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Effect of chitosan glutamate, carbomer 974P, and EDTA on the in vitro Caco-2 permeability and oral pharmacokinetic profile of acyclovir in rats

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Abstract

Background: Chitosan glutamate and polyacrylic acid (e.g., carbomer 974P) are known to modulate the tight junctions in the intestinal wall and increase permeability and blood exposure of drugs absorbed orally by the paracellular route. *Aim*: To assess the impact of chitosan glutamate and carbomer 974P on the absorption of paracellularly absorbed model drug, acyclovir, in vitro and in rat in vivo. *Methods*: The influence of chitosan glutamate and carbomer 974P (alone and in combination with EDTA–Na_2) on the *in vitro* Caco-2 permeability and oral pharmacokinetic profile in the rat of acyclovir was investigated. *Results*: In the presence of chitosan glutamate, the apparent permeability of acyclovir across Caco2 monolayer increased 4.1 times relative to control. This increase was accompanied by a significant (~60%) decrease in transepithelial electrical resistance values indicating opening of the tight junctions in the cell monolayer. In rat, chitosan glutamate doubled oral bioavailability of acyclovir and tripled the amount of acyclovir excreted unchanged into urine. In contrast, the effect of carbomer 974P was not statistically significant at 5% level. *Conclusions*: In conclusion, chitosan glutamate (1–3%) and chitosan glutamate (1%)/EDTA–Na_2 (0.01%) are effective excipients to increase permeability of acyclovir across Caco-2 cell monolayers and the oral absorption in the rat in vivo.

Key words: *Acyclovir; carbomer 974P; chitosan glutamate; EDTA; paracellular; renal recovery*

Introduction

Most commercially available drugs are administered via oral route, which is simple, cost-effective, and bears no risk of infection[1,2]. Depending on its physicochemical properties, the orally dosed drug molecule can be absorbed via several mechanisms[3–5]. The majority of drug molecules are unionized, lipophilic ($c \log P > 0$), and relatively large (Mw > 300)[3]. These molecules are absorbed via transcellular pathway—they rapidly partition from the luminal fluids into enterocytes, and subsequently migrate into blood. Some other molecules, however, are small (Mw < 300), hydrophilic ($c \log P < 0$), and often exist as charged species at the intestinal pH. Such molecules cannot easily penetrate lipophilic cellular membranes, and must rely on the paracellular absorption, which is characterized by the diffusion of hydrophilic drug molecules through water-filled tight junctions between the intestinal enterocytes. The efficiency of the paracellular absorption is limited by the low surface density of the tight junctions (<0.01% of the total surface area of the intestinal membrane) and by the fraction of the tight junctions that are open at a given time[5]. Moreover, the tight junctions between the intestinal enterocytes become smaller traveling from the duodenum toward the colon[1]. Overall, these factors lead to the low bioavailability usually reported with hydrophilic drugs that are orally absorbed across the

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paracellular pathway[1]. Not surprisingly, paracellular absorption is a minor drug absorption pathway, which is nevertheless pivotal for the oral absorption of certain drugs such as acyclovir, nadolol, nedocromil, atenolol, dazmegrel, TRH, and sumatriptan[5].

One of the commonly used approaches to improve the intestinal permeability of hydrophilic drugs across the paracellular route is the coadministration of permeability enhancers, such as surfactants, bile salts, calcium chelating agents, fatty acids, medium chain glycerides, steroidal detergents, acyl carnitine, *N*-acetylated α-amino acids, *N*-acetylated non-α-amino acids, cyclodextrins, chitosans, carbomer, and other relevant mucoadhesive polymers[6]. These permeation enhancers can be easily incorporated into traditional oral dosage forms circumventing the need for sophisticated and costlier drug delivery systems. However, many early generation penetration enhancers affected the integrity of the cellular membranes, and caused serious toxic effects, which necessitated the search for safer alternatives. Chitosan and carbomer are examples of novel macromolecular excipients that were reported to increase the paracellular transport of hydrophilic drugs[7]. Chitosan is a hydrophilic, biodegradable polysaccharide comprising copolymers of glucosamine and *N*-acetylglucosamine, which is produced by deacetylation of chitin. It is widely used in cosmetics and exhibits strong mucoadhesive properties[8]. It was shown to increase the paracellular transport of small and large hydrophilic molecules by opening the tight junctions via the interaction between the positively charged amino groups of chitosan molecule with the negatively charged surface proteins associated with the tight junctions[6]. This mechanism is corroborated by the observation that only ionized chitosan (pK_a ~ 6.5) increases the paracellular permeability of hydrophilic molecules[9]. Kotze et al. demonstrated that 0.5–1.5% chitosan chloride solutions in acidic media increased the intestinal permeability of mannitol across Caco-2 cell layer, but lost their efficiency at pH = 7.4[10]. Chitosan is being widely investigated for use as an excipient in oral and other pharmaceutical formulations. Furthermore, chitosan is generally regarded as a nontoxic and nonirritant material with a mouse oral LD_{50} of >16 g/kg[11].

Carbomer, also referred to as polycarbophil and Carbopol, is a polymer of acrylic acid crosslinked with allyl ethers of sucrose or pentaerythritol. As a polyacid, it can extract Ca^{2+} from the intestinal wall and weaken the adhesion between the adjacent enterocytes, which reversibly opens the tight junctions and increases the paracellular permeability of hydrophilic drugs[12–14]. It was also shown to inhibit the activity of the main intestinal enzymes responsible for the breakdown of various proteins, such as trypsin and carboxypeptidaze B[15,16]. In addition, this polymer has a long history of safe and effective use in various pharmaceutical dosage forms. Carbomer has been shown to have extremely low irritancy properties and is nonsensitizing with repeated usage. The use of this polymer is supported by extensive toxicological studies. For example, the LD_{50} of polycarbophil in mouse following oral dosing is equal to 4.6 g/kg (2004).

Overall, the enhancement properties of chitosan and carbomer have been well documented by in vitro (Caco-2) and in situ animal studies. However, there is little documentation regarding their promoting effect of the intestinal permeability in vivo. The results are moreover contradictory and warrant additional investigation.

Acyclovir (9-(2-hydroxyethoxymethyl)guanine) is an example of a paracellularly absorbed drug which has strong antiviral activity against herpes viruses. Its absolute rat and human oral bioavailability are in the range of 10–15% and 15–30%, respectively. This is considered poor and this was attributed to its hydrophilic physicochemical properties and its absorption through the paracellular pathway[5,17]. Furthermore, this low oral bioavailability is usually associated with high intersubject and intrasubject variability and potential development of drug resistance during prolonged usage, which would significantly increase the cost of the therapy. Thus, there have been many attempts to improve the bioavailability of acyclovir such as the development of the amino acid prodrug valacyclovir, which leads to its improved oral bioavailability[18].

The main objective of this study was to evaluate the influence of chitosan glutamate, carbomer 974P, alone and in combination with EDTA–Na_2, on the in vitro Caco-2 permeability and oral rat pharmacokinetic profile of acyclovir.

Materials and methods

Materials

Chitosan glutamate (Protasan G213) was purchased from Novamatrix (a division of FMC Biopolymer, Sandvika, Norway). Carbopol 974P was obtained from Lubrizol Corp. (Cleveland, OH, USA). EDTA–Na_2 salt was provided by Pfizer Materials Management (Groton, CT, USA). Radiolabeled [^{3}H]-acyclovir was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). [^{3}H]-mannitol was purchased from Perkin Elmer (Boston, MA, USA). Acyclovir and mannitol were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals, reagents, and solvents used were of the highest purity grade available.

Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD, USA). Cell culture medium [Dulbecco's Modified Eagles Medium (DMEM)

with 20% fetal bovine serum, 1% nonessential amino acids (NEAA), 1% GlutaMAX-1, and 0.08% gentamicin] and 0.25% trypsin–EDTA were obtained from Invitrogen (Gibco Laboratories, Grand Island, NY, USA). Hank's balanced salt solution (HBSS) plus calcium chloride (0.14 g/L) with 20 mM 2-[*N*-morpholino]ethanesulfonic acid (MES, pH 6.5) and 20 mM *N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonate (HEPES, pH 7.4) were obtained from Invitrogen (Gibco Laboratories, Grand Island, NY, USA). Transwell permeable support 12-well cell culture plates (polycarbonate membrane, 1.12 cm^2 growth area, 0.4 μm pore size) were obtained from Corning Inc. (Corning, NY, USA). Ready Safe liquid scintillation cocktail was obtained from Beckman Coulter (Fullerton, CA, USA). Scintillation counting for samples was performed on Wallac 1409 DSA (Turku, Finland, USA) via Perkin Elmer (Boston, MA, USA). Transepithelial electrical resistance (TEER) was measured using EVOM from World Precision Instruments (Sarasota, FL, USA).

Animals

Male Sprague Dawley rats (weighing 240–350 g) with surgically implanted vascular cannulas in the carotid artery and jugular vein were purchased from Charles River Laboratories (Wilmington, MA, USA). Rats were housed one per cage in an American Animal Association Laboratory Animal Care accredited facility and maintained under standard conditions of temperature (22°C ± 2°C), relative humidity (50%) and light and dark cycle (12/12 hours). Rats were allowed to acclimate to their environment for 1 week. Orally dosed rats were fasted overnight prior to dosing and had access to water ad libitum. On the day of study, food was withheld for 4 hours after administration of the initial dose. Rats that were dosed intravenously had access to food and water ad libitum. All animal experiments were in accordance with the animal care and use committee of Pfizer Inc.

Formulation

Dosing solutions were prepared as follows: For Caco-2 permeability measurements, the formulations were prepared using HBSS as a vehicle. Chitosan glutamate (1% and 3%) solutions were dissolved in the acidified HBSS (pH = 5.5). The resulting solutions were viscous and required homogenization in Thinky AR-200 mixer (Thinky Corp, Tokyo, Japan). Chitosan glutamate (1%)/EDTA–Na_2 (0.01%) solution was prepared by adding EDTA–Na_2 to chitosan glutamate (1%) solution. Carbomer 974P (1%) dissolved readily when added to HBSS but yielded an acidic solution with stirring. Upon adjusting the pH of the solution to 5.5 with sodium hydroxide, the formulation viscosity was increased significantly. Carbomer 974P (1%)/EDTA–Na_2 (0.01%) solution was prepared by adding EDTA–Na_2 to the carbomer 974P (1%) solution.

Oral dosing solutions for the in vivo experiments were prepared by dissolving acyclovir and EDTA–Na_2, where applicable, in a buffer solution at pH 5.5. Next, appropriate amounts of carbomer 974P or chitosan glutamate were added, and the resulting mixtures were homogenized using stirring and Thinky AR-200 mixer. The choice of each buffer was influenced by the dissolution kinetics of the excipients. Carbomer 974P containing formulations were based on 50 mM citrate buffer, while chitosan glutamate containing formulations utilized 10 mM acetate buffer. Being an acid, carbomer 974P necessitated the higher buffer capacity to keep the dosing solution pH close to 5.5. The control formulation did not contain the permeation-enhancing excipients and used aqueous vehicle containing 0.5% methylcellulose and 5% PEG200. Finally, the intravenous (IV) dose was prepared in DMSO:50 mM lactic acid (1:9) vehicle.

Maintenance of cell cultures

Caco-2 cells (passage #24) were cultured at 37°C with cell culture medium in an atmosphere of 10% CO_2 and 90% relative humidity in a NuAire Incubator (Plymouth, MN, USA). The cells were harvested upon reaching approximately 90% confluence using 0.25% trypsin–EDTA. Caco-2 cells were seeded onto polycarbonate membranes with 60,000 cells/well. The individual feeding tray wells received 1.5 mL of cell culture medium. The cell culture medium was changed biweekly and the confluent monolayers were used for experimentation 21 days post seeding.

Caco-2 permeability studies

Transport studies were initiated by removing the cell culture medium from the apical (AP) and basolateral (BL) sides of the cell monolayers. Cell monolayers were washed once with HBSS (37°C), which was then replaced with fresh transport buffer and equilibrated for 30 minutes. HBSS containing MES (adjusted pH to 5.5 with HCl) and HBSS containing HEPES (pH 7.4) were applied on the AP and basal (BL) compartments, respectively. Volume of AP and BL compartments in all the experiments were 0.5 and 1.5 mL, respectively. Permeation studies were carried out at 37°C with continuous agitation (60 rpm). Absorptive (AP-to-BL) transepithelial transport ($P_{app,A\rightarrow B}$) of [^{3}H]-acyclovir (10 μM; specific activity 15 Ci/mmol) and [^{3}H]-mannitol (10 μM; specific activity 13.2 Ci/mmol) were measured across Caco-2 cell monolayers in the absence and presence of chitosan glutamate (1%), chitosan glutamate (3%), chitosan

glutamate (1%)/EDTA–Na_2 (0.01%), carbomer 974P (1%), and carbomer 974P (1%)/EDTA–Na_2 (0.01%). Samples (0.2 mL) were withdrawn from receiver compartments at time intervals of 1.0, 2.0, and 3.0 hours and replaced with equal volume of fresh pre-warmed transport buffer. The drug samples were analyzed by liquid scintillation counting. All the permeation experiments were carried out in triplicate. TEER was measured across the cell membranes at the end of experiments to indicate the integrity of the Caco-2 monolayers.

Absorptive (AP-to-BL) transepithelial transport was represented as permeability value ($P_{app,A\rightarrow B} \times 10^{-6}$ cm/sec) calculated using the following equation:

$$P_{app} = \frac{1}{\text{area} \times C_D(0)} \times \frac{dM_r}{dt}, \quad (1)$$

where area is the surface area of the cell monolayer (1.12 cm^2), $C_D(0)$ the initial concentration of compound applied to the donor chamber, t the time, M_r the mass of compound in the receiver compartment, and dM_r/dt the flux of the compound across the cell monolayer.

Samples from the donor compartment were withdrawn at the end of permeation studies and analyzed to calculate the mass balance [Equation (2)].

$$\%\text{Recovery} = \left(\frac{M_R + M_D + M_S}{M_{Di}} \right) \times 100, \quad (2)$$

where M_R is the amount of compound in the receiver compartment after 3 hours, M_D the amount of compound in the donor compartment after 3 hours, M_{Di} the initial amount of compound applied to the donor compartment, and M_s the sum of amount of compound in the samples withdrawn at 1, 2, and 3 hours. Recovery of both acyclovir and mannitol were found to be between 70% and 110%.

Measurement of transepithelial electrical resistance

TEER was measured using the EVOM voltohmmeter to ensure the integrity of the monolayers formed. TEER values were measured across each cell monolayer prior to beginning the experimentation and at the last sample collection time point. The TEER ($\Omega\ cm^2$) values of the cell monolayers were calculated according to the following equation:

$$\begin{aligned}\text{Resistance of a unit area} = {} & \text{resistance } (\Omega) \\ & \times \text{effective membrane} \\ & \text{area } (cm^2). \end{aligned} \quad (3)$$

Data are represented as percentage of control.

In vivo pharmacokinetic studies

The pharmacokinetic profile of acyclovir following IV and oral administration was evaluated in Sprague Dawley rats (240–350 g). The animals were randomly distributed into seven experimental groups (n = 3). Orally dosed animals were fasted overnight prior to drug administration with free access to water, while intravenous dosed animals had access to food and water ad libitum. The oral treatment groups were dosed with one of the six formulations [chitosan glutamate (1%), chitosan glutamate (3%), chitosan glutamate (1%)/EDTA–Na_2 (0.01%), carbomer 974P (1%), carbomer 974P (1%)/EDTA–Na_2 EDTA (0.01%), or vehicle, 2.5 mg/kg], the seventh group was dosed by injection into the jugular vein as a 1-minute infusion (2.5 mg/kg). Blood samples (0.25 mL) were withdrawn from the carotid artery predose, and at the following time points after dosing 0.017 (only following IV dosing), 0.25, 0.5, 1, 2, 4, 6, 8, 12, and 20 hours for acyclovir analysis. After each blood draw, a volume of sterile normal saline (0.9% sodium chloride, USP containing 100 unit/mL heparin) equivalent to the volume of blood drawn was injected via carotid artery cannula to maintain a constant blood volume (0.25 mL). All blood samples were immediately centrifuged at 3000 rpm for 10 minutes. The resultant plasma was stored at –70°C until the acyclovir Liquid Chromatography-Mass Spectrometry (LC/MS/MS) analysis.

During the experiment, the animals were housed in metabolic cages in order to collect urine (0–20 hours). Following centrifugation to remove particulate matter, aliquots of the urine samples were injected directly onto the same LC/MS/MS as described below for the plasma extract analysis. In the literature, acyclovir is mainly eliminated in the form of unchanged drug in the urine of animals. Thus, renal recovery following oral administration could be used as another measure of drug oral bioavailability[19].

LC/MS/MS analysis of acyclovir

Equipment

LC/MS/MS analysis was carried out for acyclovir using a high-performance liquid chromatography (HPLC) system consisting of a Shimadzu binary pump (Shimadzu Scientific Instruments, Columbia, MD, USA) with CTC PAL autosampler (Leap Technologies, Carrboro, NC, USA) interfaced to an API 4000 LC/MS/MS quadrupole tandem mass spectrometer (Applied Biosystems/ MDS Sciex Inc., Ontario, Canada).

Sample preparation

A 50 µL aliquot of urine or plasma sample (including dilutions) was precipitated with 200 µL of acetonitrile containing internal standard using a VWR Multi-Tube Vortexer (VWR, Bridgeport, NJ, USA). Samples were then centrifuged at 3000 rpm with a standard laboratory

centrifuge (Eppendorf, Westbury, NY, USA). The supernatant was collected in a clean 96-well collection plate. The samples were then dried using N_2 and reconstituted with mobile phase. The plate was then vortexed for 15 seconds and 5 µL was injected onto the HPLC for LC/MS/MS analysis.

Acyclovir and one internal standard (PF-04273681) were separated on a Phenomenex Luna HILI Column (3×100 mm^2, 3 µm) by gradient elution. PF-04273681 was the internal standard used for the in vivo work performed. The mobile phase consisted of solvent A (5 mM ammonium formate in water, 0.1% formic acid) and solvent B (acetonitrile). The gradient was as follows: solvent B was held at 70% for 0.5 minute, linearly ramped from 70% to 45% in 2.5 minutes, and then immediately brought back to 70% for re-equilibration for 0.5 minute. A flow rate of 0.5 mL/min was used. The mass spectrometer was operated in positive ion ESI mode for the detection of acyclovir and the internal standard (PF-04273681). Multiple reaction monitoring was performed with the transitions m/z 226.1 → 151.9 for acyclovir and m/z 199 → 84.3 for PF-04273681 (internal standard). All raw data were processed using Analyst Software V. 1.4.2 (Applied Biosystems/MDS Sciex Inc., Ontario, Canada).

Pharmacokinetic analysis of data

All pharmacokinetic parameters were calculated using WinNonlin v. 4.1 (Pharsight, Mountain View, CA, USA). The peak plasma concentration (C_{max}) and the time of maximum concentration (t_{max}) were recorded directly from individual plasma concentration–time profiles. The terminal half-life ($t_{½}$) was calculated as Ln(2) divided by the absolute value of the slope of the terminal log-linear phase. Area under the plasma concentration–time curve (AUC) and area under the moment curve (AUMC) were calculated using the linear trapezoidal method[20]. Systemic plasma clearance (CL) was calculated as Dose/AUC, while the volume of distribution at steady state (V_{dss}) was calculated as CL × mean residence time (MRT). MRT was defined as AUMC/AUC. Absolute oral bioavailability (F) was calculated using the following equation[21]:

$$F = \frac{AUC_{PO}}{AUC_{IV}} \times \frac{Dose_{IV}}{Dose_{PO}}. \quad (4)$$

The renal recovery (Xu%) of acyclovir was estimated by dividing the total amount of acyclovir excreted unchanged into the urine during the study period by the dose administered following IV or oral dosing.

Statistical analysis

Statistical comparisons were performed by the one-way analysis of variance (ANOVA). When significant differences were found the multiple comparison Tukey's test was applied. Statistical significance was accepted for $P < 0.05$[22].

Results

The effect of absorption enhancers on Caco-2 permeability

The TEER values and mean Caco-2 permeability of acyclovir and comparative paracellular control mannitol in the absence and presence of absorption enhancers are summarized in Table 1. The $P_{app,A\to B}$ of acyclovir across Caco-2 cell monolayers under control conditions was $2.84 \pm 0.8 \times 10^{-6}$ cm/sec. The presence of chitosan glutamate (1%) increased $P_{app,A\to B}$ of acyclovir by 4.1-fold relative to the control ($P < 0.05$). Chitosan glutamate (1%)/EDTA–Na_2 (0.01%) and chitosan glutamate (3%) increased acyclovir $P_{app,A\to B}$ by 4.6- and 3.4-folds, respectively, relative to control ($P < 0.05$), and the obtained results with these absorption enhancers were not significantly different from the values obtained with chitosan glutamate (1%) ($P > 0.05$). Carbomer 974P (1%) and carbomer 974P (1%)/ EDTA–Na_2 (0.01%) had limited effect on the $P_{app,A\to B}$ of acyclovir, with

Table 1. The effect of absorption enhancers on the Caco-2 permeability of acyclovir and mannitol.

	Acyclovir		Mannitol	
Formulation	$P_{app,A\to B}$ ($\times 10^{-6}$ cm/sec)	TEER (Ω cm^2)	$P_{app,A\to B}$ ($\times 10^{-6}$ cm/sec)	TEER (Ω cm^2)
Control	2.84 ± 0.55	405 ± 35	2.53 ± 0.18	444 ± 3
Chitosan glutamate (1%)	11.6 ± 0.37*	149 ± 7*	8.43 ± 0.29*	157 ± 2*
Chitosan glutamate (1%)/EDTA–Na_2 (0.01%)	13.1 ± 0.91*	155 ± 11*	9.65 ± 1.02*	153 ± 8*
Chitosan glutamate (3%)	9.73 ± 1.21*	177 ± 28*	11.3 ± 1.82*	160 ± 9*
Carbomer 974P (1%)	3.52 ± 0.51	440 ± 45	2.59 ± 0.26	539 ± 69
Carbomer 974P (1%)/EDTA–Na_2 (0.01%)	4.40 ± 1.69	493 ± 65	3.19 ± 0.85	504 ± 22

Data were presented as mean ± SD (*$P < 0.05$ compared with control).

increased $P_{app,A\rightarrow B}$ of acyclovir by 1.2- and 1.5-folds, respectively, relative to control ($P > 0.05$).

Mannitol was tested as the comparative paracellular control for low permeability and exhibited similar trend to that obtained with acyclovir in the presence and absence of absorption enhancers. For example, the $P_{app,A\rightarrow B}$ of mannitol across Caco-2 cell monolayers under control conditions was $2.53 \pm 0.2 \times 10^{-6}$ cm/sec. The presence of chitosan glutamate (1%) increased $P_{app,A\rightarrow B}$ of mannitol by 3.3-fold relative to the control ($P < 0.05$), while chitosan glutamate (1%)/EDTA–Na_2 (0.01%) and chitosan glutamate (3%) increased acyclovir $P_{app,A\rightarrow B}$ by 3.8- and 4.5-folds, respectively ($P < 0.05$). Overall, the impact of these absorption enhancers on the $P_{app,A\rightarrow B}$ of mannitol was not significantly different compared to each other ($P > 0.05$). Carbomer 974P (1%) and carbomer 974P (1%)/ EDTA–Na_2 (0.01%) had limited effect on the $P_{app,A\rightarrow B}$ of mannitol, with increased $P_{app,A\rightarrow B}$ of mannitol by 1.0- and 1.3-folds, respectively, relative to control ($P > 0.05$).

The TEER values in Caco-2 cell monolayers were recorded prior to conducting the permeation studies and following the last sample collection time point at 3 hours (Table 1). The TEER values of control monolayers (i.e., without absorption enhancer) suggested integrity of tight junctions. However, the TEER values across the Caco-2 monolayers showed a significant reduction by over 2-fold in the presence of chitosan glutamate and its combination with EDTA–Na_2, compared to the control (TEER 444 ± 9 Ω cm^2), suggesting the opening of tight junctions (Table 1) ($P < 0.05$). Carbomer 974P (1%) and its combination with EDTA–Na_2 (0.01%) did not change the TEER values across Caco-2 monolayers ($P > 0.05$).

Pharmacokinetic profile of acyclovir in rats

The mean plasma concentration–time profiles of acyclovir following IV and oral dosing to rats are shown in Figure 1, and the corresponding mean pharmacokinetic parameters are summarized in Table 2. The plasma concentrations of acyclovir in rats decreased in a biphasic manner after the 2.5 mg/kg IV administration. The mean systemic plasma clearance of acyclovir was 44 mL/min/kg. Acyclovir had a moderate apparent volume of distribution at steady state (V_{dss}) of 1.2 L/kg, which was larger than that of the total body water of 0.67 L/kg[22,23]. The percentage of acyclovir excreted unchanged in urine (Xu%) was 79% of the total IV dose. This suggests that renal excretion is the major route of elimination of acyclovir in rats.

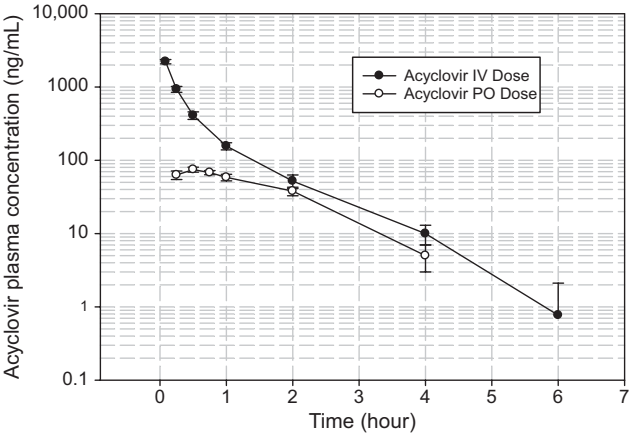

Figure 1. Plasma acyclovir concentration–time profiles after intravenous and oral administration (2.5 mg/kg) of acyclovir to Sprague Dawley rats. Acyclovir concentrations in plasma were determined by LC/MS analysis as described under section 'Materials and methods.' Plasma concentration of acyclovir was below the lower limit of quantitation (LOQ) of LC/MS assay after 4 and 6 hours following oral (PO) and IV dosing, respectively. Each point represents the mean ± SD ($n = 3$).

In the absence of intestinal permeation enhancers, acyclovir was not well absorbed following oral dosing and reached a peak plasma concentration (C_{max}) of 129 ng/mL at 0.67 hour (T_{max}) postdose. The mean

Table 2. Pharmacokinetic parameters of acyclovir determined by noncompartmental analysis.

Formulation parameters	IV ($n = 3$)	PO, control ($n = 3$)	PO, chitosan (1%) ($n = 3$)	PO, chitosan (3%) ($n = 3$)	PO, chitosan (1%)/EDTA (0.01%) ($n = 3$)	PO, carbomer (1%) ($n = 3$)	PO, carbomer (1%)/EDTA (0.01%) ($n = 3$)
AUC (ng*hours/mL)	910 ± 73	129 ± 41	202 ± 47*	261 ± 40**	216 ± 55*	158 ± 37	175 ± 28
CL (mL/min/kg)	44 ± 4	—	—	—	—	—	—
V_{dss} (L/kg)	1.2 ± 0.1	—	—	—	—	—	—
T_{max} (hours)	—	0.67 ± 0.29	0.75 ± 0.43	1.25 ± 0.66	0.42 ± 0.14	0.51 ± 0.42	0.73 ± 0.41
C_{max} (ng/mL)	—	75.6 ± 8.7	88.5 ± 18.7	82.8 ± 34.3	69.8 ± 23.9	88.8 ± 18.9	80 ± 25
$t_{½}$ (hours)	0.5 ± 0.1	1.4 ± 0.4	1.9 ± 0.7	1.1 ± 0.2	1.6 ± 0.5	1.7 ± 0.4	1.6 ± 0.3
F (%)	—	14 ± 5	22 ± 5*	29 ± 4**	24 ± 6*	17 ± 4	19 ± 3
Xu% (mg/kg)	79 ± 5	10 ± 1	16 ± 2*	30 ± 2**	14 ± 2	13 ± 1	11 ± 1

The pharmacokinetic parameters were obtained by noncompartmental analysis using WinNonlin. Data were presented as mean ± SD (*$P < 0.05$, **$P < 0.01$ compared with PO control).

acyclovir Xu% following oral dosing was 10%. The mean absolute oral bioavailability of acyclovir from an oral solution was 14%, indicating low oral bioavailability. After acyclovir oral dosing, the terminal apparent $t_{1/2}$ was 1.4 hour and longer than that reported following IV dosing ($t_{1/2}$ = 0.52 hour). This may suggest that oral acyclovir followed 'flip-flop' kinetics, with absorption being much slower than elimination of the drug from the body. Overall, these findings are in accordance with that reported by other research groups[18,24,25].

Effect of chitosan glutamate (1–3%) and chitosan glutamate (1%)/EDTA (0.01%) formulations on the oral pharmacokinetic profile of acyclovir in rats

The effect of chitosan glutamate (1–3%) and chitosan glutamate (1%)/EDTA–Na_2 (0.01%) formulations on the oral pharmacokinetic profile of acyclovir is shown in Figure 2 and summarized in Table 2. Chitosan glutamate (1–3%) and chitosan glutamate (1%)/EDTA–Na_2 (0.01%) formulations had a significant impact on the pharmacokinetic parameters of acyclovir ($P < 0.05$). Chitosan glutamate (3%) resulted in a statistically significant 2-fold increase in the acyclovir oral AUC ($P < 0.01$) relative to vehicle-treated rats (control). Chitosan glutamate (1%) and chitosan glutamate (1%)/EDTA–Na_2 (0.01%) formulations increased acyclovir oral AUC by 1.6- and 1.7-folds, respectively relative to control ($P < 0.05$).

In addition to providing the highest increase in the oral AUC of acyclovir, chitosan glutamate (3%) provided the highest increase in acyclovir Xu% relative to control (3.1-fold) ($P < 0.01$). Chitosan glutamate (1%) and chitosan glutamate (1%)/EDTA–Na_2 (0.01%) increased acyclovir Xu% by 1.6- and 1.5-folds, respectively, relative to control ($P < 0.05$). Overall, chitosan glutamate (1–3%) and chitosan glutamate (1%)/EDTA–Na_2 (0.01%) formulations did not have a significant impact on acyclovir $t_{1/2}$, T_{max}, and C_{max} relative to control ($P > 0.05$).

Effect of carbomer 974P (1%) and carbomer 974P (1%)/EDTA–Na_2 (0.01%) formulations on the oral pharmacokinetic profile of acyclovir in rats

The effect of carbomer 974P (1%) and carbomer 974P (1%)/EDTA–Na_2 (0.01%) formulations on the oral pharmacokinetic profile of acyclovir is shown in Figure 3 and summarized in Table 2. Carbomer 974P (1%) and carbomer 974P (1%)/EDTA–Na_2 (0.01%) formulations had a small impact on the pharmacokinetic parameters of acyclovir. Carbomer 974P (1%) and carbomer 974P (1%)/EDTA–Na_2 (0.01%) increased acyclovir oral AUC by 1.2- and 1.4-folds, respectively, relative to control but this did not reach statistical significance ($P > 0.05$).

Carbomer 974P (1%) and carbomer 974P (1%)/EDTA–Na_2 (0.01%) formulations increased acyclovir Xu% by 1.3- and 1.1-folds, respectively, relative to control ($P > 0.05$). Furthermore, carbomer 974P (1%) and carbomer 974P (1%)/EDTA–Na_2 (0.01%) formulations did not have a significant impact on acyclovir $t_{1/2}$, T_{max}, and C_{max} relative to control ($P > 0.05$).

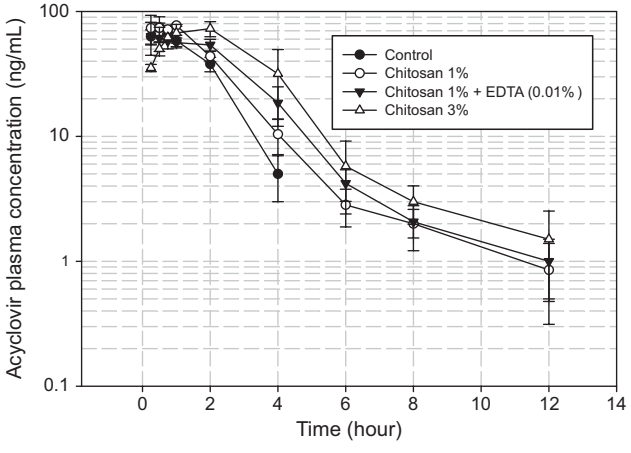

Figure 2. Plasma acyclovir concentration–time profiles after oral administration of 2.5 mg/kg of acyclovir to Sprague Dawley rats. Acyclovir concentrations in plasma were determined by LC/MS/MS analysis as described under section 'Materials and methods.' Plasma concentration of acyclovir was below the LOQ of LC/MS assay after 4 and 12 hours following control and treated formulations, respectively. Each point represents the mean ± SD ($n = 3$).

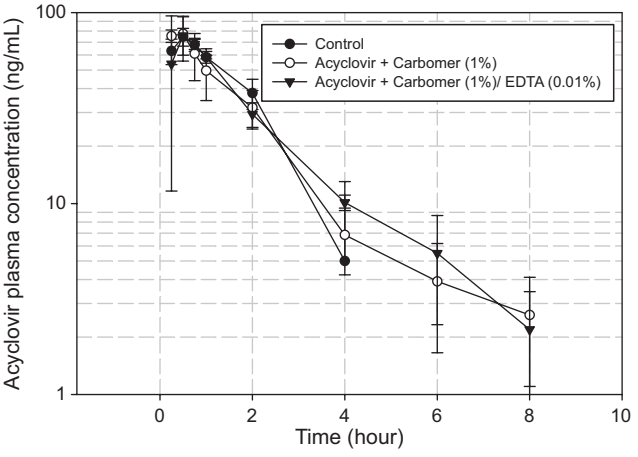

Figure 3. Plasma acyclovir concentration time profiles after oral administration of 2.5 mg/kg of acyclovir to Sprague Dawley rats. Acyclovir concentrations in plasma were determined by LC/MS/MS analysis as described under section 'Materials and methods.' Plasma concentration of acyclovir was below the LOQ of LC/MS assay after 4 and 8 hours following control and treated formulations, respectively. Each point represents the mean ± SD ($n = 3$).

Discussion

The objective of this work was to evaluate the effect of novel macromolecular excipients on paracellular absorption and bioavailability of acyclovir in vitro and in vivo. Acyclovir is an excellent model for paracellularly absorbed drugs and possesses good aqueous solubility (2.5 mg/mL at 37°C), low lipophilicity (log P = –0.73), small molecular weight (Mw = 225), and is ionized at physiological pHs (pK_{a1} = 2.27, pK_{a2} = 9.25)[26]. Moreover, acyclovir is well tolerated by animals, minimally metabolized and once orally absorbed, it is mainly eliminated by renal excretion[5]. Therefore, any alteration in the amount excreted in the urine (as measured by changes in the renal recovery 'Xu%') and AUC, following oral dosing of acyclovir with absorption permeability enhancers was used to determine the impact of these enhancers on acyclovir oral absorption[27].

The intestinal permeation enhancers selected for this study were chitosan glutamate, carbomer 974P, and EDTA–Na_2. Chitosan salts, such as glutamate, combine the permeation enhancing effect with good solubility. Chitosan glutamate is a polymer with a good aqueous solubility and safety profile[11]. According to the literature data, tight junction opening by chitosan saturates in vitro at 0.4%[28]. To compensate for the salt form and to evaluate the saturation effect in vivo, the formulations for this study contained 1% and 3% chitosan glutamate (w/v). Carbomer 974P is the second generation of carbomer 934P with a safer residual solvent profile (Lubrizol product information). It was only dosed at 1% level because the formulations with higher concentrations were too viscous for oral dosing. EDTA–Na_2 was used as traditional permeation enhancer for comparison. EDTA–Na_2 chelates Ca^{2+} ions from the intestinal cells, which leads to loosening and opening of the tight junctions of the intestinal enterocytes[10]. While efficient, EDTA–Na_2 exhibits serious toxic effects, which limits its applications. In our experiments, the concentration of EDTA–Na_2 was set at a low concentration of 0.01%, so as to minimize its potential toxic effect[29].

The pH of all the formulations used was kept at 5.5 to ensure that the amino groups of chitosan are protonated, thereby enhancing the intestinal permeation activity. All other formulation pHs were kept low to ensure consistency in experimentation.

In Caco-2 experiments, acyclovir demonstrated poor $P_{app,A\rightarrow B}$, which indicates poor intestinal permeability consistent with the hydrophilic nature of the molecule. The limited permeability likely explains the poor oral AUC, F, low Xu%, and is in agreement with the literature data stating that acyclovir has permeability limited absorption (BCS class III)[24,25].

$P_{app,A\rightarrow B}$ of acyclovir across Caco-2 monolayer was significantly increased in the presence of chitosan glutamate. This increase was paralleled by the decrease in TEER values indicative of opening of the tight junctions. Furthermore, the standard probe for the paracellular absorption, mannitol, exhibited a permeability profile similar to acyclovir. This collected data establishes that acyclovir is absorbed via the paracellular pathway, and that chitosan glutamate enhances the opening of the tight junction thus increasing its permeability in vitro. EDTA–Na_2 coadministered with chitosan glutamate failed to offer statistically significant improvement over chitosan glutamate alone.

Overall, the Caco-2 in vitro results suggest that the increase in paracellular permeability of acyclovir in the presence of an absorption permeability enhancer, such as chitosan glutamate, would lead to higher oral exposure in vivo exemplified by higher AUC, F, and Xu% values.

In vivo experiments were carried out in the rat, which is a good model for human paracellular bioavailability. He et al. demonstrated a good correlation between the bioavailability of a few hydrophilic substances (including acyclovir), which are predominantly excreted unchanged following IV administration in humans and rats[5].

Chitosan glutamate and its combination with EDTA–Na_2 had a significant impact on the oral exposure of acyclovir in rats ($P < 0.05$) (Figure 3). For example, chitosan glutamate (1–3%) and chitosan glutamate (1%)/EDTA–Na_2 (0.01%) formulations led to 1.7- to 2-fold increase in AUC and F of acyclovir and 1.5- to 3.1-fold increase in Xu% of acyclovir compared to control ($P < 0.05$). It is interesting to observe a good correlation between the AUC and F of acyclovir and the chitosan concentration (1%–3%) in the oral formulation (r^2 = 0.949 and 0.982 for AUC and Xu% versus chitosan concentration, respectively) (Figure 4). These in vivo findings were different

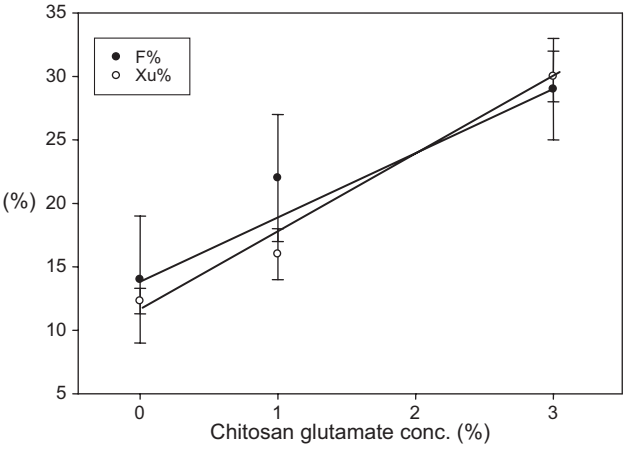

Figure 4. The relationship between chitosan glutamate concentration and acyclovir oral bioavailability (F%) and renal recovery (Xu%) following 2.5 mg/kg PO dosing of acyclovir to Sprague Dawley rats using 0%, 1%, and 3% chitosan glutamate as permeation enhancer. Each point represents the mean ± SD (n = 3).

from the in vitro data reported in the literature that suggested saturation of the chitosan effect on the intestinal permeability of paracellular absorbed drugs at chitosan levels of 0.4–1%. Overall, the in vivo findings disagree with the concern that chitosan will not be ionized and effective at intestinal pH. This is attributed to the fact that the pH in vicinity of the intestinal wall is around 6 and lower than the bulk pH of 6.5 and the pK_a of chitosan (6.5)[30]. Furthermore, even at a pH value of 6.5, a fraction of the chitosan amino groups will still be protonated and effective in reversibly perturbing the intestinal membrane and increase the permeability of paracellularly absorbed drugs. It should be emphasized that instead of the modest changes in C_{max} and T_{max} values of acyclovir in the presence of chitosan alone and in combination with EDTA–Na_2, the enhancers seemed to extend period of absorption and sustain plasma concentrations of acyclovir close to C_{max} up to 2–4 hours post oral dosing. This resulted in the significant increase in the AUC values of acyclovir in the presence of these enhancers.

Carbomer 974P (1%) and carbomer 974P (1%)/EDTA–Na_2 (0.01%) did not result in significant increase in the AUC, F, and Xu% of acyclovir relative to control ($P > 0.05$). In vitro, carbomer 974P (1%) and carbomer 974P (1%)/EDTA–Na_2 (0.01%) did not significantly increase the permeability of acyclovir across Caco-2 monolayer (1.2- and 1.5-folds, respectively, relative to control) ($P > 0.05$). Carbomer 974P did not decrease the TEER values, which is consistent with the literature.

It is interesting to note the smaller increase in the AUC, F, and Xu% following the peroral dosing of acyclovir as compared to the permeability values of acyclovir obtained across Caco-2 cell-line in vitro in the absence/presence of various absorption permeation enhancers studied. This phenomenon can be attributed to many reasons. For example, the volume of the fluid present in the gastrointestinal tract, the gastric emptying, and intestinal motility may dilute the absorption permeation enhancer and spread it over a large surface area. This may lead to a shorter residence time of both the drug and absorption permeation enhancer at any particular site. In addition, the presence of mucus layer barrier on the intestinal wall may act as a barrier to drug diffusion, which may further limit the usefulness of the absorption permeation enhancer[6]. Overall, it is well established in the literature that lower concentration of absorption enhancer is needed to reversibly perturb the intestinal barrier and increase drug permeation across Caco-2 cell line as compared to the intestinal enterocyte in vivo[6,10].

Conclusions

Acyclovir has poor oral bioavailability that is attributed to its hydrophilic nature and absorption through paracellular pathway. In this study, chitosan glutamate had an impact on its oral exposure. Chitosan glutamate was found to be an efficient enhancer of paracellular absorption both in vitro and in vivo. The enhancement demonstrated by carbomer 974P was not statistically significant.

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