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Effect of the six-mer synthetic peptide (AT1002) fragment of zonula occludens toxin on the intestinal absorption of cyclosporin A

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Abstract

Zonula occludens toxin (Zot) and its biologically active fragment, delta G, have been shown to reversibly open tight junctions (TJ) in endothelial and epithelial cells. Recently, a six-mer synthetic peptide H-FCIGRL-OH (AT1002) was identified and synthesized that retains the Zot permeating effect on intercellular TJ. The objective of this study was to evaluate the biological activity of AT1002 on enhancing the oral administration of cyclosporin A (CsA). The intestinal permeability enhancing effect of AT1002 on the transport of CsA across Caco-2 cell monolayers was examined after the following treatments, i.e., CsA, CsA/protease inhibitors (PI), CsA/PI/benzalkonium chloride (BC), CsA/AT1002, CsA/PI/AT1002, and CsA/PI/BC/AT1002 (CsA 0.5 μCi/ml, PI (bestatin 15 mM and E-64 5 mM), BC 0.005 w/v%, and AT1002 5 mM, respectively). Apparent permeability coefficients (P_{app}) were calculated for each treatment. In addition, four treatments, i.e., CsA, CsA/PI/BC, CsA/AT1002, and CsA/PI/BC/AT1002 (CsA 120 μCi/kg, PI (bestatin 30 mg/kg and E-64 10 mg/kg), BC 0.1 w/v%, and AT1002 doses of 5, 10 or 40 mg/kg, respectively) were prepared and administered intraduodenally to male Sprague–Dawley rats (230–280 g, $n = 4$–5). Blood samples were collected at 0, 20, 60, and 120 min post-dosing and CsA plasma concentrations were determined subsequently using a Beckman Liquid Scintillation Counter. No significant increases in CsA transport were observed in the Caco-2 cell culture experiments following pre-treatment with AT1002 (5 mM). Even though, AT1002 appeared to increase the P_{app} of CsA in each treatment (CsA/AT1002, $1.54 \pm 0.13 \times 10^{-6}$ cm/s and CsA/PI/AT1002, $1.76 \pm 0.05 \times 10^{-6}$ cm/s) compared to each control (CsA and CsA/PI), respectively. The plasma concentration of CsA was significantly increased over a range of 1.55–2.50 at 10 and 40 mg/kg dose of AT1002. Also, $AUC_{0-120\,min}$ of CsA over a range of 1.64–2.14 and the C_{max} of CsA over a range of 1.77–2.56 was statistically and significantly increased at 10 and 40 mg/kg of AT1002 after the intraduodenal administration of CsA/PI/BC/AT1002 to Sprague–Dawley rats. AT1002 significantly increased the *in vivo* oral absorption of CsA in the presence of PI. This study suggests that AT1002-mediated tight junction modulation, combined with metabolic protection and stabilization, may be used to enhance the low oral bioavailability of certain drugs when administered concurrently.

Keywords: Absorption enhancer; Delta G; Zonula occludens toxin; Intestinal absorption

1. Introduction

The low bioavailability (BA) of efficacious pharmacotherapeutic drugs continues to be a major obstacle in drug development and in many instances may be the deciding factor on whether or not a potent agent is developed. These therapeutic agents experience low BA after oral administration due to poor absorption or susceptibility to first pass metabolism. The search for an efficient novel drug delivery system to overcome this problem cannot be overemphasized. A means of enhancing the gastrointestinal absorption of these drugs would significantly extend their therapeutic usefulness as well as decreasing the dose required to produce efficacy.

Absorption enhancers, including surfactants, fatty acids, and chitosan derivatives, have been used to modify bioavailability by either disruption of the cell membrane or modulation of the tight junctions (TJ) (Aungst, 2000). In general, the optimal absorption enhancer should possess the following qualities;

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1	MSIFIHHGAP	GSYKTSGALW	LRLLPAIKSG	RHIITNVRGL	NLERMAKYLK	MDVSDISIEF
61	IDTDHPDGRL	TMARFWHWAR	KDAFLFIDEC	GRIWPPRLTA	TNLKALDTPP	DLVAEDRPES
121	FEVAFDMHRH	HGWDICLTTP	NIAKVHNMIR	EAAEIGYRHF	NRATVGLGAK	FTLTTHDAAN
181	SGQMDSHALT	RQVKKIPSPI	FKMYASTTTG	KARDTMAGTA	LWKDRKILFL	FGMVFLMFSY
241	SFYGLHDNPI	FTGGNDATIE	SEQS**EPQSKA**	**TAGNAVGSKA**	**VAPASFG** $\boxed{\textbf{FCIGRL}}$ **CVQDGFV**	
301	**TVGDERYRLV**	**DNLDIPYRGL**	**WATGHHIYKD**	**KLTVFFETES**	**GSVPTELFAS**	**SYRYKVLPLP**
361	**DFNHFVVFDT**	**FAAQALWVEV**	**KRGLPIKTEN**	**DKKGLNSIF**		

Fig. 1. Amino acid sequence of Zot. Highlighted (265–399) is delta G, the biologically active fragment of Zot, and box (288–293) is AT1002, active domain of Zot.

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 TJ. Zonula occludens toxin (Zot), a 44.8 kDa protein (399 amino acids; AA) located in the cell envelope of the bacterial strain *Vibrio cholerae*, is capable of reversibly opening the TJ between cells and increasing the paracellular transport of many drugs in a non-toxic manner (Fasano et al., 1991, 1995, 1997a, 1997b; Cox et al., 2001, 2002). Intensive investigation of the biological activity of Zot as an absorption enhancer was triggered by reports of effective oral administration of insulin with Zot in diabetic rats (Fasano et al., 1997a). Recently, a smaller 12 kDa fragment (AA 265–399, Fig. 1) of Zot, referred to as delta G (ΔG), was introduced as the biologically active fragment of Zot (Di Pierro et al., 2001). Amino acid comparison between Zot active fragment and Zonulin, combined with site-directed mutagenesis experiments, confirmed the presence of an octapeptide receptor-binding domain toward the amino terminus of the processed Zot.

Studies in our laboratory have shown that Zot enhances the intestinal transport of drug candidates of varying molecular weight (mannitol, PEG4000, inulin, and sucrose) or low BA (paclitaxel, acyclovir, cyclosporin A, and doxorubicin) across Caco-2 cell monolayers (Cox et al., 2001, 2002) and the transport enhancing effect of Zot is reversible and non-toxic (Fasano et al., 1991; Cox et al., 2002). In addition, ΔG significantly increased the *in vitro* transport of paracellular markers (mannitol, PEG4000, and inulin) in a non-toxic manner and the *in vivo* absorption of low bioavailable therapeutic agents (cyclosporin A, ritonavir, saquinavir, and acyclovir) (Salama et al., 2003, 2004, 2005). Even though promising results were obtained with the use of ΔG with therapeutic agents, the isolation/purification process did not yield sufficient amounts of biologically active ΔG to allow for conduct of *in vivo* studies at higher doses. In an attempt to resolve this issue, several modifications of ΔG sequence were evaluated (Di Pierro et al., 2001). It was noted that the amino acid sequence ‘IGRL’, identified as part of the binding domain in Zot/delta G is the same as that observed in the PAR-2 agonists (fur-L*IGRL*, FC*IGRL*) (Di Pierro et al., 2001). PAR-2 agonists have been reported to increase paracellular permeability (Cenac et al., 2004). As such, this lead to the hypothesis that Zot/ΔG may act at these receptors and produce an increase in the paracellular permeability. Recently, AT1002, a six-mer synthetic peptide H-FCIGRL-OH, was isolated from the active fragment of ΔG, subsequently synthesized and assumed to retain ΔG or Zot permeating effect on intercellular TJ. FCIGRL is identical to the AA residues 288–293 of Zot and the XX-IGRL sequence is part of the putative receptor-binding motif of Zot/ΔG, thus the peptide was expected to have similar properties as Zot/ΔG (Fig. 1). Hence, this is the first study to evaluate the effectiveness of AT1002 as an absorption enhancer after oral co-administration with a low bioavailable therapeutic agent across Caco-2 cell monolayers and after intraduodenal dosing in rodents. Cyclosporin A (CsA) as a low bioavailable therapeutic agent is a potent immunosuppressant agent with high molecular weight, efflux properties, and low oral BA (<20%) (Ogino et al., 1999). Increases in the absorption of CsA would suggest that AT1002 could be used to improve the BA for novel therapeutic macromolecules (e.g., proteins, peptides, and peptidomimetics).

2. Materials and methods

2.1. Materials

AT1002 (>98%) was purchased from Alba Therapeutics Corp. (Baltimore, MD). [^{3}H]-cyclosporin A (CsA; 8 Ci/mM, 1 mCi/ml) was purchased from Amersham Radiochemicals (Piscataway, NJ). Ketamine HCl injection, USP, was purchased from Bedford Laboratories (Bedford, OH). [^{14}C]-mannitol (46.6 mCi/mM, 60 μCi/ml), benzalkonium chloride (BC), xylazine, captopril, protease inhibitors (PI; bestatin and E-64) were purchased from Sigma Chemical Co. (St. Louis, MO). All chemicals were of analytical grade. All surgical supplies were purchased from World Precision Instruments (Sarasota, FL). Polyethylene 50 (PE50) tubing was obtained from Clay Adams (Parsippany, NJ). Universol Scintillation counting cocktail was purchased from ICN (Cost Mesa, CA). The Caco-2 cell line was obtained from American Tissue Culture Collection (ATCC; Rockville, MD). Caco-2 cell culture supplies (Dulbecco’s modified Eagle medium, phosphate buffer saline (PBS), non-essential amino acids, 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). Transwell clusters, 12-well (0.4 μm pores, surface area 1 cm^2) were purchased from Corning Costar (Cambridge, MA).

2.2. Caco-2 transport studies of CsA with AT1002

Caco-2 cells, a human colon adenocarcinoma cell line, were grown as monolayers for 21 days in Dulbecco’s Modified Eagle’s medium (1×) containing 10% fetal bovine serum, 1% non-essential amino acid solution, 1% penicillin–streptomycin and 2% glutamine at 37 °C in an atmosphere of 5% CO_2 and 90% relative humidity. Caco-2 cells from passage numbers of 51–52 were seeded on permeable polycarbonate inserts (1 cm^2, 0.4 μm pore size) in 12 Transwell plates at a density of 80,000 cells/cm^2. The inserts were fed with media every other day until they were used for experiments 21 days after the initial seeding. The integrity of the cell monolayers was evaluated by measuring the transepithelial electrical resistance (TEER) values before the study using a Millicell®-ERS meter (Millipore Corp., Bedford, MA) with chopstick electrodes. The transport of [^{14}C]-mannitol was also performed prior to the transport studies. The cell monolayers were considered to be tight when the apparent permeability coefficients (P_{app}) value of [^{14}C]-mannitol was $<1 \times 10^{-6}$ cm/s. The cell monolayers were washed twice with PBS prior to the transport experiments. After the wash, the plates were incubated for 30 min at 37 °C, and the integrity of the cell monolayers was evaluated by measurement of TEER. The cell inserts were used in transport experiments when the TEER values reached >300 Ω cm^2.

To measure the apical to basolateral transport of CsA, 0.5 ml of each CsA treatment, i.e., (1) PBS solution of CsA, (2) PBS solution of CsA/PI, (3) PBS solution of CsA/PI/BC, (4) PBS solution of CsA/AT1002, (5) PBS solution of CsA/PI/AT1002, and (6) PBS solution of CsA/PI/BC/AT1002. The following concentrations were used in the previously mentioned treatments: CsA 0.5 μCi/ml, PI (bestatin 15 mM and E-64 5 mM), BC 0.005 w/v%, and AT1002 5 mM. Each solution was added to the apical side, and 1.5 ml of PBS was added to the basolateral side of the insert. The insert was moved to a well containing fresh PBS every 10 min for 40 min. Samples were collected from the basolateral side of each well, and the radioactivity of CsA transported was measured by Beckman Coulter LS 6500 multi-purpose Scintillation counter.

2.3. Cytotoxicity study of AT1002 on Caco-2 cell monolayers

Cell viability in the presence of AT1002 was evaluated using a commercially available kit (Cytotoxicity Detection Kit^{PLUS} (LDH), Roche, Mannheim, Germany), based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells. LDH is a stable cytoplasmic enzyme present in all cells. An increase in the amount of dead or plasma membrane-damaged cells results in an increase of LDH activity. An increase of LDH activity directly correlates to the amount of formazan formed during the incubation. The formazan dye formed has an absorption maximum at 490 nm. For these studies, Caco-2 cell monolayers were incubated with AT1002 (2.5 and 5 mM) in PBS for 30 or 60 min. LDH activity was assessed as a function of time by the addition of LDH kit reagents to an aliquot of the solution in the 96 Transwell plate at the end of each incubation period following the manufacturer's manual. PBS and Triton X-100 (5%) were used as negative and positive controls, respectively, and the absorbance was measured at 490 nm.

2.4. Animals

Male Sprague–Dawley rats (230–280 g) were purchased from Harlan Laboratories (Indianapolis, IN). Rats were housed individually in cages and allowed to acclimate at least 2 days after arrival. Rats were fed rat chow and water *ad libitum* and maintained on a 12-h light:12-h dark cycle. The protocol for the animal studies was approved by the School of Pharmacy, University of Maryland IACUC.

2.5. Intraduodenal administration of CsA with AT1002 to rats

Peptides like AT1002, when administered orally, are likely to undergo substantial degradation in the stomach and gastrointestinal tract. In order to exclude the variability in effect as a result of gastric degradation, AT1002 was administered intraduodenally to rats, and plasma concentrations of CsA were monitored for 120 min. Rats were anesthetized with an intra-peritoneal injection of ketamine (80 mg/kg) and xylazine (12 mg/kg) and both the duodenum and jugular vein were cannulated as previously described (Salama et al., 2003, 2004, 2005). Male Sprague–Dawley rats were fasted overnight prior to and during the study with free access to water. Cannulated animals were administered one of the following four treatments, i.e., (1) a distilled water solution of CsA (120 μCi/kg), (2) a distilled water solution of CsA/PI/BC (120 μCi/kg, PI (bestatin 30 mg/kg and E-64 10 mg/kg), BC 0.1 w/v%, respectively), (3) a distilled water solution of CsA/AT1002 (120 μCi/kg and 40 mg/kg, respectively), and (4) distilled water solutions of the CsA/PI/BC/AT1002 (120 μCi/kg, PI (bestatin 30 mg/kg and E-64 10 mg/kg), BC 0.1 w/v%, AT1002 doses of 5, 10 or 40 mg/kg) in each group of study and CsA (5) CsA/PI/BC/control protein, 10 mg/kg (120 μCi/kg, PI (bestatin 30 mg/kg and E-64 10 mg/kg), respectively). The doses selected for the study were determined in a preliminary study. Doses were then slowly administered to intraduodenally cannulated rats with a volume dose of 2 ml/kg rat. Blood samples (250 μl) were drawn via the jugular cannula into heparinized syringes at 0 (actual time point was −5 min before the administration), 20, 60, and 120 min into polypropylene tubes, centrifuged (13,000 rpm for 10 min) immediately and plasma was obtained. Scintillation cocktail was added and samples were analyzed for radioactivity by Beckman Coulter LS 6500 multi-purpose Scintillation counter.

2.6. Data analysis

P_{app} was calculated according to the following equation:

$$P_{app} = \frac{dQ}{dt}\frac{1}{AC_0} \quad (1)$$

where dQ/dt is equal to the linear appearance rate of mass in the receiver solution, A the cross-sectional area (1 cm^2), and C_0 is equal to the initial concentration in the donor compartment.

In the *in vivo* study, the amount of radiolabelled CsA absorbed was converted to concentrations using the specific activity of the radiolabelled stock solution. The area under the plasma concentration–time curve (AUC_{0-t}) was calculated using the linear trapezoidal rule. The highest observable concentration was defined as maximum concentration (C_{max}). The percent enhancement ratio (ER) for the pharmacokinetic parameters was calculated from the formula:

$$ER = \frac{PK_{\text{parameter (treatment)}}}{PK_{\text{parameter (control)}}} \quad (2)$$

All data were expressed as the mean and standard error of the mean of the values (mean ± S.E.M.). The statistical significance of differences between treatments and/or controls was evaluated using the Student's t-test and ANOVA followed by Dunnett's post hoc test where appropriate (SPSS for Windows versions 12.0, SPSS Inc., Chicago, IL) and the level of significance was set at $p < 0.05$.

3. Results

3.1. Caco-2 transport studies of CsA with AT1002

Table 1 summarizes the permeability coefficients (P_{app}) associated with the various transport studies performed with AT1002 and CsA. The mean P_{app} determined for CsA was 1.28 ± 0.10, 1.54 ± 0.13, 1.59 ± 0.07, 1.76 ± 0.05, 1.60 ± 0.03, and 1.52 ± 0.06 ($\times 10^{-6}$ cm/s, mean ± S.E.M., $n = 3$), for the following treatments CsA, CsA/AT1002, CsA/PI, CsA/PI/AT1002, CsA/PI/BC, and CsA/PI/BC/AT1002, respectively. The fold increases of CsA across Caco-2 cell monolayers were minimal after each AT1002 treatment as compared to control treatments as seen in Table 1. In addition, there were no significant differences observed in P_{app} or fold increase for the transport of CsA across Caco-2 cell monolayers between treatments and each control. Mannitol permeability was found to be $6.86 \pm 0.57 \times 10^{-7}$ cm/s suggesting integrity of the tight junctions in the Caco-2 cells.

3.2. Cytotoxicity study of AT1002

The LDH assay was used to investigate the toxic effects of AT1002 on the plasma membrane of cells. Caco-2 cell monolayers were incubated with PBS, AT1002, and Triton X-100 (5%) for a period of 30 and 60 min. Statistical comparisons of the viability of the cells after incubation with AT1002 for 30 and 60 min were not statistically different from the negative control, PBS after incubation. However, both PBS and AT1002 incubation results were significantly different ($p < 0.01$) from Triton X-100 (5%) as a positive control. These results suggest that at a concentration up to 5 mM, AT1002 was not cytotoxic to Caco-2 cells over that period time.

Table 1
The apparent permeability coefficient (P) of mannitol, CsA, and CsA treatments across Caco-2 cell monolayers

	P_{app} ($\times 10^{-6}$ cm/s)	ER (%)
Mannitol	0.69 ± 0.06	–
CsA	1.28 ± 0.10	–
CsA/AT1002	1.54 ± 0.13	120
CsA/PI	1.59 ± 0.07	–
CsA/PI/AT1002	1.76 ± 0.05	111
CsA/PI/BC	1.60 ± 0.03	–
CsA/PI/BC/AT1002	1.52 ± 0.06	95

Mannitol 0.5 μCi/ml, CsA 0.5 μCi/ml, PI (bestatin 15 mM and E-64 5 mM), BC 0.005 w/v%, and/or AT1002 5 mM, respectively. Data presented as mean ± S.E.M. ($n = 3$).

3.3. Intraduodenal administration of CsA with AT1002 to rats

Fig. 2 illustrates the mean (±S.E.M.) plasma concentration versus time profile for CsA in jugular vein cannulated Sprague–Dawley rats following the ID administration of four treatments of CsA, i.e., CsA, CsA/PI/BC, CsA/AT1002, and CsA/PI/BC/AT1002 (at AT1002 doses of 5, 10, or 40 mg/kg). The plasma concentration of CsA from CsA/PI/BC/AT1002 with the dose of 40 mg of AT1002 was 1.78 and 1.55 significantly ($p < 0.05$) higher than those from CsA/PI/BC as the control at 20 and 60 min time period, respectively. Further, the plasma concentration of CsA was significantly increased by 2.01 ($p < 0.05$), 2.05 ($p < 0.01$), and 2.50 ($p < 0.05$) from the dose of 10 mg/kg of AT1002 compared to the control at each 20, 60, and 120 min time period, respectively. Also, the plasma concentration of CsA from the dose of 5 mg of AT1002 was 1.34 significantly ($p < 0.05$) higher than the control at 20 min time period, indicating a significant enhancement in absorption of CsA by AT1002. In comparison, no significant differences were found in the plasma concentration of CsA between the CsA, CsA/PI/BC, and CsA/AT1002 solutions at time points evaluated.

The AT1002 treatments (CsA/PI/BC with AT1002 10 or 40 mg/kg) were found to significantly ($p < 0.01$) increase the extent ($AUC_{0-120\,min}$) and rate (C_{max}) of absorption compared to the control treatment (CsA/PI/BC) without AT1002. AT1002 at a dose of 5 mg/kg (CsA/PI/AT1002 5 mg/kg) produced a 1.45-fold increase in the $AUC_{0-120\,min}$ (34.28 ± 3.23 min ng/ml) and 1.46

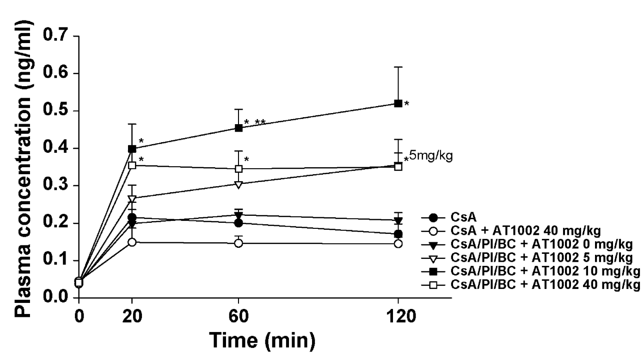

Fig. 2. Average plasma concentration vs. time profile for CsA in jugular cannulated Sprague–Dawley rats following the ID administration of four treatments, i.e., CsA (●), CsA/AT1002 (○), CsA/PI/BC (▼), and CsA/PI/BC/AT1002 (CsA 120 μCi/kg, PI (bestatin 30 mg/kg and E-64 10 mg/kg), BC 0.1 w/v%, and/or AT1002 5 mg/kg (▽), 10 mg/kg (■) or 40 mg/kg (□)). Each data point represents the mean ± S.E.M. of four to five rats. *Significant at $p < 0.05$ compared to CsA/PI/BC of each same time point and **significant at $p < 0.05$ compared to CsA/PI/BC + AT1002 5 mg/kg of same time point.

Table 2
Mean ± S.E.M. bioavailability parameters for CsA (120 μCi/kg) after ID administration to jugular vein cannulated Sprague–Dawley rats (n = 4–5) alone and/or with PI/BC (PI (bestatin 30 mg/kg and E-64 10 mg/kg) and BC 0.1 w/v%) and/or AT1002

	$AUC_{0–120\,min}$ (min ng/ml)	ER	C_{max} (ng/ml)	ER
CsA	21.97 ± 3.79	–	0.22 ± 0.04	–
CsA + AT1002 40 mg/kg	16.56 ± 1.81	0.75	0.18 ± 0.03	0.83
CsA/PI/BC + AT1002 0 mg/kg	23.70 ± 1.79	–	0.24 ± 0.02	–
CsA/PI/BC + AT1002 5 mg/kg	34.28 ± 3.23	1.45	0.36 ± 0.03	1.46
CsA/PI/BC + AT1002 10 mg/kg	50.70 ± 1.78*	2.14	0.62 ± 0.03*	2.56
CsA/PI/BC + AT1002 40 mg/kg	38.81 ± 4.27*	1.64	0.43 ± 0.06*	1.77
CsA/PI/BC + control protein 10 mg/kg	22.26 ± 2.11	–	0.22 ± 0.03	–

* Significant (p < 0.01) compared to CsA and CsA/PI/BC.

(0.36 ± 0.03 ng/ml) increase in C_{max} of CsA. However, these increases were found to be non-significant as compared to the control treatment (CsA/PI/BC). When CsA was co-administered with only AT1002 at a dose of 40 mg/kg, no significant changes were observed in $AUC_{0–120\,min}$ or C_{max} when compared to the control treatment. Further, the increase in $AUC_{0–120\,min}$ and C_{max} was not statistically different for the CsA/PI/BC without AT1002 treatment or the control protein regimen as compared with those of CsA administered alone (Table 2).

The influence of increasing doses of AT1002 (0, 5, 10, and 40 mg/kg) on the plasma concentration of CsA is shown in Fig. 3. The plasma concentrations of CsA at each sampling time point correlated well with the dose range over 0–10 mg/kg of AT1002, with r^2 of 0.9665 at 20 min, 0.9731 at 60 min, and 0.9991 at 120 min. No significant increase in the plasma concentration of CsA was observed for the 40 mg/kg dose of AT1002 versus the 10 mg/kg dose of AT1002. This suggests that that the increase of CsA may have reached a plateau at the 10 mg/kg of AT1002.

4. Discussion

Many therapeutically active agents experience low bioavailability after oral administration due to poor absorption or susceptibility to first pass metabolism. Transient opening of TJ to improve paracellular drug transport and increase oral absorption would be beneficial to enhancing the therapeutic effect. Absorption enhancers are capable of modulation of TJ to improve the transport or absorption of low bioavailable drugs. However, some absorption enhancers cause serious damage to the epithelial integrity, morphology and function (Thanou et al., 2000). Our studies examined the effect of AT1002 as an absorption enhancer of CsA, one of the major potent immunosuppressive drugs which exhibits a low therapeutic index and a poor BA with a mean of ∼20% (Ogino et al., 1999), on Caco-2 cell monolayers and after intraduodenal administration in male Sprague–Dawley rats.

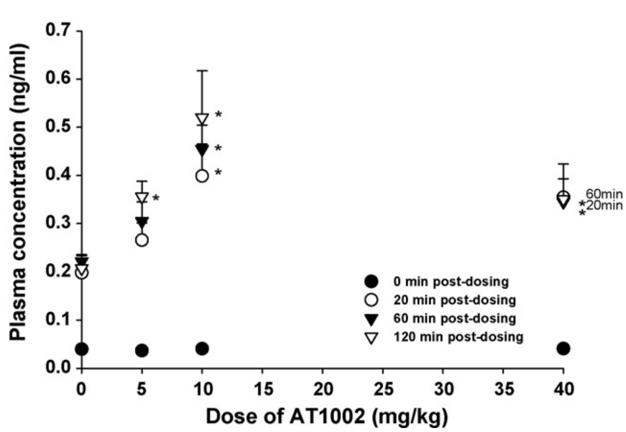

Fig. 3. Average plasma concentration of CsA vs. AT1002 dose profile in jugular cannulated Sprague–Dawley rats following the ID administration of each dose of AT1002 (0, 5, 10, and 40 mg/kg) with CsA/PI/BC (CsA 120 μCi/kg, PI (bestatin 30 mg/kg and E-64 10 mg/kg) and BC 0.1 w/v%, respectively). Each bar is expressed as the mean ± S.E.M. for four to five rats. *Significant p < 0.05 compared to CsA/PI/BC of each same time point.

In previous studies with Zot, bioavailability of oral insulin co-administered with Zot (4.4×10^{-10} mol/kg) was sufficient to lower serum glucose concentrations to levels comparable to those obtained after parenteral injection of the hormone in diabetic rats (Fasano et al., 1997a). Zot (0.45×10^{-10} and 0.89×10^{-10} mol/ml) increased the permeability of molecular weight markers (sucrose and inulin) over a range of 1.30–1.95 and chemotherapeutic agents (paclitaxel and doxorubicin) across the bovine brain microvessel endothelial cells (BBMEC) (Karyekar et al., 2003). In addition, Zot ($(0.22–0.89) \times 10^{-10}$ mol/ml) enhanced the transport of molecular weight markers (mannitol, PEG4000, and inulin) or low bioavailability therapeutic agents (doxorubicin, paclitaxel, acyclovir, cyclosporin A, and enaminones) by as much as 30-fold across Caco-2 cell monolayers, without modulating transcellular transport (Cox et al., 2001, 2002).

Also, studies have shown that ΔG ($(0.83–1.50) \times 10^{-8}$ mol/ml) increased the transport of paracellular markers (mannitol, inulin, and PEG4000) by 1.2–2.8-fold across Caco-2 cells relative to the transepithelial transport of markers in its absence (Salama et al., 2003, 2004). Further, after ID administration to rats, ΔG ($(3.48–6.00) \times 10^{-8}$ mol/kg) displayed high intrinsic biological activity with paracellular markers (mannitol, inulin, and PEG4000) and low bioavailable drugs (CsA, ritonavir, saquinavir, and acyclovir) (Salama et al., 2003, 2004, 2005). Moreover, the *in vivo* studies with ΔG displayed up to 57- and 50-fold increased in C_{max} and AUC as seen with CsA after metabolic protection was provided (Salama et al., 2005).

A significant enhancement in the absorption of CsA was observed in this study after dosing with AT1002, suggesting that the six-mer peptide retained the Zot domain directly involved in the protein permeating effect. AT1002 statistically and significantly increased $AUC_{0–120\,min}$ of CsA over a range of 1.64–2.14, and C_{max} of CsA over a range of 1.77–2.56 at 10 mg/kg (1.41×10^{-5} mol/kg) and 40 mg/kg (5.65×10^{-5} mol/kg) dose of AT1002 ($p < 0.01$) from the treatment of CsA/PI/BC/AT1002 compared to CsA/PI/BC as control. Also, the plasma concentration of CsA was statistically and significantly increased over a range of 2.01–2.50 from CsA/PI/BC/AT1002 10 mg/kg compared to the CsA concentration of CsA/PI/BC at every time point examined in rats. In addition, the enhancement of CsA at 40 mg/kg seemed to be lower than the 10 mg/kg of AT1002 versus control. It would appear that at an AT1002 dose of 10 mg/kg, the biological effect is maximal and this dose may represent the maximum effective dose for this therapeutic agent under this study protocol.

It was reported that protease inhibitors (a mixture of bestatin, captopril, and leupeptin) are needed to minimize enzymatic degradation of ΔG secondary to proteases or peptidases and to display a high intrinsic biological activity of drug with ΔG (Salama et al., 2003, 2005). Similarly, based on the low molecular weight and the peptide nature of AT1002, it would be expected that AT1002 would be extensively metabolized in the gastrointestinal track by enzymes and intestinal flora. When CsA was co-administered intraduodenally to rats with AT1002 excluding PI, the plasma concentration at each time point and bioavailability parameters ($AUC_{0–120\,min}$, C_{max}) were not significantly changed compared to those of CsA. PI which was composed of bestatin and E-64 was selected in one of the treatment arms to minimize enzymatic degradation secondary to proteases or peptidases because of their inhibitory effect on leucine aminopeptidase, alanyl aminopeptidase, serine, and cysteine proteases. In addition, previous studies have evaluated the use of BC in stabilizing peptide (Song et al., 2002). Systematic investigations are underway to optimize the use of BC and AT1002 by LC–MS as well as to identify more stable analogues of the peptide. Studies of the effect of PI/BC showed that PI/BC caused no significant difference in the absorption of CsA. Therefore, administration of PI/BC did not result in significant absorption improvement, and PI/BC/AT1002 absorption enhancement is due to metabolic protection and/or stabilizing effect of AT1002.

In the transport study across Caco-2 cells, no treatments showed statistically increase in their transport in the presence of AT1002 across cell monolayers compared to each control. The Caco-2 cell monolayers have been reported to have lower paracellular permeability than the intestinal epithelium (Artursson and Borchardt, 1997; Rubas et al., 1996) due to anatomical differences between intestinal segments and by noting the colonic origin of this cell line (Wilson and Dietschy, 1972), and there are differences in the level of expression and substrate specificity of transporters and enzymes (Artursson and Borchardt, 1997; Audus et al., 1990; Borchardt, 1995; Brayden, 1997). The enhancement of CsA by AT1002 is assumed to be related to protease activated receptor-2 (PAR-2) receptor. PAR-2 agonists are 6-mer peptides, with four of the amino acids being identical to that of the Zot/Zonulin receptor-binding motif (XX-IGRL) (Di Pierro et al., 2001). This suggests that AT1002 (H-FCIGRL-OH) may possess similar biological activity at PAR-2 receptors. The PAR-2 receptor belongs to a class of G-protein-coupled receptors that are activated by cleavage of their N-terminal by a proteolytic enzyme. Following the cleavage the newly unmasked N-terminal acts as a tethered ligand and activates the receptor (Nystedt et al., 1995). Intracolonic infusion of a 5 μg dose of the PAR-2 agonist, SLIGRL, resulted in a twofold increase in the paracellular permeability of $[^{51}C]$-EDTA (Cenac et al., 2004). Thus, the difference in the *in vitro* versus *in vivo* extent of enhancement observed in our studies might arise from differences in the expression of PAR-2 receptors along the gastrointestinal tract versus cultured Caco-2 cells. Studies are underway to determine the level of expression of PAR-2 receptors in Caco-2 cells as a function of passage number.

5. Conclusion

This study provided information on the effectiveness of the active fragment of Zot and/or ΔG, AT1002, in enhancing *in vivo* oral absorption. The enhancing effects observed *in vivo* (CsA/PI/BC/AT1002) were found to be significantly higher than our controls (CsA or CsA/PI/BC), however, its effect in the *in vitro* model was not apparent. The *in vivo* intraduodenal absorption study illustrates AT1002's potential usefulness in enhancing oral drug delivery. Formulations of the peptide to minimize the adverse effects related to the physiology of the GI tract will be useful and lead to the development of a practically relevant drug delivery technology for low bioavailable drugs.

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