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Pharmacological and Pharmacokinetic Evaluation of Celecoxib Prodrugs in Rats

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ABSTRACT: This study demonstrates the utility of an *in vitro – in vivo* correlative approach in the selection and optimization of a prodrug candidate of celecoxib (CBX), a COX_2 inhibitor. As an initial screening step, a comparative single oral dose pharmacokinetic study was conducted in rats for CBX and its three aliphatic acyl water-soluble prodrugs viz., CBX-acetyl (CBX-AC), CBX-propionyl (CBX-PR) and CBX-butyryl (CBX-BU) at high equimolar dose, 100 mg/kg. Only CBX-BU and CBX-PR converted rapidly to CBX and yielded approximately five-fold greater systemic exposure of CBX than CBX alone or CBX-AC. Rank order of systemic exposure of prodrugs in its intact form was CBX-AC > CBX-PR > CBX-BU. Further *in vitro* hydrolysis studies of CBX prodrugs in intestinal mucosal suspensions and liver homogenates indicated that CBX-BU is rapidly and completely converted to CBX, whereas CBX-PR and CBX-AC require longer incubation period for complete conversion to CBX. There was a very good correlation of the *in vitro* and *in vivo* data supporting CBX-BU as the prodrug of choice. Further i*n vitro* pharmacological studies showed that COX_2 selective inhibition is improved for CBX-BU as compared to CBX-AC and CBX-PR. Dose proportionality in pharmacokinetic studies of CBX-BU and CBX at equimolar oral doses confirmed that relative oral bioavailability of CBX was improved following CBX-BU administration and there was linearity in pharmacokinetics of CBX over a wide dose range (10–100 mg/kg), whereas CBX in its conventional form showed poor bioavailability and lack of dose linearity in pharmacokinetics. The oral bioavailability of CBX from CBX-BU was dose independent and was in the range 78–96%. At a 50% reduced molar dose, CBX-BU showed an equivalent efficacy to that of CBX in the *in vivo* carrageenan model. Based on the study, water-soluble CBX-BU prodrug can be considered for clinical development in view of its potential advantages.

Key words: celecoxib; prodrugs; pharmacokinetics

Introduction

The discovery that the enzyme cyclooxygenase (COX), exists in two isoforms namely the constitutive cyclooxygenase (COX-1) and the inducible isoform cyclooxygenase (COX-2), opened up a novel approach to treat inflammatory disorders. It is well documented that inducible form, COX-2, is associated with inflammatory conditions and pain, whereas the constitutively expressed enzyme, COX-1, is responsible for the cytoprotective effects of prostaglandins [1]. Differential role of COX isoforms in inflammatory processes and gastric ulceration led to discovery of selective COX-2 inhibitors, which are thought to be devoid of gastric

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disturbances [2]. Over expression of COX-2 enzyme is not only limited to inflammatory process, but has also been observed in various types of cancer (colon, lung, breast, prostate, bladder, pancreatic, skin and gastric) [3]. Clearly, intervention with selective COX-2 inhibitors, is gaining momentum with the demonstration of active role of COX-2 enzyme in the broader range of diseases/disorders.

Celecoxib (CBX), 4-[5-(4-methyl)-3-trifluoromethyl-1H-pyrazoyl-1-yl]benzene sulphonamide (**1**), is the first specific inhibitor of COX-2 approved by US FDA for the treatment of patients with rheumatoid arthritis, osteoarthritis, and pain management. CBX has proven to be equi-efficacious when compared with other commonly used non-sterodial anti-inflammatory drugs, including diclofenac, naproxen and ibuprofen [4]. Recently, it has been shown in clinical trials that CBX 400 mg twice a day for 6 months is efficacious in regressing the intestinal tumors and polyps [5].

In general, COX-2 inhibitors of the diarylheterocycle class such as celecoxib, rofecoxib and valdecoxib possess modest aqueous solubility. This physicochemical parameter has a profound influence on the oral absorption as well as on the design of other dosage forms. Previously, Talley *et al*. [6] employed prodrug approach to develop water-soluble parecoxib for parenteral administration for management of acute pain and it is currently under clinical evaluation. CBX exhibits dose-dependent pharmacokinetics at oral doses higher than 200 mg in humans, because of poor solubility in gastric environment. As a result, the systemic exposure of CBX increases less than proportional to the administered dose [7].

In this paper, we have attempted synthesis of various water-soluble prodrugs of CBX and demonstrated the advantages of oral administration of prodrugs compared to conventional CBX based on the release kinetics of CBX in both *in vitro* and *in vivo* systems. Based on the release kinetics, one of the prodrugs was further considered for a detailed pharmacokinetic characterization including oral bioavailability and dose proportionality studies in rats. Our criteria of evaluation of an ideal prodrug were based on water solubility, efficacy parameter (comparable or superior to CBX), complete and rapid cleavage to the parent drug (CBX) *in vivo*.

Materials and Methods

Materials

CBX (B.No: ECO-5RRC, purity 99.8%) was procured from Dr Reddy's Laboratories Ltd., Hyderabad, India. NADP, glucose-6-phosphate (G-6-P) and G-6-P dehydrogenase were purchased from Sigma Chemical Company (St Louis, MO, USA). All other chemicals and solvents used were of the highest quality commercially available. Thin-layer chromatography was performed on silica gel plates (60 F254, Merck, Germany).

Instruments

Flash chromatograph (Aldrich, USA), melting point apparatus (Veego, India), NMR spectrometer 200 MHz (Varian Gemini, Germany), FT-IR spectrometer (Perkin-Elmer, UK) and HPLC system with UV detector (Shimadzu, Japan) were used in the synthesis, characterization and analysis of test compounds.

Methods

Synthesis of Prodrugs. CBX (**1**) was acylated using acetic and propionic anhydrides in the presence of triethyl amine, to yield **2** and **3**, respectively, whereas **4** was prepared by treating **1** with a mixed anhydride obtained from butyric acid and pivaloyl chloride in the presence of triethyl amine (Figure 1). These acylated products were then converted into corresponding sodium salts i.e. CBX-AC (**5**), CBX-PR (**6**) and CBX-BU (**7**) by treating each of them with sodium bicarbonate in methanol. The yields in both the steps were very good and the purity of the final compounds was uniformly >98%, as determined by HPLC with UV detection at 255 nm. The products were characterized by spectroscopic methods.

Animals. Male Wistar rats, 10 to 12 weeks old (weight range, 200–220 g) were obtained from National Institute of Nutrition, Hyderabad, India. Animals were quarantined and acclimatized to our laboratory conditions for a period of 7

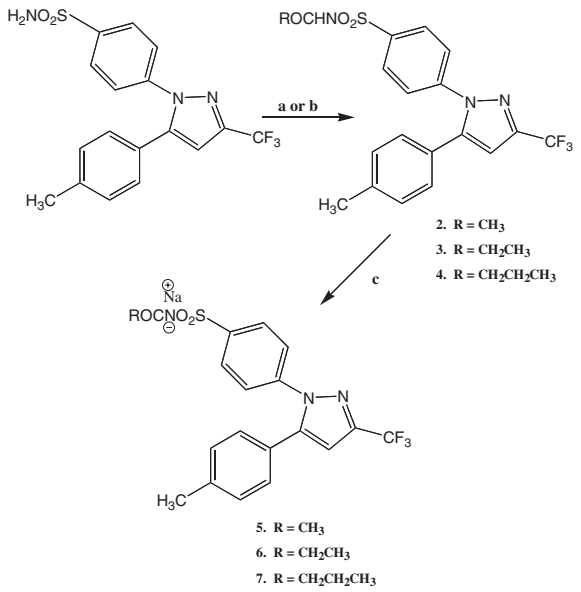

Figure 1. Synthesis of celecoxib prodrugs. Reagents: (a) $(RCO)_2O$, TEA, CH_2Cl_2; (b) n-Butyric acid, pivaloyl chloride, TEA, CH_2Cl_2; (c) $NaHCO_3/CH_3OH$

days. Feed and water were given *ad libitum*. Dr Reddy's Research Foundation Institutional Ethics Committee approved all procedures and protocols in the experimental design.

Solubility determination by HPLC. Test compound was added in excess quantities to water and kept under constant shaking in a water bath maintained at 37°C for 24 h. Saturated solution was obtained by separating the undissolved solute by filtration through 0.22 μM Millipore (Millex GV4) filter unit and concentration of test compound in the resultant solution was determined by HPLC. Separate calibration curves were obtained by plotting the peak areas of standards as a function of drug concentration.

Single dose pharmacokinetics in Wistar rats. After an overnight fast, CBX, CBX-AC, CBX-PR and CBX-BU (100 mg/kg, CBX equivalent) were administered orally either as a suspension in 0.25% sodium carboxy methyl cellulose (in case of CBX) or as solution in water (prodrugs of CBX). Blood samples (0.25 ml) were collected from retro-orbital plexus at designated time points (0.5, 1, 2, 3, 5, 8, 12 and 24 h) into glass tubes containing 5% EDTA (20 μl per ml of blood). Plasma was obtained by centrifuging the blood using a tabletop centrifuge (Remi Instruments, R-24, Mumbai, India) and concentrations of CBX and its prodrug were determined on-line. In a separate experiment, single dose intravenous pharmacokinetics of CBX was carried out at 10 mg/kg dose by administering CBX through tail vein and blood samples were collected at designated time points (0.17, 0.5, 1, 2, 4, 6, 8 and 12 h) and plasma was obtained by centrifugation of blood and stored at −20°C until the completion of analysis. In another experiment, dose proportionality pharmacokinetic study was carried out with CBX and CBX-BU at two different doses (10 and 30 mg/kg, p.o equivalent to CBX) in overnight fasted rats. Blood samples (0.25 ml) were collected at designated time points (0.5, 1, 2, 3, 5, 8, 12 and 24 h). Plasma was obtained by centrifugation of blood, processed and analyzed on-line to determine the concentrations of CBX and CBX-BU.

Preparation of in vitro test systems. After an overnight fast, animals were anaesthetized (pentobarbitone sodium, 60 mg/kg, i.p) and whole blood was collected by cardiac puncture into glass tubes containing 5% EDTA solution (20 μl per ml of blood). Plasma was separated by centrifuging the blood using a tabletop centrifuge and was immediately used for the hydrolysis studies. Standard surgical procedure was employed to remove duodenum and liver for the preparation of intestinal mucosal suspension and liver homogenate. After flushing with saline, duodenum (5 cm piece) was cut open and mucosa was scraped off with a glass slide and suspended in 5.0 ml of 0.85% NaCl–10 mM phosphate buffer, pH 7.4. This suspension was used for the hydrolysis studies. The liver was perfused through the portal vein with ice-cold saline to drain off blood and minced with five volumes of 1.15% KCl solution, and finally centrifuged at 10000*g* for 30 min to obtain the liver homogenate and was used on the same day for *in vitro* studies [8].

Hydrolysis studies. Hydrolysis studies for the three prodrugs were carried out in freshly

collected rat plasma, intestinal mucosal preparations and in rat liver homogenates in order to determine the rate as well as site of hydrolysis. The reaction mixture consisting of the prodrug solution (1 mg/ml methanol, 50 µl), biological matrix (1.0 ml of plasma/intestinal mucosal suspension/liver homogenate preparation) and 0.1 M phosphate buffer, pH 7.4, (0.5 ml) was incubated at 37°C. At appropriate time intervals (0, 0.25, 0.5, 1, 2 and 3 h), a 100 µl aliquot was withdrawn and the samples were processed and analyzed online by HPLC. CBX and corresponding prodrugs present in the reaction mixture were measured at each time point and hydrolytic rate profiles were generated.

In vitro inhibition studies with COX-1 and COX-2 enzymes. Microsomal fraction of ram seminal vesicles was used as a source of COX-1 enzyme and microsomes from sf-9 cells infected with baculovirus expressing human COX-2 cDNA was used as a source of COX-2 enzyme. Enzymatic activities were measured using a chromogenic assay based on oxidation of N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) spectrophotometric method [9] during the reduction of PGG_2 to PGH_2. The assay mixture (500 µl) contained 100 µM Tris pH 8.0, 3 mM EDTA, 15 µM hematin, ~150 units enzyme and 1% DMSO. The mixture was incubated at 25°C for 15 min before initiation of enzyme reaction in presence of compound/vehicle. The reaction was initiated by the addition of 10 µM arachidonic acid and 120 µM TMPD. The enzyme activity was measured by estimation of the initial velocity of TMPD oxidation over the first 25 s of the reaction as followed from the increase in absorbency at 605 nm.

Carrageenan-induced paw edema. CBX and CBX-BU were either suspended in 0.25% CMC or dissolved in water and administered orally to male Wistar rats (weight range: 120–140 g) at different doses (1, 3, 10 and 30 mg/kg) 2 h before carrageenan injection. Hind paw edema was induced in rats by intradermal injection of 50 µl of 1% λ-carrageenan in saline into the plantar aponeurosis of the right hind foot pad [10]. Paw volume was measured before and 3 h after carrageenan injection using plethysmometer (Ugo-Basile, Italy). Paw edema was compared with the vehicle control group and percent inhibition was calculated with reference to control and ED_{50} values were calculated using linear regression plot.

Bioanalysis. A validated HPLC method (to be published elsewhere) was employed for the simultaneous measurement of CBX and its prodrugs in the plasma, intestinal mucosal suspension and liver homogenate. Aliquots of blood collected during the pharmacokinetic studies were immediately centrifuged to obtain plasma and were extracted with dichloromethane/ethyl acetate (1:1, v/v) mixture. After evaporation of the organic solvent, the residue was reconstituted in mobile phase and analyzed by C-18 reverse phase HPLC (Kromasil, KR100, C-18, 5 µm, 4.6 mm × 250 mm) column. The mobile phase [35% of 0.01 M KH_2PO_4 buffer (pH 3.2): 65% of acetonitrile mixture, v/v] was delivered at 1 ml/min, and effluents were monitored at 255 nm. Another diaryl pyrazole derivative (DRF-6726) was used as an internal standard (IS). Retention times of CBX, CBX-AC, CBX-PR, CBX-BU and IS in HPLC are 8.2, 9.3, 11.6, 14.4 and 22.3 min, respectively (Figure 2). Plasma sample collection and processing were carried online in order to avoid plasma amidase activity during retention on bench and to obtain reliable and accurate data of hydrolytic rate between the CBX prodrugs.

Data analysis. Pharmacokinetic parameters were calculated by non-compartmental model analysis. The peak plasma concentration (C_{max}) and the corresponding time (T_{max}) were directly obtained from the raw data. The area under the plasma concentration versus time curve up to the last quantifiable time point, $AUC_{(0-t)}$ was obtained by the linear and log – linear trapezoidal summation. The $AUC_{(0-t)}$ extrapolated to infinity (i.e. $AUC_{(0-\infty)}$) by adding the quotient of C_{last}/K_{el}, where C_{last} represents the last measurable time concentration and K_{el} represents the apparent terminal rate constant. K_{el} was calculated by the linear regression of the log-transformed concentrations of the drug in the terminal phase. The half-life of the terminal elimination

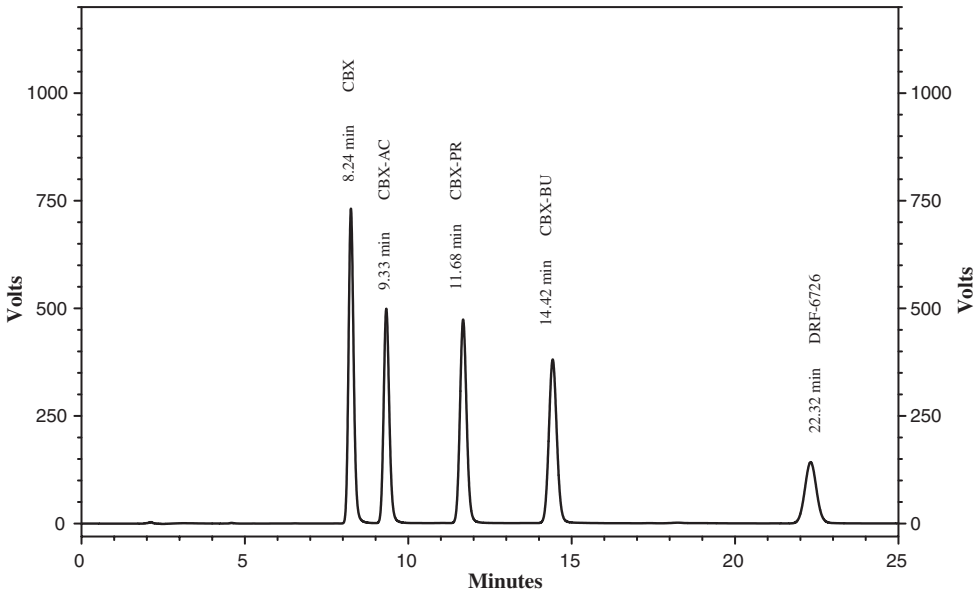

Figure 2. HPLC chromatogram showing Celecoxib (CBX), CBX-acetyl (CBX-AC), CBX-propionyl (CBX-PR) and CBX-butyryl (CBX-BU) and DRF-6726 (Internal standard)

phase was obtained using the relationship $t_{1/2} = 0.693/K_{el}$. Area under the first moment curve ($AUMC_{(0-t)}$) obtained from equation ${}_0\Sigma^t[(t_n - t_{n-1}) * (C_n * t_n + C_{n-1} * t_{n-1})]/2$ and is extrapolated to infinity ($AUMC_{(0-\infty)}$) by adding the quotient $C_n * t_n/K_{el}^2 + C_n/K_{el}$. Mean residence time (MRT) is calculated from the ratio of AUMC/AUC. Oral bioavailability (f) was calculated with the equation: $(AUC_{oral} * Dose_{i.v.} * 100)/(AUC_{i.v.} * Dose_{oral})$, where oral doses were expressed as CBX equivalents.

Statistical analysis. The data was statistically analyzed using Sigma Stat (Scientific software, Jandel Scientific, ver: 2.0, USA). A minimum p-vlaue of 0.05 was used as the significant level for all tests. One-way analysis of variance (ANOVA), Student t-test and Bonferroni's method were performed on pharmacokinetic parameters of CBX and its prodrugs. Data are reported as Mean $\pm$ S.D. Linear regression analysis was performed on the results obtained from dose proportional pharmacokinetic studies.

Results

Single dose pharmacokinetics of CBX and its prodrugs

Plasma concentrations and pharmacokinetic parameters of CBX after oral administration of CBX, CBX-AC, CBX-PR and CBX-BU at the same dose (equivalent to 100 mg/kg) are shown in Figure 3 and Table 1, respectively. Pharmacokinetic parameters of prodrugs as such (in its intact form) following administration of CBX-AC, CBX-PR and CBX-BU are in Table 1. Pharmacokinetic parameters of CBX following *intravenous* administration are given in Table 2.

In vitro hydrolysis of prodrugs

An essential pre-requisite for prodrug effectiveness is its ability to rapidly and completely release the parent drug after oral administration. To characterize the tissues or organs capable of hydrolyzing acyl bond of the prodrugs, hydrolytic activity was measured using plasma, intestinal mucosal suspension and the liver

homogenate. Hydrolytic profiles of CBX prodrugs in plasma, liver and intestinal mucosa are given in Figure 4(a)–(c).

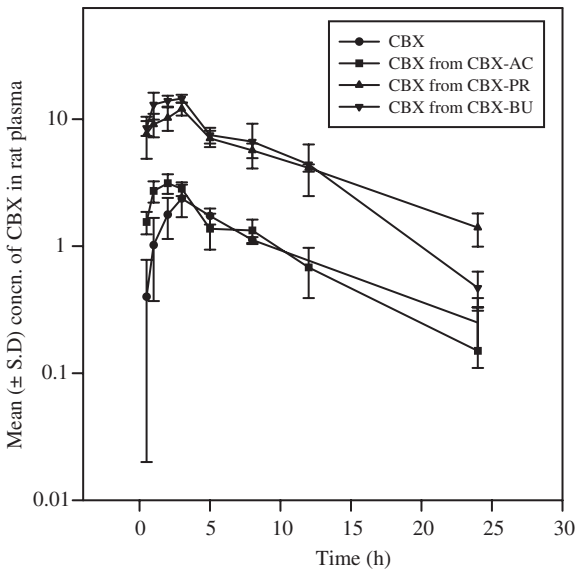

Figure 3. Semi logarithmic plots of mean plasma concentration time curves of celecoxib (CBX) in rats following oral administration of CBX, CBX-acetyl (CBX-AC), CBX-propionyl (CBX-PR) and CBX-butyryl (CBX-BU) at 100 mg/kg (Molar equiv. of CBX)

Dose proportionality in pharmacokinetics of CBX

CBX declines in a parallel fashion across the dose panels for both CBX alone and CBX-BU administration. Pharmacokinetic parameters of CBX following administration of CBX and CBX-BU are given in Table 3.

In vitro inhibitory activity against COX-1 and COX-2 enzymes

CBX prodrugs, CBX-AC, CBX-PR and CBX-BU, showed negligible to modest inhibitory activity against recombinant isoforms of cyclooxygenases, ram COX-1 and h COX-2, at 10 μM. Percent inhibitory activities of CBX-AC, CBX-PR and CBX-BU against COX-2 isoform are 0, 9 and 55, respectively, and against COX-1 isoform are 11, 22 and 0, respectively. CBX exhibited 100% inhibition of COX-2 isoform, without any inhibition of COX-1 isoform at 1 μM concentration.

In vivo carrageenan-induced rat paw edema

Comparative evaluation of CBX and CBX-BU was carried out in the carrageenan model following oral administration. The ED_{50} of CBX and CBX-BU was found to be 10 and 6.2 mg/kg (equivalent to 4.9 mg/kg of CBX on a molar basis), respectively.

Table 1. Mean (± S.D.) pharmacokinetic parameters of celecoxib (CBX) and its prodrugs, CBX-acetyl (CBX-AC), CBX-propionyl (CBX-PR) and CBX-butyryl (CBX-BU) following administration to male Wistar rats at 100 mg/kg[a]

Parameters for CBX	CBX	CBX released from CBX-AC	CBX released from CBX-PR	CBX released from CBX-BU
$AUC_{(0-t)}$ (μg h/ml)	23.2 ± 1.2	24.7 ± 3.4	118 ± 9*	129 ± 24*
$AUC_{(0-\infty)}$ (μg h/ml)	28.2 ± 3.4	26.2 ± 4.9	134 ± 13*	132 ± 24*
C_{max} (μg/ml)	2.5 ± 0.6	3.3 ± 0.5	12.1 ± 1.4*	16.0 ± 1.6*
T_{max} (h)	3.2 ± 1.3	2.0 ± 0.8	3.0 ± 0.0	2.5 ± 1.0
K_{el} (h^{-1})	0.13 ± 0.03	0.16 ± 0.05	0.09 ± 0.02	0.16 ± 0.03
$T_{1/2,\beta}$ (h)	5.8 ± 1.8	4.8 ± 2.2	7.9 ± 1.5	4.4 ± 0.7
$AUMC_{(0-t)}$ (μg h^2/ml)	173 ± 39	163 ± 38	928 ± 51*	856 ± 232*
$AUMC_{(0-\infty)}$ (μg h^2/ml)	181 ± 47	169 ± 42	945 ± 57*	871 ± 232*
MRT (h)	6.8 ± 0.8	6.4 ± 0.5	7.0 ± 0.3	6.5 ± 0.5
f (%)	16	15	79	78
Parameters for CBX prodrugs		CBX-AC	CBX-PR	CBX-BU
$AUC_{(0-t)}$ (μg h/ml)		21.1 ± 2.9	18.4 ± 5.6	5.0 ± 1.0**
C_{max} (μg/ml)		2.9 ± 0.4	20.4 ± 8.6**	4.5 ± 2.4
t_{max} (h)		2.0 ± 0.8**	0.5 ± 0.0	0.5 ± 0.0

[a] $n = 3-4$ animals. *$p < 0.05$ from the CBX treated (control) group. **$p < 0.05$ in pairwise multiple comparison procedures by Bonferroni's method.

Table 2. Mean ($\pm$ S.D.) pharmacokinetic parameters of celecoxib following *intravenous* administration to male Wistar rats at 10 mg/kg[a]

Parameter	Mean $\pm$ S.D
$AUC_{(0-t)}$ (μg h/ml)	15.5 $\pm$ 1.7
$AUC_{(0-\infty)}$ (μg h/ml)	16.9 $\pm$ 2.3
C_0 (μg/ml)	5.1 $\pm$ 0.3
K_{el} (h^{-1})	2.0 $\pm$ 0.8
$t_{1/2,\beta}$ (h)	3.5 $\pm$ 0.7
Cl (l/h/Kg)	0.6 $\pm$ 0.1
V_d (l/Kg)	3.0 $\pm$ 0.3

[a] $n = 4$ animals.

Discussion

In the present study, an *in vivo – in vitro* correlative approach was used to identify an optimal prodrug candidate of CBX. CBX and related diaryl heterocycle class of drugs possess modest aqueous solubility and because of this property, CBX exhibits dose-dependent pharmacokinetics at oral doses higher than 200 mg in humans. Talley *et al*. [6] demonstrated that the prodrug approach to achieve water solubility needed for parenteral administration of valdecoxib.

We devised a prodrug approach for CBX, whereby we could potentially take advantage of the occurrence of pre-systemic metabolism, to enable both rapid and complete conversion of the prodrug to the active parent moiety. The produrg approach has been commonly reported in the literature for other agents [11–13]. The design of our experiments was aimed to unequivocally identify the best prodrug with excellent water solubility properties and hydrolytic cleavage kinetics, which then would translate into an enhanced bioavailability and well-behaved pharmacokinetics of CBX *in vivo*. However, it is essential, that the pharmacological activity of CBX should not be compromised, as a result of delivering CBX via the prodrug approach.

Solubility of these prodrugs in water was similar and was found to be $\sim$15 mg/ml, whereas CBX is practically insoluble in water ($<$50 μg/ml). Although prodrugs have similar water solubility, their *in vivo* CBX releasing properties are different. The plasma levels of CBX after oral administration of CBX as such and CBX-AC were lower than compared with those after administration of CBX-PR and CBX-BU. It is evident from Figure 2, that the appearance of CBX at the first time points (0.5 h) in plasma, regardless of the prodrug, suggested that the hydrolytic cleavage is happening pre-systemically. Systemic exposures of CBX following administration of CBX and CBX-AC were similar, whereas systemic exposure of CBX increased by four-fold following its administration of CBX-PR and CBX-BU. Therefore, if one computes the relative bioavailability of CBX when given alone to those after CBX-PR and CBX-BU prodrug administrations, several fold increase in the parameter was noted. The relative bioavailability of CBX from CBX-AC, CBX-PR and CBX-BU were 99, 508 and 500%, respectively, relative to CBX administered. Regardless of prodrugs or CBX administration, the half-life of CBX was unchanged, suggesting that the terminal disposition of CBX was not altered, among the various treatments.

Based on the preliminary pharmacokinetic studies, it was difficult to select between prodrugs CBX-PR and CBX-BU for further studies. Overall pharmacokinetic parameters of CBX released from both the prodrugs appeared to be very similar. However, there was a significant difference in systemic exposure of the intact CBX-PR compared to CBX-BU. Apparently, CBX-PR releases CBX slowly compared to CBX-BU, resulting in higher levels of CBX-PR relative to CBX-BU at any given time following oral administration. Preliminary *in vivo* pharmacokinetics of prodrugs were evaluated at a high dose i.e. 100 mg/kg, p.o., in order to obtain greater clarity between the prodrugs in their *in vivo* hydrolytic cleavage properties and its effective translation into systemic exposure of CBX. We believe, pharmacokinetic data gathered from the stress conditions are useful for the unambiguous selection of prodrugs. Although this dose is much higher than the efficacious dose of CBX for anti-inflammatory properties, it is very relevant to the efficacious doses of CBX for its anticancer properties in rodent model [14]. Systemic exposure of CBX-BU was very less and CBX-BU levels in plasma were seen only up to 1 h. Though the *in vivo* comparative pharmacokinetic study clearly showed CBX-BU as the

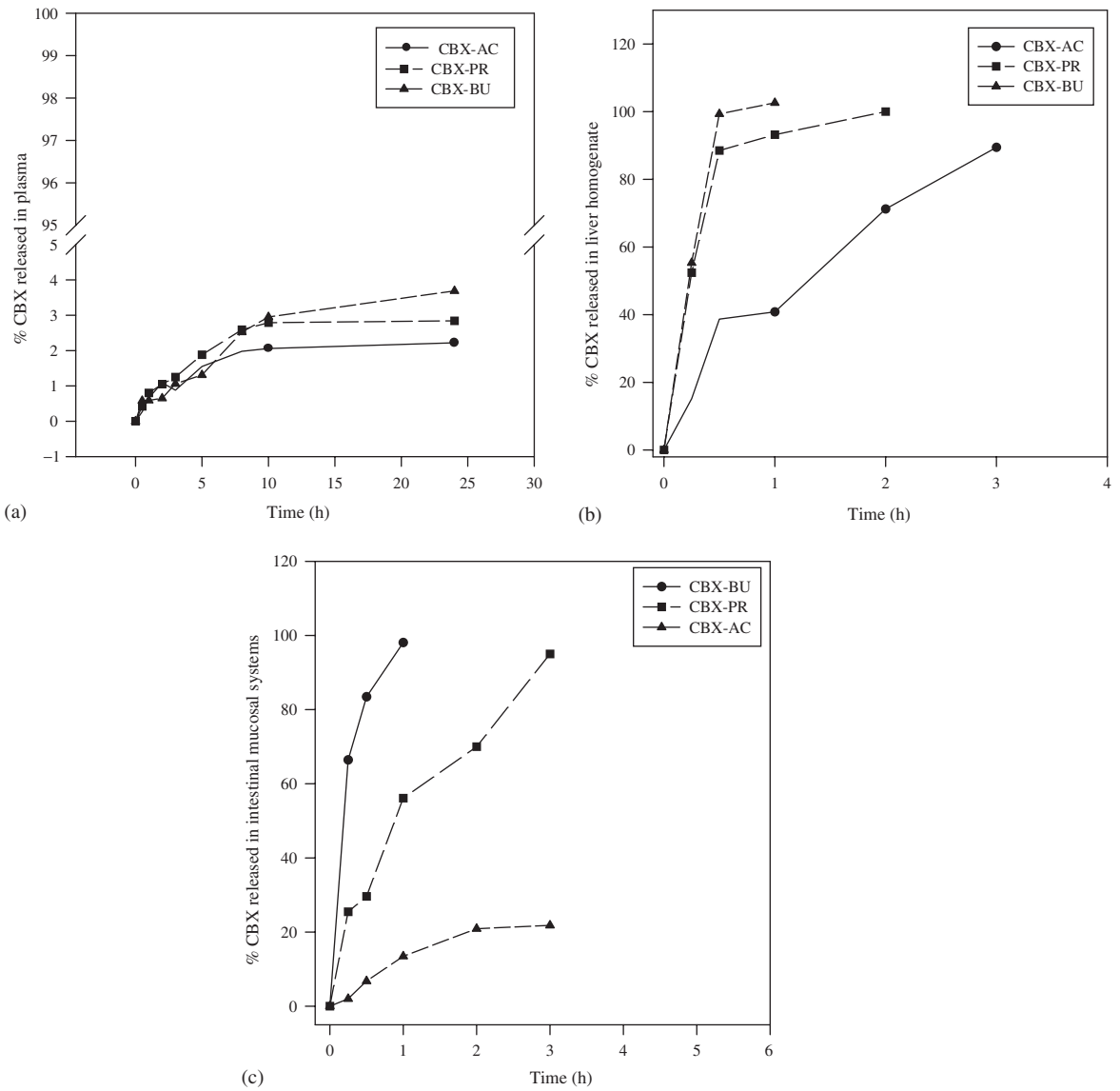

Figure 4. (a) Time courses for CBX-acetyl (CBX-AC), CBX-propionyl (CBX-PR) and CBX-Butyryl (CBX-BU) during *in vitro* hydrolysis in rat plasma at 37°C; (b) time courses for CBX-acetyl (CBX-AC), CBX-propionyl (CBX-PR) and CBX-butyryl (CBX-BU) during *in vitro* hydrolysis in rat liver homogenate; (c) time courses for CBX-acetyl (CBX-AC), CBX-propionyl (CBX-PR) and CBX-butyryl (CBX-BU) during *in vitro* hydrolysis in rat intestinal mucosal preparations

ideal prodrug, we wanted to substantiate further by *in vitro* hydrolysis studies. CBX-AC and CBX-PR were slowly hydrolyzed compared to CBX-BU in all the matrices *in vitro*. Hydrolyzing capacity of the matrices were in the order liver > intestinal mucosa > plasma. Percent release of CBX following 24 h incubation in plasma was negligible (<5%) for all the three prodrugs. The release of CBX from CBX-BU in the gastric mucosal suspension was complete within 1 h of incubation whereas % release of CBX from CBX-PR and CBX-AC was about 56 and 14%, respectively. Similarly in liver homogenates, CBX-BU hydrolyzed very rapidly and released

Table 3. Mean (± S.D.) pharmacokinetic parameters of CBX following oral administration of CBX and CBX-BU to male Wistar rats at 10, 30 and 100 mg/kg (dose equiv. to CBX)[a]

Parameters	CBX (mg/kg)			CBX released from CBX-BU (mg/kg)		
	10	30	100	10	30	100
$AUC_{(0-t)}$ (μg h/ml)	9.7 ± 0.6	11.8 ± 0.5	23.2 ± 1.2	16.0 ± 1.7*	41 ± 7*	129 ± 24*
$AUC_{(0-\infty)}$ (μg h/ml)	13.5 ± 2.9	12.3 ± 0.5	28.2 ± 3.4	16.2 ± 1.7	44 ± 9*	132 ± 24*
C_{max} (μg/ml)	1.6 ± 0.2	1.5 ± 0.3	2.5 ± 0.6	2.2 ± 0.4*	4.6 ± 1.5*	16.0 ± 1.6*
T_{max} (h)	2.8 ± 0.4	2.7 ± 0.5	3.2 ± 1.3	2.5 ± 1.0	3.0 ± 0.0	2.5 ± 1.0
K_{el} (h^{-1})	0.11 ± 0.03	0.13 ± 0.00	0.13 ± 0.03	0.20 ± 0.03*	0.12 ± 0.02	0.16 ± 0.03
$t_{1/2,\beta}$ (h)	6.7 ± 2.6	5.2 ± 0.04	5.8 ± 1.8	3.5 ± 0.5	5.8 ± 0.9	4.4 ± 0.7
f (%)	80	24	16	96	86	78

[a] $n = 4$ animals. C_{max}, $AUC_{(0-t)}$ and $AUC_{(0-\infty)}$ at similar doses were compared by Students t-test and t_{max}, K_{el} and $t_{1/2,\beta}$ across the dose levels were compared by one-way ANOVA (pairwise multiple comparison by Bonferroni's method).
* $p < 0.05$.

CBX completely within 30 min of incubation and % release of CBX from CBX-PR and CBX-AC at 30 min was 88 and 38%, respectively. It took 2 h for CBX-PR to completely hydrolyze and release the CBX, whereas hydrolysis of CBX-AC was not even complete even after 3 h incubation in liver homogenates.

Further, these results were supported by *in vitro* inhibitory activity against COX-1 and COX-2 enzymes and *in vivo* carrageenan-induced rat paw edema experiments. *In vitro* COX-2 inhibitory potencies of CBX prodrugs were CBX-BU > CBX-PR > CBX-AC. As expected, in the *in vitro* potency studies CBX prodrugs showed relatively less inhibition than the CBX. Based on the relative merits in terms of rapid and complete hydrolytic cleavage and higher *in vitro* potency among the tested prodrugs, CBX-BU was selected as a prodrug candidate. CBX-BU was evaluated for its *in vivo* efficacy by carageenan-induced rat paw edema model and efficacy equivalent to that of CBX was observed at only half of the dose of CBX.

Present dose proportionality studies of CBX in rats clearly showed lack of proportionality in the pharmacokinetic parameters with the increments in the dose. Our results using CBX-BU, showed a dose-independent pharmacokinetics of CBX across the wide dose range 10–100 mg/kg, by virtue of its water solubility, rapid and complete conversion to CBX. When the doses increased in a ratio 1:3:10, the C_{max} and AUC for CBX following the parent drug administration increased by 1:1:1.5 and 1:1:2.1, respectively. In sharp contrast, for a similar dose increment ratio following CBX-BU administration C_{max} and AUC for CBX increased by 1:2.1:7.2 and 1:2.7:8.1, respectively. The plasma levels of CBX following CBX administration are less than proportional to the administered dose, whereas following administration of CBX-BU, both C_{max} and AUC are linearly proportional with the administered dose ($r^2 > 0.9$). This is further substantiated with the absolute oral bioavailability of CBX, where by increasing the oral dose of CBX (alone) there was a decrease in absolute bioavailability whereas administering increasing doses CBX-BU from 10–100 mg/kg absolute oral bioavailability remained unchanged, within a range of 78–96%. None of the plasma samples following administration of 10 and 30 mg/kg CBX-BU showed detectable concentrations of CBX-BU (LOQ = 50 ng/ml). At 100 mg/kg dose, CBX-BU concentrations were observed in plasma up to 2 h. At the lowest tested dose, 10 mg/kg, pharmacokinetic parameters of CBX from CBX-BU and CBX alone were comparable, but the higher efficacy of CBX-BU observed in inflammation models could be attributable to a cohort of factors: a possible differential distribution of CBX, a somewhat higher exposure to CBX (approximately 30%), and an inherent COX-2 activity of CBX-BU.

In conclusion, the pharmacokinetic behavior of CBX following oral dosing of the CBX and CBX-BU was dramatically different, with much higher plasma levels of CBX released from the CBX-BU compared with those after CBX alone. CBX-BU was completely hydrolyzed to CBX by

gastrointestinal mucosa and liver, whereas hydrolyzing capacity of plasma was very poor. Other prodrug forms, CBX-AC and CBX-PR, did not meet the pre-set criteria and hence, they were not evaluated further. Absolute oral bioavailability of CBX was improved by CBX-BU administration and pharmacokinetics of CBX were dose proportional over a wider dose range 10–100 mg/kg. Owing to its improved oral bioavailability, it may be possible to reduce the clinical dose size of CBX via the prodrug approach. From a formulation perspective, water-soluble prodrug, CBX-BU, gives various dosage options. This is particularly important with increasing role of therapeutic potential of CBX in a broad range of diseases like cancer, rheumatoid arthritis, etc. Overall, based on our investigations, CBX-BU offers several advantages over conventional CBX. Nevertheless, further evaluation of CBX-BU is required before its possible usage in the clinic.

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