

Zanamivir Oral Delivery: Enhanced Plasma and Lung Bioavailability in Rats

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Abstract

The objective of this study was to enhance the oral bioavailability (BA) of zanamivir (ZMR) by increasing its intestinal permeability using permeation enhancers (PE). Four different classes of PEs (Labrasol[®], sodium cholate, sodium caprate, hydroxypropyl β -cyclodextrin) were investigated for their ability to enhance the permeation of ZMR across Caco-2 cell monolayers. The flux and P_{app} of ZMR in the presence of sodium caprate (SC) was significantly higher than other PEs in comparison to control, and was selected for further investigation. All concentrations of SC (10-200 mM) demonstrated enhanced flux of ZMR in comparison to control. The highest flux (13 folds higher than control) was achieved for the formulation with highest SC concentration (200 mM). The relative BA of ZMR formulation containing SC (PO-SC) in plasma at a dose of 10 mg/kg following oral administration in rats was 317.65% in comparison to control formulation (PO-C). Besides, the AUC_{0-24h} of ZMR in the lungs following oral administration of PO-SC was 125.22 ± 27.25 ng hr ml⁻¹ with a C_{max} of 156.00 ± 24.00 ng/ml reached at 0.50 ± 0.00 h. But, there was no ZMR detected in the lungs following administration of control formulation (PO-C). The findings of this study indicated that the oral formulation PO-SC containing ZMR and SC was able to enhance the BA of ZMR in plasma to an appropriate amount that would make ZMR available in lungs at a concentration higher (>10 ng/ml) than the IC_{50} concentration of influenza virus (0.64-7.9 ng/ml) to exert its therapeutic effect.

Key Words: Zanamivir oral delivery, Permeation enhancer, Bioavailability enhancement, Sodium caprate, Influenza

INTRODUCTION

Influenza is a highly contagious respiratory tract infection that is caused by influenza type A and B viruses (Elliott, 2001). Influenza can cause serious morbidity and increased mortality, particularly in the elderly or the immunocompromised and those with underlying diseases such as chronic obstructive pulmonary disease, cardiovascular disease, and diabetes (Dunn and Goa, 1999; Freund *et al.*, 1999; Elliott, 2001; Colman, 2005). Neuraminidase inhibitors, oseltamivir and zanamivir (ZMR), are considered to be the drug of choice in the treatment of influenza principally due to their low toxicity and high efficacy (Freund *et al.*, 1999; Boonyapiwat *et al.*, 2011; Gupta *et al.*, 2011). Recently, there are many reported incidences of viral resistance against oseltamivir that restricts the

use in that patient population. However, there are no reports of resistance to ZMR to date due to its structural similarity with sialic acid (de Jong *et al.*, 2005; Le *et al.*, 2005; Fick *et al.*, 2007; Stephenson *et al.*, 2009). The lack of resistance coupled with high efficacy and low toxicity makes ZMR a highly sought drug in the treatment of influenza including oseltamivir-resistant variants (de Jong *et al.*, 2005; Le *et al.*, 2005; Fick *et al.*, 2007; Stephenson *et al.*, 2009).

Commercially, ZMR (Fig. 1) is available as a dry powder formulation for oral inhalation applied using Diskhaler[™] (a dry powder inhaler) due to its poor oral bioavailability (2%; range 1 to 5%) in human (Dunn and Goa, 1999; Caballero *et al.*, 2000; Elliott, 2001; Colman, 2005). The major drawback with this route of administration is lack of patient compliance, and therapy is not recommended in patients with chronic re-

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spiratory conditions such as asthma, COPD, etc. (Calfee *et al.*, 1999). Besides, it is difficult to administer this drug by inhalation to the pediatric population which is at high risk for influenza infection (Shelton *et al.*, 2011). Thus, other routes of administration such as oral route is highly warranted considering patients for whom inhalation may be difficult or may not effectively deliver the drug to the sites of viral replication.

Until now, oral delivery of ZMR has been a problem due to its strong hydrophilic nature that limits its transport across the intestinal epithelium (Li *et al.*, 1998; von Itzstein, 2007; Miller *et al.*, 2010). Recently, carrier-mediated prodrug and liposome approaches were made by two different researchers to enhance the permeability of ZMR in Caco-2 cell monolayers (*in vitro*) with no investigation performed *in vivo* (Boonyapiwat *et al.*, 2011; Gupta *et al.*, 2011). Besides, both approaches involved cumbersome method of preparation and stability problems such as liposome stability or prodrug chemical stability (hydrolysis). Generally, the lipophilic drugs use the transcellular pathway to cross the epithelial barrier, while hydrophilic drugs are limited to use the paracellular pathway of the intestinal epithelium (Cano-Cebrian *et al.*, 2005; Gomez-Orellana, 2005; Sharma *et al.*, 2005b; Salama *et al.*, 2006). The tight junctions (TJ) which connects the adjacent epithelial cells provide an extra barrier to the permeation of hydrophilic drugs like ZMR (Salama *et al.*, 2006).

Permeation enhancers (PEs) are compounds that aid the oral drug absorption of drug substances by altering the structure of the cellular membrane (transcellular route) and/or the TJs between cells (paracellular route) of the intestinal epithelium (Yee, 1997; Lindmark *et al.*, 1998; Sharma *et al.*, 2005a; Whitehead *et al.*, 2008a). PEs have been successfully applied to the development of oral dosage forms of various biopharmaceuticals including heparin, insulin, calcitonin, parathyroid hormone, and growth hormone, all of which are advancing through clinical trials (Lee *et al.*, 1994; Baughman *et al.*, 1998; Nissan *et al.*, 2000; Leone-Bay *et al.*, 2001; Buclin *et al.*, 2002; Goldberg and Gomez-Orellana, 2003; Kidron *et al.*, 2004). Accordingly, numerous potential PEs have been identified including surfactants, fatty acids and their derivatives, bile acids, cyclodextrins, etc (Whitehead *et al.*, 2008b).

The objective of this study was to enhance the oral bioavailability (BA) of ZMR by increasing its intestinal permeability using permeation enhancers (PE). For this reason, we have investigated the permeation of ZMR across the Caco-2 cell monolayers in the presence of four different classes of PEs and evaluated their efficacy in terms of flux, apparent permeability coefficient (P_{app}), and enhancement ratio (ER) in comparison to control. The PE with significantly higher flux, P_{app} ,

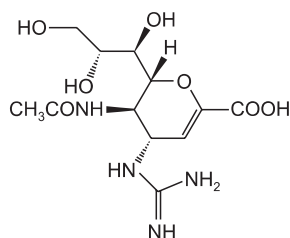


Fig. 1. Chemical structure of ZMR (5-(acetylamino)-4-[(aminomethyl)-amino]-2,6-anhydro-3,4,5-trideoxy-D-glycero-D-galacto non-2-enonic acid).

and ER among others was used for *in vivo* BA study in rats after concentration optimization. The concentration profile of ZMR in lungs was also investigated in rats to understand the possible therapeutic effect in its site of action.

MATERIALS AND METHODS

Materials

The following materials were purchased from various companies and then used as received. Zanamivir was purchased from Shanghai Fulland Chemicals Ltd. (Shanghai, China). Labrasol® (LA) was purchased from Gattefossé (Saint-Priest Cedex, France). Sodium cholate (SCH), sodium caprate (SC), and hydroxypropyl β -cyclodextrin (HPCD) were purchased from Sigma Chemicals Co. Ltd. (St. Louis, USA). The adenocarcinoma cell line Caco-2 was obtained from the American Type Culture Collection (Rockville, MD, USA). Transwell® polycarbonate permeable membrane inserts (12 well, 0.4 μ m pore size, 1.12 cm² growth surface area) were purchased from Corning Incorporated Life Sciences (Lowell, USA). Dulbecco's Modified Eagles Medium (DMEM), fetal bovine serum, streptomycin/penicillin, and trypsin/EDTA were all purchased from Lonza (Walkersville, USA). Glucose, sodium bicarbonate, Hank's Balanced Salt Solution (HBSS), and other cell-culture related materials were all purchased from Sigma Chemicals Co. Ltd. (St. Louis, USA). Acetonitrile, ammonium acetate, and all other chemicals were of HPLC grade and were used without further purification.

Preparation of ZMR formulations

The liquid formulations containing ZMR with or without PEs for *in vitro* Caco-2 cell monolayer study and *in vivo* BA study were prepared using HBSS and purified water as solvent carrier, respectively. The compositions used in the preparation of various formulations were listed in Table 1, Table 2, and Table 3. The ZMR formulations were prepared by adding appropriate amount of ZMR in HBSS or purified water. The mixture was then stirred well to obtain a clear ZMR solution. The ZMR formulations containing PEs were prepared by adding an appropriate amount of PE to the ZMR solution. The mixture was again stirred well to get a clear solution. All samples were pre-

Table 1. The compositions used to investigate the effect of various PEs on transepithelial transport of ZMR (50 μ M) across the Caco-2 cell monolayers and their calculated flux and ER

PE	PE concentration (% w/v)	Flux ($\times 10^{-8}$ μ mol cm ² sec ⁻¹)	ER
Control	-	1.32 \pm 0.53	1.00
LA	0.5	0.94 \pm 0.27	0.71
SC	0.5	7.47 \pm 0.73*	5.66
HPCD	0.5	9.11 \pm 10.14	6.89
SCH	0.5	2.33 \pm 1.41	1.77

HPCD: hydroxypropyl β -cyclodextrin, LA: labrasol, PE: permeation enhancer, SC: sodium caprate, SCH: sodium cholate, ZMR: zanamivir. Control is 50 μ M ZMR without PE in Hank's balanced salt solution (HBSS). ER enhancement ratio calculated as a ratio of P_{app} of ZMR from formulations with PE to that of control.

*Statistically significant (p -value $<$ 0.05) in comparison to control.

pared freshly before the start of experiments. The intravenous reference solution (IV-R) containing ZMR for *in vivo* study in rats was prepared similarly using saline as a solvent carrier instead of HBSS or purified water.

Caco-2 cell culture

The Caco-2 cells were cultured in an incubator with 37°C temperature and 95% relative humidity (RH) and 5% CO₂. The medium used was Dulbecco's Modified Eagles Medium containing (DMEM) 4.5 g/L glucose supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin/streptomycin. The cells were grown as permanent cultures in 75 cm² culture flasks with twice a week medium change, and were subcultured using trypsin/EDTA when they reach 90% confluency. The Caco-2 cells were then seeded on to 12-well Transwell® polycarbonate filter inserts with a density of 5x10⁵ cells/cm². The medium was changed every other day and the cell cultures were at no time found to be infected. All the related experiments were done with cell passage number between 28 to 40 and confluent monolayers between 21 to 30 days after seeding on filters.

Transepithelial transport studies

The transport of ZMR from apical to basolateral was investigated using Hank's Balanced Salt Solution (HBSS, pH 7.4) at 37°C. Before the start of experiment, the DMEM was removed completely from the Transwell® plate containing Caco-2 monolayer and was washed twice with pre-warmed phosphate buffer saline (37°C). The plate was then filled with 0.5 ml and 1 ml of pre-warmed HBSS (37°C) both apically and basolaterally, respectively. The transepithelial electrical resistance (TEER) of the monolayers before and after the experiments was measured. Monolayers with TEER value of more than 250 Ω cm² were only selected for the experiment, and were incubated for 30 minutes in an orbital shaking incubator (RPM 50 and temperature 37 ± 0.5°C). At the start of the experiment (time 0), apical HBSS was replaced with the relevant formulations containing ZMR with or without PEs. The transport across the monolayers was measured by sampling at basolateral well at 30, 60, 90, and 120 min. At each sampling, the inserts were transferred to a new plate containing 1 ml of fresh pre-warmed HBSS basolaterally. The initial and final apical concentration (donor) of the sample solution was also sampled for analysis.

Table 2. The composition of formulations containing various amounts of SC to investigate the effect of SC concentration on transepithelial transport of ZMR (50 μM) across the Caco-2 cell monolayers and their calculated flux and ER

Formulation	SC concentration (%)	Flux (x10 ⁻⁸ μmol cm ⁻² sec ⁻¹)	ER
Control	-	1.16 ± 1.39	1.00
SC-1	0.2	5.69 ± 2.83*	4.90
SC-2	1.0	8.88 ± 2.41*	7.65
SC-3	2.0	13.2 ± 1.68*	11.36
SC-4	4.0	15.5 ± 2.39*	13.34

SC: sodium caprate, PE: permeation enhancer, ZMR: zanamivir. ER enhancement ratio calculated as a ratio of Papp of ZMR from formulations with PE to that of control. *Statistically significant (p-value<0.05) in comparison to control.

Mean values of three samples (n=3 wells) were calculated for the apical to basolateral solutions at each time point. The samples were analyzed by a validated LC-MS/MS method. The flux (μmol cm⁻² sec⁻¹) of ZMR across the Caco-2 cell monolayers was determined from the slope of the plot of the cumulatively transported amount of ZMR versus time using linear regression analysis. The apparent permeability coefficient (P_{app}) and the permeation enhancement ratio (ER) of the PE were calculated as follows,

$$\text{Permeability coefficient (P}_{\text{app}}) = \frac{dQ/dt}{C_0 A}$$

Where, dQ/dt is the rate of permeation of the ZMR across the Caco-2 cell monolayer, C₀ is apical concentration at time zero obtained from analysis of the dosing solution at the start of experiment, and A is the area of the Caco-2 cell monolayer.

$$\text{Enhancement ratio (ER)} = \frac{\text{P}_{\text{app}} \text{ of formulation with PE}}{\text{P}_{\text{app}} \text{ of control (without PE)}}$$

LC-MS/MS analysis of ZMR

Analysis of ZMR was performed using Agilent™ 1,100 series (USA). ZMR was separated on an Atlantis dC18 (2.0x100 mm, 5 μm) stationary phase. The isocratic mobile phase consisted of acetonitrile/10 mM ammonium acetate: 20/80 (v/v, %). The flow rate of the mobile phase and the column oven temperature were set at 0.3 ml/min and 30°C, respectively.

The LC system was coupled to an API 4000 Qtrap mass spectrometer equipped with turbo ion spray ionization source (AB MDS Sciex, Toronto, Canada). The turbo ion spray ionization source was operated in a positive mode. The curtain gas, nebulizer gas, and the turbo gas (nitrogen) pressures were set at 10, 50, and 40 psi, respectively. The turbo gas temperature was set at 600°C, and the ion spray needle voltage was adjusted to 5,500 V. The mass spectrometer was operated at a unit resolution for both Q1 and Q3 in the multiple reaction monitoring (MRM) mode with a dwell time of 300 ms in each transition. The transition of the precursors to the product ion was monitored at 333.2→60.1 for ZMR, 278.2→152.0 for entecavir (internal standard). The collision energy was set at medium. Data acquisition was performed with the Analyst 1.4 software (AB MSD Sciex, Toronto, Canada). Validation for a concentration range of 2.0 ng/mL to 2,000 ng/mL was per-

Table 3. The composition of various formulations, route, and dose used for the *in vivo* BA study in rats

Parameters	IV-R	PO-C	PO-SC
Route of administration	i.v.	p.o.	p.o.
ZMR dose (mg/kg)	1	10	10
SC dose (mg/kg)	-	-	100
ZMR concentration (%)	0.1	1.0	1.0
SC concentration (%)	-	-	10
Medium	Saline	Water	Water

ZMR: zanamivir, SC: sodium caprate, i.v.: intravenous, p.o.: oral, IV-R: i.v. reference formulation of ZMR in saline solution, PO-C: p.o. control formulation of ZMR, PO-SC: p.o. test formulation of ZMR with SC.

formed and found linear with a correlation coefficient of 0.995.

In vivo BA study

Animals: All animal treatment protocols were in accordance with National Institute of Health (NIH) guidelines, South Korea. Male Sprague-Dawley (SD) rats weighing about 300 ± 15 g were purchased from Oriental Bio (Seoul, Korea). The rats were kept in plastic cages with free access to Purina Certified Rodent Chow No. 5002 meal (Ralston Purina, St. Louis, USA) and tap water. Animals were maintained on a 12-h photoperiod (light on at 08:00 and off at 20:00) in our animal facility at $23 \pm 2^\circ\text{C}$ and 50-80% RH (TECNIPLAST, Italy). All the animals received five days of acclimation period in our animal facility before the experiment.

In vivo protocol

The rats were randomly divided into three groups for the *in vivo* BA study to investigate the enhancing effect of SC on the BA of ZMR. All the rats were anaesthetized by intraperitoneal injection of ketamine and xylazine (90:10 mg/kg) and cannulated with a polyethylene (PE) tubing (0.58 mm i.d., 0.96 mm o.d., Natsume, Tokyo, Japan) in the left femoral artery and vein. Formulations PO-SC (ZMR with SC for p.o.) and PO-C (ZMR control solution for p.o.) were administered orally at a ZMR dose of 10 mg/kg, and IV-R (reference ZMR saline solution for i.v.) was administered i.v. at a dose of 1 mg/kg to rats under conscious condition. Blood samples were collected prior to and at 0.5, 1, 2, 3, 4, 6, 8, and 24 hr after administration. At each sampling point, three rats from each group were sacrificed after blood collection to extract the lungs. The lungs were cleansed with saline after extraction of lungs from the rats through a chest incision. The lungs were then transferred into E-tube and stored in the freezer (-80°C) until analysis. Plasma samples were harvested by centrifugation at $1,500 \times g$ for 10 min and stored at -20°C until analysis. The analysis of ZMR in both plasma and lungs was performed using before-mentioned LC-MS/MS method.

Plasma and lung ZMR extraction protocol

ZMR in the plasma samples was analyzed by adding 20 μl of 500 ng/ml of entacavir (internal standard) to 100 μl of plasma. The mixture was then vortexed for about 30 seconds and was centrifuged for 5 min at $10,000 \times g$. The supernatant was filtered using Millipore Millex-GV filter (0.22 μm , PVDF, 33 mm) and was analyzed using the LC-MS/MS method described earlier. To determine the concentration of ZMR in lungs, the lungs were added to 4% bovine serum albumin solution (5 time dilution) after weighing and were homogenized using Ultra-Turrax™ homogenizer at 21,000 rpm for 15 min under ice bath. The resultant mixture was then centrifuged at $4,000 \times g$ for 10 min. The supernatant solution was then analyzed by the LC-MS/MS method described earlier.

Data analyses

PK parameter analyses: The plasma concentration of ZMR versus time profile was analyzed by a non-compartmental method using WinNonlin Professional Version 2.1 program for windows (Pharsight, Cary, NC, USA). The relative and absolute bioavailabilities (BA) of ZMR from orally administered test formulation (PO-SC) were calculated by comparing the BA of orally administered control formulation (PO-C) or i.v. administered reference formulation (IV-R), respectively using the

following equation:

$$\text{BA (\%)} = \frac{\text{AUC}_{\text{test}}}{\text{AUC}_{\text{reference}}} \times \frac{\text{Dose}_{\text{reference}}}{\text{Dose}_{\text{test}}} \times 100$$

Where, AUC is the area under plasma drug concentration curve from time zero to the last sampling time (24 h) of the test formulation.

Statistical analyses: Statistical analyses were carried out using SPSS statistical software (SPSS Statistics ver. 17.0). Multiple comparisons between different formulation groups and their statistical significance were analyzed using ANOVA followed by LSD posthoc test. Confidence interval of 90% was used to calculate the statistical significance in all analyses performed.

RESULTS AND DISCUSSION

This study was a critical step towards making an oral inhalation antiviral drug, ZMR, an oral drug for the treatment of influenza by enhancing its oral BA using PEs to increase its intestinal permeation.

Effect of PEs on ZMR permeation

In this experiment, the ability of PEs (efficacy) to enhance the permeation of ZMR across the Caco-2 cell monolayers was investigated with Labrasol® (LA), sodium caprate (SC), hydroxypropyl β -cyclodextrin (HPCD), and sodium cholate (SCH), which is chemically caprylocaproyl macrogol-8 glyceride, sodium salt of medium chain fatty acid (MCFA) capric acid (C10), hydroxypropyl substituted β -cyclodextrin, and sodium salt of cholic acid, a bile acid, respectively. All these PEs were reported to enhance the permeation of various hydrophilic and/or lipophilic drugs across the intestinal layer, both *in vitro* and *in vivo* (Lindmark *et al.*, 1995; Lindmark *et al.*, 1998; Cano-Cebrian *et al.*, 2005; Sharma *et al.*, 2005a; Whitehead and Mitragotri, 2008; Whitehead *et al.*, 2008a, b).

The composition, flux, and ER of PEs for ZMR across the Caco-2 cell monolayers were shown in Table 1. The transport of ZMR (50 μM) as a function of time and experimental P_{app} of ZMR with or without PEs across the Caco-2 cell monolayers were shown in Fig. 2 and Fig. 3, respectively. TEER values of the Caco-2 cell monolayers that correlate to the integrity of the monolayers measured before and after the transport study

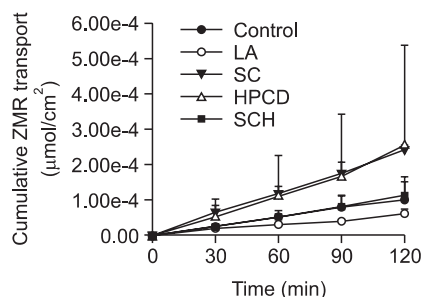


Fig. 2. Effect of various PEs on transepithelial cumulative transport of ZMR (50 μM) across the Caco-2 cell monolayers as a function of time. Data represented as mean \pm SD of three replicates.

were shown in Fig. 4, and a decrease in the TEER value after the experiment is indicative of the disruption of the tight junctions (Yee, 1997; Lindmark *et al.*, 1998; Artursson *et al.*, 2001; Press and Di Grandi, 2008; Smetanová *et al.*, 2011). The TEER values of monolayers included in the transport study had TEER values $\geq 250 \Omega \text{ cm}^2$.

A linear accumulation ($r^2 > 0.98$) of ZMR with time was obtained in the receiver (basolateral) chamber up to 120 min for all the compositions tested with or without PEs. It was expected that all PEs would enhance the permeation of ZMR across the Caco-2 cell monolayers. However, it was disappointing to see from the Fig. 2 and Fig. 3 that the transport rate and P_{app} of ZMR in the presence of LA or SCH was either lower or similar to control. Among the PEs tested, the flux of ZMR in the presence of SC was significantly higher with a value of $7.47 \pm 0.73 \times 10^{-8} \mu\text{mol cm}^{-2} \text{sec}^{-1}$ (Table 1), which was about six-fold higher than the control. The mean experimental P_{app} values of ZMR were $0.26 \pm 0.11 \times 10^{-6} \text{ cm sec}$, $0.19 \pm 0.05 \times 10^{-6} \text{ cm sec}$, $1.49 \pm 0.47 \times 10^{-6} \text{ cm sec}$, $1.82 \pm 2.02 \times 10^{-6} \text{ cm sec}$, and $0.47 \pm 0.28 \times 10^{-6} \text{ cm sec}$ for control, LA, SC, HPCD, and SCH, respectively. The P_{app} of ZMR in the presence of SC was significantly higher than the other tested PEs with ER of 5.66 compared to control.

This result is similar to the previous results that SC increased the flux of various kinds of poorly permeable drugs across the GIT (Mori, 1953). While the mode of action of SC

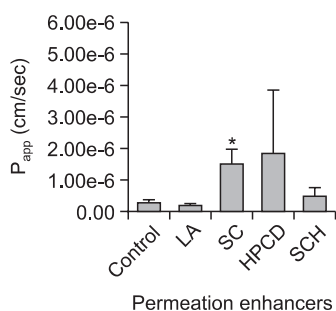


Fig. 3. Effect of various PEs on P_{app} of ZMR (50 μM) across the Caco-2 cell monolayers. Data represented as mean \pm SD of three replicates. *Statistically significant (p -value < 0.05) in comparison to control.

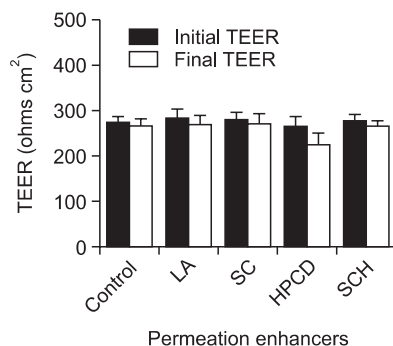


Fig. 4. TEER values of the Caco-2 cell monolayers calculated before and after the drug transport study to investigate the effect of various PEs on transepithelial transport of ZMR (50 μM) across the Caco-2 cell monolayers. Data represented as mean \pm SD of three replicates.

is not yet fully understood, reports of various *in vitro* and *in vivo* studies suggested that SC acts on both transcellular and paracellular pathways (Maher *et al.*, 2009). SC is thought to promote paracellular permeability through modulation of TJs in cell culture (*in vitro*) at lower concentrations, and promotes transcellular permeability through modulation of transcellular membranes in animals and humans (*in vivo*) through its additional surfactant properties at higher concentrations (Gomez-Orellana, 2005; Salama *et al.*, 2006; Maher *et al.*, 2009).

The reduction in TEER values of all the Caco-2 cell monolayers after the experiment was possibly due to alteration of TJs by the PEs. Among the PEs tested, HPCD-tested monolayers showed the highest reduction with a TEER value of $224 \Omega \text{ cm}^2$ (Fig. 4). Although the mean flux and P_{app} of HPCD was slightly higher than SC, it demonstrated a huge variation in the values between samples, which rendered it statistically insignificant (p -value > 0.05) in comparison to control. This huge variation could be due to the membrane solubilizing property of HPCD at the concentration (0.5% w/v) tested in this study. Besides, this observation was similar to the previous research reports that ranked the toxicity of the cyclodextrins as high compared to bile salts and medium chain fatty acids. The reported ranking order of the toxicity in decreasing order is as follows: cyclodextrins $>$ bile salts \approx medium chain fatty acids (Aungst, 2000; Sharma *et al.*, 2005a; Whitehead *et al.*, 2008b). Based on the results, SC was chosen for further studies because of its significantly higher flux, P_{app} , and ER compared to control at the tested concentration.

Effect of SC concentration on ZMR permeation

This experiment was aimed to investigate the effect of SC concentration on the permeation of ZMR across the Caco-2 cell monolayers. As shown in Table 2, formulations SC-1, SC-2, SC-3, and SC-4 containing various concentrations of SC with ZMR (50 μM) were employed in the current study and comparisons were made with the control. The flux and ER of ZMR for various concentrations of SC across the Caco-2 cell monolayers were shown in Table 2. The cumulative transport as a function of time and P_{app} of ZMR (50 μM) in the absence or presence of various concentrations of SC across the Caco-2 cell monolayers were shown in Fig. 5 and Fig. 6, respectively. The TEER values of the Caco-2 monolayers measured before and after the transport study was shown in Fig. 7. The TEER values of all the monolayers after the experiment were

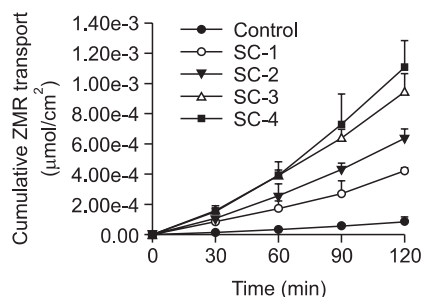


Fig. 5. Effect of SC concentration on transepithelial cumulative transport of ZMR (50 μM) across the Caco-2 cell monolayers as a function of time. Data represented as mean \pm SD of three replicates.

reduced with highest reduction achieved for formulation SC-4 with a value of 222 Ω cm².

All the formulations containing ZMR with or without SC produced a linear accumulation ($r^2 > 0.98$) of ZMR with time in the receiver (basolateral) chamber up to 120 min. It is easily understood from the Fig. 5 that all the concentrations of SC demonstrated significant concentration-dependent enhancement of ZMR cumulative transport in the Caco-2 cell monolayers compared to control. The highest cumulative transport of ZMR across the Caco-2 cell monolayers was achieved for SC-4, the formulation with highest SC concentration (4.0%), with a flux of $15.50 \pm 2.39 \times 10^{-8} \mu\text{mol cm}^{-2} \text{sec}^{-1}$ (Table 2). The flux enhancement of the formulations in comparison to control was about 5 folds, 8 folds, 11 folds, and 13 folds for SC-1, SC-2, SC-3, and SC-4, respectively. The enhanced permeation of ZMR across the Caco-2 monolayers could be due to the reason that the SC concentrations used in this study were either similar or higher than 10-13 mM, which is the minimum reported concentration of SC required to increase the flux of drugs across the Caco-2 cell monolayers (Mori, 1953; van Hoogdaem *et al.*, 1991a; Cano-Cebrian *et al.*, 2005; Salama *et al.*, 2006; Whitehead and Mitragotri, 2008; Maher *et al.*, 2009).

Fig. 6 depicted the P_{app} of ZMR in the presence or absence of various concentrations of SC in Caco-2 cell monolayers. The mean experimental P_{app} values of the tested formulations were $0.23 \pm 0.08 \times 10^{-6} \text{ cm sec}$, $1.14 \pm 0.57 \times 10^{-6} \text{ cm sec}$, 1.78

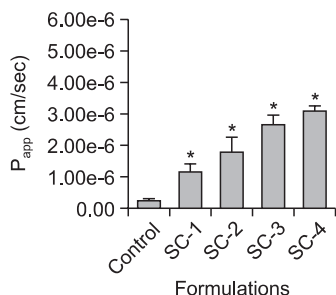


Fig. 6. Effect of SC concentration on P_{app} of ZMR (50 μM) across the Caco-2 cell monolayers as a function of time. Data represented as mean ± SD of three replicates. *Statistically significant (p -value < 0.05) in comparison to control.

$\pm 0.48 \times 10^{-6} \text{ cm sec}$, $2.64 \pm 0.34 \times 10^{-6} \text{ cm sec}$, and $3.10 \pm 0.17 \times 10^{-6} \text{ cm sec}$ for control, SC-1, SC-2, SC-3, and SC-4, respectively. The P_{app} of control formulation was comparable with our earlier study (*in vitro* PE screening) suggesting that the results are reproducible. The highest P_{app} was achieved for formulation SC-4 containing highest concentration of SC (4.0%). All the formulations containing various concentrations of SC demonstrated excellent ER in comparison to control (SC-4 > SC-3 > SC-2 > SC-1) with the highest ER being 13.34 for SC concentration of 4.0% (SC-4) (Table 2). This could be due to the concentration-dependent permeation effects of SC which induces the transcellular packing reorganization in both lipid and protein domains (Whitehead *et al.*, 2008b; Maher *et al.*, 2009). Also, SC partitions into lipid bilayers with increased concentrations and disrupts intermolecular forces between membrane phospholipids to decrease resistance to transcellular permeation and eventually enhanced permeability (Maher *et al.*, 2009). However, SC causes a rapid reversible concentration-dependent reduction in TEER across Caco-2 cell monolayers simultaneously. The use of SC in humans should be safe for use in high concentrations in oral dosage forms that may need to be given repeatedly even on a long-term basis (Sawada *et al.*, 1991; Lindmark *et al.*, 1998; Sharma *et al.*, 2005a; Whitehead *et al.*, 2008b; Maher *et al.*, 2009). Besides, SC is

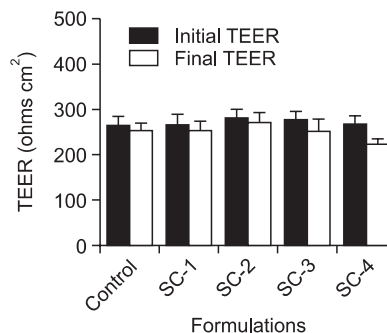


Fig. 7. TEER values of the Caco-2 cell monolayers calculated before and after the drug transport study to investigate the effect of various SC concentration on transepithelial transport of ZMR (50 μM) across the Caco-2 cell monolayers. Data represented as mean ± SD of three replicates.

Table 4. The PK parameters of ZMR in rats following administration of ZMR formulations with calculated BA

Formulations	AUC _{0-24h} (ng.h/ml)	C _{max} (ng/ml)	T _{max} (h)	Absolute BA%**	Relative BA% [†]
<i>Plasma PK Parameters</i>					
IV-R	2,588.50 ± 364.83	-	-	-	-
PO-C	5,28.30 ± 189.28	136.80 ± 25.24	2.00 ± 1.0	2.04	-
PO-SC	1,678.1 ± 247.05*	857.37 ± 51.65*	0.50 ± 0.0*	6.48	317.65
<i>Lung PK Parameters</i>					
IV-R	417.50 ± 49.98	-	-	-	-
PO-C	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-	-
PO-SC	125.22 ± 27.25	156.00 ± 24.00	0.50 ± 0.00	2.99	-

All data are expressed as mean value (n=3); AUC_{0-24 h} area under the concentration-time curve from the time of dosing (0 h) to last observation (24 h); BA: bioavailability, C_{max}: maximum measured plasma concentration, T_{max}: time of maximum plasma concentration. *Statistically significant in comparison to PO-C ($p < 0.05$). **Absolute BA of PO-C and PO-SC following oral administration of 10 mg/kg ZMR was calculated in reference to IV-R following i.v. administration at a dose of 1 mg/kg of ZMR. [†]Relative BA of PO-SC following p.o. administration of 10 mg/kg ZMR was calculated in reference to PO-C following p.o. administration at a dose of 10 mg/kg.

approved by the FDA as a direct food additive for human consumption and is currently in clinical trials as a key component of several proprietary oral formulations (Maher *et al.*, 2009).

In vivo BA in rats

The intestinal membranes of animals and humans are often found to be more resistant to toxicity effects of PEs than Caco-2 cell monolayers and therefore, the membrane disruption effect of PEs might not be observed *in vivo* for the same concentrations (Aungst, 2000; Rege *et al.*, 2001). Besides, the effectiveness of PEs is dependent on its concentration at the site of drug absorption. However, the dilution of PEs occurs *in vivo* by GI fluids that will reduce the enhancer effectiveness (Sawada *et al.*, 1991; Sharma *et al.*, 2005a; Whitehead *et al.*, 2008b). Thus, a relatively higher concentration of PE is expected to produce a desired permeation enhancement with comparatively lower toxicity in animals. Since, the repeated oral administration of capric acid (500 mg/kg) to rats for 150 days was well tolerated and did not lead to gross morphological stomach damage (Mori, 1953), 100 mg/kg dose of the SC was selected for the *in vivo* studies. Oral inhalation by Diskhaler™ (dry powder inhaler) delivers ZMR to the site of infection of influenza virus in the lungs for effective treatment. Thus, for successful oral delivery of ZMR *in vivo*, it should reach the lungs in an amount necessary to produce the therapeutic effect. For this reason, the present study was designed to evaluate plasma as well as lung PK of ZMR in rats.

The composition and route of administration of all formulations used in this study were shown in Table 3. The mean plasma concentration-time profiles and the PK parameters of ZMR following PO-SC, PO-C, and IV-R were shown in Fig. 8 and Table 4, respectively. The dose of oral formulations and i.v. formulation was fixed at 10 mg/kg and 1 mg/kg of animal body weight, respectively. The ZMR concentrations in plasma were detectable for 4 h and 8 h following i.v. and oral formulations, respectively. The AUC_{0-24 h} of ZMR following i.v. administration of the formulation IV-R in rats at a ZMR dose of 1 mg/kg was 2,588.50 ± 364.83 ng h ml⁻¹. Following oral administration, the C_{max} and T_{max} were 857.37 ± 51.65 ng/ml and 0.5 h for PO-SC and 136.80 ± 25.24 ng/ml and 2 h for PO-C, respectively. The AUC_{0-24 h} of ZMR following the oral administration of PO-SC was 1,678.1 ± 247.05 ng hr ml⁻¹, which was significantly (*p*-value < 0.05) higher than PO-C (528.30 ± 189.28 ng h ml⁻¹). The mean absolute BA% of ZMR following oral administration of PO-SC (10 mg/kg dose) was 6.48% in comparison to i.v. administration of IV-R (1 mg/kg dose), which was more than

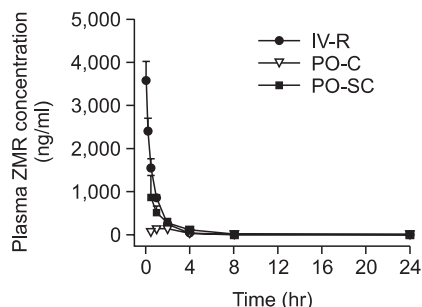


Fig. 8. Mean plasma concentration-time profile of ZMR following p.o. or i.v. administration of formulations containing ZMR with or without SC in SD rats. Mean ± S.D. (n=6).

three folds higher than that of PO-C (2.04%) following oral administration (10 mg/kg dose). The relative BA% of ZMR from PO-SC in comparison to PO-C was 317.65% following oral administration of both at a dose of 10 mg/kg in rats (Table 4). Relatively high BA and C_{max} of ZMR following oral administration of PO-SC were obviously due to the permeation enhancing effect of SC. Besides, the shorter T_{max} of ZMR following PO-SC formulation was probably the rapid and reversible absorption enhancement of SC *in vivo*. This is possibly because of the reason that SC is rapidly absorbed with a T_{max} of less than 10 min (Takahashi *et al.*, 1994; Jorgensen *et al.*, 1998; Raof *et al.*, 2002).

The mean lung concentration-time profile and PK parameters of ZMR following PO-SC, PO-C, and IV-R were shown in Fig. 9 and Table 4, respectively. ZMR concentrations in lungs were detectable up to 1 h following both routes of administration. The AUC_{0-24 h} of ZMR in lungs following IV-R administration was 417.50 ± 49.98 ng hr ml⁻¹, which was about 16.13% of plasma ZMR concentration. There was no ZMR detected in the rat lungs following oral administration of PO-C (control formulation) in rats, which precluded the calculation of PK parameters of ZMR in lungs. The AUC_{0-24 h} of ZMR following oral administration of PO-SC was 125.22 ± 27.25 ng hr ml⁻¹ with a C_{max} of 156.00 ± 24.00 ng/ml reached at 0.50 ± 0.00 h. The AUC_{0-24 h} of ZMR in lungs following PO-SC administration was 7.45% of the plasma ZMR concentration. It appeared that the lung concentration of ZMR increased proportionately with increasing plasma ZMR concentration. The calculated absolute BA% of ZMR in lungs following PO-SC was around 3% (Table 4).

The findings of this study indicated that ZMR was distributed to lungs, which is the target site of its action following oral administration of ZMR. Given that the estimated IC₅₀ for ZMR is 0.64-7.9 ng/ml and the ZMR concentrations of 10 ng/ml have been shown to produce at least a 1.0 log₁₀ TCID₅₀/ml (tissue culture infective dose) decrease in yield for clinical influenza A virus isolates in human respiratory tract epithelial cells (Dunn and Goa, 1999; Reece, 2010; von Itzstein, 2007; Shelton *et al.*, 2011), it appears that the formulation PO-SC containing ZMR and SC would provide concentrations exceeding the IC₅₀ for influenza virus (>10 ng/ml) to produce its therapeutic effect in lungs. However, efficacy studies in animals and/or humans are warranted to understand the optimal SC concentration to achieve the required plasma/lung ZMR concentrations to produce the necessary therapeutic effect without viral resistance. Besides, it is possible that PEs that are effective in rats may have little or no promoting activity in larger animals and hu-

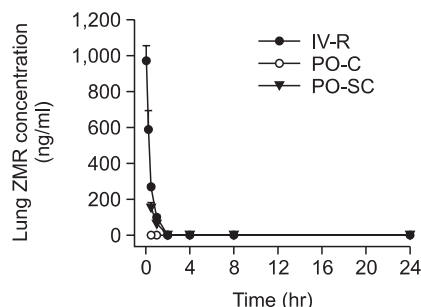


Fig. 9. Mean lung concentration-time profile of ZMR following p.o. or i.v. administration of formulations containing ZMR with or without SC in SD rats. Mean ± S.D. (n=3).

mans (van Hoogdalem *et al.*, 1991b). Thus, further investigations on the safety, BA, and efficacy in larger animals and/or humans are warranted for successful oral delivery of ZMR. Nevertheless, this study provided a critical step towards oral delivery of ZMR for the treatment of influenza by enhancing its oral BA using PE to increase the intestinal permeation of ZMR.

CONCLUSION

The current study demonstrated that the strategy of delivering ZMR orally in combination with SC (sodium caprate, one of the intestinal PEs) to increase the intestinal permeability of ZMR has been successful in significantly enhancing the oral BA of ZMR *in vivo*. Besides, the findings of this study indicated that SC at the tested concentration was able to enhance the BA of ZMR in plasma to an appropriate amount that would make ZMR available in lungs at a concentration higher than the IC₅₀ concentration of influenza virus (>10 ng/ml) to exert its therapeutic effect following oral administration of ZMR with SC. However, efficacy studies in animals and/or humans are warranted to understand the optimal SC concentration to achieve the required plasma/lung ZMR concentrations to produce the necessary therapeutic effect without viral resistance.

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