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Research paper

Enhancing the permeation of marker compounds and enaminone anticonvulsants across Caco-2 monolayers by modulating tight junctions using zonula occludens toxin

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Abstract

Zonula occludens toxin (Zot), a protein elaborated from *Vibrio cholerae*, has been shown to be capable of reversibly opening tight junctions between intestinal cells The objective of this study was to examine the effect of Zot on the flux of various molecules across Caco-2 cell monolayers. In addition, the transport of a series of anticonvulsants, the enaminones was also evaluated in the presence of Zot. The flux of [^{14}C]mannitol, [^{14}C]inulin and various enaminones across Caco-2 cell monolayers ($n = 6$) was examined after pre-incubation for 1 h with Zot (0 or 4000 ng/ml) or phosphate-buffered saline (PBS). At the end of the incubation period, the flux of radiolabeled compounds or enaminones (1×10^{-4} M) was assessed over a 2-h period. In addition, dose–response studies with Zot (0, 1000, 2000 or 4000 ng/ml) were performed using mannitol. The flux of both mannitol and inulin significantly increased ($P < 0.05$) in the presence of Zot. The transport of the enaminones with Zot ranged from 9.42 to 26.83×10^{-5} cm/s vs. 4.68 to 13.83×10^{-5} cm/s without Zot. Zot significantly increased the transport of all agents tested. This suggests that the co-administration of drugs with Zot may be a useful delivery strategy to increase the intestinal permeability and hence oral absorption of poorly bioavailable agents.

Keywords: Tight junctions; Enaminones; Zonula occludens toxin; Paracellular transport; Bioavailability

1. Introduction

In the drug development process, the interrelationship between drug structure, drug receptor affinity and drug bioavailability plays a significant role in the viability of a drug candidate. An orally administered drug must possess not only intrinsic activity, but also favorable biopharmaceutical properties, which allow drug molecules to cross the intestinal membrane. In recent years, agents such as protease inhibitors, which possess low bioavailability, have been developed and marketed due to the importance of their pharmacological properties. From this example, it is clear that novel technologies, which enhance oral drug delivery are needed, since minimal oral bioavailability may be observed for promising drug candidates.

In addition to first pass metabolism, one of the limitations to systemic bioavailability is the barrier that comprises the intestinal mucosa, the tight junctions, which control paracellular transport [1–3]. Tight junctions are generally reported to be impermeable to molecules with radii larger than 11–15 Å and represent the major barrier toward the paracellular transport of compounds [1]. Studies have been performed to examine the ability of endogenous and exogenous factors to modify the junctions. Some of these factors referred to as absorption enhancers include the chitosan analogs and acylcarnitines [4–10]. Chitosan has been demonstrated to promote the nasal absorption of insulin in rats and sheep [8] and enhance the paracellular transport of peptides in vitro and in vivo by opening of tight junctions [5,8]. However, either unacceptable side effects or poor solubility at physiological pH has hampered development of agents falling within these two general classes of absorption enhancers (i.e. calcium chelators and surfactants). Nonetheless, the search for agents able to reversibly modulate tight junctions that are non-toxic to the body and can be supplied in appropriate pharmaceutical dosage forms are still under investigation for the enhancement of poorly absorbable agents. Thus, the approach for increasing the permeability of agents via the paracellular pathway may be to co-administer agents that

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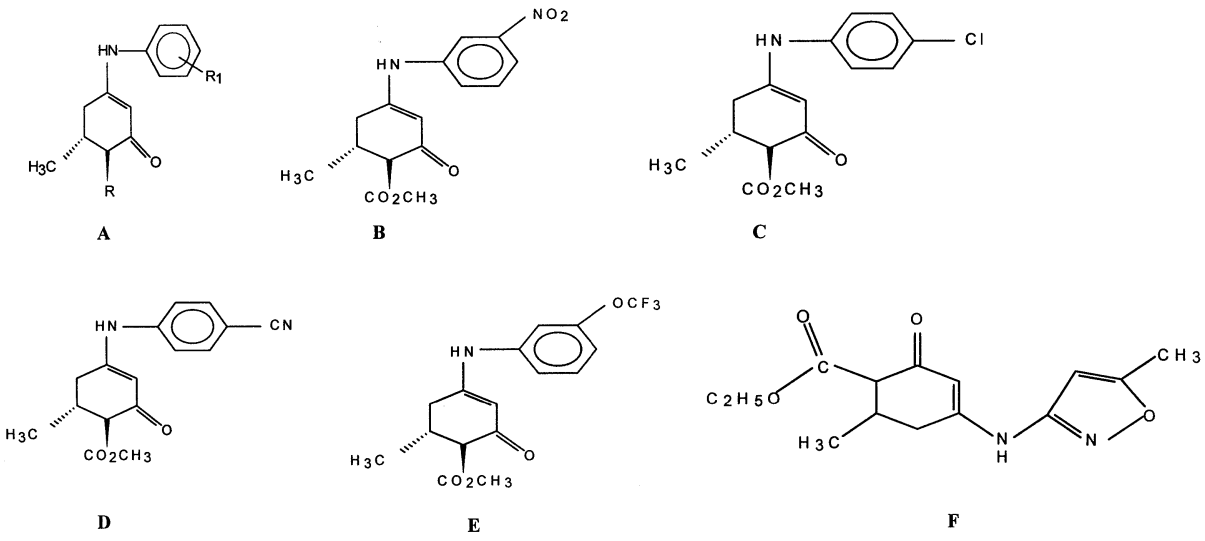

Fig. 1. Structure of enaminone esters. General enaminone structure (A) and DM44 (B), DM5 (C), LS20 (D), DM49 (E) and ON2 (F) [15–18].

will 'loosen' the tight junction and hence allow an increase in the transport of poorly bioavailable agents.

Fasano et al. [11] 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. 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 [12,13]. Studies conducted by Fasano and co-workers 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 [13,14].

The objective of this study was to evaluate the influence of Zot on the intestinal permeability of two molecular weight markers (mannitol, inulin) and a series of hydrophobic anticonvulsant agents, the enaminones (Fig. 1 and Table 1), using Caco-2 cell monolayers. The enaminones series, synthesized by Scott et al. [15–18], were selected based on their differences in lipophilicity ($C\log P$), molecular weight, substituents and efficacy (Table 1). The prototype anticonvulsant enaminone of the series, methyl 4-[(4′-chlorophenyl)amino]-6-methyl-2-oxo-3-cyclohexene-1-carboxylate (DM5), was found to be active intraperitoneally (ip) in mice and orally (po) in rats when evaluated using, maximal electroshock seizure (MES), subcutaneous pentylenetetrazol (scMET) and neurological toxicity (Tox) [15–18]. Quantitative structure–activity relationship (QSAR) evaluation indicated that the most potent analogs are those which are electron withdrawing ($+\sigma$) and lipophilic ($+\pi$). The lack of activity for certain enaminones administered orally within this series, which possess positive QSAR attributes may suggest limited systemic oral bioavailability. For this reason, these compounds were selected to determine if their intestinal permeability could be enhanced with the co-administration of Zot.

2. Materials and methods

2.1. Materials

The Caco-2 cell line was obtained from American Type

Table 1
General chemical and pharmacological properties of enaminone analogs [15–18]

Compound	R[a]	Formula	M.W.	$C\log P$	ED_{50}	
					Mouse, ip	Rat, po
ON2[b]	–	$C_{14}H_{17}N_2O_4$	277	1.29	68.4	4.5
LS20	4′-CN	$C_{16}H_{16}N_2O_3$	284.36	2.35	248.8	–
DM44	3′-NO_2	$C_{15}H_{16}N_2O_5$	304.33	2.61	NA[c]	–
DM49	3′-OCF_3	$C_{16}H_{16}NO_4F_3$	343.32	2.88	ND[d]	50
DM5	4′-Cl	$C_{15}H_{16}NO_3Cl$	293.77	3.23	26.2	5.79

[a] R for all compounds is CO_2CH_3 except ON2.
[b] ON2 placed in miscellaneous chemical series.
[c] ND = not determined, class 1 (active at 100 mg/kg or less).
[d] NA = not active at dosages up to 300 mg/kg.

Culture Collection (Rockville, MD, USA) at passage 19. Cells used in this study were between passage numbers 35 and 40. Dulbecco's modified Eagle medium (DMEM), Dulbecco's modified phosphate-buffered saline with and without Ca^{2+} and Mg^{2+} (PBS), non-essential aminoacids (NEAA), fetal bovine serum (FBS), 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) and [^{14}C]inulin (14.5 mCi/g) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Kenneth R. Scott (Howard University, Washington, DC, USA) synthesized enaminone compounds. Alessio Fasano supplied the Zot (4000 ng/ml). All other chemicals were of analytical grade from Sigma Chemical Co. (St. Louis, MO, USA) and Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Caco-2 cell culture system

The growth, maturation and seeding of Caco-2 cells have been previously described [2]. Caco-2 cells with a passage number between 25 and 35 were used in an effort to derive Caco-2 monolayers with consistent morphological and biochemical properties. 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 utilized for transport studies between days 21 and 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. Prior to experiments, TEER values were assessed for Caco-2 cell monolayers and were in the order of 250–350 Ω cm^2.

2.3. Caco-2 transport studies with mannitol and inulin

The transport of two compounds (mannitol and inulin) was evaluated based on their molecular weights (160 and 5000 Da, respectively), 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 assess the dose–response effect of Zot on paracellular transport, cell monolayers ($n = 6$) were pre-incubated for 30 min with Zot (0, 1000, 2000 and 4000 ng/ml). [^{14}C]mannitol was added and at 5, 10, 15, 20, 30, 45, 60, 75, 90 and 120 min, the insert was transferred to fresh solutions (2.6 ml) of PBS in the basolateral chamber. TEER measurements were taken at the end of the 30-min pre-incubation period prior to the addition of [^{14}C]mannitol. [^{14}C]inulin was added to cell monolayers ($n = 6$) pre-incubated with only one concentration of Zot (4000 ng/ml). 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 PBS in the basolateral chamber. Samples were collected over 120 min from the basolateral chamber. All incubations with Zot were maintained at 37°C and the monolayers were agitated orbitally at 50–60 rpm during the course of the permeability study. Sample analysis was performed by radiographic methods.

2.4. Caco-2 transport studies with enaminones

Five enaminone compounds (DM5, DM44, DM49, LS20 and ON2, Table 1) were evaluated in this study in the presence or absence of Zot. Caco-2 cells were grown to confluency and transport studies were conducted as described above. To assess the absorption enhancing influence of Zot on the enaminones, cell monolayers ($n = 6$) were pre-incubated for 30 min with Zot (4000 ng/ml). At the end of the incubation period, each enaminone (1×10^{-4} M) was added and at 5, 10, 15, 20, 30, 45, 60, 75, 90 and 120 min, the insert was transferred to fresh solutions (2.6 ml) of PBS in the basolateral chamber. The incubations were maintained at 37°C and the monolayers were agitated orbitally at 50–60 rpm during the course of the permeability study. Sample analysis was performed by a HPLC–UV method.

2.5. Enaminone HPLC method

A selective and specific high-performance liquid chromatography method was used to quantitate enaminones in transport media [19]. Reverse phase chromatography with ultraviolet detection ($\lambda = 307$ nm) was utilized to quantitate the eluate. The chromatographic system for enaminone analysis included the following: (1) Model 515 liquid chromatograph (Waters-Millipore, Milford, MA, USA), (2) 717 Waters autosampler, (3) Waters Model 486 UV detector and (4) 3390A Hewlett Packard Integrator (Avondale, PA, USA). Enaminone standards ranged from 0.1 to 50 μg/ml and the internal standard used was carabamazepine (5 μg/ml). The analytical column was an ODS C_{18} (250×4.6 mm^2, Phenomenex, Torrance, CA, USA). 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 ultrasound and vacuum for 15 min. The mobile phase was delivered at a flow rate of 1.0 ml/min. The mobile phase consisted of acetonitrile, 0.05 M phosphate buffer (65:35, v/v). The assay was linear in the concentration ranges of 0.1–50 μg/ml ($r \geq 0.999$) and inter-day precision ranged from 0.5 to 5.5%.

2.6. Data analysis

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

$$P_{app} = \frac{\Delta Q}{\Delta t}\frac{1}{AC_{(0)}} \quad (1)$$

where $\Delta Q/\Delta t$ is the linear appearance rate of mass in the receiver solution, A the cross-sectional area and $C_{(0)}$ the initial enaminone 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. Transport enhancement ratios R were calculated from P_{app} values:

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

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 one-way analysis of variance (ANOVA) using Dunnett's test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Transport of mannitol and inulin

To assess monolayer integrity and the 'tightness' of the junctions, the permeability of mannitol alone across Caco-2 cells was determined. As seen in Fig. 2A (insert), cell monolayer integrity (i.e. tight junction) was within the normal range (<0.05%/h). The dose–response studies to examine the influence of various concentrations of Zot on the flux of the paracellular marker, [^{14}C]mannitol are illustrated in Fig. 2A. The flux of mannitol in the presence of Zot (1000, 2000 and 4000 ng/ml) was shown to significantly increase in the first and second hours as compared to mannitol alone. Mean P_{app} values for mannitol were 7.6, 16.4, 75 and 95.7×10^{-7} cm/s for Zot concentrations of 0, 1000, 2000 and 4000 ng/ml. The TEER values ranged from 250 to 300 Ω following pre-incubation without Zot and ranged from 175 to 240 Ω after 30 min of pre-incubation with 1000, 2000 and 4000 ng/ml of Zot.

Since the largest increase in flux was observed with Zot at a concentration of 4000 ng/ml, this concentration was used in all subsequent studies with inulin and the enaminones. Fig. 2B exhibits the effect of Zot (4000 ng/ml) on the flux of [^{14}C]inulin across Caco-2 monolayers. Zot was found to significantly increase the flux of inulin at only the second

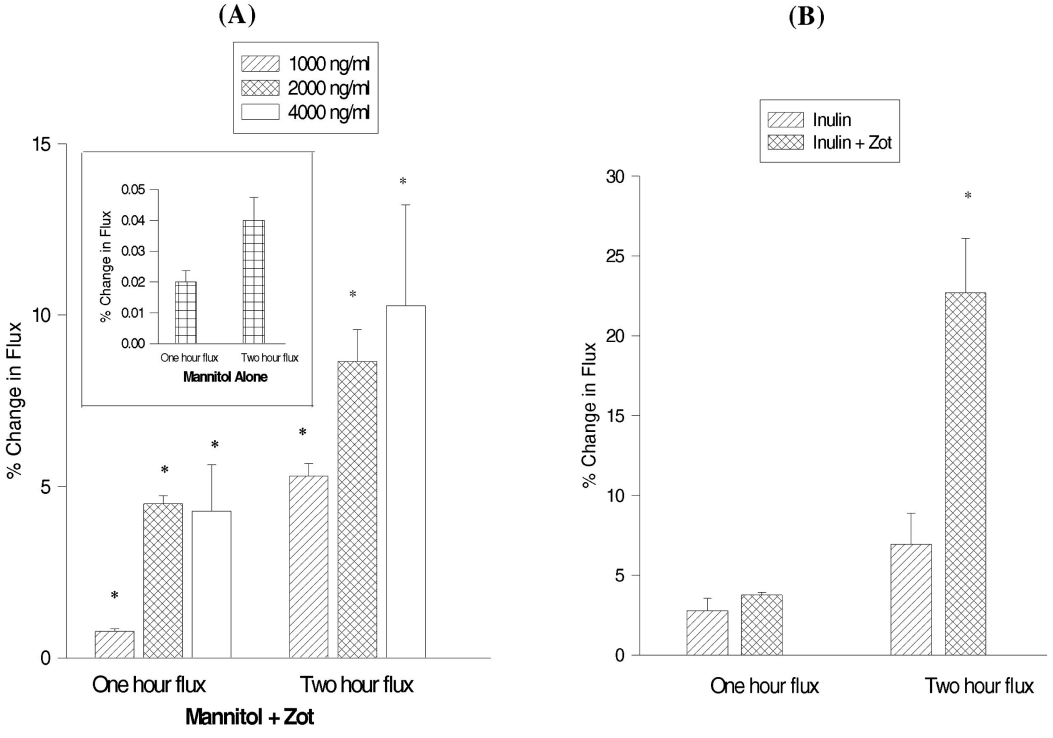

Fig. 2. Transport of molecular weight markers across Caco-2 monolayers in the presence and absence of Zot. (A). Dose–response effect of Zot (1000, 2000 and 4000 ng/ml) on the 1- and 2-h flux of mannitol across Caco-2 cell monolayers. Insert is the 1 and 2 h flux of mannitol alone across Caco-2 cell monolayers. Data listed as the mean at $n = 6$. *Significant difference at $P < 0.05$ compared to permeability alone. (B) The effect of Zot (0, 4000 ng/ml) on the flux of inulin across Caco-2 cell monolayers. Data listed as the mean at $n = 6$. *Significant difference at $P < 0.05$ compared to permeability alone.

Table 2
Permeability coefficients ($P_{app} \times 10^{-5}$ cm/s) of enaminones across Caco-2 monolayers in the absence and presence of Zot (4000 ng/ml)

Compound	AP → BL[a]	AP → BL + Zot	Fold ↑ (*R*)
LS20	4.68 (±0.64)[c]	11.38[b] (±0.62)[c]	2.43
DM44	6.78 (±0.09)	9.42[b] (±0.29)	1.39
DM49	8.28 (±0.42)	21.86[c] (±0.37)	2.64
DM5	4.93 (±0.51)	11.05[b] (±0.34)	2.24
ON2	13.83 (±0.22)	26.83[b] (±1.59)	1.93

[a] P_{app} coefficients listed as 10^{-5} cm/s in the apical (AP) to basolateral (BL) direction.
[b] Significant difference, $P < 0.05$.

hour as compared to permeability of inulin alone. An approximate three-fold increase in the permeability of inulin in the presence of Zot was observed at 2 h as compared to inulin transport alone.

3.2. Transport of the enaminones

The potential intestinal permeability of select enaminones was evaluated across Caco-2 cell monolayers. Table 2 summarizes the apparent permeability coefficients for each enaminone. The permeability of the compounds ranged from 4.68 to 13.83×10^{-5} cm/s and the rank order of transport was as follows: ON2 > DM49 (–OCF_3) > DM44 (–NO_2) > DM5 (–Cl) > LS20 (–CN). It would appear that the lipophilicity as indicated by *C* log *P* (Table 1) was not a predictor of permeability. The *C* log *P* of ON2 was 1.29 yet its P_{app} was 13.83×10^{-5} cm/s as compared to DM5 with a *C* log *P* of 3.23 displayed a P_{app} of 4.93×10^{-5} cm/s.

In order to assess the effects of an absorption enhancer on the permeability of enaminones, studies were also performed with Caco-2 cell monolayers that were pre-incubated with Zot. Table 2 presents the permeability coefficients of the enaminones in the presence and absence of Zot. The transport of the enaminones with Zot ranged from 9.42 to 26.83×10^{-5} cm/s vs. 4.68–13.83×10^{-5} cm/s without Zot.

Zot significantly increased the transport of the enaminones, further the enhancement ratio *R* was found to be greater than 2 for three enaminones (LS20, DM49, DM5). The rank order of permeability enhancement was DM44 (–NO_2) < ON2 < DM5 (–Cl) < LS20 (–CN) and <DM49 (–OCF_3).

4. Discussion

The permeability of active agents across the intestinal mucosa as well as the bioavailability after oral administration has been found to be a limiting property for a number of highly efficacious agents. Most agents are administered orally, therefore a high systemic availability is a required property. In general, the systemic bioavailability of drugs is dependent on a number of various factors including drug solubility, intestinal and hepatic first pass metabolism, drug stability as well as permeability across the intestinal membranes. One of the rate-limiting factors in drug availability at its site of absorption can be the permeation across a biological membrane. Drug permeability across most biological membranes is mediated by passive, transcellular or active transport mechanisms.

The objective of this work was to examine the influence of Zot on enhancing the flux of agents in an in vitro cell culture system. Zot is potentially effective in modulating tight junctions found in the intestine and possibly enhancing intestinal drug transport. The impermeable nature of the paracellular pathway is due to apical intercellular attachments, the most important being the zonula occludens or tight junction [3,20]. Zot, a molecule elaborated by *V. cholerae*, can reversibly open the tight junctions between intestinal epithelia, allowing passage of water and solutes between the lumen and the basolateral side of the intestine [12]. This activity appears to be modulated by a protein kinase C-mediated reorganization of the cytoskeleton. Fasano and co-workers [13,14] have performed numerous studies exhibiting the reversible and dose-dependent effect of Zot in vitro and in vivo. This is the first study that successfully shows the increase in the flux of two molecular weight markers (mannitol and inulin) across Caco-2 cell monolayers.

To evaluate the influence of Zot's absorption enhancing properties in light of other agents such as the chitosan analogs, comparisons can be made between changes in P_{app} values of mannitol in the presence of the absorption enhancer. Thanou et al. [10] examined the change in transport of mannitol across Caco-2 cells using *N*-trimethyl chitosan analogs with moderate (TMC40) and extensive (TM60) substitution. Baseline mannitol mean P_{app} values were 0.7×10^{-7} cm/s. At TMC40 concentrations of 0.05, 0.1, 0.25, 0.5 and 1.0% (w/v), mean P_{app} values were 7.3, 8.2, 9.1, 17.0 and 19.0×10^{-7} cm/s, respectively. At TM60 concentrations of 0.05, 0.1, 0.25, 0.5 and 1.0% (w/v), mean P_{app} values were 18.1, 18.5, 18.9, 23.1 and 27.3×10^{-7} cm/s, respectively. In comparison, mean P_{app} values with Zot at 0, 1000, 2000 and 4000 ng/ml were found to be 7.6, 16.4, 76.0 and 95.7×10^{-7} cm/s. It would appear from these results that Zot has greater transport enhancing properties when evaluated in the context of the chitosan analogs.

Studies were also performed with Caco-2 cell monolayers pre-treated with Zot for 30 min followed by an assessment of enaminone transport. The enaminones are highly hydrophobic compounds (*C* log *P* 1.29–3.23), which would suggest that they are transported via the transcellular route. This is supported by their lipophilicity as well as their permeability coefficients across Caco-2 cell monolayers (4.9–13.8×10^{-5} cm/s). Previous studies examining

their transport across bovine brain microvessel endothelial cells (BBMEC) also observed a high permeability ($5.8–31.9 \times 10^{-5}$ cm/s) suggesting transcellular transport [21].

Even though the transport of the enaminones is most likely due to transcellular permeation, their molecular size (mol. wt. 277–343 Da) does not preclude minimal paracellular transport. The apparent permeability coefficients for the enaminones in the presence of Zot were found to be significantly higher than the values observed in the absence of Zot. The significant increase observed for these agents in the presence of Zot may be due to enhanced permeation of the paracellular pathway suggesting that the intestinal transport of the enaminones can be modulated by the effect of Zot on tight junctions.

In summary, this research focuses on the potential of a novel absorption enhancer, Zot, to enhance the paracellular transport of molecular weight markers and highly lipophilic compounds across Caco-2 monolayers. These studies showed that Zot enhanced the permeability of mannitol, a paracellular marker, and inulin, a compound with a molecular weight of 5000 Da across Caco-2 cell monolayers. In addition, the presence of Zot increased the permeation of a series of lipophilic agents, the enaminones. Research is underway to evaluate the in vivo absorption enhancing properties of Zot using agents of varied molecular weight, lipophilicity and bioavailability. These studies will be extremely useful in determining if co-administration of Zot with therapeutic agents may be a useful drug delivery strategy to increase the intestinal permeability and hence bioavailability of agents administered orally.

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