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Beta-casein nanocarriers of celecoxib for improved oral bioavailability

Abstract: Beta-casein (bCN) micelles were developed as a platform for improved oral bioavailability (BA) of poorly water-soluble drugs. Here we demonstrate a proof-of-concept using the NSAID celecoxib (Cx) loaded into bCN micelles (Cx/bCN). In a crossover pharmacokinetic (PK) study in pigs (n=4), dosed intraduodenally with either the commercial Cx formulation Celebra[®] or Cx/bCN, the C_{max} obtained after administration of Cx/bCN was 2.3-fold higher and the T_{max} was 1.57-fold faster, leading to a 1.76-fold increase in the BA of Cx, compared to the Celebra[®] formulation. It is suggested that this BA enhancement was caused by improvement of Cx solubility in intestinal fluids by bCN micelles, which maintained their Cx cargo in an amorphous state.

Keywords: beta-casein; bioavailability; celecoxib; micelles.

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Introduction

Celecoxib (Cx) is a selective COX-2 inhibitor widely used in the management of osteoarthritis, rheumatoid arthritis and acute pain. It is a small lipophilic molecule (MW=381, logP=3.683) (1). As a weak acid (pKa of 11.1), Cx is uncharged and practically insoluble (7 µg/mL at 25°C) at intestinal pH (2). According to the biopharmaceutical classification system (BCS) (3), Cx is a class II compound due to its low solubility and high gastrointestinal (GI) permeability. Therefore, its bioavailability (BA) after oral administration is governed by its dissolution rate in intestinal fluids, and may be affected by the formulation. Thus, development of an appropriate oral vehicle for Cx, and for other BCS class II compounds, is an important challenge that can affect their clinical use.

Celebra[®] (Pfizer Inc.), the commercial Cx product, is available as oral capsules in a lactose-based formulation. It has been reported that the oral BA of Cx in Celebra[®] can be increased by 40% following its administration with a high-fat meal (4). Studies in dogs (5) showed that the BA of Cx in Celebra[®] capsules is low (22%–40%) and that the drug is extensively metabolized (primarily by CYP2C9).

Several formulation approaches have been reported for increasing the water solubility of Cx (which may enhance BA through the GI tract). Examples include the use of co-solvents (1), cyclodextrin (6), a self-microemulsifying drug delivery system (7), salt formation combined with precipitation inhibitors (8), reduction in particle size (9, 10) and formation of amorphous nanoparticles (10–13).

In an attempt to increase the oral BA of Cx, we developed a novel formulation based on beta-casein (bCN) as an encapsulating agent (14, 15). Characterization of our Cx/bCN formulation has been reported previously (15). In this study, in direct continuation, our goal was to examine, in a pig model, whether Cx/bCN can increase Cx BA compared to the Celebra[®] formulation.

Beta-casein (bCN) is a 209 amino acid (~24 kDa), open-structure, proline-rich phosphoprotein found in mammalian milk. It is biocompatible and biodegradable and is generally recognized as safe (GRAS). The N-terminal region of bCN is highly charged while the

remainder of the molecule is neutral and hydrophobic (16). At concentrations above its critical micellization concentration (CMC, 0.5–2 mg/mL at pH~7, 25°C), bCN self-assembles in water into nanometric micelles (~20–25 nm in diameter) having a hydrophobic core and a hydrophilic corona (17). The bCN structural and physicochemical properties facilitate its application in drug delivery (18–20), and bCN has been used for encapsulation of hydrophobic chemotherapeutics (21–23). However, our formulation is quite different from these, and also from the Cx and casein-based formulation described recently (11). We assumed that in our formulation, bCN can allow high drug loading and encapsulation efficiency since the drug is encapsulated within bCN micelles present in significantly higher concentrations than its CMC. We achieved high drug loading within small, uniform and stable micelles, in the absence of any additional polymers or cryoprotectants. In our previous characterization studies (15), direct-imaging cryogenic-transmission electron microscopy (cryo-TEM) and light microscopy supported by dynamic light scattering showed that Cx/bCN consists of well-ordered, homogenous, nano-sized assemblies. Cx loading leads to formation of larger (increase from ~20 to ~30–35 nm), spherical and uniform micelles, and after freeze-drying, the micelles' dimensions and morphology remained almost unchanged. Zeta potential measurements showed that the negative charge of bCN micelles slightly increases upon Cx loading and also after freeze-drying. HPLC analysis demonstrated the high Cx-loading capacity of the bCN micelles and wide-angle X-ray diffraction (WAXS) and NMR show the amorphous state of Cx in Cx/bCN (submitted manuscript, Turovsky 2014). Additional characterization was performed by solution DSC (submitted manuscript, Perlstein 2014).

Materials

Bovine β -casein (cat. no. C6905) and Dowex 1x8-200 (cat. no. 217425) anion exchange resin (100–200 mesh) were purchased from Sigma-Aldrich. Celecoxib was obtained from Teva (Israel). All other reagents were of analytical grade. The non-labeled hard gelatin capsules (DBCaps, size AAA, Code 22.307) were purchased from Capsugel, Germany.

Animals

Female pigs (n=4, 21–24 kg) were purchased from Kibbutz Lahav (Israel). The pigs were housed in the Hadassah Ein Karem animal

facility (Jerusalem, Israel), an AAALAC International accredited institute, in individual cages without litter, to ascertain fasting conditions during the pharmacokinetic (PK) studies, at 21–24°C, with 12 h-light/dark cycles. The pigs had free access to water and received approximately 1 kg standard pig chow per day. The pig studies were conducted in accordance with the Principles of Laboratory Animal Care (NIH publication #85-23, revised 1985). The study protocol was approved by the joint Ethics Committee (IACUC) of the Hebrew University and Hadassah Medical Center for animal welfare (#MD-11-13049).

Methods

Cx formulations

Solution for intravenous (IV) injection of Cx was prepared by dissolving 1.11 mg/mL Cx in PEG 400/saline solution (2:1, v/v) followed by aseptic filtration through a 0.45- μ m polyvinylidene fluoride (PVDF) filter.

Cx/bCN dry formulations were prepared by dissolving bCN (1% and 2%) in 20 mM HEPES buffer (pH 7.0) containing 1 mM $MgCl_2$, 2 mM EGTA and 10 mM NaCl, followed by overnight stirring at 4°C and then filtering through a 0.45- μ m PVDF filter. Cx was dissolved in ethanol and a volume corresponding to a specific Cx:bCN mole ratio was added drop-wise to the bCN dispersions while stirring, keeping the ethanol concentration in the final dispersions at 5% v/v. After additional stirring for 30 min at room temperature, the Cx/bCN micellar dispersions were frozen in liquid nitrogen, lyophilized and stored at -20°C. For the dissolution and pig studies, the formulation was optimized by preparing increased volumes of individual dispersions (5, 10 and 25 mL). After lyophilization, 21 individual powders were selected based on their Cx content, mixed well to generate a uniform powder mixture, and analyzed for final content of Cx and bCN (see results of analysis in Table 1). For dissolution testing, 66 mg of the lyophilized powder was filled into hard gelatin capsules (4 mg Cx per capsule). For the pig studies, 820 mg of the lyophilized powder was filled into hard gelatin capsules (50 mg Cx per capsule). The contents of the Celebra® capsules (equivalent to 4 mg Cx for the dissolution studies, 50 mg Cx for the pig studies) were emptied and repacked into unmarked hard gelatin capsules.

Quantitative analysis of bCN and Cx

bCN protein was quantified using Bradford assay, based on Coomassie Blue dye (24). UV absorbance (595 nm) of the tested samples

Table 1 Encapsulated Cx weight and mole ratios in Cx/bCN (average of 4 measurements).

%Cx encapsulated	Cx content (mg/g formulation)	bCN content (mg/g formulation)	Cx:bCN weight ratio	Cx:bCN mole ratio
100%	61.0	402	0.152	9.54

was determined using Synergy 4 HT Multi-Mode Microplate Reader (BioTek), employing a calibration curve with a linearity range of 0–60 µg/mL bCN.

Analysis of Cx in the buffer solution was conducted by HPLC (Hewlett Packard 1050, equipped with UV/Vis detector), using LiChro-CART 100 RP-18 column (250 mm×4 mm, 5-µm particle size, Merck) and a guard column LiChroCART 4-4. Flow rate was 0.5 mL/min, UV detection was at 254 nm at ambient temperature and injection volume was 50 µL. The mobile phase consisted of acetonitrile: methanol: 0.5% glacial acetic acid in water adjusted to pH 3.5 with NaOH (45:45:10, v/v/v) (25). Total and non-encapsulated Cx in the samples were measured in triplicate as follows: (A) for total Cx amount, 140 µL of cold acetonitrile was added to 120 µL of Cx/bCN sample, vortexed for 1 min and immersed for 20 min in crushed ice; (B) for non-encapsulated Cx, non-encapsulated (free) Cx was separated from the encapsulated Cx using an anionic exchange resin (Dowex 1x8-200), which binds the negatively charged bCN, with or without Cx, while the charge-less free drug does not bind to the resin. Both total (A) and Dowex-treated (B) samples were centrifuged for 10 min at 3000g at 4°C. Supernatants were collected, diluted with the mobile phase, and 300 µL was transferred into HPLC vials for analysis. Linearity of calibration curve was maintained in Cx concentration range of 0.3–10.0 µg/mL.

HPLC analysis of Cx in pig plasma was conducted as described above, except that a Jupiter 4-µm Proteo 90 Å 30×4.6 mm column (Phenomenex) was used. The drug was extracted from plasma as follows: 300-µL aliquots of plasma were mixed with 50 µL (5 µg) of mefenamic acid (MA) as internal standard, followed by addition of 350 µL of cold acetonitrile. The samples were vortexed for 1 min and immersed for 20 min in crushed ice, followed by 10 min centrifugation at 3000g at 4°C. From the supernatant, 550 µL was collected and evaporated to dryness with filtered air at 60°C using Eppendorf Concentrator 5301. The residue was re-dissolved in 200 µL of mobile phase, vortexed for 1 min and centrifuged for 2 min at 14,000 rpm at room temperature before transferring to HPLC vials. Calibration curves were prepared by spiking pig plasma with Cx and MA solutions, and linearity of calibration curves was determined for the whole range of analyzed Cx plasma concentrations.

Turbidity measurements

Change of turbidity was used to assess the effect of incubation at an elevated temperature (37°C) on bCN and Cx/bCN micellar dispersions (26). The increase in turbidity was assessed from OD measurements at 600 and 430 nm, which are significantly above the absorbance wavelengths of the protein and the drug. Increase in turbidity was expressed as OD ratio: 600 nm/430 nm. Samples were measured, in triplicate, before and after 60 min of incubation at 37°C with continuous stirring at 50 rpm. The absorbance measurements were performed using Synergy 4 HT Multi-Mode Microplate Reader (BioTek). The encapsulated Cx:bCN mole ratio of Cx/bCN was determined as previously described (15).

Dissolution studies

The dissolution studies of Cx capsules were conducted in two types of buffered media:

(a) Simulated intestinal fluid (SIF, USP, without pancreatin) with 1% sodium lauryl sulphate (27) (SIFSLs 1%). Cx solubility in SIFSLs 1% was determined to be 457 µg/mL (measured after excess Cx was mixed with SIFSLs 1% in triplicate, maintained at 37°C with stirring at 50 rpm for up to 24 h and filtered through a 0.2-µm PVDF filter). The SIFSLs 1% (500 mL per vessel, n=3) was maintained at 37°C with stirring at 50 rpm. The hard gelatin capsules, each containing 4 mg Cx, were placed in the stirred buffer and 2-mL samples were withdrawn at 0, 5, 10, 15, 20, 30, 40, 50, 60 and 100 min, and replaced by 2 mL of fresh medium. The samples were immediately filtered through a 0.2-µm PVDF filter and appropriately diluted for Cx analysis.

(b) Fasted state simulated intestinal fluid (FaSSIF-V2) (28), containing 152 mg/L of lecithin, 1.61 g/L of sodium taurocholate, 2.22 g/L of maleic acid, 1.39 g/L of NaOH and 4.01 g/L of NaCl. Cx solubility in FaSSIF-V2 was determined to be ~3 µg/mL. FaSSIF-V2 (200 mL per vessel, n=2) was maintained at 37°C while stirring at 50 rpm. The hard gelatin capsules, each containing 4 mg Cx, were placed in the stirred medium and 2-mL samples were withdrawn at 0, 5, 10, 15, 20, 25, 30, 35 and 40 min, and replaced by 2 mL fresh medium. Samples were immediately filtered through a 0.45-µm PVDF filter and appropriately diluted for Cx analysis. The filter and bCN 1 mg/mL did not affect Cx concentrations in SIFSLs 1% and FaSSIF-V2 media, as verified in preliminary experiments (data not shown).

Pig studies

In separate studies, each pig received in a crossover fashion, either IV injection of Cx (0.5 mg/kg), or intraduodenal Celebra® formulation (2 mg/kg), or Cx/bCN (2 mg/kg) with a 1-week washout period between individual phases. Intraduodenal administration was carried out to avoid a possible effect of gastric conditions on bCN. Before each phase, the pigs were fasted overnight. For the intraduodenal phases the pigs were fed with a liquid diet (Osmolite, Abbott, USA) 24 h before fasting. Prior to drug administration, the pigs were anesthetized with ketamine/xylazine (150/15 mg/kg) followed by 5% iso-flurane (for 6 h in the IV phases and 3 h in the intraduodenal phases).

For the IV administration, a central venous catheter was inserted into the jugular vein of the animals, and a pre-dosing blood sample (0 min) was collected, after which the Cx solution was injected through the same catheter. Approximately 2.5-mL blood samples were collected into chilled tubes (containing K₃EDTA) at 2.5, 5, 10, 15, 30, 45, 60, 75, 90, 120, 150, 210 and 360 min.

For the intraduodenal administrations, the various Cx capsules were administered directly into the pig duodenum by an endoscope guided through the pylorus using Pillcam Express® (a video capsule delivery device by Given Imaging Ltd., Israel). Approximately 2.5-mL blood samples were collected before the dosing and at 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12 and 24 h after dosing. Plasma was separated by centrifuging at 3000g for 10 min at 4°C immediately after each blood collection, followed by storage at -20°C until HPLC analysis. After drug administrations, and twice a day during the washout period, the pigs were monitored for loss of appetite, loss of weight, diarrhea, vomiting or signs of pain or discomfort.

Pharmacokinetic analysis

For each study, Cx concentration at each time point was measured (mean of triplicate determinations) and a concentration versus time

curve was plotted. Maximum plasma concentration ($C_{\max}$) and $T_{\max}$ (time to reach $C_{\max}$) were determined directly from each plot. Other PK parameters were calculated using PK Solver 2.0 software (29), after normalizing to pig weight. The area under the curve from time zero to the last measurement time point (AUC_{0-t}) was calculated using the linear trapezoid method. Linear regression analysis of the last three Cx plasma concentration points (for the intraduodenal administrations) or last four Cx concentration points (for the IV administrations) was used to estimate the elimination rate constant (λ), which was used for calculation of the terminal half-life ($t_{1/2}$ terminal) as follows:

$$t_{1/2} = \frac{\ln 2}{\lambda} \quad (1)$$

The absolute bioavailability (F) was calculated based on dose/weight normalized plasma concentrations as follows:

$$F = \frac{AUC_{0-t(\text{intraduodenal})}}{AUC_{0-t(IV)}} \times 100 \quad (2)$$

The relative bioavailability (F') was calculated based on dose/weight normalized plasma concentrations as follows:

$$F' = \frac{AUC_{0-t(\text{bCN})}}{AUC_{0-t(\text{Celebra})}} \times 100 \quad (3)$$

Results and discussion

Cx encapsulation efficiency of Cx/bCN

Quantitative analysis of Cx/bCN revealed an exceptionally high Cx encapsulation efficiency of 100%, resulting in a Cx:bCN mole ratio of 9.54:1 (see Table 1).

Temperature effect on Cx/bCN

Turbidity measurements (Figure 1) show that while turbidity was similar at the beginning of each study, after 60 min of incubation at 37°C, the Cx/bCN micellar dispersion became turbid (the 600/430 nm OD ratio increased to 0.9). This increase in turbidity is attributed to a heat-induced Cx release from bCN micelles. Verification by HPLC analysis indicated that 96% of the encapsulated drug was released from the bCN micelles after incubation at 37°C for 60 min (Table 2). This finding implies that Cx can be efficiently released from Cx/bCN upon exposure to a physiologically-relevant temperature and become available for absorption following intraduodenal administration of Cx/bCN.

In vitro dissolution studies

We compared the rate and extent of Cx release from Cx/bCN to those of the Celebra® formulation. Figure 2 shows

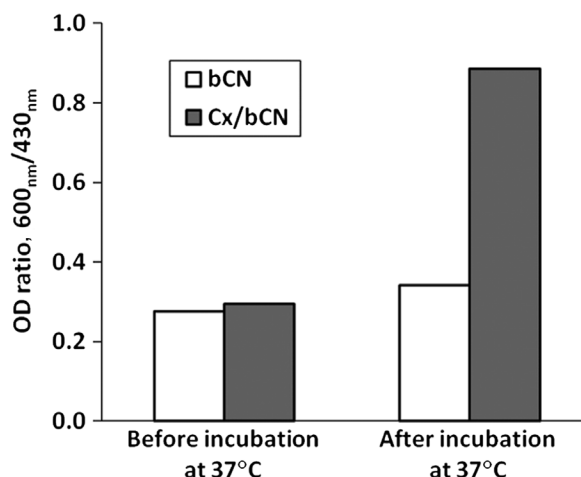


Figure 1 Effect of incubation at 37°C on turbidity of bCN and Cx/bCN.

that the release kinetics in both formulations was dependent on Cx solubility in the dissolution medium. The addition of the surface active agent, SLS, to SIF increased profoundly (5-fold) Cx release rate in both formulations. However, the dissolution test in the more physiologically-relevant medium (FaSSIF-V2, which can solubilize lower amounts of Cx, as compared to SIF/SLS 1%), showed reduced Cx release from both formulations (Figure 2B).

The 10-min lag phase in the dissolution process of both formulations could be attributed to the time required for the capsule shell to dissolve. In presence of SLS, Cx release from Cx/bCN micelles was slower (43% and 72% of the drug released at 15 and 20 min, respectively). While Cx release from Cx/bCN was completed within 30 min, release from the Celebra® formulation was completed within 20 min (Figure 2A). This may be explained by increased amount of SLS in the Celebra® dissolution system due to the presence of SLS in the Celebra® formulation. The outcomes of the dissolution studies should be interpreted with caution (30). In our studies, there was no clear correlation between the outcomes of the in vitro dissolution studies and the outcomes of the in vivo pig study.

Table 2 Encapsulated Cx:bCN mole ratio before and after incubation at 37°C for 60 min (average of 3 measurements).

Cx/bCN	Encapsulated Cx		bCN ^a		Encapsulated Cx:bCN mole ratio
	mg/mL	mM	mg/mL	mM	
Before incubation	0.968	2.54	10	0.42	6.09
After incubation	0.039	0.10	10	0.42	0.25

^aCalculated value.

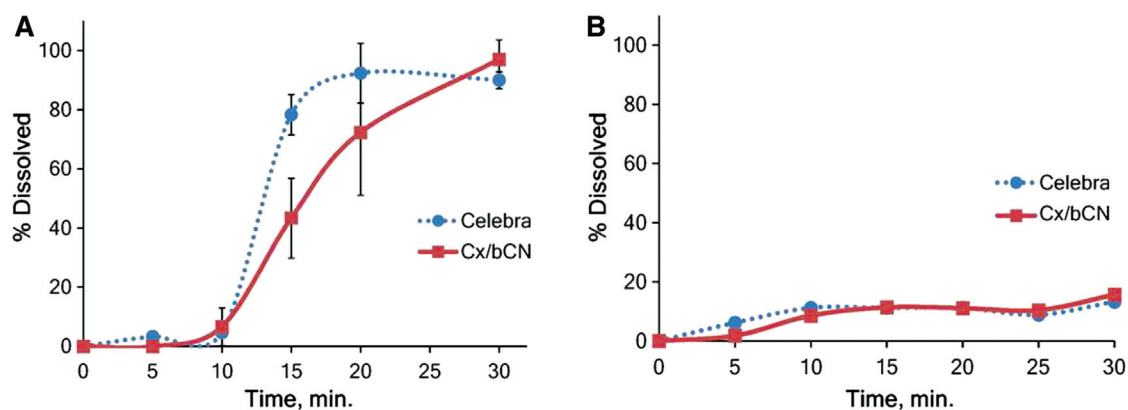


Figure 2 Release of Cx from Cx/bCN and from Celebra® formulations. (A) Release into SIFSLs 1% (sink conditions). (B) Release into FaSSIF-V2.

In vivo studies in pigs

The resolution of the HPLC method used in this study (see Figure 3) allowed reliable quantification of Cx in the samples and subsequent calculation of PK parameters. The rapid weight gain of the pigs during the study led to the need to normalize the PK parameters to each individual animal weight. After IV injection of 0.5 mg/kg Cx in PEG 400/saline solution to the pigs, Cx pharmacokinetics was biphasic (Figure 4A). Plasma concentration declined rapidly within an initial distribution phase (up to 30 min), and much slower at later time points, suggesting extensive tissue distribution. This observation is in agreement with a previous report in which a two-compartment model was found to best describe Cx plasma levels after IV injection in pigs (31). Overall, the IV data are characterized by high inter-animal variability. The estimated mean C_0 was 1.96 kg/L (Table 3), indicating a mean initial volume of distribution (V_0/D) of 0.53 L/kg, again suggesting substantial

extravascular distribution of Cx in the experimental animals. High inter-animal variability of terminal half-lives ($t_{1/2}$ terminal) precluded reliable estimation of $AUC_{0-\infty}$ values (and of the clearance and the steady-state volume of distribution values). Therefore, the subsequent calculation of bioavailability (see below) was based on AUC_{0-t} values (area under the curve from time zero to last measurement time points).

After intraduodenal administrations of Celebra® and Cx/bCN formulations, Cx plasma concentration vs. time curves differed profoundly between the individual pigs (Figure 4B and C). Nevertheless, some prominent trends could be observed. For each individual pig (except for pig #251, which may be an outlier based on the IV data), Cx plasma concentrations reached their maximum values faster after administration of Cx/bCN compared to Celebra® formulation. Mean T_{max} values of Cx/bCN and the Celebra® formulation were 330 min (5.5 h) and 210 min (3.5 h), respectively (Table 4). This 1.57-fold difference

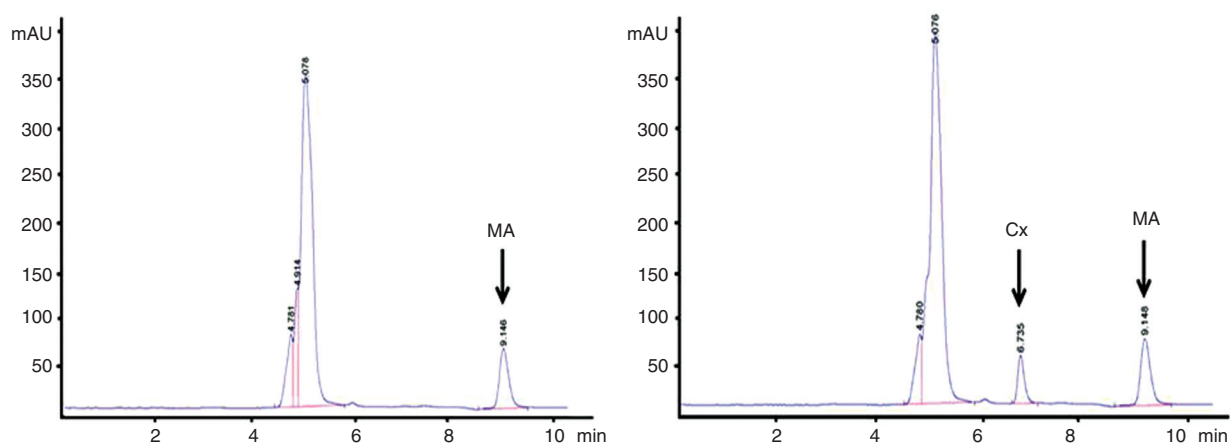


Figure 3 Representative HPLC chromatograms of pig plasma with MA as an internal standard without Cx (left panel) and with spiking of Cx 500 ng into pig plasma sample (right panel).

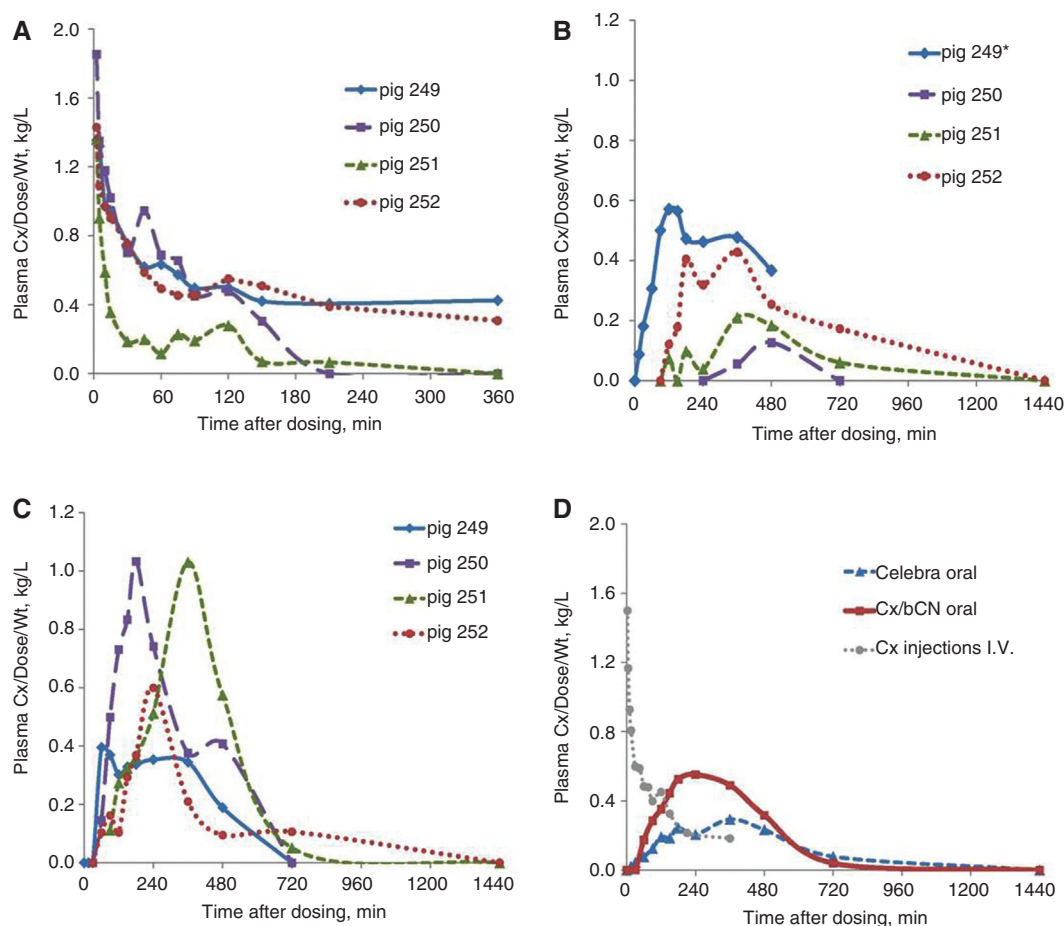


Figure 4 Cx individual PK profiles (normalized to dose and weight) in pigs. After: A. IV administration of 0.5 mg/kg Cx; B. Intraduodenal administration of 2 mg/kg of Celebra® formulation; C. Intraduodenal administration of 2 mg/kg of Cx/bCN formulation; D. Average curves of Figure 4A, B and C.

*Terminated at 9 h after dosing due to problematic recovery from anesthesia.

in mean $T_{\max}$ values reflects a faster in vivo dissolution of Cx from Cx/bCN vs. the Celebra® formulation. Mean maximum plasma concentration ($C_{\max}$) was 2.3-fold higher for Cx/bCN (0.77 kg/L) than the Celebra® formulation (0.34 kg/L). Similarly, a 1.76-fold higher mean AUC_{0-t} value was obtained following intraduodenal administration of Cx/bCN compared to the Celebra® formulation (215 and 122 kg*min/L, respectively), indicating more efficient absorption of the drug from Cx/bCN.

Bioavailability analysis

Unfortunately, statistically significant differences in the analyzed PK parameters could not be found due to high variability between pigs and the small number of pigs used in the study ($n=4$). Nevertheless, data analysis (Table 5) indicates that absolute BA of Cx was higher following intraduodenal administration of Cx/bCN (171%) as compared to Celebra® formulation (97%). These values are

Table 3 Cx PK parameters (normalized to pig weight) following IV injection of 0.5 mg/kg Cx.

Pig #	$T_{\max}$, min	$C_{\max}$, kg/L	C_0 , kg/L	V_0 , L	V_0/D , L/kg	$t_{1/2}$ terminal, min	AUC_{0-t} , kg*min/L
249	2.5	1.35	1.39	15.1	0.72	1690 ^a	182.8
250	2.5	1.85	2.54	9.0	0.39	81	102.9
251	2.5	1.36	2.05	11.7	0.49	62	44.7
252	2.5	1.43	1.87	12.8	0.53	287	171.7
Mean±SD	2.5±0	1.50±0.3	1.96±0.4	12.2±1.9	0.53±0.1	143 ^a ±125	125.5±64

^aNot used for calculations.

Table 4 Cx PK parameters (normalized to pig weight) following intraduodenal administration of 2 mg/kg Celebra® or Cx/bCN formulations to pigs.

Formulation	Pig #	$T_{\max}$, min	$C_{\max}$, kg/L	AUC_{0-t} , kg*min/L
Celebra®	249	120	0.57	205.8
	250	480	0.13	20.6
	251	360	0.21	81.3
	252	360	0.43	178.4
	Mean±SD	330±151	0.34±0.2	121.5±86
Cx/bCN	249	60	0.40	144.5
	250	180	1.03	250.3
	251	360	1.03	319.0
	252	240	0.60	146.3
	Mean±SD	210±125	0.77±0.3	215.0±85

higher than expected; in a similar study in dogs, Celebra® had a much lower absolute BA of 22%–40% (5). In our study, the absolute BA, which exceeded 100% after administration of Cx/bCN, was exceptional and unexpected. Unless the IV exposure was underestimated, the only way to explain this observation is that bCN altered the PK profile of Cx, similar to findings reported by Nassar and co-workers (32) for polymer-based nanoparticles. Alteration of PK profiles by internalization of intact micelles can contribute to increased oral BA (33–35), as demonstrated for modified chitosan or modified PLGA micelles. For protein-based nanocarriers, their hydrophilic nature is expected to induce rapid drug release, as demonstrated by our turbidity measurements (Figure 1, Table 2). In addition, bCN is biodegradable and is expected to be digested in the GI tract, further promoting drug release. Therefore, in order to determine the reason for the high absolute BA of Cx/bCN, further investigations into the fate of Cx/bCN micelles in vivo are required. It is possible that the capsules have transited through “dry segments” in the pig intestines, similar to those found in humans (36), leading to the significant variations observed in the individual plasma concentration vs. time profiles. An additional potential contributing factor could be the prolonged

Table 5 The calculated absolute and relative BA of the various Cx formulations in pigs (n=4).

Formulation	Dose- and weight-normalized AUC_{0-t} Mean±SD, kg*min/L	Absolute F, %	Relative F', %
Cx, IV bolus	126±64	100	–
intraduodenal Celebra®	122±86	97	100
intraduodenal Cx/bCN	215±85	171	176

presence of the formulations in the pigs' GI tracts. Based on an estimated general intestinal transit time of 3–4 h in pigs (37), it is possible that the capsules could have reached the colon, which may be more susceptible to absorption enhancement.

The relative bioavailability of Cx/bCN was 1.76-fold higher than that of the Celebra® formulation (Table 5). This increase in systemic exposure, which was accompanied by lower $T_{\max}$ values of Cx/bCN, indicates that this formulation was characterized by more rapid and efficient Cx dissolution in the GI tract. This outcome may stem from the fact that the Celebra® formulation contained micron-sized crystalline drug (11, 38) while Cx in Cx/bCN is nano-sized and amorphous (15). Enhanced dissolution rate and oral bioavailability of Cx in an amorphous nano-formulation was previously reported (12). It should be noted that intraluminal concentration of a drug is not necessarily limited by its thermodynamic solubility in gastrointestinal fluids, as the drug could be presented in a supersaturated state (8, 39) which can contribute to increased bioavailability (39).

Another possibility that should be investigated is a possible direct effect of bCN (below and/or above its CMC) on the intestinal epithelium. Surfactants are routinely employed to increase the solubility of poorly-soluble drugs, yet the impact of micellar solubilization on intestinal membrane permeability of a drug is often overlooked. Increased uptake of polymeric micelles as a result of direct effects, such as increase in membrane permeability and/or inhibition of P-gp efflux pump, has been reported (35). Membrane fluidity could also be affected, as demonstrated for pluronic micelles (40). Soluble amphiphiles, or detergents, are known to produce structural and dynamic effects on membranes, even at low concentrations. This includes transmembrane lipid motion (flip-flop), breakdown of the membrane permeability barrier (leakage), and vesicle lysis/reassembly (41).

With respect to tolerability, according to the pigs' physical and behavioral parameters monitored throughout the study, no signs of stress or disease were detected. The pigs recovered well from anesthesia (except for pig #249, which, in the last study phase showed signs of discomfort upon waking up until it died 9 h after Celebra® administration). Overall, the pigs showed good tolerability to all formulations, including Cx/bCN.

Conclusions

Beta-casein micelles were developed as a platform for improved oral BA for poorly water-soluble drugs, as

demonstrated for Cx in this study. High encapsulation of Cx in the Cx/bCN formulation was achieved, indicating feasibility of a larger scale production than previously done. In vitro dissolution testing for Cx/bCN vs. Celebra® formulations under sink and non-sink conditions demonstrated rapid release of Cx from both formulations. Incubating the Cx/bCN dispersion in aqueous buffer at 37°C showed almost complete drug release. Cx/bCN significantly enhanced Cx bioavailability, as compared to the Celebra® formulation, although the results could not be supported statistically due to high variability and small number of pigs in this study. The 1.76-fold increase in systemic exposure (AUC) of Cx and the 1.57-fold smaller $T_{\max}$ values of Cx/bCN indicate a higher rate and extent of Cx dissolution in vivo, compared to the Celebra® formulation. Further investigation of the mechanism(s) of BA enhancement and development of alternative dissolution methods may lead to further optimization of the new Cx/bCN formulation.

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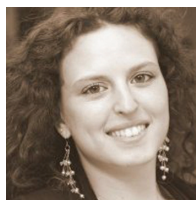
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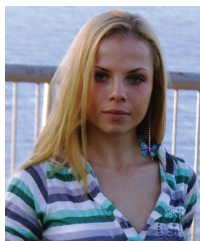
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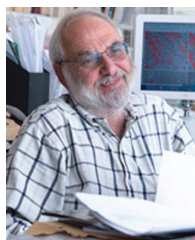
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