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Research paper

The effect of chitosan on the bioaccessibility and intestinal permeability of acyclovir

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ABSTRACT

Chitosan is object of pharmaceutical research as a candidate permeability enhancer. However, chitosan was recently shown to reduce the oral bioavailability of acyclovir in humans. The effect of chitosan on two processes determining the oral bioavailability of acyclovir, bioaccessibility and intestinal absorption, was now investigated. Acyclovir's bioaccessibility was studied using the dynamic TNO gastro-Intestinal Model (TIM-1). Four epithelial models were used for permeability experiments: a Caco-2 cell model in absence and presence of mucus and both rat and porcine excised intestinal segments. Study concentrations of acyclovir (0.8 g/l) and chitosan (1.6 g/l and 4 g/l) were in line with those used in the aforementioned human study. No effect of chitosan was measured on the bioaccessibility of acyclovir in the TIM-1 system. The results obtained with the Caco-2 models were not in line with the *in vivo* data. The tissue segment models (rat and porcine intestine) showed a negative trend of acyclovir's permeation in presence of chitosan. The Ussing type chamber showed to be the most biopredictive, as it did point to an overall statistically significantly reduced absorption of acyclovir. This model thus seems most appropriate for pharmaceutical development purposes, in particular when interactions between excipients and drugs are to become addressed.

1. Introduction

Chitosan is an unbranched binary heteropolysaccharide consisting of the two units N-acetyl-D-glucosamine and D-glucosamine, obtained by partial deacetylation of the natural product chitin [1–3]. Besides its employment as a conventional excipient (e.g. filler, disintegrant, release modifier), chitosan has been tested as a candidate drug bioavailability modulator. Several studies has showed that chitosan is able to enhance the permeability of diverse low permeability compounds, such as acyclovir, or other hydrophilic markers mannitol and fluorescein isothiocyanate dextran 4000 [4–8]. Particularly, chitosan salts increased the *in vitro* permeability of acyclovir across Caco-2 monolayers and the oral absorption of acyclovir in the rat at concentrations varying from 0.1% to 3% [9,10]. However, the data from those *in vitro* and animal experiments were not confirmed by human data tested at identical dose levels.

The mechanism for permeation enhancement is postulated to occur through an interaction of the positively charged chitosan molecules and negative charges in the cavity of the epithelial tight junctions resulting in opening of these tight junctions [8,11,12]. However, the presence of different anions along the gastrointestinal tract might change in the *in vivo* situation. For instance, both polyanionic macromolecules, heparin and mucin, has demonstrated to interact with chitosan decreasing its absorption enhancement effect [13,14]. Furthermore, luminal contents as bile salts may also provide an additional potential source of interactions [11,15].

A recent study in healthy human volunteers performed by the authors showed how chitosan hydrochloride actually reduced the

Abbreviations: AUC, area under the curve; C_{max}, maximum concentration; DD, degree of deacetylation; FD4, fluorescein isothiocyanate-dextran; GI, gastrointestinal; MW, molecular weight; P_{app}, apparent permeability; PBPK, physiologically-based pharmacokinetic; Rf, transsegmental electrical resistance; TEER, transepithelial electrical resistance; T_{max}, time at which C_{max} is reached

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bioavailability of 200 mg acyclovir when orally administered at a concentration of 0.16% or 0.4% (1.6 g/l or 4 g/l; given as 400 mg and 1000 mg chitosan hydrochloride respectively in 250 ml water) [16]. Although apparent inconsistent results emerge from the human and preclinical studies, data are often difficult to compare as the characteristics of the applied chitosan (molecular weight, degree of deacetylation (DD), salt form), as well as the test compounds and dose levels differ between studies. In the current work, we used five different *in vitro* methods to study effect of chitosan on acyclovir absorption. As a highlight, all experiments were performed with chitosan of the same or very similar quality at identical dose levels as applied in the human study. These studies improve the understanding of chitosan's effects on two kinetic processes underlying the oral bioavailability of the high solubility and low permeability model drug acyclovir (BCS class III, [17]). In addition, this research allows evaluation of the different intestinal permeability models for pharmaceutical development or biowaiver purposes.

2. Materials and methods

2.1. Materials

Chitosan hydrochloride was obtained from Heppe Medical GmbH, Halle, Germany. The following characteristics applied: DD 92.7%; viscosity 4–5 mPa s at 1% in water at 20 °C for TIM-1 studies and Ussing type rat model and DD 93.05%, viscosity 5.9 mPa s at 1% in water at 20 °C for Caco-2 and the inTESTine. Zovirax 200 mg dispersible tablets (GlaxoSmithKline, UK) were purchased in the Netherlands. Acyclovir was obtained from Fagron (Fagron, The Netherlands). For the Caco-2 studies, Hanks' balanced salt solution (HBSS), Dulbecco's modified Eagle's medium (DMEM), 10,000 IU/ml penicillin and 10,000 µg/ml streptomycin, nonessential amino acid medium (100×) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were obtained from Lonza (Verviers, Belgium). Fetal bovine serum (FBS) was purchased from Biological Industries (Beit Haemek, Israel). 2-(N-morpholino)ethanesulfonic acid (MES) was obtained from Sigma–Aldrich (St. Louis, MO, United States). For rat ligated loop studies, ketamin (Ketavet, Pfizer, Germany), and xylazin (Rompun, Bayer, Germany) were obtained via the Pharmacy of the Medical Center of the Johannes Gutenberg University, Mainz, Germany. For the InTESTine study, ^{14}C-Antipyrine (55 mCi/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, Missouri, United States). ^{3}H-Atenolol (1.79 Ci/mmol), and ^{14}C-acyclovir (440 mCi/mmol) were purchased from Moravek biochemicals Inc. (Brea, California, United States). All other chemicals were purchased at Sigma-Aldrich, Schnelldorf, German.

2.2. TIM-1 model

The *in vitro* dynamic TIM-1 system (TNO Triskelion, The Netherlands) consists of one gastric compartment and three intestinal compartments (duodenum, jejunum and ileum) connected with valves simulating the gastric and small intestinal passage of food and pharmaceutical products. The TIM-1 systems, previously well described [18–20], have a simulated pyloric sphincter for controlled gastric emptying of liquids and solids (particles less than 3–5 mm) with specific settings for fasted and fed conditions. The conditions in the compartments are computer-controlled via pH electrodes, temperature and pressure sensors. The secretions into the gastric compartment consist of artificial saliva with electrolytes, α-amylase and gastric juice with hydrochloric acid, pepsin and lipase. In the small-intestinal compartments, the secretion fluids consist of bicarbonate, electrolytes, pancreatic juice with digestive enzymes, and bile.

The TIM-1 studies were performed with acyclovir (0.8 g/l) in absence or presence of chitosan (1.6 and 4.0 g/l) to provide information on the effect of chitosan on the bioaccessibility (i.e. availability for absorption) of acyclovir during gastrointestinal passage. Preparation of the dispersions containing Zovirax tablets in presence and absence of the two levels of chitosan took place in line with the instructions applied during the human study [16]: a total volume of 250 ml water was introduced in the gastric compartment. The model conditions simulated the fasted state including gastric pH profile, enzyme levels, gastric emptying etc. A gastric emptying half-time of 20 min (default fasted state) was used for the three conditions (acyclovir in absence of chitosan, in presence of 1.6 g/l or in presence of 4.0 g/l chitosan). The jejunum and ileum compartments were connected with dialysis membranes (cut-off 5 kDa) to remove the released and water-dissolved compounds. Jejunum and ileum dialysate samples were collected every 30 min during the first three hours and every 60 min during the next 2 h till a total of five hours. The amount of acyclovir in these dialysate samples was considered as the fraction available for absorption from the upper gastrointestinal tract, i.e. the bioaccessible amount, within a given time period. In addition, ileum effluent was sampled every hour. These ileum effluent samples provide information on the non-bioaccessible fraction during transit through the upper GI tract, and which will enter the colon. After five hours, the experiments were ended and the residues were collected to be able to calculate the mass balance of acyclovir in each individual TIM-1 experiment. All samples were stored at or below −18 °C until analysis.

2.3. Caco-2 cell permeation studies (n = 3 wells)

Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA) and were grown in DMEM^{+} at 37 °C in an atmosphere of 5% CO_2 and 90% relative humidity. Cells were passaged every 3–4 days (at 80–90% confluence) at a split ratio of 1:6. For transport experiments, cells were seeded at a density of 90,000 cells/cm^2 on Costar Transwell membrane inserts (3 µm pore diameter, 12 mm diameter; Corning Inc., Corning, NY, United States) and were used for experiments 17–18 days after seeding. Only monolayers with transepithelial electrical resistance (TEER) values higher than 400 Ω * cm^2 were used for transport studies.

Caco-2 cell culture medium consisted of DMEM supplemented with 10% FBS, 1% nonessential amino acids, 100 IU/ml penicillin and 100 µg/ml streptomycin (DMEM^{+}).

Transport medium consisted of HBSS containing 25 mM glucose and was buffered with 10 mM MES to pH 6.0 (donor compartment) or with 10 mM HEPES to pH 7.4 (acceptor compartment).

Three conditions were tested in absence or presence of mucus: 0.8 g/l acyclovir, 0.8 g/l acyclovir + 1.6 g/l chitosan and 0.8 g/l acyclovir + 4 g/l chitosan based on the concentrations for chitosan which effects have been described previously [9,10,16]. The mucus used as a protective barrier in the Caco-2 assay consisted of type III mucin derived from porcine stomach dissolved in HBSS^{+} pH 6.0. Mucus was used in a concentration of 50 mg/ml [21].

Prior to the transport study, Caco-2 cells were washed twice with pre-warmed HBSS^{+} pH 7.4 and placed in a shake incubator (Thermostar, BMG Labtech, Offenburg, Germany) at 37 °C and 300 rotations per minute (rpm) for 30 min. After the pre-incubation, 100 µl of mucus were applied to the apical compartment of the transwell plates for the corresponding conditions; in the basolateral compartment fresh HBSS^{+} pH 7.4 was added. The transport experiment was initiated by adding 0.5 ml of the corresponding incubation medium at pH 6.0 to the donor compartment. Plates were incubated in the shake incubator at 300 rpm for 2 h at 37 °C. 200 µl samples were taken from the basolateral compartment at t = 15, 30, 45, 60, 90 and 120 min and were replaced by fresh buffer. 10 µl apical samples were taken at t = 0 and 120 min and diluted 100× in HBSS^{+} pH 7.4. Samples were analyzed immediately. Monolayer integrity after the transport experiment was confirmed by comparing the measured TEER at t = 0 min with the TEER at t = 120 min.

2.4. Ussing-type chamber permeation studies using rat jejunal segments (5–6 rats)

Rats were purchased from Charles River (Sulzfeld, Germany). Rat excised jejunal segments were obtained and permeation studies in an Ussing-type chamber were performed as described by Heinen et al. 2013 [22]. On the apical side, a 5 ml Krebs-Ringer-Bicarbonate-Buffer (KRB) containing MES at pH 6.0 was used. Same three conditions mentioned above were tested. Samples of 600 µl were taken at 30, 60, 90 and 120 min from the acceptor chamber, each replaced with fresh KRB buffer and at 0 and 120 min from the donor chamber. Similar as Caco-2 experiments, transsegmental electrical resistance (Rf) was measured at both the beginning and end of each experiment assessing the integrity of the tissue. Acyclovir permeability experiments were performed in the presence of chitosan (0, 1.6, and 4.0 g/l). Additional electrical resistance measurements were performed at higher chitosan concentrations up to 50.0 g/l.

2.5. Porcine excised segment InTESTine permeation studies (2 individual studies each with 4 replicates)

Porcine excised jejunal segments were obtained from healthy pigs, mounted in a newly developed InTESTine™ system, and permeability studies were performed as described by Westerhout et al. [23]. On the apical side, a 1 ml pre-warmed (37 °C) Krebs-Ringer Bicarbonate buffer (containing 10 mM glucose, 25 mM HEPES, 15 mM sodium bicarbonate, 2.5 mM calcium chloride, pH 7.4, and saturated with oxygen using a 95%/5% O_2/CO_2 mixture by gassing for 120 min, further indicated as KRB-HEPES) dose solutions containing 10 µM acyclovir (containing [^{14}C]-acyclovir, 2 kBq/ml) in the absence and presence of chitosan (0, 1.6, 4.0 g/l) and 50 µM fluorescein isothiocyanate-dextran (FD4) as a membrane integrity marker was used. The basolateral compartment contained 7.5 ml pre-warmed KRB buffer (37 °C). In parallel, the permeability of ^{3}H-atenolol and ^{14}C-antipyrine (both 10 µM) was determined in the absence and presence of chitosan as a control for the permeability of a low and high permeability marker, respectively. The InTESTine samples were taken from both the apical and basolateral compartment after a pre-incubation time of 45 min in order to measure the linear phase of the transport over 60 min incubation. Recovery of active substance compared to added quantity at the beginning of the study was determined at the end of the studies by determination of the mass balance. It was previously shown that intestinal permeability of a wide range of compounds is comparable between (adult) human and porcine intestinal tissue [23].

2.6. Analytical methods

2.6.1. Acyclovir by HPLC-Fluorescence Detection (TIM-1)

Acyclovir in ileum and jejunum dialysate, ileum effluent and residue samples from the TIM-1 experiment was analyzed by HPLC (Jasco PU-980, Jasco, Germany) with fluorescence detection (Shimadzu, RF-551, Shimadzu, Germany) (excitation wavelength 260 nm, emission wavelength 375 nm). A Waters Sunfire 150 × 3.0 mm 3.5 µm column was used at ambient conditions, with an injection volume of 10 µl. A gradient elution method was applied involving solvent A (0.1% formic acid in acetonitrile) and solvent B (0.1% formic acid in purified water) at a flow rate of 0.6 ml/min. Solvent A and B were used in a ratio of 99:1 during the first 5 min, followed by 2 min of 100% solvent B. The run time of 14 min was completed using the 99:1 ratio again. Low, middle and high QC samples in two TIM matrices were analyzed in duplo in parallel with the samples. Linearity was demonstrated in a range of 25 µg/ml to 250 µg/ml ($R^2 \geq 0.998$). Regarding precision, intraday variation coefficients per concentration level varied from 0.03 to 5.1%, which is acceptable considering the complex matrix. The results for accuracy showed for QC low (25 µg/ml) an average deviation of +15% in both matrices. Considering that the deviation varied from +5% to +23% and that the results for overall recovery are limited to 110%, a correction factor was not applied. The mean QC middle (100 µg/ml) and high (250 µg/ml) showed an average deviation of the nominal values of −1% and 4%, respectively. LOQ was set at 25 µg/ml while LOD was 10 µg/ml.

2.6.2. Acyclovir by HPLC-UV (Caco-2)

Acyclovir concentration in media samples from the Caco-2 cell experiments were analyzed by HPLC consisting of a Waters 600 pump and a Waters 717 auto injector (Waters, Milford, MA). For chromatographic separation a Waters Novapak C18 column under radial compression was used. UV absorbance was monitored using a Waters 2487 detector at 254 nm. The observed peaks were integrated using Empower Pro (Empower 2) software. The mobile phase consisted of a 25 mM acetate buffer (pH 3.5) (95%)/methanol (5%) and a flow rate of 1 ml/min was applied. Retention time of acyclovir was 6.0 min. The calibration curve of acyclovir was linear over the concentration range of 0.12–1000 µM. The assessment of repeatability at concentrations of 250, 25, 2.5 and 0.25 µM resulted all in RSD's below 2.5%. Samples from the acceptor compartment were not diluted; the 10 µl samples from the apical department were diluted with 990 µl HBSS+ pH 7.4. The injection volume amounted to 50 µl. LOQ was set at 0.12 µM and LOD was 0.05 µM, respectively.

2.6.3. Acyclovir by HPLC-UV (Ussing-type chamber)

Acyclovir concentration in media samples from Ussing-type chamber experiments were determined using isocratic HPLC with UV detection at 254 nm (equipment see above). A Lichrospher 10 RP 18 (5 µm), 250-4 column was used for chromatographic separation, at 40 °C and ~133 bar. The mobile phase consisted of 10 mM acetic acid and acetonitrile (95:5; V/V) and a flow rate of 1 ml/min was applied (retention time acyclovir ~4.6 min). Linearity was demonstrated in a range of 0.050 µg/ml to 50.0 µg/ml acyclovir. Intraday precision resulted in RSD values < 5.5% for concentrations ≥0.50 µg/ml and < 11% for the lower concentrations. Interday precision resulted in RSD values < 5.5% for concentrations ≥0.10 µg/ml and 13% for the lowest concentration of 0.050 µg/ml. Samples from the acceptor compartment were not diluted; 10 µl of the samples from the apical department were diluted with 390 µl buffer. Injection volumes were 50 µl.

2.6.4. Liquid scintillation counting (InTESTine)

Concentrations of radioactive labeled compounds in The InTESTine samples were measured on a Tri-Carb 3100TR Liquid Scintillation counter (LSC, Perkin Elmer, Boston, Massachusetts, United States) after adding scintillation liquid (Ultima Gold, Perkin Elmer Inc., Boston, Massachusetts, United States) to samples of the InTESTine experiments.

2.6.5. Fluorescence spectrophotometry (InTESTine)

FD4 levels in media samples from both the apical and basolateral compartments of the InTESTine system were determined using a FLUOstar OPTIMA fluorescence spectrometer (BMG Labtech, Ortenberg, Germany) at excitation wavelength 490 nm and emission wavelength 520 nm.

2.7. Data analysis

2.7.1. Bioaccessibility

The amount of acyclovir in each sample collected during the TIM-1 experiment was calculated by multiplying the measured concentration by the total volume of the individual samples collected in the time periods. The bioaccessibility of acyclovir is given as percentage of the intake dose of acyclovir and expressed as the mean and range of duplicate TIM-1 experiments. The mass balance (recovery) was calculated as the sum of the bioaccessible fraction (ileum and jejunum), the ileum effluent and the residues in the system after ending the experiments.

2.7.2. Apparent permeability

The apparent permeability coefficient (P_{app}) was calculated based on the linear part of the curves according to the following equation:

$$P_{app} = dQ/dt * 1/A * C_0$$

where Q is the amount of drug appearing in the acceptor compartment as a function of time (t), A is the surface area of the Transwell membrane (1.13 cm^2) or the exposed surface of the intestinal segment in the Ussing-type chamber (0.67 cm^2), or InTESTine system (0.79 cm^2), and C_0 is the initial drug concentration in the donor (apical) compartment.

2.8. Statistical analysis

The TIM-1-results for cumulative bioaccessibility were compared using unpaired t-tests at each time point. Differences were considered statistically significant when $p < 0.05$.

Statistical analysis of the permeation studies was performed using GraphPad Prism 6.03. The mean P_{app} values obtained from the Caco-2 study, Ussing-type chamber and InTESTine experiments were compared with the references using one-way ANOVA and Dunnett's multiple comparison test. P-values below 0.05 were considered significant at a confidence level of 95%.

3. Results

3.1. Bioaccessibility in TIM-1

Fig. 1 shows the jejunal, ileal and total bioaccessible fractions of acyclovir tested in absence (reference) and presence of chitosan (1.6 g/l and 4.0 g/l). The total bioaccessibility of the high solubility compound acyclovir tested under fasted state conditions in TIM-1 was found to be high, as values above 90% were measured. No effect of chitosan on the bioaccessibility of acyclovir was observed. Equal bioaccessible fractions were measured in the TIM-1 runs in absence of chitosan (93.6% ± 0.9%, mean ± range expressed as % of intake, n = 2); in presence of 1.6 g/l chitosan (90.6% ± 6%, mean ± range, n = 2) and in presence of 4.0 g/l chitosan (93.2% ± 3.2%, mean ± range, n = 2), respectively. Overall recovery of acyclovir in the six TIM-1 runs was 108.6% ± 5.1% (mean ± rsd; n = 6).

Values above 100% could be related to possible overestimation of the acyclovir content at low concentrations as observed in the analysis of the low QC samples (in contrast to the mid and high levels).

3.2. Intestinal permeability

Table 1 shows an overview of the results of the intestinal permeability of acyclovir measured in results of the five different models. The mean relative effect on the absorption is reflected by the ratio of test versus reference condition. The p-values indicate the statistical significance of the observed effect compared to the reference.

3.3. Caco-2 cell permeation studies

Monolayer integrity was measured through the TEER values, see Table 2. In absence of chitosan, the monolayer integrity was well preserved in both absence and presence of a mucus layer. In the unmodified model, the addition of 1.6 g/l and 4 g/l chitosan resulted in a complete loss of monolayer integrity after 120 min of incubation. Due to this loss of barrier function, the transport of acyclovir from apical to the basolateral compartment increased at these conditions. This effect was confirmed in a second study at the University of Mainz with the same test substances at the same concentrations following the same study protocol (data not shown): a reduction of TEER values to 2–3% of the original value was found in presence of chitosan, accompanied by a

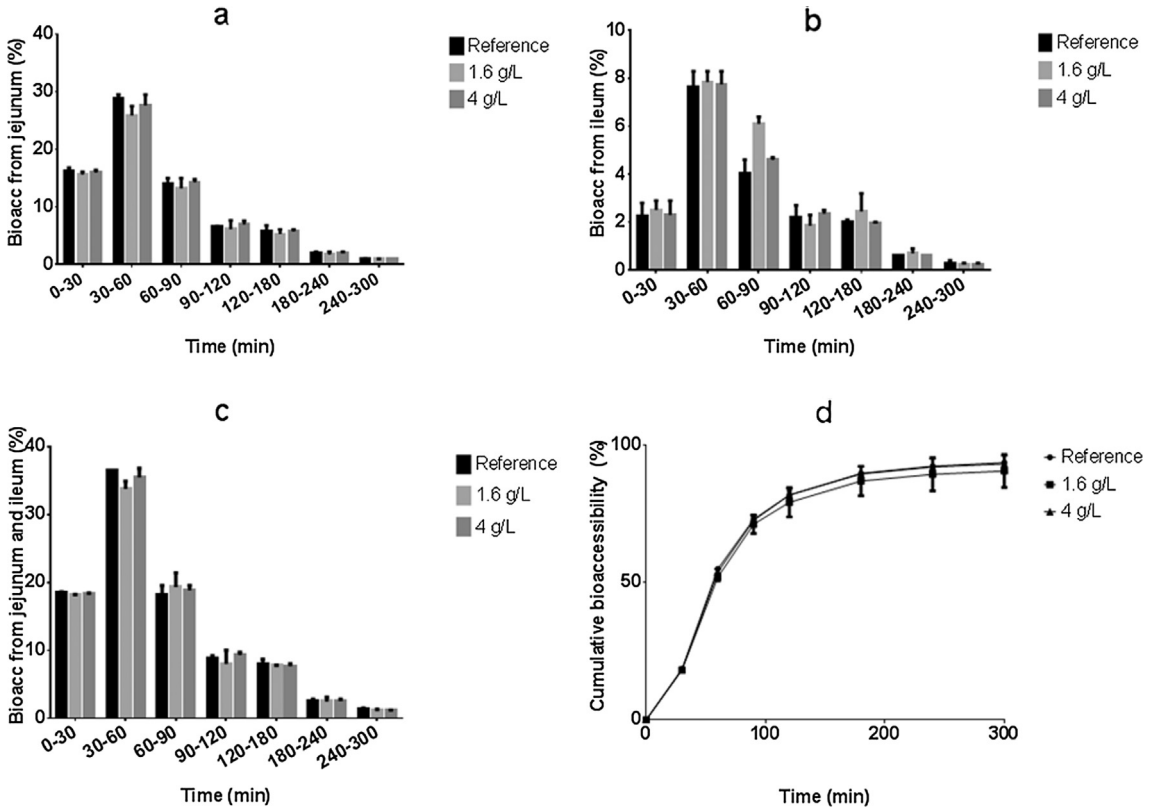

Fig. 1. Bioaccessibility of acyclovir in absence (reference) or presence of chitosan (1.6 and 4.0 g/l) as measured in TIM-1 in hourly time intervals. a. jejunal bioaccessibility (% of intake, mean ± range, n = 2); b. ileal bioaccessibility (% of intake, mean ± range, n = 2); c. total bioaccessibility (=sum of jejunum and ileum; % of intake, mean ± range, n = 2); d. cumulative total bioaccessibility (% of intake, mean ± range, n = 2).

Table 1
Apparent permeability values (P_{app}) of acyclovir in the presence of chitosan as measured in different permeability models.

Model and concentration chitosan hydrochloride[#]		Permeation of acyclovir		Significant result			
Caco-2	Nr of inserts	P_{app} in 10^{-6} cm/s (sd)	Ratio test vs reference	$p < 0.05$, ANOVA + Tukey multiple comparison	$p < 0.05$, ANOVA + Dunnett's multiple comparison	*t*-test individual values compared to reference, Bonferroni correct alpha ($p = 0.05/3 = 0.02$)	*t*-test individual values
0 g/l	3	0.17 (0.01)					
1.6 g/l	3	21 (1.08)	124	y	y	y	y
4 g/l	3	24 (1.31)	143	y	y	y	y
Caco-2 + mucus	Nr of inserts	P_{app} in 10^{-6} cm/s (sd)	Ratio				
0 g/l	3	0.12 (0.01)					
1.6 g/l	3	0.13 (0.003)	1.08	n	n	n	n
4 g/l	3	0.28 (0.15)	2.33	n	n	n	n
Ussing type	Nr of segments (rat)	P_{app} in 10^{-6} cm/s (sd)	Ratio				
0 g/l	5	7.4 (1.5)					
1.6 g/l	5	5.4 (0.9)	0.73	n	y	n	y
4 g/l	5	6.2 (1.0)	0.84	n	n	n	n
InTESTine	Nr of segments (pig)[$]	P_{app} in 10^{-6} cm/s (sd)	Ratio				
0 g/l	8	0.54 (0.10)					
1.6 g/l	8	0.49 (0.15)	0.91	n	n	n	n
4 g/l	8	0.38 (0.13)	0.70	n	n	n	y

[#] All conditions included 0.8 g/l acyclovir.
[$] From two pigs, each 4 slices.

Table 2
Effect of acyclovir and the addition of chitosan on monolayer integrity of Caco-2 cells as measured in the absence and presence of mucus.

Concentration chitosan hydrochloride (g/l)	TEER (0 min) (%)	TEER (120 min) (%)	SD (%)	RSD (%)
Caco-2				
0	100	115.0	6.0	5.3
1.6	100	4.1	0.2	5.9
4	100	2.8	0.5	18.4
Caco-2 + mucus				
0	100	124.5	2.4	1.9
1.6	100	132.4	1.6	1.2
4	100	55.7	27.8	49.9

30- to 40-fold increase in acyclovir's permeability.

In absence of chitosan, P_{app} values of acyclovir in the Caco-2 system in absence and presence of mucus were comparable. This indicates that the mucus layer had no influence on the permeation of acyclovir. When 1.6 g/l chitosan was added in the apical compartment of the Caco-2 model in presence of a mucus layer, the monolayer integrity was maintained during the experiment. Addition of 4 g/l chitosan showed that the monolayer integrity was partly compromised after 120 min, which was accompanied by increased variation in TEER results (Table 2). In presence of mucus, 1.6 g/l chitosan resulted in a P_{app} value (i.e. $0.13 * 10^{-6}$ cm/s) similar to the reference value (i.e. $0.12 * 10^{-6}$ cm/s). Increasing the concentration of chitosan to 4 g/l led to an elevated P_{app} value (i.e. $0.28 * 10^{-6}$ cm/s) compared to the reference conditions, although this difference was not statistically significant.

3.4. Rat jejunal tissue mounted in the Ussing-type chamber

Two concentrations of chitosan hydrochloride (1.6 g/l and 4 g/l) were applied in the Ussing type chamber with rat intestinal segments to test their effect on the permeability of acyclovir (0.8 g/l). The results were compared with acyclovir alone (reference). At the end of all incubations, the transsegmental electrical resistance (Rf) values decreased during the experiment as presented in Table 3. The Rf decreased by 20% in absence of chitosan, however this effect was attenuated by the co-incubation with chitosan either at 1.6 or 4.0 g/l (Table 3). A relatively high P_{app} value for the permeability of acyclovir in absence of chitosan was found (i.e. $7.4 * 10^{-6}$ cm/s) in the Ussing type chamber with rat intestinal segments. This mean P_{app} was reduced by both concentrations of chitosan, which was statistically significant only for 1.6 g/l chitosan in the donor solution.

3.5. Porcine intestinal tissue

The permeability of acyclovir, atenolol and antipyrine was measured across jejunal porcine tissue mounted in the InTESTine system. The tissue was subjected to the same test concentrations with respect to chitosan used in the rat tissue Ussing-type chamber experiments. Recovery of all compounds based upon media alone were > 95%. FD4 leakage remained below 1% indicating no effect of the test solutions on intestinal integrity. The P_{app} values of acyclovir, atenolol, and antipyrine in the absence of chitosan were $0.54 * 10^{-6}$, $0.46 * 10^{-6}$, and $6.58 * 10^{-6}$ cm/s, respectively. Low concentrations of chitosan (1.6 and 4 g/l) showed a negative trend on the absorption of acyclovir across porcine jejunal segments mounted in the InTESTine system, although not statistically significantly different from the control incubations. The permeability of the paracellular transport marker atenolol enhanced with higher concentrations of chitosan from $0.46 * 10^{-6}$ cm/s up to > $1 * 10^{-6}$ cm/s at 30 and 50 g/l (data not shown). No effect of chitosan was observed on the permeability of the transcellular transport marker antipyrine, which varied in the range of $6–9 * 10^{-6}$ cm/s (data not shown).

Table 3
Effect of acyclovir and the addition of chitosan on monolayer integrity of rat intestinal tissue as mounted in Ussing-type chamber.

Concentration chitosan hydrochloride (g/l)	Rf (0 min) (%)	Rf (120 min) (%)	SD (%)	RSD (%)
0	100	79.7	6.6	8.3
1.6	100	97.3	5.6	5.8
4	100	88.2	6.6	7.5
10	100	128.5	13.0	10.1
30	100	45.9	15.0	32.7
50	100	8.4	3.6	43.3

4. Discussion

Excipients such as chitosan may exert their effect on oral bioavailability through modulation of different elements of the absorption process including the dissolution of the active substance from the dosage form, the gastrointestinal transit process and interactions in the intraluminal compartment or at the permeation site. In this study, two of these individual processes were investigated using different *in vitro* techniques that could provide a mechanistic understanding of previously obtained *in vivo* observations [16]. Chitosan decreased both acyclovir's mean area under the plasma concentration-time curve (AUC_{0-12} and $AUC_{0-\infty}$) and maximal plasma concentration (C_{max}) following concomitant oral intake of 400 and 1000 mg chitosan (administered as 1.6 and 4.0 g/l in water) in humans (Table 4). Meanwhile, the T_{max} of acyclovir increased significantly for the treatment combinations with chitosan; however, the effect was statistically significant for the 1000 mg of chitosan co-administration only. Overall, the negative effect of chitosan on acyclovir's absorption was variable between the human subjects but statistically significant [16].

In the human study, the test products were dissolved prior to use and as such, an effect of chitosan on dissolution of acyclovir from the dosage form can be ruled out [16]. In the present study, various *in vitro* intestinal models were used to investigate whether chitosan has an effect on the bioaccessibility and intestinal permeability of acyclovir at the same dose levels as applied in the human study. The selected models have previously been described as tools to study the individual processes underlying oral bioavailability and identify the critical process(es) hampering the oral bioavailability of a specific compound. Based on its physico-chemical properties and available data, acyclovir was previously classified as a BCS III compound indicating that intestinal permeability, not solubility, is the rate limiting process in oral bioavailability [17].

Table 4
Oral bioavailability data of acyclovir as measured in humans [16].

C_{max} (mg/ml)		Ratio[#]
Reference Zovirax 200	0.37 ± 0.21	
Ref + 400 mg chitosan	0.21 ± 0.09*	0.56
Ref + 1000 mg chitosan	0.24 ± 0.26*	0.63
$AUC_{0-\infty}$ (mg·h/ml)		
Reference Zovirax 200	1.53 ± 0.63	
Ref + 400 mg chitosan	1.13 ± 0.42	0.74
Ref + 1000 mg chitosan	1.07 ± 0.05*	0.70
T_{max} (h)		
Reference Zovirax 200	1.2 ± 0.4	
Ref + 400 mg chitosan	1.5 ± 0.9	1.25
Ref + 1000 mg chitosan	1.8 ± 0.9*	1.50

[#] Ratio of absolute values of test vs reference.
* $p < 0.05$ comparing 90% confidence intervals of the ratio of test and reference product.

The TIM-1 system simulating the human physiological conditions in the stomach and the three parts of the small intestine is an *in vitro* tool mainly used to study biorelevant dissolution. Since acyclovir tablets were dissolved before the administration, this tool was applied to investigate the effect of chitosan rather on the bioaccessibility of acyclovir than on its dissolution (Fig. 1). A high bioaccessibility of acyclovir was measured (> 90%) in absence and presence of chitosan which indicates a large fraction of the acyclovir dose added to the gastric compartment appears to be available for absorption irrespective of the presence of chitosan. These observations are in line with acyclovir's high solubility, 2.33 mg/ml at biorelevant pH [17,24]. Luminal interactions affecting both acyclovir and chitosan might possibly explain the *in vivo* results. For instance, an interaction of chitosan with bile acids in the gut lumen, reducing the solubility of acyclovir was hypothesized [15,16]. Nevertheless, no effect of chitosan on the bioaccessibility of acyclovir was observed in this study, suggesting that it is highly unlikely that luminal interactions may affect the oral bioavailability of acyclovir.

The current set-up of the TIM-1 experiments simulated the average human adult fasted state conditions concerning enzyme levels, pH values, transit times, etc. Gastric emptying time was fixed and identical in the TIM-1 runs testing the three applied experimental conditions. Even though the computer-controlled TIM-1 system allows changes in gastric emptying time to study the effect of chitosan on this specific parameter, it was not tested in the current study. In fact, 5 mg/kg of chitosan, corresponding to 1.4 g/l, did not delay gastric emptying of several drugs with different absorption/physicochemical characteristics. Furthermore, even higher chitosan amounts (7 g/l) had also no effect on residence time of the low permeability cephalexin [11]. The recently performed human study showed an increased T_{max} value at 4 g/l chitosan, however associated with high interindividual variability.

Chitosan is thought to act as a potential permeability modulator through interaction with the tight junctions between the epithelial cells, resulting in redistribution of cytoskeletal F-actin and translocation of tight junction proteins ZO-1 and occludin from the membrane to the cytoskeleton [8,25]. Smith et al. showed activation of PKC-dependent signal transduction pathways using a Caco-2 model [12]. Although, the mucus layer was also reported to play a role in the access of chitosan to the epithelial membrane and the subsequent effect on the paracellular permeation route [14]. Therefore, the effect of chitosan causing a change in permeation of acyclovir was tested in four different permeability models with and without a mucus layer. As described in methods, the chitosan effect was assessed in buffered solutions (pH = 6.0) preventing that the polymer precipitates. Selection of this pH also resembles the proximal small intestinal pH (5.9–6.3) [26] and also because this allows the comparison with previous studies (pH = 5.5–6.2) [4,6,9,10].

In the unmodified human intestinal Caco-2 cell model, chitosan concentrations enhanced the permeability of acyclovir 124- and 143-fold, respectively (Table 1). This was caused by the disruption of the monolayer integrity by the reduction of the TEER values as it is shown in Table 2 and several other studies [7,9,10,27]. Although the reference Caco-2 P_{app} in this study was slightly lower than some previously reported values [9,10,28], this does not fully explain the relatively large increase in presence of chitosan. For instance, Merzlikine et al. showed an enhancement on acyclovir Caco-2 permeability of only approx. 10-fold by co-administrating even much higher chitosan concentrations (10–30 g/l) than those used in the present work [9]. However, the chitosan tested in that study had a lower degree of deacetylation (DD = 75–90%) compared to the one used in the present study (DD = 93.05%). It has been proposed that chitosan positive charges are critical for binding to membrane proteins and to exert its subsequent permeability enhancer effect [5,8]. Considering that chitosan exhibits a pKa around 6.3–6.5 [29,30], the charge density of polymer molecules will mainly depend on DD at the studied pH (=6.0). Therefore, discrepancies regarding to the magnitude of the enhancing effect could be related to the physicochemical characteristics of chitosan. In fact, Shah et al. showed that similar concentrations (1–5 g/l) of chitosan (DD = 85%) only enhanced acyclovir Caco-2 P_{app} 5.8–10.2-fold, accompanied by a TEER reduction of 46.6–58.5% [10]. Consistently, chitosan displayed a much greater TEER reduction in this study (Table 2), which could be also due to the high deacetylation degree. Finally, both the low pH of the current study, applied to overcome precipitation of chitosan, and the general interlaboratory variability of the Caco-2 system [31,32], may also contribute to explain the differences found.

The modified Caco-2 model with mucus demonstrated that the disruptive effect of chitosan on the integrity of the epithelial cells is much lower in presence of mucus [14,33]. Both TEER values and acyclovir's permeation were less affected than in the unmodified model when 1.6 g/l chitosan was added, suggesting that the mucus layer prevented damage to cell monolayer integrity and consequential increased permeation of acyclovir. The higher concentration of chitosan (4 g/l) caused reduced monolayer integrity accompanied by an elevated permeation of acyclovir, although this was not statistically significant. Likewise, such reduction of monolayer integrity was not accompanied by an increase in acyclovir P_{app}. Therefore, in the presence of mucus, no effect of chitosan (1.6 and 4.0 g/l) on the absorption of acyclovir was detected in this modified Caco-2 cell model.

Rat intestine mounted in an Ussing chamber has shown to be a useful model in predicting human intestinal absorption too, although differences in transporter expression and metabolic characteristics apply as well [34–36]. Even though apparently larger permeability values were observed in this model (Table 1), they were comparable with P_{app} of other low permeability compounds across rat jejunum mounted in this same experimental set-up [36]. Permeability of acyclovir was statistically significantly reduced in presence of a concentration of 1.6 g/l chitosan, while the reduction remained a non-significant trend at 4.0 g/l. The Rf values decreased during the experiment and the limited reduction in presence of chitosan suggested a stabilizing effect (Table 3). Interactions of chitosan with mucus may again explain these observations. The mucus production of intestinal tissue segments in the Ussing-type chamber is enhanced by the experimental conditions [22], as was also observed in the present study. Charge interactions of chitosan in the mucus-producing model may neutralize the reactivity of chitosan and prevent an absorption enhancing effect.

Acyclovir permeability through porcine intestine was slightly lower than previously reported for other low permeability compounds by using this same system [23], consistent with its classification as BCS class III [17]. The results of same concentrations of chitosan (1.6 and 4 g/l) suggested a negative effect on the absorption of acyclovir across porcine jejunal segments mounted in the InTESTine system (Table 1). However, neither the negative nor the positive effect were statistically significantly different from the reference. The effect of chitosan on paracellular transport marker compound atenolol was in line with that for acyclovir, which confirmed an effect of chitosan on the paracellular absorption route. The absence of an effect of chitosan on transport of antipyrine confirmed the absence of an effect on the transcellular route and of a non-specific effect on the mucus barrier (data not shown).

Despite of the chitosan effect has been already studied in diverse *in vitro* systems, they do not necessarily resemble the true anatomic intestinal lining due to the lack of a mucus layer. Especially the differences in protective mucus layer and tight junctions are of relevance, as the modulation of membrane transport by cationic chitosan is postulated to occur through an interaction with negative charges in the cavity of the tight junctions [8]. In this line, Schipper et al. studied the permeability of the well-known permeability marker mannitol through HT29-H mucus-secretory cells in presence of chitosan. As expected, chitosan co-administration increased mannitol P_{app} when the mucus layer was removed. However, the presence of a mucus layer prevented such enhancement through unwashed cells [14]. In the present study,

three out of four permeability experiments were performed in mucus containing set-ups. Consistently, the expected enhancing effect of chitosan on acyclovir P_{app} in all mucus containing models was at least prevented. Furthermore, acyclovir permeability across rat and pig intestinal tissues was decreased when co-incubated, being statistically significant in rat at 1.6 g/l of chitosan, in good agreement with the *in vivo* situation. The protective effect of mucus on barrier integrity was also observed in electrical resistance measurements, where mucus-containing models maintained TEER and Rf values in presence of chitosan (Tables 2 and 3). Fluorescence microscopy experiments showed that highly deacetylated chitosan did not reach the membrane of mucus-secreting cells [14]. Although mucus displayed similar magnitudes of protective effect for different types of chitosan (DD and MW), it is still possible that the interaction depends on those physicochemical features. A key factor of this present work is that the same chitosan from the clinical trial was used. Therefore, the chitosan mucus interaction could provide an explanation for controversial bioavailability estimates obtained from different *in vitro* models for the human situation.

Being a polycation, chitosan might bind to any other polyanion present in the system studied. For example, the enhancing chitosan effect observed in Caco-2 cells was reverted by addition of heparin, demonstrating the un-specificity of such ionic interaction [8]. Acyclovir tablets studied here declare the presence of sodium starch glycolate, a polyanion with pKa 4.8, likely charged at biorelevant pHs. Hence, a possible excipient-excipient interaction can be speculated. Nevertheless, clear solutions were observed when tablets were dissolved in water, regardless of the presence of chitosan [16]. Moreover, acyclovir mass balance > 90% after TIM-1 experiment (Fig. 1), suggests that even when an excipient-excipient interaction occurred, it would not affect the amount of acyclovir available for absorption. On the other hand, the chitosan effect could be also attenuated by slightly alkaline media because of its low solubility above its pKa [30]. However, experimental media were well-buffered 0.5 units below the chitosan pKa, which was enough to achieve the needed solutions before running permeability experiments. In contrast, precipitation was observed after sample addition to Caco-2 cells supplemented with mucus, suggesting a direct interaction of chitosan with the mucus, rather than other possible causes, thus reducing the available concentration of the modulator in solution. Taken together, the results presented here provide additional evidence supporting the protective role of mucus, and suggest that its presence in the *in vivo* GI tract might have prevented chitosan to exert an overall enhancing effect on the bioavailability of acyclovir. The presence of a mucus layer thus seems an important factor in determining the biorelevance of permeation models and might also be considered for other excipients effects on intestinal permeability, in particular when they are positively charged.

Our human study showed a relatively high intra-individual and interindividual variability of the absorption of acyclovir itself, which augmented in presence of chitosan. Both increased and decreased AUC and C_{max} values were observed when acyclovir was co-administered with chitosan. Moreover, four out of twelve volunteers showed a higher AUC in presence of one of the two doses of chitosan and a lowered AUC in presence of the other dose [16]. Further evaluation of potential intra- and interindividual differences in physiological processes in the absorption of acyclovir and the potential effect of chitosan on these processes, may thus be relevant. Integration of different *in vitro* datasets in a PBPK model can also be helpful in this perspective and will be the subject of a follow-up study.

The observed results on bioaccessibility and intestinal permeability could also be important in the context of biowaiving. Based on human studies with acyclovir and cimetidine, Vaithianathan et al. recently proposed widened biowaiver possibilities for changes in the content of 12 common excipients combined with BCS class III substances [37]. In the current case of potential excipient chitosan, the contrary can be concluded. The unmodified Caco-2 cell model showed a loss of membrane integrity, which has no correlation to the *in vivo* human situation. The mucus-containing Caco-2 model showed a non-significant positive trend, which is not line with the *in vivo* data. These models thus seem unsuitable to replace *in vivo* testing. The trends observed for the tissue segment models (rat and porcine intestine) were negative. Among the models explored in the present study, the Ussing type chamber showed to be the most biopredictive as it did point to an overall statistically significantly reduced absorption of acyclovir. This model thus seems most appropriate for pharmaceutical development purposes. However, it is not considered suitable to fully exempt from *in vivo* testing e.g. for biowaiver purposes. An *in vivo* pharmacokinetic study is needed to determine the magnitude of the clinical effect on AUC and C_{max} and its consequential relevance for drug absorption.

5. Conclusion

This study presents for the first time a comparison of various preclinical intestinal permeability models run under comparative conditions with the aim of testing the predictive power of each model. The overall *in vivo* effects of chitosan pointed to a reduced rate and extent of the absorption of acyclovir. Acyclovir's bioaccessibility in TIM-1 was not affected by chitosan; this model thus confirmed absence of intraluminal interactions hampering the solubility and availability for absorption of acyclovir. Chitosan's influence on intestinal permeability of acyclovir differed per model. The results obtained with the Caco-2 models were not in line with the *in vivo* data. The tissue segment models (rat and porcine intestine) showed a negative trend of acyclovir's permeation in presence of chitosan. The Ussing type chamber showed to be the most biopredictive of models studied, as it did point to an overall statistically significantly reduced absorption of acyclovir. This model thus seems most appropriate for pharmaceutical development purposes. As a follow-up, PBPK modelling is currently applied to more specifically correlate the outcome of the models to the *in vivo* data. In absence of an established correlation, *in vivo* pharmacokinetic studies remain necessary to determine the actual clinical effect of chitosan on the absorption of acyclovir.

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