JPP JOURNAL OF Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY
JPP Journal of Pharmacy And Pharmacology
Research Paper

The influence of AT1002 on the nasal absorption of molecular weight markers and therapeutic agents when co-administered with bioadhesive polymers and an AT1002 antagonist, AT1001

Keon-Hyoung Song[a,b] and Natalie D. Eddington[a]

[a]Pharmacokinetics-Biopharmaceutics Laboratory, Department of Pharmaceutical Science, School of Pharmacy, University of Maryland, Baltimore, MD, USA and [b]College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul, Republic of Korea

Keywords
AT1001; AT1002; carrageenan; Na-CMC

Correspondence
Natalie D. Eddington, Pharmacokinetics-Biopharmaceutics Laboratory, Department of Pharmaceutical Science, School of Pharmacy, University of Maryland, Baltimore, MD 21201, USA.
E-mail: neddingt@rx.umaryland.edu

Received March 8, 2011
Accepted September 22, 2011

doi: 10.1111/j.2042-7158.2011.01381.x

Abstract

Objectives The purpose of this study was to demonstrate the effects of the tight junction permeation enhancer, AT1002, on the nasal absorption of molecular weight markers and low bioavailable therapeutic agents co-administered with bioadhesive polymers or zonulin antagonist.

Methods The bioadhesive polymers, carrageenan and Na-CMC, were prepared with AT1002 to examine the permeation-enhancing effect of AT1002 on the nasal absorption of inulin, calcitonin and saquinavir after nasal administration to Sprague–Dawley rats. Blood samples were collected over a 6-hour period from a jugular cannula. In addition, we determined whether AT1002 exerts a permeation-enhancing effect via activation of PAR-2 specific binding to a putative receptor of zonulin. To examine this zonulin antagonist, AT1001, was administered 30 min prior to dosing with an AT1002/inulin solution and blood samples were collected over a 6-hour period.

Key findings The bioadhesive polymers did not directly increase the absorption of inulin, calcitonin and saquinavir, but promoted the permeation-enhancing effect of AT1002 when delivered nasally, thereby significantly increasing the absorption of each drug. Pre-treatment with AT1001 antagonized the zonulin receptor and significantly minimized the permeation-enhancing effect of AT1002.

Conclusion These findings will assist in understanding the permeation-enhancing capability of and the receptor binding of AT1002. Further, combining AT1002 with carrageenan supports the development of the mucosal delivery of therapeutic agents that have low bioavailability even with bioadhesive agents.

Introduction

Low bioavailability of effective therapeutic agents continues to be a major problem in drug development. To address this issue, drug delivery research continues to focus on identifying new approaches to enhance drug absorption with permeation enhancers. As a new class of tight junction permeation enhancers, several proteins or peptides (e.g. *Zonula occludens* toxin (Zot), HIV-1 Tat,[1] PN159,[2] Ephrin-A2[3]) have emerged. Among them, the biologic activity of Zot and its fragments (delta G and AT1002) have been extensively studied[4–13] since it was found that Zot modulates intercellular tight junctions.[14–17]

Previous studies have shown that Zot (44.8 kDa, 399 amino acids) enhanced the permeation of various molecular weight markers (mannitol, PEG4000, inulin, sucrose) or low bioavailability agents (paclitaxel, acyclovir, cyclosporin A, doxorubicin) across Caco-2 cells and blood brain barrier cells.[4–6] Delta G (12-kDa, amino acid residues 265–399 of Zot, 135 amino acids), a biologically active fragment of Zot, increased the transport of paracellular markers (mannitol, inulin, PEG4000) across Caco-2 cells and displayed high intrinsic biological activity with paracellular markers and drugs with low bioavailability (cyclosporin A, ritonavir,

saquinavir, acyclovir) after intraduodenal administration.[7–10] Zot and delta G were capable of reversibly opening the tight junctions between cells and increasing the paracellular transport of many drugs in a non-toxic manner.[4–10]

AT1002, the six-amino acid fragment, FCIGRL, of Zot (amino acids, 288–293) was isolated from delta G. Unlike delta G, AT1002 did not require extensive isolation and purification. It is assumed that AT1002 retains the delta G permeating effect on intercellular tight junctions because: (1) AT1002 has the same amino acid sequence '-IGRL-' as delta G and this is part of the binding domain in delta G molecule, and (2) this same sequence is found in the protease-activated receptor-2 (PAR-2) agonists.[18,19] PAR-2 may be the target receptor for zonulin,[20] which opens the tight junctions via PAR-2 activation.[21] 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. PAR-2 activation increases paracellular permeability and *zonula occludens*-1 disruption through calmodulin activation, which can bind to and activate MLC kinase, and then provokes tight junction opening by perijunctional ring myosin phosphorylation. PAR-2 is activated by trypsin, mast cell tryptase, trypsin-like proteins and synthetic peptides, so-called PAR-activating peptides such as SLIGRL. Thus, PAR-2 agonists have been reported to increase paracellular permeability.[22]

Our studies have shown that AT1002 is capable of increasing the paracellular transport of inulin, PEG4000 and cyclosporin A in a non-toxic manner after intranasal and intraduodenal administration to rats, respectively.[11,12] These promising results led to the investigations presented herein to: (1) examine the permeation-enhancing capability of AT1002 when co-administered with bioadhesive polymers in the enhancement study and (2) examine the receptor binding of AT1002 with pre-treatment of a zonulin antagonist in the inhibition study.

For the enhancement study, we examined the effect of AT1002 on nasal absorption when co-administered with a bioadhesive allowing the permeation enhancer to remain on the nasal mucosa for an extended period of time. Bioadhesives reduce mucociliary clearance, thus co-administration should increase the AT1002's membrane adhesion and enhance permeation.[23,24] Among bioadhesive polymers tested, carrageenan and Na-carboxymethyl cellulose (CMC) have been used to increase the membrane residence time of drugs. Carrageenan, an anionic polymer derived from seaweed, has a high solution viscosity and good bioadhesion,[25,26] and Na-CMC has a high mucoadhesion formed by water uptake.[27]

For the inhibition study, the nasal membranes were pre-treated with AT1001, an octa-peptide inhibitor of paracellular permeability[28] and a zonulin binding antagonist.[20] AT-1001 inhibits gliadin-induced intestinal epithelial cells' cytoskeleton rearrangement, tight junction disassembly and peak F-actin increment. Pre-treatment with AT-1001 the peptide blocks paracellular permeability in monolayers induced by Zot analogues.[28]

This study was also designed to determine whether AT1002 exerts its permeation-enhancing effect through activation of PAR-2 via specific binding to the putative receptor of zonulin. We hypothesized that pre-treatment with a zonulin antagonist (AT1001) would inhibit the specific binding of AT1002 to the tight junction receptor and produce less enhancement of paracellular permeability as compared to AT1002 administration alone.

The purpose of this paper was therefore to evaluate the permeation-enhancing capability of AT1002 when co-administered with bioadhesive polymers and with pre-treatment with a zonulin antagonist, AT1001.

Materials and Methods

Materials

AT1002 (FCIGRL) and AT1001 (GGVLVQPG; FZI/0) (>98%) were obtained from Alba Therapeutics Corp. (Baltimore, MD). Salmon calcitonin, [^{14}C]-inulin (1.2 mCi/g) and [^{3}H]-saquinavir (1 mCi/μM) were purchased from Calbiochem (San Diego, CA), American Radiolabeled Chemicals, Inc. (St Louis, MO) and Moravek Biochemicals (Brea, CA), respectively. Iota(ι)-carrageenan was purchased from FMC BioPolymer (Newark, DE). Dextrose injection solution was purchased from Baxter Healthcare Corporation (Deerfield, IL). Ketamine HCl injection, USP, was purchased from Bedford Laboratories (Bedford, OH). Xylazine and Na-CMC were purchased from Sigma Chemical Co. (St Louis, MO). Polyethylene tubing (PE-50) was obtained from Clay Adams (Parsippany, NJ). All surgical supplies were purchased from World Precision Instruments (Sarasota, FL). HPLC-grade acetonitrile and methanol were obtained from American Bioanalytical (Natick, MA). All solutions and buffers were prepared with HPLC-grade water and doubly purified with a Barnstead D3750 (Ellicott City, MD) system and cellulose nitrate membrane filters (47 mm, 0.2 μm, Whatman, Maidstone, UK). Universol scintillation counting cocktail was purchased from ICN (Cost Mesa, CA). All other reagents were of the highest grade commercially available.

Animals

The protocol for the animal studies was approved by the School of Pharmacy, University of Maryland Institutional Animal Care and Use Committee. Male Sprague–Dawley (SD) rats (280–290 g) were purchased from Harlan Laboratories (Indianapolis, IN). Rats were housed individually in cages and allowed to acclimatise for 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; rats were fasted overnight prior to the study, with free access to water.

Preparation of formulations for intranasal administration

Inulin solutions were obtained by mixing [^{14}C]-inulin with 5% dextrose solution prior to the addition of each of the bioadhesive polymers [inulin/carrageenan (1%w/v) or inulin/Na-CMC (0.5% w/v)]. AT1002 (5 or 10 mg/kg) was added to a 5% dextrose solution of the inulin control and the final solutions were prepared as either inulin/carrageenan/AT1002 or inulin/Na-CMC/AT1002. The formulations for each of the low-bioavailability drug (salmon calcitonin, [^{3}H]-saquinavir) were prepared with mixing calcitonin or saquinavir with 5% dextrose solution, respectively (calcitonin alone, saquinavir alone). After addition of an appropriate amount of AT1002 (5 or 10 mg/kg) to each drug alone solution, AT1002 treatment solutions were prepared as calctonin/AT1002 and saquinavir/AT1002. Solutions, including 1 w/v% carrageenan, were obtained by mixing carrageenan with each drug solution and the AT1002 treatment solution, respectively (calcitonin/carrageenan, calcitonin/carrageenan/AT1002, saquinavir/carrageenan, saquinavir/carrageenan/AT1002).

For the inhibition study, a solution of [^{14}C]-inulin in 5% dextrose (control) or inulin and AT1002 in 5% dextrose (treatment) was used. The pretreatment solution was prepared by mixing AT1001 with 5% dextrose solution (5% dextrose solution of AT1001).

The 5% dextrose is usually used for nasal administration solution during *in vivo* studies.[29] The concentrations of 1 w/v% carrageenan and 0.5 w/v% Na-CMC, which have similar viscosities, were determined based on the viscosity, the dosing volume of the solutions and the pharmaceutical dose levels of carrageenan in topical formulations up to 2%.[30] The doses of the solutions were determined based on our previous AT1002 studies to compare the effect of bioadhesive polymers or pre-treatment of AT1001 (30 μCi/kg of [^{14}C]-inulin, 80 μCi/kg of [^{3}H]-saquinavir, 5 mg/kg of AT1002 in the enhancement study; 30 μCi/kg of [^{14}C]-inulin, 10 mg/kg of AT1002, and 10 mg/kg of AT1001 in the inhibition study).[11,12] The dose of calcitonin (15 mg/kg) was determined based on the detection levels in the blood sample.[31] All dosing solutions were prepared immediately prior to intranasal administration to jugular-cannulated rats.

Intranasal administration of formulations of AT1002 in the presence of bioadhesive polymers

Male SD rats were anesthetized with an intra-muscular injection of ketamine (80 mg/kg) and xylazine (12 mg/kg), and the jugular vein was cannulated using PE-50 as previously described.[8–10] The following formulations were administered intranasally to rats ($n = 3$–4 per group) per molecular weight marker and low-bioavailability drug: 5% dextrose solutions of (1) inulin/carrageenan/AT1002, (2) inulin/carrageenan, (3) inulin/Na-CMC/AT1002 and (4) inulin/Na-CMC for the enhancement-inulin study; 5% dextrose solutions of (1) calcitonin/carrageenan/AT1002, (2) calcitonin/carrageenan, (3) calctonin/AT1002 and (4) calcitonin alone for the enhancement-calcitonin study; and 5% dextrose solutions of (1) saquinavir/carrageenan/AT1002, (2) saquinavir/carrageenan, (3) saquinavir/AT1002 and (4) saquinavir alone for the enhancement-saquinavir study.

The rats were manually restrained in a supine position and the head was tilted back slightly while the formulations were instilled into the nostril. The dosing solutions were administered via micropipette (Eppendorf, Germany) in a maximum administered volume (50 μl/nostril[29,32]) considering the solubility of bioadhesive polymers and AT1002. Extreme care was taken to avoid membrane damage that could result from contact with the intranasal mucosa.

Intranasal administration of inulin with pre-treatment of zonulin antagonist

For the inhibition study, the nasal membranes of jugular vein-cannulated rats were pre-treated with 5% dextrose solution of zonulin antagonist (AT1001) by intranasal administration. After 30 min of pre-treatment with AT1001, the following formulations were then administered intranasally to the pre-treated rats ($n = 3$ per group): 5% dextrose solutions of (1) inulin/AT1002 as an AT1002 solution group and (2) inulin as a control group. All dosing solutions, including the pre-treatment AT1001 solution, were administered in the same administration volume to rats as described above.

Sample analysis

Blood samples (250 μl) were drawn via the jugular cannula into heparinized syringes at 0, 10, 20, 40, 60, 120, 240 and 360 min for inulin, at 0, 10, 20, 30, 40, 60 and 90 min for the calcitonin study and at 0, 10, 20, 30, 40, 60, 120, 240 and 360 min for the saquinavir study, respectively, and centrifuged (13 000 rpm for 10 min) immediately to obtain the plasma (100 μl). Scintillation cocktail for the samples of radiolabeled inulin and saquinavir was added and samples were analysed for radioactivity by Beckman Coulter LS6500 multi-purpose scintillation counter. For the samples of calcitonin, deproteinization was then performed by the addition of 300 μl of acetonitrile to each tube, followed by vortexing for 10 s and centrifuging at 8000 *g* for 20 min. The resulting supernatants were analysed by a HPLC-MS system (TSQ Quantum Discovery MAX mass spectrometer system (Finnigan MAT, San Jose, CA) as previously described.[33]

Data analysis

The area of the mass peak of salmon calcitonin in SIM chromatograms was plotted against a calibration curve

of calcitonin in standard solutions and subsequently converted to concentration of calcitonin. The amount of each radiolabeled compound absorbed (inulin, saquinavir) was converted to a concentration using the specific activities of serially diluted solutions of each radiolabeled stock solution. The pharmacokinetic parameters were calculated using the non-compartmental analysis Winnolin® pharmacokinetic software package (Pharsight Inc., Mountain View, CA). The area under the plasma concentration–time curve ($AUC_{0\text{-}t}$) was calculated using the linear trapezoidal method. Peak plasma concentration of each compound (C_{max}) and time to reach the peak (T_{max}) following intranasal administration was determined from the observed data. The enhancement ratio (ER, fold times), for the pharmacokinetic parameters was calculated from the formula,

$$ER = \frac{PK_{parameter\cdot(treatment)}}{PK_{parameter\cdot(control)}} \quad (1)$$

All data were expressed as the mean and standard error of the mean of the values (mean ± SEM). The statistical significance of differences between treatments and/or controls were 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 $P < 0.05$ or $P < 0.01$.

Results

Intranasal administration of inulin with AT1002 in the presence of bioadhesive polymers

In the enhancement-inulin study, male SD rats cannulated in the jugular vein were randomly assigned to receive 5% dextrose solutions of (1) inulin/carrageenan/AT1002, (2) inulin/carrageenan, (3) inulin/Na-CMC/AT1002 and (4) inulin/Na-CMC. The pharmacokinetic profiles were characterized for each group and the parameters were calculated using non-compartmental analysis. Figure 1 shows that the mean (±SEM) plasma concentration of inulin following administration with AT1002 in the presence of each bioadhesive polymer (inulin/carrageenan/AT1002, inulin/Na-CMC/AT1002) was significantly higher than each control (inulin/carrageenan, inulin/Na-CMC) at each time point over 360 min, representing a significant enhancement in the intranasal absorption of inulin with AT1002 in the presence of each bioadhesive polymer. Table 1 lists the pharmacokinetic parameters for each group. Statistically significant increases in C_{max} and $AUC_{0\text{–}360\ min}$ of inulin were observed for each AT1002 group. The administration of inulin/carrageenan/AT1002 led to a 6.89-fold increase in C_{max} (5.10 ± 0.45 μg/ml) and a 6.60-fold increase in $AUC_{0\text{–}360\ min}$ (1336 ± 57.51 min μg/ml) compared with the control group of inulin/carrageenan without AT1002 (0.74 ± 0.06 μg/ml of

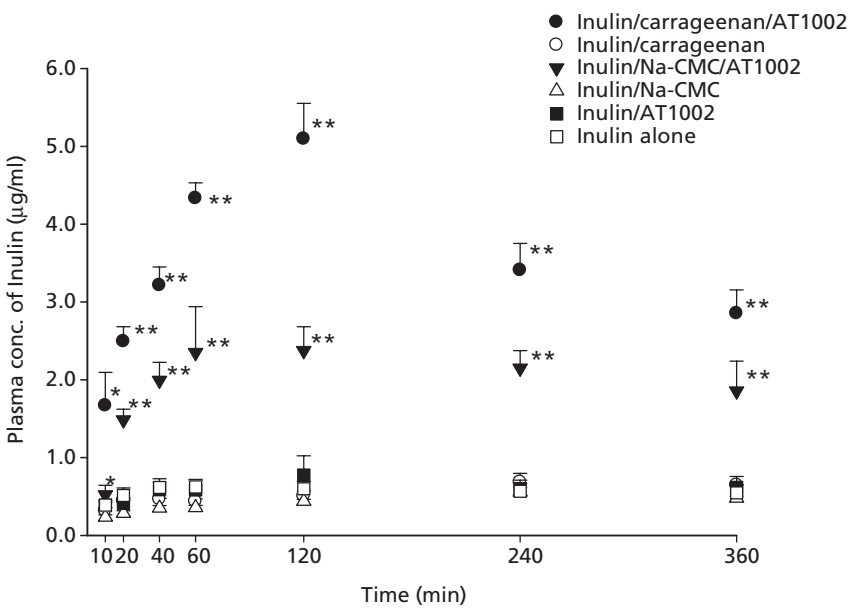

Figure 1 Average plasma concentration of [^{14}C]-inulin versus time in jugular-cannulated SD rats following the intranasal administration of each formulation in 5% dextrose solution. Each data point represents the mean ± SEM of four rats. Significant difference compared with each control (*$P < 0.05$, **$P < 0.01$). (30 μCi/kg of [^{14}C]-inulin, 5 mg/kg of AT1002, 1 w/v% of ι-carrageenan, 0.5 w/v% of Na-CMC) (inulin/AT1002 and inulin alone were cited from reference [12]).

Table 1 Pharmacokinetic parameters in jugular vein-cannulated SD rats after intranasal administration of [^{14}C]-inulin, salmon calcitonin or [^{3}H]-saquinavir with AT1002. Each data represents the mean ± SEM of three to four rats. Each value in the bracket represents the enhancement ratio of each pharmacokinetic parameter compared with each control (30 μCi/kg of [^{14}C]-inulin, 15 mg/kg of salmon calcitonin, 80 μCi/kg of [^{3}H]-saquinavir, 5 mg/kg of AT1002, 1 w/v% of ι-carrageenan, 0.5 w/v% of Na-CMC)

	C_{max} (μg/ml)	$AUC_{0–360\ min}$ (min μg/ml)	T_{max} (min)
Inulin/carrageenan/AT1002 (n = 4)	5.10 ± 0.45** (6.89-fold)	1336 ± 57.51** (6.60-fold)	100.0 ± 20.00* (0.36-fold)
Inulin/carrageenan (n = 4)	0.74 ± 0.06	202.5 ± 7.37	280.0 ± 40.00
Inulin/Na-CMC /AT1002 (n = 4)	2.63 ± 0.49* (4.61-fold)	744.2 ± 89.07** (4.55-fold)	180.0 ± 91.65 (0.64-fold)
Inulin/Na-CMC (n = 4)	0.57 ± 0.06	163.4 ± 3.04	280.0 ± 40.00
Inulin/AT1002[12] (n = 4)	0.83 ± 0.26 (1.26-fold)	224.2 ± 56.00 (1.10-fold)	152.0 ± 53.67 (3.20-fold)
Inulin alone[12] (n = 4)	0.66 ± 0.10	203.4 ± 33.94	47.50 ± 7.50
	C_{max} (μg/ml)	$AUC_{0–90\ min}$ (min μg/ml)	T_{max} (min)
Calcitonin/carrageenan/ AT1002 (n = 3)	5.88 ± 0.54** (6.06-fold)	227.6 ± 46.16* (4.08-fold)	20.00 ± 0.00 (0.60-fold)
Calcitonin/carrageenan (n = 3)	0.97 ± 0.19	55.83 ± 12.34	33.33 ± 6.67
Calcitonin/AT1002 (n = 4)	1.83 ± 0.31 (1.49-fold)	85.54 ± 19.26 (1.43-fold)	27.50 ± 4.79 (0.85-fold)
Calcitonin alone (n = 4)	1.23 ± 0.25	59.82 ± 13.53	32.50 ± 2.50
	C_{max} (ng/ml)	$AUC_{0–360\ min}$ (min μg/ml)	T_{max} (min)
Saquinavir/carrageenan/AT1002 (n = 4)	23.66 ± 3.53** (5.77-fold)	4.32 ± 0.47** (3.51-fold)	15.00 ± 2.89 (0.19-fold)
Saquinavir/carrageenan (n = 4)	4.10 ± 0.35	1.23 ± 0.10	77.50 ± 26.58
Saquinavir/AT1002 (n = 3)	6.13 ± 0.24 (1.02-fold)	1.52 ± 0.09 (1.02-fold)	30.00 ± 5.77 (1.00-fold)
Saquinavir alone (n = 3)	6.03 ± 0.26	1.49 ± 0.03	30.00 ± 5.77

*P < 0.05, **P < 0.01.

C_{max}, 202.5 ± 7.37 min μg/ml of $AUC_{0–360\ min}$) (P < 0.01). The enhancement of inulin by AT1002 in the presence of a bioadhesive polymer was also apparent in the administration with Na-CMC. The administration of inulin/Na-CMC/AT1002 increased C_{max} and $AUC_{0–360\ min}$ of inulin by 4.61-fold (2.63 ± 0.49 μg/ml, P < 0.05) and 4.55-fold (744.2 ± 89.07 min μg/ml, P < 0.01), respectively, over the control group of inulin/Na-CMC without AT1002 (0.57 ± 0.06 μg/ml of C_{max}, 163.4 ± 3.04 min μg/ml of $AUC_{0–360\ min}$) (Table 1).

Intranasal administration of calcitonin with AT1002 in the presence of carrageenan

In the enhancement-calcitonin study, the 5% dextrose solutions of (1) calcitonin/carrageenan/AT1002, (2) calcitonin/carrageenan, (3) calctonin/AT1002 and (4) calcitonin alone were intranasally administered to male jugular vein-cannulated SD rats as described above. Figure 2 illustrates that the mean (±SEM) plasma concentration of calcitonin following administration with AT1002 in the presence of carrageenan (calcitonin/carrageenan/AT1002) was significantly higher than other groups (calcitonin/carrageenan, calctonin/AT1002 and calcitonin alone) at each time point over 40 min, and no statistical differences were found between each group except calcitonin/carrageenan/AT1002, indicating a considerable enhancement in the intranasal absorption of calcitonin with AT1002 in the presence of carrageenan. The intranasal absorption enhancement of calcitonin was clearer in the pharmacokinetic parameters of each group. The administration of calcitonin/carrageenan/AT1002 led to a 6.06-fold increase in C_{max} (5.88 ± 0.54 μg/ml, P < 0.01) and a 4.08-fold increase in $AUC_{0–90\ min}$ (227.6 ± 46.16 min μg/ml, P < 0.05) compared with the control group of calcitonin/carrageenan without AT1002 (0.97 ± 0.19 μg/ml of C_{max}, 55.83 ± 12.34 min μg/ml of $AUC_{0–90\ min}$). In addition, the administration of calcitonin/AT1002 increased C_{max} and $AUC_{0–90\ min}$ of calcitonin by only 1.49-fold and 1.43-fold with no statistical differences, respectively, over the control group of calcitonin alone (Table 1).

Intranasal administration of saquinavir with AT1002 in the presence of carrageenan

The formulations of saquinavir with or without AT1002 in the presence or absence of carrageenan were intranasally administered to male jugular-cannulated SD rats. The mean (±SEM) plasma concentration versus time profiles following the intranasal administration of saquinavir formulations are shown in Figure 3. Statistically higher absorption of saquinavir was found in the group of saquinavir/carrageenan/AT1002 compared with the control group of saquinavir/carrageenan without AT1002 at each sampled time point, representing a significant enhancement in the intranasal absorption of saquinavir with AT1002 in the presence of carrageenan (P < 0.01). The administration of saquinavir with AT1002 in the presence of carrageenan resulted in a 5.77-fold increase in C_{max} (23.66 ± 3.53 ng/ml)

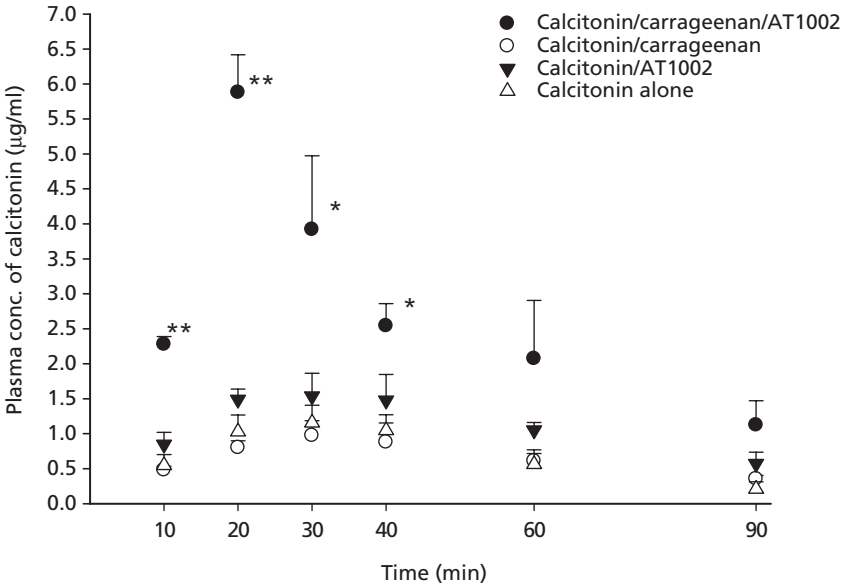

Figure 2 Average plasma concentration of salmon calcitonin versus time in jugular-cannulated SD rats following the intranasal administration of each formulation in 5% dextrose solution. Each data point represents the mean ± SEM of three to four rats. Significant difference compared with each control (*$P < 0.05$, **$P < 0.01$) (15 mg/kg of salmon calcitonin, 5 mg/kg of AT1002, 1 w/v% of ι-carrageenan).

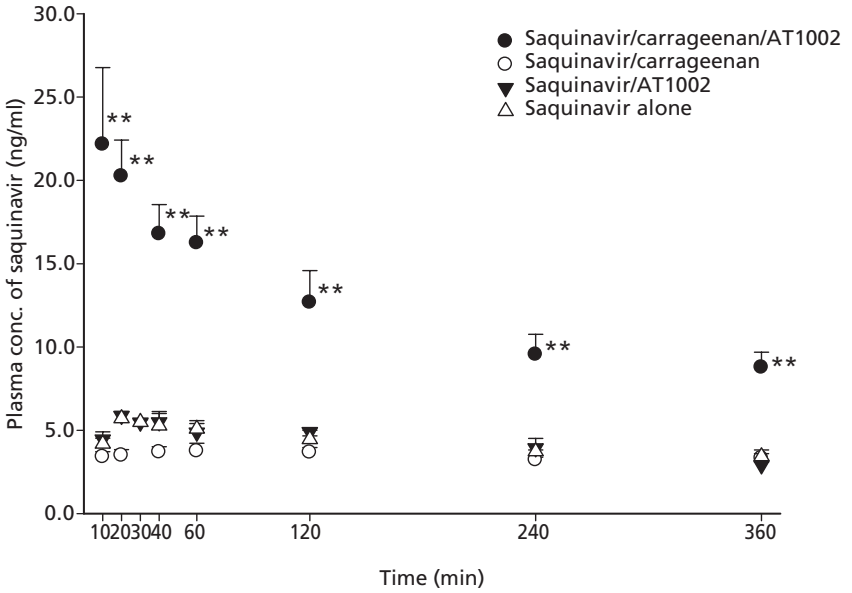

Figure 3 Average plasma concentration of [^{3}H]-saquinavir versus time in jugular-cannulated SD rats following the intranasal administration of each formulation in 5% dextrose solution. Each data point represents the mean ± SEM of three to four rats. (80 μCi/kg of [^{3}H]-saquinavir, 5 mg/kg of AT1002, 1 w/v% of ι-carrageenan; **$P < 0.01$).

and a 3.51-fold increase in $AUC_{0–360\ min}$ (4.32 ± 0.47 min μg/ml) compared with the control group of saquinavir/carrageenan (4.10 ± 0.35 ng/ml of C_{max}, 1.23 ± 0.10 min μg/ml of $AUC_{0\text{-}360\ min}$) ($P < 0.01$). However, AT1002 in the absence of carrageenan did not produce a statistic enhancement in the permeation of saquinavir in the group of saquinavir/AT1002 over the control group of saquinavir alone (1.02-fold increase in both C_{max} and $AUC_{0–360\ min}$) (Table 1).

Intranasal administration of inulin with pre-treatment of zonulin antagonist

In the inhibition study, formulations of inulin/AT1002 and inulin alone were administered intranasally to male jugular vein-cannulated SD rats at 30 min after pre-treatment with zonulin antagonist (AT1001) solution. As shown in Figure 4, the nasal administration of inulin/AT1002 with pre-treatment of AT1001 resulted in increased concentrations of inulin compared with the formulation of inulin without AT1002 after pre-treatment of AT1001 (inulin alone with pre-treatment) at each time point over 360 min. In addition, the administration of inulin/AT1002 with pre-treatment produced 1.56-fold and 1.55-fold increases in C_{max} and $AUC_{0\text{-}360\ min}$ of inulin, respectively, over inulin alone with pre-treatment.

When the administration of inulin/AT1002 with pre-treatment was compared with the reported results from our previous inulin study[12] with the same dose of inulin and AT1002 but no pre-treatment, there was significantly less enhancement of inulin than in the administration of inulin/AT1002 without pre-treatment.[12] In addition, there was no statistical difference of inulin concentration from the administration of inulin without pre-treatment.[12] In addition, the administration of inulin alone with pre-treatment showed no statistical difference in inulin concentration at each time point except 10 min, but showed a tendency for less inulin enhancement at all sampled time points compared with the administration of inulin alone without pre-treatment[12] (Table 2, Figure 4).

Discussion

The goal of permeation enhancers is to overcome the tight junction barriers across cellular membranes to significantly increase the amounts of therapeutic agents that can reach the systemic circulation and/or target tissue after dosing. In previous studies Zot and delta G were found to be promising permeation enhancers that modulated tight junctions in a non-toxic manner.[4–10] Recent studies have shown that AT1002, the fragment of delta G, also possesses permeation-enhancing effects with no toxicity.[11–13] Promising results were observed when AT1002 (5–10 mg/kg) enhanced the transport of inulin by 1.10–2.92-fold in $AUC_{0–t}$ after intranasal administration to rats.[12] For this reason an enhancement study using bioadhesive polymers was conducted to investigate the effect of AT1002 on the nasal absorption of molecular weight markers and low bioavailable agents.

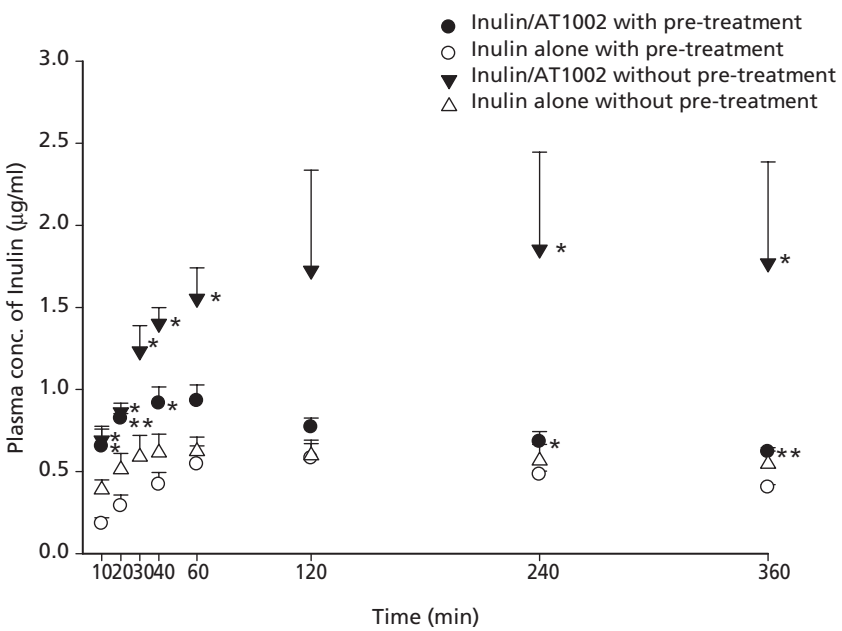

Figure 4 Average plasma concentration of [^{14}C]-inulin versus time in jugular-cannulated SD rats following the intranasal administration of each formulation. Rats were pre-treated with zonulin antagonist (AT1001) for 30 min before the intranasal administration. Each data point represents mean ± SEM of three rats. Significant difference compared with each control (*$P < 0.05$, **$P < 0.01$). (30 μCi/kg of [^{14}C]-inulin, 10 mg/kg of AT1002, 10 mg/kg of AT1001) (inulin/AT1002 and inulin alone without pre-treatment were cited from reference[12]).

Table 2 Mean ± SEM of pharmacokinetic parameters of [^{14}C]-inulin, following intranasal administration with or without AT1002 to jugular vein-cannulated SD rats ($n = 3$). Rats were pre-treated with zonulin antagonist (AT1001) for 30 min before each intranasal administration. Significant difference compared with each control (30 μCi/kg of [^{14}C]-inulin, 10 mg/kg of AT1002, 10 mg/kg of AT1001)

	C_{max} (ng/ml)	$AUC_{0-360\ min}$ (min μg/ml)	T_{max} (min)
Inulin/AT1002 with pre-treatment	959.5 ± 77.41* (1.56-fold)	262.7 ± 18.19* (1.55-fold)	40.00 ± 11.55 (0.28-fold)
Inulin alone, with pre-treatment	615.3 ± 90.36	169.8 ± 17.79	143.3 ± 50.44
Inulin/AT1002[12] (no pre-treatment)	2066 ± 517.8* (3.15-fold)	594.9 ± 168.9* (2.92-fold)	167.5 ± 80.35 (3.53-fold)
Inulin alone[12] (no pre-treatment)	656.5 ± 100.7	203.4 ± 33.94	47.50 ± 7.50

*$P < 0.05$.

The enhancement study showed that the nasal administration of molecular weight marker and low-bioavailability drugs with AT1002 in the presence of bioadhesive polymers resulted in significant increases in C_{max} and AUC_{0-t} over each control, respectively. The administration of inulin with AT1002 led to statistically significant 6.89- and 6.60-fold increases in C_{max} and $AUC_{0-360\ min}$ ($P < 0.01$) in the presence of carrageenan (inulin/carrageenan/AT1002) over the control (inulin/carrageenan), and produced significant 4.61- and 4.55-fold increases in C_{max} ($P < 0.05$) and $AUC_{0-360\ min}$ ($P < 0.01$) in the presence of Na-CMC (inulin/Na-CMC/AT1002) over the control (inulin/Na-CMC), respectively. Furthermore, each nasal administration of calcitonin and saquinavir with AT1002/carrageenan (calcitonin/carrageenan/AT1002, saquinavir/carrageenan/AT1002) resulted in statistically significant 6.06-, 5.77-fold increases in C_{max} ($P < 0.01$) and 4.08-, 3.51-fold increases in AUC_{0-t} ($P < 0.05$ for calcitonin, $P < 0.01$ for saquinavir) over the control (calcitonin/carrageenan, saquinavir/carrageenan), respectively. In addition, the permeation enhancement ratios of inulin (30 μCi/kg, 6.60-fold increase of $AUC_{0-360\ min}$) or saquinavir (80 μCi/kg, 3.51-fold increase of $AUC_{0-360\ min}$) with AT1002 (5 mg/kg, 7.06×10^{-6} mol/kg) in the presence of carrageenan were comparable or superior to the ratios with Zot or delta G when the differences of the administration doses and routes were disregarded. Zot (0.89×10^{-10} mol/ml) increased the permeability of inulin by 6.24-fold across Caco-2 cell monolayers,[5] and delta G (6.00×10^{-8} mol/kg) enhanced the transport of inulin (30 μCi/kg) and saquinavir (120 μCi/kg) by 6.56- and 1.03-fold of AUC_{0-t}, respectively, after intraduodenal administration with protease inhibitors to rats.[9,10]

However, the permeation of the same dose of inulin with AT1002 but no bioadhesive polymer (inulin/AT1002,[12]) or with bioadhesive polymers but no AT1002 (inulin/carrageenan, inulin/Na-CMC) was increased by only 1.26-, 1.13-, 0.87-fold of C_{max} and 1.10-, 1.00-, 0.80-fold of $AUC_{0-360\ min}$ over the control (inulin alone,[12]), respectively. These results are in accordance with the results of a calcitonin and saquinavir study which showed that the permeation of calcitonin or saquinavir with AT1002 or carrageenan (calcitonin/AT1002, calcitonin/carrageenan, saquinavir/AT1002, saquinavir/carrageenan) was not increased statistically (0.68-fold–1.49-fold of C_{max}, 0.83-fold to 1.43-fold of AUC_{0-t}) over each control (calcitonin alone, saquinavir alone).

These results obviously indicate that AT1002 alone (5 mg/kg) and bioadhesive polymer alone did not sufficiently increase the permeation of inulin, calcitonin or saquinavir, but the bioadhesive polymer enhanced the permeation-enhancing effect of AT1002, thereby significantly increasing the absorption of each drug. The significant enhancement of drugs with AT1002 in the presence of the bioadhesive polymer and the temporal decreased tendency of drugs in the presence of bioadhesive polymer alone (without AT1002) could be explained if carrageenan and Na-CMC have enzyme inhibitory activity together with bioadhesive activity. It is known that some bioadhesive polymers like chitosan and polyacrylic acids have enzyme inhibitory activity.[34,35] Thus, the bioadhesive activity of carrageenan and Na-CMC might increase the membrane adhesion of drugs and the permeation enhancer (AT1002). This may extend the release and delay drug absorption, which is in accordance with these results, while increasing the specific binding of AT1002 to the putative receptor of zonulin on the tight junctions. Also, the enzyme inhibitory activity of the bioadhesive polymer might protect the nasal enzymatic degradation of AT1002 (six-mer peptide), although the nasal mucosa has lower enzymatic activity than the gastrointestinal tract.[36,37]

Figures 1 to 3 display significant enhancement of drugs by AT1002/bioadhesive polymer, and also illustrate no statistical differences between drug/AT1002, drug/bioadhesive polymer and drug alone at each time point. Furthermore, no statistical difference in drug concentrations between the formulations of drug/bioadhesive polymer and drug alone suggests that the bioadhesive polymer itself did not alter the nasal membranes, so the concentrations of bioadhesive polymers used may not have had any cytotoxic effects on the nasal membrane. The safety of carrageenan and Na-CMC at the nasal membrane is also supported by previous reports. Carrageenan compounds are on the US Food and Drug Administration GRAS (Generally Recognized as Safe) list of products for topical applications,[38] and are used in topical formulations at dose levels of up to 2% (~30 mg) in the pharmaceutical industry.[30] ι-Carrageenan

was also reported to be a safe compound in the form of nasal spray.[30] CMC caused no ciliary erosion in the nasal cavity, and CMC is known to be an intranasal mucoadhesive platform for use in non-chronic and sub-chronic disease conditions.[39,40]

In the inhibition study, the nasal administration of inulin/AT1002 with pre-treatment produced only 1.56-fold and 1.55-fold increases in C_{max} and $AUC_{0\text{-}360\ min}$ of inulin over the administration of inulin without AT1002 with pre-treatment (inulin alone with pre-treatment). The administration of inulin alone with pre-treatment resulted in a decrease of 0.46-fold and 0.44-fold decreases in C_{max} and $AUC_{0\text{–}360\ min}$ of inulin compared to our previously reported results for inulin/AT1002 without pre-treatment.[12] In addition, the plasma concentrations of inulin at each time point after the administration of inulin/AT1002 with pre-treatment were not statistically different from those of the formulation of inulin without pre-treatment (inulin alone without pre-treatment[12]). Thus, the permeation-enhancing effect of AT1002, which was significant in non-pre-treatment, diminished with pre-treatment of AT1001, thereby reducing the enhancement of inulin. This supports the hypothesis that AT1002 exerts its permeation-enhancing effect through activation of PAR-2 via specific binding to the putative receptor of zonulin. Namely, the four amino acid sequence '-IGRL-' of AT1002, identified as part of the binding domain in zonulin receptor,[7] was identical to that observed in the PAR-2 agonists (i.e. SLIGRL, fur-LIGRL),[18,19] and the receptor of zonulin action involving PAR-2 activation,[20] which increases the permeability, was inhibited by zonulin antagonist,[21] like AT1001.[20] These results are also supported by a study which showed that pre-treatment with the zonulin antagonist AT1001 blocked increased monolayer permeability[41] and its intranasal administration prevented ZOT-induced immune responses.[42]

Conclusions

The results of this paper demonstrate that bioadhesive polymers like carrageenan and Na-CMC do not sufficiently increase the absorption of molecular weight markers when administered alone, but enhance the permeation when co-administered with AT1002, thereby significantly increasing the absorption of molecular weight marker and low bioavailable therapeutic agents. Pre-treatment of AT1001 antagonized the zonulin receptor and significantly decreased the permeation-enhancing effect of AT1002. These findings will assist in understanding of the permeation-enhancing capability and the receptor binding of AT1002. Further, the approach of the administration of AT1002 with the bioadhesive polymer, carrageenan, supports the development of mucosal drug delivery systems for low bioavailable therapeutic agents co-formulated with bioadhesive agents to increase systemic bioavailability.

Declarations

Conflict of interest

The Authors declare that they have no conflicts of interest to disclose.

Acknowledgements and funding

This work was supported by NIH (Grant number 2-R01 EB02771) and was supported, in part, by the Korea Research Foundation [Grant number M01-2004-000-20396-0].

References

1. Andras IE *et al.* HIV-1 Tat protein alters tight junction protein expression and distribution in cultured brain endothelial cells. *J Neurosci Res* 2003; 74: 255–265.
2. Johnson PH, Quay SC. Advances in nasal drug delivery through tight junction technology. *Expert Opin Drug Deliv* 2005; 2: 281–298.
3. Larson J *et al.* Endothelial EphA receptor stimulation increases lung vascular permeability. *Am J Physiol Lung Cell Mol Physiol* 2008; 295: L431–L439.
4. Cox DS *et al.* Enhancing the permeation of marker compounds and enaminone anticonvulsants across Caco-2 monolayers by modulating tight junctions using zonula occludens toxin. *Eur J Pharm Biopharm* 2001; 52: 145–150.
5. Cox DS *et al.* Enhanced permeability of molecular weight markers and poorly bioavailable compounds across Caco-2 cell monolayers using the absorption enhancer, zonula occludens toxin. *Pharm Res* 2002; 19: 1680–1688.
6. Karyekar CS *et al.* Zonula occludens toxin increases the permeability of molecular weight markers and chemotherapeutic agents across the bovine brain microvessel endothelial cells. *J Pharm Sci* 2003; 92: 414–423.
7. Di Pierro M *et al.* Zonula occludens toxin structure-function analysis. Identification of the fragment biologically active on tight junctions and of the zonulin receptor binding domain. *J Biol Chem* 2001; 276: 19160–19165.
8. Salama NN *et al.* Effect of the biologically active fragment of zonula occludens toxin, delta G, on the intestinal paracellular transport and oral absorption of mannitol. *Int J Pharm* 2003; 251: 113–121.
9. Salama NN *et al.* The effect of delta G on the transport and oral absorption of macromolecules. *J Pharm Sci* 2004; 93: 1310–1319.
10. Salama NN *et al.* The impact of DeltaG on the oral bioavailability of low bioavailable therapeutic agents. *J Pharmacol Exp Ther* 2005; 312: 199–205.

11. Song KH *et al*. Effect of the six-mer synthetic peptide (AT1002) fragment of zonula occludens toxin on the intestinal absorption of cyclosporin A. *Int J Pharm* 2008; 351: 8–14.
12. Song KH *et al*. Enhanced nasal absorption of hydrophilic markers after dosing with AT1002, a tight junction modulator. *Eur J Pharm Biopharm* 2008; 69: 231–237.
13. Gopalakrishnan S *et al*. Mechanism of action of Zot-derived peptide AT-1002, a tight junction regulator and absorption enhancer. *Int J Pharm* 2009; 365: 121–130.
14. Fasano A *et al*. Vibrio cholerae produces a second enterotoxin, which affects intestinal tight junctions. *Proc Natl Acad Sci USA* 1991; 88: 5242–5246.
15. Fasano A *et al*. Zonula occludens toxin modulates tight junctions through protein kinase C-dependent actin reorganization, in vitro. *J Clin Invest* 1995; 96: 710–720.
16. Fasano A *et al*. The enterotoxic effect of zonula occludens toxin on rabbit small intestine involves the paracellular pathway. *Gastroenterology* 1997; 112: 839–846.
17. Fasano A, Uzzau S. Modulation of intestinal tight junctions by zonula occludens toxin permits enteral administration of insulin and other macromolecules in an animal model. *J Clin Invest* 1997; 99: 1158–1164.
18. Camerer E *et al*. Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. *Proc Natl Acad Sci USA* 2000; 97: 5255–5260.
19. Kawabata A *et al*. 2-Furoyl-LIGRL-NH2, a potent agonist for proteinase-activated receptor-2, as a gastric mucosal cytoprotective agent in mice. *Br J Pharmacol* 2005; 144: 212–219.
20. Fasano A *et al*. P0517 Zot/Zonulin-mediated regulation of intestinal tight junctions involves the proteinase-activated receptor 2 (Par-2). *J Pediatr Gastroenterol Nutr* 2004; 39: S253–S254.
21. Tripathi A *et al*. Identification of human zonulin, a physiological modulator of tight junctions, as prehaptoglobin-2. *Proc Natl Acad Sci USA* 2009; 106: 16799–16804.
22. Cenac N *et al*. PAR2 activation alters colonic paracellular permeability in mice via IFNgamma-dependent and -independent pathways. *J Physiol* 2004; 558(Pt.3): 913–925.
23. Schipper NG *et al*. The nasal mucociliary clearance: relevance to nasal drug delivery. *Pharm Res* 1991; 8: 807–814.
24. Merkus FW *et al*. Nasal mucociliary clearance as a factor for nasal drug delivery. *Adv Drug Deliv Rev* 1998; 29: 13–38.
25. Madsen F *et al*. A rheological examination of the mucoadhesive/mucus interaction: the effect of mucoadhesive type and concentration. *J Control Release* 1998; 50: 167–178.
26. Bertram U, Bodmeier R. In situ gelling, bioadhesive nasal inserts for extended drug delivery: in vitro characterization of a new nasal dosage form. *Eur J Pharm Sci* 2006; 27: 62–71.
27. Piao J *et al*. Development of novel mucoadhesive pellets of metformin hydrochloride. *Arch Pharm Res* 2009; 32: 391–397.
28. Paterson BM *et al*. The safety, tolerance, pharmacokinetic and pharmacodynamic effects of single doses of AT-1001 in coeliac disease subjects: a proof of concept study. *Aliment Pharmacol Ther* 2007; 26: 757–766.
29. Sharp PE, La Regina MC. *The Laboratory Rat*. Boca Raton, FL: CRC Press, 1998.
30. Eccles R *et al*. Efficacy and safety of an antiviral iota-carrageenan nasal spray: a randomized, double-blind, placebo-controlled exploratory study in volunteers with early symptoms of the common cold. *Respir Res* 2010; 11: 108.
31. Song KH *et al*. Enhanced intestinal absorption of salmon calcitonin (sCT) from proliposomes containing bile salts. *J Control Release* 2005; 106: 298–308.
32. Kuboyama Y *et al*. Changes in nasal cavity volume and target area of exposure to nasal drops with age in rats. *Exp Anim* 1996; 45: 15–22.
33. Song KH *et al*. Simple liquid chromatography–electrospray ionization mass spectrometry method for the routine determination of salmon calcitonin in serum. *J Chromatogr B Analyt Technol Biomed Life Sci* 2002; 775: 247–255.
34. Luessen HL *et al*. Bioadhesive polymers for the peroral delivery of peptide drugs. *J Control Release* 1994; 29: 329–338.
35. Luessen HL *et al*. Multifunctional polymers for the peroral delivery of peptide drugs. In: Mathiowitz E *et al*., ed. *Bioadhesive Drug Delivery Systems. Fundamentals, Novel Approaches, and Development*. New York: Marcel Dekker, 1999: 299–339.
36. Illum L. Nasal drug delivery: new developments and strategies. *Drug Discov Today* 2002; 7: 1184–1189.
37. Illum L. Nasal drug delivery-possibilities problems and solutions. *J Control Release* 2003; 87: 187–198.
38. US Food and Drug Administration. Generally Recognized as Safe (GRAS). [Online] Available at: http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/default.htm.
39. Ugwoke MI *et al*. Toxicological investigations of the effects carboxymethylcellulose on ciliary beat frequency of human nasal epithelial cells in primary suspension culture and in vivo on rabbit nasal mucosa. *Int J Pharm* 2000; 205: 43–51.
40. Murakami T *et al*. Enhanced rectal and nasal absorption of human epidermal growth factor by combined use of the absorption promoter and the synthetic polymer in rats. *Eur J Drug Metab Pharmacokinet* 1991; Spec 3: 125–131.
41. Drago S *et al*. Gliadin, zonulin and gut permeability: effects on celiac and non-celiac intestinal mucosa and intestinal cell lines. *Scand J Gastroenterol* 2006; 41: 408–419.
42. Marinaro M *et al*. Zonula occludens toxin acts as an adjuvant through different mucosal routes and induces protective immune responses. *Infect Immun* 2003; 71: 1897–1902.

