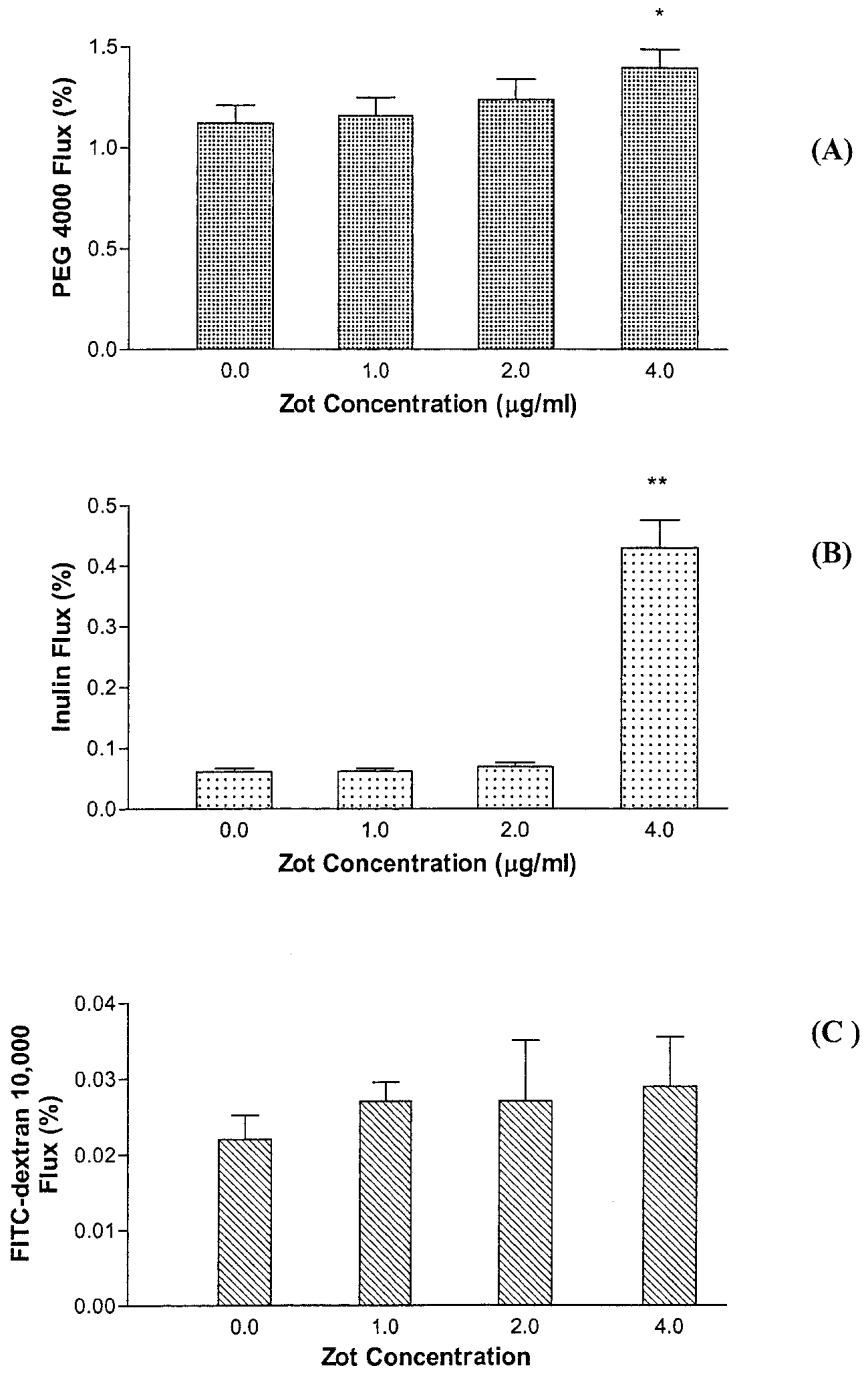

Fig. 2. Percent flux of molecular weight markers observed in the presence (1, 2, 4 μg/mlL) and absence (0 μg/mL) of Zot. (A) PEG 4000 flux at two hours across Caco-2 cell monolayers (n = 6) in the apical to basolateral direction. (B) Inulin flux at 2 h across Caco-2 cell monolayers (n = 6) in the apical to basolateral direction. (C) FITC-4000 flux at 1 and 2 h across Caco-2 cell monolayers (n = 6) in the apical to basolateral direction. (* $p < 0.05$ vs. control).

1.5, 1.6, and 1.8, respectively. The percent flux of [^{14}C]cyclosporin (Fig. 3B), a lipophilic cyclic polypeptide with a molecular weight of 1203 Da, was significantly enhanced with the 2 and 4 μg/mL Zot treatments at 2 h vs. control in Caco-2 cell monolayers. There was a significant increase ($p < 0.05$) in P_{app} for cyclosporin A at the Zot 4 μg/mL treatment (8.1×10^{-7} cm/s) vs. control (6.7×10^{-7} cm/s).

Figure 3 presents the percent flux of [^{14}C]doxorubicin (Fig. 3C) and [^{14}C]paclitaxel (Fig. 3D) in the presence (1, 2, 4 μg/mL) and absence (0 μg/mL) of Zot at 2 h across Caco-2 cell monolayers in the apical to basolateral direction. As seen in Fig. 3C, the percent flux of doxorubicin was significantly higher in the presence of Zot (1, 2, 4 μg/mL) vs. control when measured at 2 h across Caco-2 cell monolayers. Zot significantly increased paclitaxel transport across Caco-2 cells as compared with control with an enhancement ratio of 3.13.

To determine whether the enhancement effect of Zot may have been influenced by the modulation of the transcel-

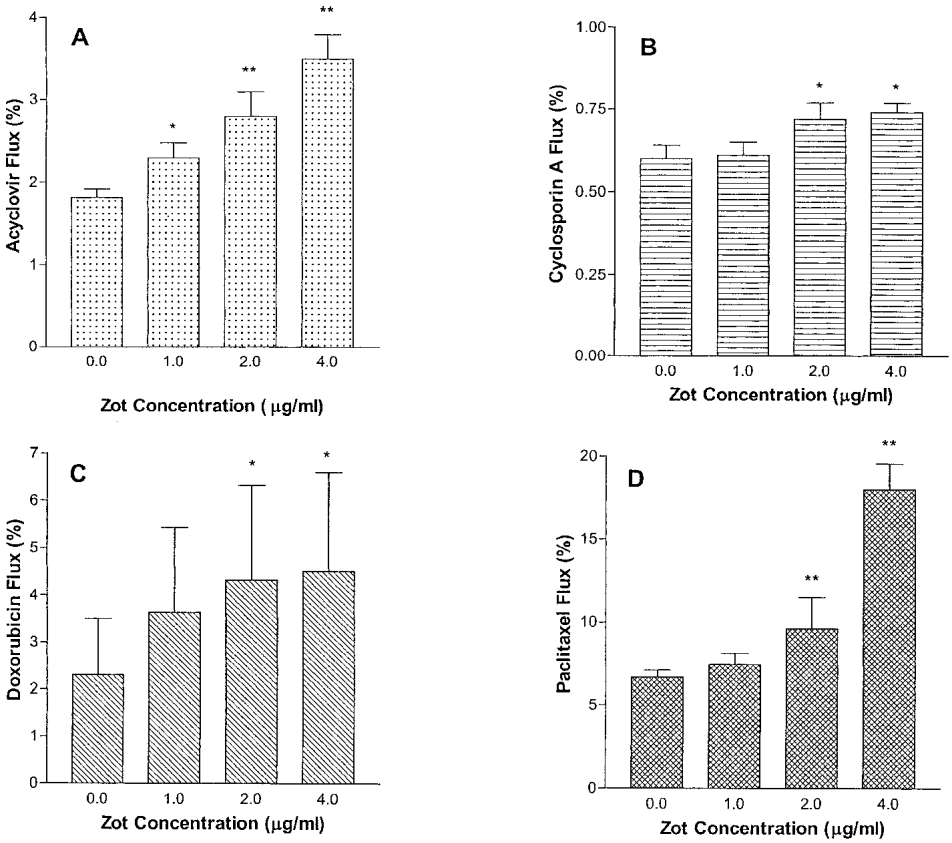

Fig. 3. Percent mean (±SD) flux at 2 h of therapeutic agents observed in the presence (1, 2, 4 μg/mL) and absence (0 μg/mL) of Zot. (A) Acyclovir flux across Caco-2 cell monolayers (n = 6) in the apical to basolateral direction. (B) Cyclosporin A flux across Caco-2 cell monolayers (n = 6) in the apical to basolateral direction. (C) Doxorubicin flux across Caco-2 cell monolayers (n = 6) in the apical to basolateral direction. (D) Paclitaxel flux across Caco-2 cell monolayers (n = 6) in the apical to basolateral direction. (*$p < 0.05$ vs. control, **$p < 0.01$ vs control).

lular pathway, Caco-2 transport studies were conducted with [^{14}C]propranolol, a transcellular marker. No significant increase in [^{14}C]propanolol flux or P_{app} values (Table I) were observed suggesting that Zot does not significantly modulate the transcellular pathway.

TEER

Figure 4 presents the time-course and reversal of Zot (0, 1, 2, and 4 μg/mL) effects on the transepithelial electrical resistance in Caco-2 cells. The Zot treatments displayed significantly lower TEER values vs. control treatment from approximately 0.5 to 2 h regardless of concentration. At 30, 45, and 60 min, there was a 30–36.5, 32–35, and 32–35% decrease, respectively, in TEER values from baseline after the 1, 2, and 4 μg/mL Zot treatment, respectively. The nadir TEER values occurred at 0.5 h, which supports the 30 min pre-incubation time interval for optimal opening of the tight junction with Zot. The reversal of the opening of the tight junctions with Zot was assessed by removing Zot at 2 h and replacing it with DMEM-HEPES (pH 7.4). TEER values reached baseline levels in approximately 30 min after Zot was removed from the system. The reversibility of the effect of Zot on TEER across Caco-2 cell monolayers suggests that its effect is not secondary to cytotoxicity. To further address this the viability of the Caco-2 cell monolayers were evaluated after 3-h incubation with Zot (0–4 μg/mL) using the LDH dehydrogenase assay. No significant difference in LDH activity for Zot-treated cells vs. untreated cells was observed.

DISCUSSION

There are numerous absorption enhancers that have been evaluated to assess their ability to increase intestinal permeability and hence oral bioavailability. In general, the optimal absorption enhancer should possess the following qualities; its effect should be reversible, it should provide a rapid permeation enhancing effect on the intestinal cellular membrane, it should be non-cytotoxic at the effective concentration level without deleterious and/or irreversible effects on the cellular membrane or cytoskeleton of the tight junctions. The categories of permeation enhancers include surfactants, fatty acids, glycerides, acyl carnities and chitosan derivatives (19,21). In general these agents act by either disruption of the cell membrane or modulation of the tight junctions. Evidence now exists that tight junctions, once regarded, as static structures are dynamic structures and readily adapt to a variety of developmental, physiologic, and pathologic circumstances (22).

Zot, a toxin produced by the bacterial strain *V. cholerae*, activates a complex intracellular cascade of events that regulate tight junction permeability (14). Zot, a 45-kDa protein, is

capable of binding to a specific receptor on the luminal surface of the intestine and reversibly opening the tight junctions between intestinal epithelial cells (15,16). Studies conducted by Fasano and coworkers have shown that the tight junctions can be perturbed enough to allow for the transport of agents across the intestinal mucosa to achieve higher concentrations in the systemic circulation (16,17).

We have examined the utility of this permeation enhancer, Zot, using an *in vitro* model of the intestinal membrane (Caco-2 cell monolayers) to obtain an initial indication of the extent of permeation expected for model compounds in this system within a defined concentration range. It should be noted that the Zonula Occludens Toxin receptor has been purified and characterized from Caco-2 cell lines used in this study as well as the IEC6 intestinal cell lines (23). Determination of the stability of Zot was important since it is a protein produced by *V. cholerae*. Zot was found to be stable over a 10-day period when stored at 4°C as indicated by consistent [^{14}C]mannitol flux values. In addition, Zot was found to be nontoxic in our Caco-2 cell culture system similar to previous reports using isolated rabbit ileum (14). Another indication of the lack of cytotoxicity was displayed in the reversibility observed in the Caco-2 cell culture TEER studies.

Zot produces changes in the cytoskeletal organization that leads to the opening of tight junctions related to protein kinase Ca-dependent polymerization of actin monomers into actin filament. This change has been reported to be time-dependent and reversible (14–17). In our studies, we observed a significant non-dose dependent reduction in TEER, however permeability of the solutes evaluated with Zot displayed a dose related enhancement in transport. This "phenomenon" has been observed with other absorption enhancers, where at certain concentrations there are non-significant differences in TEER values with significant changes in solute Papp values (24–26). Specifically, these findings were reported with Caco-2 permeability studies evaluating the permeation enhancing effect of the non-ionic absorption enhancer, hydrogenated castor oil, on mannitol and PEG 4000 transport (24). Varying concentrations of hydrogenated castor oil, produced small changes in TEER values with significant increases in mannitol and PEG4000 permeability. Other investigators have hypothesized and reported that there may be a recruitment phenomenon associated with the changes in TEER and solute permeability (25,26). The relationship between TEER values, solute flux and number of days in culture was evaluated using rat alveolar epithelial cells (26). Initially a large reduction in TEER values occurred with little or no change in mannitol or urea flux. As the population of junctional pores increased in size, solute flux increases dramatically with only a slight change in TEER.

The changes in TEER values were also accompanied by a significant enhancement in transport of a variety of hydrophilic markers that were assessed. Studies were performed with Caco-2 cell monolayers pre-treated with Zot (4 μg/mL) for 30 min followed by an assessment of marker transport over a two-hour period. The apparent permeability coefficients for specific markers in the presence of Zot were found to be significantly higher than the values observed in the absence of Zot. [^{14}C]Mannitol displayed a significant enhancement in permeability in the presence of Zot (~19 fold) as compared with control values. Investigations with the absorption enhancer, N-trimethyl chitosan chloride (1.5–2.5%), have also reported a significant increase in mannitol transport, however with a high order of enhancement (> 32 fold; 27).

Larger molecular weight hydrophilic markers such as FITC-dextran, inulin, and PEG have been used to assess the potential of absorption enhancers. The transport of FITC-dextran (molecular weight [MW] 4400) across Caco-2 mon-

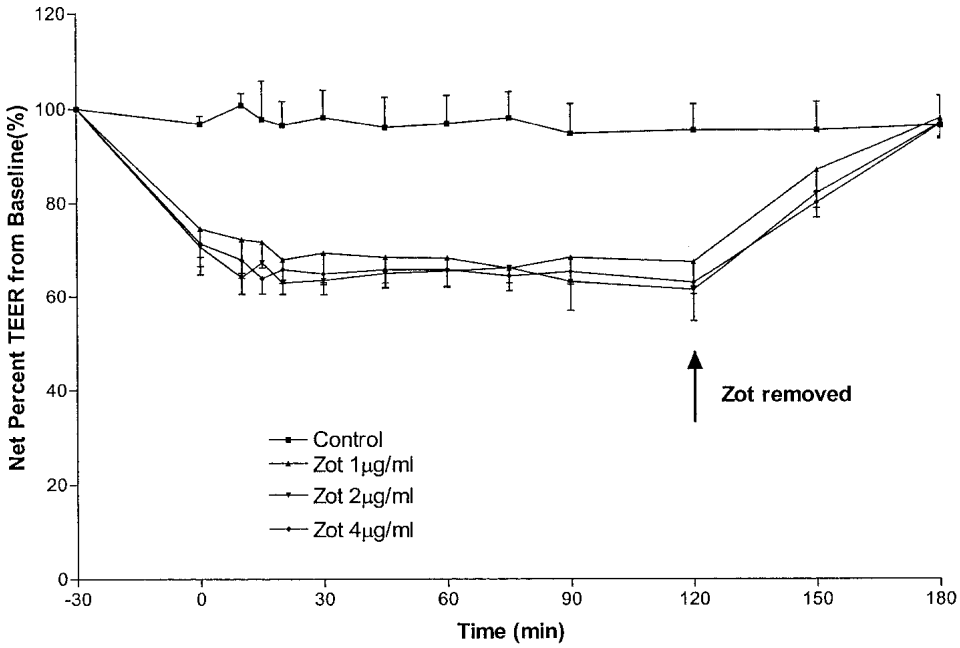

Fig. 4. Effect of Zot (0, 1, 2, 4 μg/mL) on TEER in Caco-2 cell monolayers. The TEER values measured for control, 1, 2, and 4 μg/mL at the beginning of the experiment were 294 ± 10.1, 283 ± 4.58, 318 ± 7.55, and 285 ± 7.65 Ω·cm^2, respectively. Each point represents the mean ± SD of at least 6 experiments. (Reversibility experiment started as indicated after 120 min of incubation).

layers has been studied in the presence of sodium caprate, sodium deoxycholate and N-trimethyl chitosan chloride (27–29). Sodium deoxycholate alone had no effect on the transport of FITC-dextran 4000, but both N-trimethyl chitosan chloride and sodium caprate displayed significant increases in permeability. At the concentrations studied, sodium caprate was cytotoxic in the cell culture system using the trypan blue exclusion test (26). Our results provided a non-significant increase in P_{app} for FITC-dextran 10,000. Hence, it may be possible to produce a significant increase in FITC-dextran transport with higher Zot (>4 μg/mL) concentrations, since the effects of an absorption enhancers are related to their concentration at the site of absorption (21).

Even though the presence of Zot did not significantly enhance FITC permeation, we did note an increase in transport for both hydrophilic markers inulin (MW 5,000 da) and PEG-4000 (MW 4,000 da) at the highest concentration of Zot. Zot produced a significant increase in the permeability of inulin from 7.0×10^{-7} cm/s (control) to 4.37×10^{-6} cm/s (4 μg/mL Zot). This increase in permeability of a large molecular weight hydrophilic compound resulted in a 6.3-fold enhancement of transport inulin, whereas the increase in fold enhancement with PEG-4000 was much lower. Two factors that may explain the higher permeation effect that Zot (4 μg/mL) has on inulin vs. PEG 4000 are structural configuration and actual molecular weight. Studies have suggested that inulin has a more compact cylindrical configuration relative to the extended chain configuration associated with PEG 4000. (30,31). Size exclusion chromatography analysis found retention times of 44.8, 48.2, and 49.6 min for PEG 4000, inulin, and PEG 900, respectively, suggesting that despite the higher molecular weight of inulin, it has a molecular configuration that contributes to its retardation in size exclusion chromatography (32). In addition, these investigators have also suggested the presence of low molecular weight polysaccharide impurities in inulin. Taken together, the higher permeability of inulin vs. PEG 4000 with Zot may be a result of its compact cylindrical configuration as well as the presence of low molecular weight components.

In addition to the molecular weight markers, the effect of Zot on the permeability of a series of hydrophobic therapeutic molecules was examined. In general, Zot-induced treatment at the highest concentration (4 μg/mL) produced a significant increase in transport for the therapeutic agents. The rank order of permeability enhancement observed with the therapeutic agents was paclitaxel > doxorubicin > acyclovir > cyclosporin A. These *in vitro* results are promising since they display a range of permeation increase from 20 (cyclosporin A) to approximately 300 (paclitaxel)%. The oral bioavailability of paclitaxel and cyclopsorin A has long been a problem due to their low solubility and P-glycoprotein substrate status, which significantly minimizes oral absorption (33). The P_{app} enhancement ratio for paclitaxel was found to be 3.13, which suggests that when co-administered with Zot, there is the potential of increasing the oral bioavailability of paclitaxel. Furthermore, the Papp enhancement of cyclopsorin A was 1.20, which at best represents a possible increase in bioavailability when this agent is co-administered with Zot. It should be noted that the concentration of Zot evaluated in this study was within a finite range (1–4 μg/mL) and higher concentrations may provide for higher permeation.

In summary, Zot provides a significant and rapid decrease in the TEER measurements and an enhancement of the permeability of a variety of hydrophilic markers and therapeutic agents across Caco-2 cell monolayers. Its permeability enhancing properties are associated with a reversible modulation of the tight junction cytoskeleton in a manner that is also nontoxic to the cellular membrane. These qualities make Zot a promising drug delivery technology to be used to enhance drug transport across the intestinal mucosa. Future studies will focus on the enhancing properties of Zot in animal studies to determine if the *in vitro* results observed are evident in an *in vivo* system.

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