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Identification of carboxylesterases expressed in rat intestine and effects of their hydrolyzing activity in predicting first-pass metabolism of ester prodrugs

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Carboxylesterases (CESs) located in the intestine play an unique role in the absorption of many drugs especially ester prodrugs. In order to determine the expression and hydrolyzing activity of CESs isozymes (CES1 and CES2) located in rat intestine, the activities of CES1 and CES2 were evaluated by the intestinal S9 incubation with imidapril and irinotecan (CPT-11), the substrates of CES1 and CES2, respectively. The distribution characteristics of CES1, CES2, Pregnane X Receptor (PXR) and Constitutive Androstane Receptor were analyzed by real-time polymerase chain reaction (RT-PCR) or Western blot. Imidaprilat metabolized from imidapril by CES1 was too low to be detected in rat intestinal S9 fractions, while there was little and even no expression of CES1 mRNA in intestinal segments. In contrast, V_{max} values for CPT-11 diminished gradually from proximal to distal segments within the rat intestine which was consistent with the mRNA expression level of CES2. These results indicated that CES2 represents the major CESs isoform in the rat complete intestine and decreased from duodenum to colon, whereas the expression of CES1 was too low to influence the metabolism of ester prodrugs. The expression of PXR and CAR decreased slightly along the entire intestine on both mRNA and protein levels which indicated that PXR and CAR may be one of the major factors which contribute to the expression of CES1 and CES2. Thus, the knowledge about the characteristic and site-specific expression of CES1 and CES2 in rat intestine will help to predict the oral bioavailability of ester prodrugs.

1. Introduction

The first-pass metabolism of drugs when passing through the intestinal epithelium has attracted a lot of attention in recent years, since the small intestine is well recognized to have numerous functions, such as absorption, metabolism, and exsorption (Lin et al. 1999). The intestine definitely performed some important metabolic functions due to its possession of an array of metabolic enzymes. The contribution of cytochrome P450 (CYP) isoforms on intestinal absorption were well documented. As we all know, the metabolism and absorption of ester prodrugs is limited by esterases, and more specifically, carboxylesterases (CESs).

Mammalian CESs belong to the α,β-hydrolase-fold family and efficiently catalyze the hydrolysis of a variety of ester and amide-containing drugs (including prodrugs), such as antitumor drugs (CPT-11 and Capecitabin)(Danks et al. 1998; Guichard et al. 1998; Humerickhouse et al. 2000; Sanghani et al. 2003; Tabata et al. 2004), angiotensin-converting enzyme inhibitors (temocapril, cilazapril, quinapril, and imidapril) and narcotics (cocaine, heroin and meperidine)(Brzezinski et al. 1997; Furihata et al. 2004; Geshi et al. 2005; Kamendulis et al. 1996; Takai et al. 1997; Zhang et al. 1999). According to the classification of esterases by Aldridge, CESs are classified into five groups denominated CES1∼CES5 (Satoh and Hosokawa 1998, 2006). Among them, CES1 and CES2 were identified to be the majority of CESs. The expression of CESs is ubiquitous with high level in various tissues, such as liver, intestine, kidney and lung (Hosokawa et al. 2001; Morgan et al. 1994). It has reported that CESs are located in the endoplasmic reticulum (ER) of the intestinal endothelium (Pelham 1990). The CESs located in the intestine play a key role in the absorption and metabolism of many ester drugs especially ester prodrugs. For example, Barthel et al. (2008) reported that Pentyl PABC Doxazolidine (PPD) was selectively hydrolyzed to the active metabolite (doxazolidine) via human intestinal CESs. Imai et al. (2005) predicted the human intestinal absorption of temocapril through a Caco-2 cell monolayer. The results revealed that temocapril was almost completely metabolized to temocaprilat during transport across Caco-2 cells by CESs. Various kinds of ester prodrugs are hydrolyzed by CESs during the process of absorption across the intestinal endothelium. Therefore, it was thought that CESs are major determinants for the pharmacokinetics and pharmacodynamics of ester prodrugs.

Several studies about the expression characteristic of CESs were conducted. For instance, Taketani et al. (2007) performed an experiment to evaluate the expression of CESs in liver and intestine among different animals and humans. Masaki et al. (2007) showed that the relative mRNA of CES2 was approximately 1.5 higher in the jejunum than in ileum. Although CES1 and CES2

have attracted increasing attention in terms of metabolism activity, little information exists regarding their expression in the gut wall, especially in respect of the site-specific along the whole length of the intestine.

To the best of our knowledge, some nuclear receptors (NR) could regulate the expression of CES1 and CES2. Emerging knowledge of Pregnane X Receptor (PXR) and Constitutive Androstane Receptor (CAR) has shown that PXR and CAR, two members of the NR superfamily of ligand-activated transcription factors, mediated the regulation of CESs isozymes in liver. For instance, Shi et al. (2008) showed that cotransfection of PXR stimulated the promoter of hydrolase B and hydrolase C, belonging to CES1 isozymes, in response to dexamethasone at micromolar concentrations. Actually, a previous study suggested that suppression of CES2 gene expression was achieved by four triazol fungicides and was probably dependent on PXR/CAR mediated gene activation pathways (Tully et al. 2006). Additionally, the experiment of human hepatocytes with 10 μM rifampicin, the prototypical human PXR activating compound, caused a moderate induction of hCES1 and hCES2 gene expression (Goetz et al. 2006). Definitive reports about PXR and CAR activations to CESs gene expressed in the intestine are currently lacking.

In the present study, imidapril and irinotecan (CPT-11), specific substrates of CES1 and CES2, were adopted to calculate the hydrolysis effect of four selected intestinal segments by detecting the metabolites (imidaprilat, 7-ethyl-10-hydroxy-camptothecin (SN-38)) respectively. Consequently, intestinal S9 incubation and RT-PCR were conducted to investigate the activity and expression level of CES1 and CES2 in rat intestinal segments systemically. In addition, the expression characteristics of PXR and CAR in selected intestinal segments were measured by RT-PCR and Western Blot to explore the relationship between NR (PXR and CAR) and CESs (CES1 and CES2).

2. Investigations and results

2.1. *Hydrolase activity in S9 fraction from rat duodenum, jejunum, ileum and colon*

The formation of imidaprilat and SN-38 were detected to investigate the activity level of CES1 and CES2 in rat all intestinal regions with the respect to the hydrolysis of CES1 and CES2 on imidapril and CPT-11. Firstly, the phenomemon that imidaprilat could not be detected in the intestinal segments S9 fractions demonstrated that scarce hydrolase activity of CES1 existed in the whole rat intestine. On the contrary, CPT-11 could be rapidly hydrolyzed into SN-38 after incubation in the intestinal segments S9 fractions. The time-dependent formation of SN-38 in homogenates of rat intestine from duodenum to colon is presented in Fig. 1: a significant site-specific hydrolysis was observed at all time points ($P < 0.05$). Correspondingly, the kinetic parameters of CES2 were calculated on the basis of Michaelis-Menten equation by nonlinear least-squares analysis (Table 1). Interestingly, the V_{max} value for CPT-11 was highest in duodenum, and was 4.7-, 3.6-fold higher in the jejunum and ileum than in the colon. Table 2 shows the K_m of CES2 in the four intestinal segments with no pronounced difference.

2.2. *Inhibition effect of BNPP on hydrolysis by rat intestine S9 fractions*

The effects of BNPP on the hydrolysis of imidapril and CPT-11 in rat intestinal segments S9, as measured by the formation of the hydrolysis product, were conducted by adding different doses of BNPP into the reaction system. Imidaprilat was still not detected in the intestinal homogenates with different concentrations of BNPP, whereas the formation of SN-38 hydrolyzed from CPT-11 was inhibited by BNPP, a special CESs inhibitor (Fig. 2). The percentage of CPT-11 hydrolyzed was reduced to 67%, 61%, 69% and 52% of the control in rat duodenum, jejunum, ileum and colon S9 fractions with 0.1 μM BNPP. The formation of SN-38 with 1 μM BNPP decreased to 41%, 37%, 42% and 32% relative to the control value. Meanwhile, the hydrolysis of CPT-11 decreased to 24%, 25%, 20% and 18% when co-incubated with 10 μM BNPP. Furthermore, the residual hydrolytic activities for CPT-11 were 14%, 13%, 11% and 10%, respectively, with 100 μM BNPP. The results obtained from the different inhi-

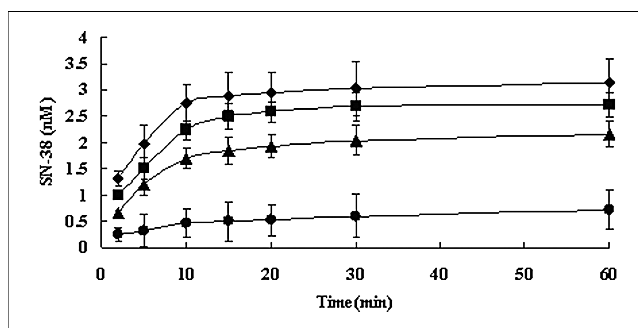

Fig. 1: Rate of formation of SN-38 following incubation of 10 μM CPT-11 with S9 fraction of rat intestines (♦ duodenum, ■ jejunum, ▲ ileum, ● colon, respectively). The protein concentration was diluted with HEPES buffer (50 mM) to 100 μg/mL in all cases. Each time point presents mean ± S.D. (n = 3)

Table 1: Kinetic parameters for hydrolysis of CPT-11 in rat intestinal S9 K_m and V_{max} were calculated according to the Michaclis-Menten equation for a single enzyme using Sigmaplot. Values represent means ± S.D. (n = 3)

Segments	V_{max} (pmol/min/mg protein)	K_m μM
Duodenum[a]	15.64 ± 5.41*	8.62 ± 1.72
Jelunum[a]	12.88 ± 3.97*	5.76 ± 2.03
Ileum[a]	9.81 ± 3.74*	6.83 ± 1.21
Colon [a]	2.75 ± 0.86*	6.67 ± 1.05

[a] The intestinal S9 fractions were diluted with HEPES buffer (50 mM) to 100 μg/ml in all cases. The CPT-11 concentration was 0.5 to 200 μM.
* Statistical significance refers to the different activity in rat duodenum, jejunum, ileum and colon. Hydrolysis activity of rat intestinal segments with the general order of decreasing activities: duodenum > jejunum > ileum > colon ($P < 0.05$).

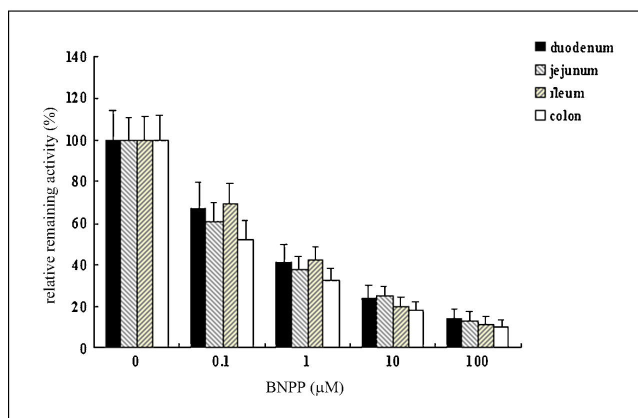

Fig. 2: Inhibition of the hydrolysis activity for CPT-11 in rat intestinal segments S9 fraction with BNPP 0.1, 1, 10, 100 μM, respectively. The protein concentration was diluted with HEPES buffer (50 mM) to 100 μg/mL in all cases. Each point presents mean ± S.D. (n = 3)

Table 2: The forward and reverse primers of NM 001024365, AB010635, AY034877, PXR, CAR and β-actin

Primer	Forward Sequence	Reverse Sequence
β-actin	5'-CGTTGACATCCGTAAAGACCTC	5'-TAGGAGCCAGGGCAGTAATCT
AB010635	5'-GCTGGCTGAATGCTGTGCTC	5'-CGTGGTCAAGCTTTCCTCG
AY034877	5'-GGAGTGGTGTGAGAGATGCG	5'-CAGGTTAGAGCCCTCACGG
NM_001024365	5'-AGGTCCTGGGGAAGTATGCC	5'-TGCATCTTGGGAGCACATAGG
PXR	5'-CAAGCGGAAGAAAAGTGAACG	5'-CACAGATCTTTCCGGACCTG
CAR	5'-ACCAGATCTCCCTTCTCAAG	5'-CTCGTACTGGAACCCTA

bition of BNPP suggested that CESs were mainly responsible for the hydrolysis of CPT-11 in rat intestinal segments.

2.3. Expression level of CESs isozymes, PXR and CAR mRNA in the selected intestinal segments

For a calculation of the relative expression of CESs, along the complete intestine, the β-actin-normalized intensity of the AB010635 on colon was set to 1, and the intensities of the other segments and other CESs isozymes were set relative to it.

The relative expression levels of CESs isozymes in rat intestine were determined using total RNA isolated from duodenum, jejunum, ileum and colon (Fig. 3). Compared with AB010635 and AY034877, NM_001024365 belonged to the CES1 isozymes, with only minor amounts expressed in the upper intestine and disappearing in the colon. Taking data from different selected regions, AB010635 abundance had a similar expression pattern with AY034877, which showed a decrease from proximal to distal regions of the intestine. In the whole intestine, the expression level of AB010635 was the highest compared with NM_001024365 and AY034877. Furthermore, significant differences of the expression of CES1 and CES2 were observed in rat duodenum, jejunum, ileum and colon ($P < 0.05$).

In addition to CES1 and CES2, the expression of PXR and CAR mRNA were presented along the whole intestine. Both PXR and CAR showed a similar expression profile insofar as they had high expression level in the upper intestine and low expression in the lower intestine (Fig. 4). In contrast to CES1 and CES2, the amount of PXR expressed in whole intestine was about the same as CAR.

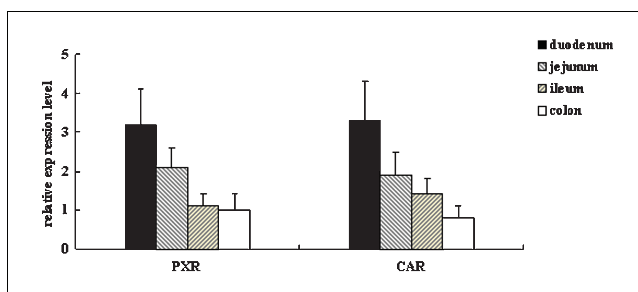

Fig. 4: The RT-PCR of PXR and CAR in rat duodenum, jejunum, ileum and colon. Data represented the mRNA expression level of PXR and CAR in the selected intestinal regions normalized by that of PXR in colon (n = 4). The mRNA level for both PXR and CAR in the rat whole intestine with a decreasing order: duodenum > jejunum > ileum > colon ($p < 0.05$)

2.4. Expression level of PXR and CAR protein in the selected intestinal segments

Fig. 5 showed the result of a typical full-scan Western blot of PXR and CAR in selected intestinal regions obtained with antibodies against PXR, CAR, and β-actin. The PXR protein was

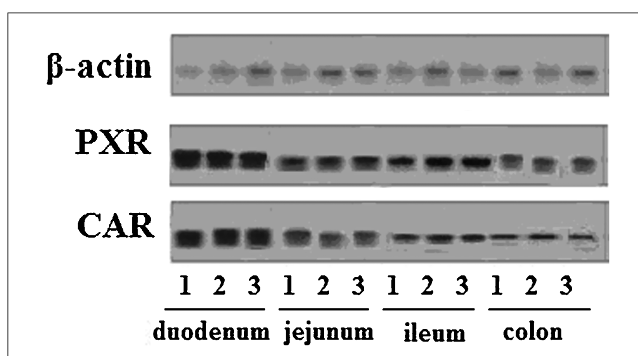

Fig. 5: The expression of PXR and CAR protein in rat selected intestinal segments. Western blot analysis was performed to determine the expression levels of PXR and CAR, and β-actin protein, respectively. Each lane represents an individual animal

expressed at the highest level in duodenum, with lower but easily detectable levels in jejunum, ileum, and colon. Meanwhile, relative expression of CAR protein decreased continuously along the entire intestine. The absolute protein expression of PXR and CAR was in comparable amount with a declining expression pattern in the selected intestinal segments.

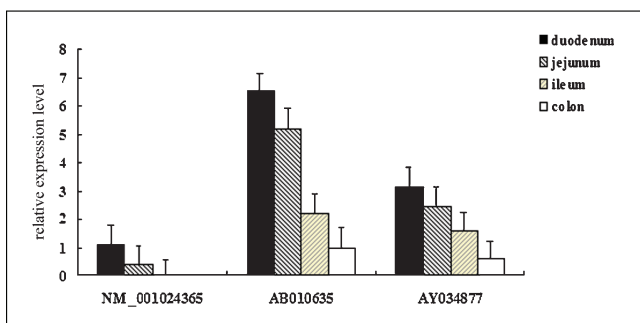

Fig. 3: The RT-PCR of CES1 and CES2 in rat duodenum, jejunum, ileum and colon. Data represented the mRNA expression level of each CES isozymes in the selected intestinal regions normalized by that of AB010635 in colon (n = 4). The mRNA level for both CES1 and CES2 isozymes in the rat whole intestine with a decreasing order: duodenum > jejunum > ileum > colon ($P < 0.05$)

3. Discussion

The intestinal first-pass metabolism of ester prodrugs has received increasing attention since the intestine was recognized as contributing substantially to drug metabolism due to the presence of the CESs isozymes, CES1 and CES2 especially. A variety of ester prodrugs, including several angiotensin-converting enzyme inhibitors, antitumor drugs, and narcotics, were acted on by CESs enzymes hydrolysis. In this regard, CESs were believed to be major determinants of the metabolism and disposition of ester prodrugs through their hydrolysis in intestine.

Many studies demonstrated that the CES1 subfamily mainly hydrolyzed substrates with small alcohol and large acyl groups. Previous studies reported that oseltamivir and the methyl ester

of cocaine could be hydrolyzed by CES1 rapidly (Pindel et al. 1997; Shi et al. 2006). In this regard, imidapril with "bulky" acyl chains is a good substrate for CES1. On the other hand, the CES2 isozymes recognize substrates with large alcohol and small acyl groups. Pindel et al. (1997) reported that CES2 had high catalytic efficiencies for the hydrolysis of 4-methylumbelliferyl acetate, heroin, and 6-monoacetylmorphine. Accordingly, CPT-11 with "small" acyl chains can be hydrolyzed by CES2 with a high hydrolysis activity. Imidapril and CPT-11, as substrates of CES1 and CES2 respectively, were used to evaluate the activity levels of CES1 and CES2 in rat intestine.

In order to quantity mRNA expression of CES1 and CES2, we established a reliable and reproducible RT-PCR method. For exact quantification, it is crucial to use an appropriate reference gene, which was expressed constantly and independently of the experimental conditions. Recently, β-actin was regarded to be the most frequently used housekeeping gene and consequently used for quantification of CES1 and CES2 mRNA successfully (Cecchin et al. 2005; Hamalainen et al. 2001; Radonic et al. 2004; Schmittgen and Zakrajsek 2000; Zhong and Simons 1999).

It is now well established that CESs were glycoproteins localized within the lumen of the endoplasmic reticulum (Crow et al. 2007). Reports showed that BNPP specially inhibited CESs without any inhibition of aminopeptidase activity and affect neither active transport, e.g., P-glycoprotein and peptide transporters, nor passive diffusion (Masaki et al. 2007). Accordingly, *in vitro* hydrolysis of CESs in rat intestinal segments S9 fraction was conducted with or without BNPP. In our study, imidaprilat was not detected after incubation in rat intestinal segments S9 with or without BNPP, suggesting that merely CES1 was expressed in rat intestine. The result showed that low expression level of CES1 mRNA was determined by RT-PCR. In addition, CES1 mRNA showed a decreasing expression from duodenum to ileum and disappeared in colon. These results implied that the expression of CES1 in intestine segment was too small to impact the ester prodrugs metabolism.

The formation of SN-38 in intestinal homogenate can be used as surrogate marker of CES2 activity. CPT-11 was hydrolysed rapidly in rat intestinal segments S9, and the formation rate of SN-38 was highest in duodenum followed by jejunum, ileum and colon (15.64 ± 5.41, 12.88 ± 3.97, 9.81 ± 3.74 and 2.75 ± 0.86 pmol/min/mg protein, $P < 0.05$). It was obvious that CES2 activity showed a pronounced decrease along the intestine axis from proximal to distal parts. Furthermore, the hydrolysis of CPT-11 reduced sharply in rat selected intestinal S9 fractions with 0.1 μM BNPP (Fig. 2). The activity of CES2 was inhibited almost completely by 100 μM BNPP (14%, 13%, 11%, 10%, respectively). These results revealed that CES2 hydrolysed CPT-11 to SN-38, which confirmed that the activity of CES2 could be determined by detecting the formation of SN-38. Additionally, the expression of mRNA for CES2 was highest in duodenum and decreased toward lower regions ($P < 0.05$). The finding from the mRNA expression of CESs implied that there was an abundance expression of CES2 but little expression of CES1. All in all, high levels of CES2 were expressed in rat intestine with a decreasing order: duodenum > jejunum > ileum > colon investigated by intestinal S9 incubation and RT-PCR.

In spite that the expression of CES2 was much more abundant in intestine than that of CES1, the declining expression of the CES1 and CES2 at lower intestinal regions may be related to the expression of PXR and CAR. To our knowledge, PXR and CAR, two members of the NR superfamily of ligand-activated transcription factors, play a positive role in the modulation of CESs isozymes in rats and humans. On the other hand, it is now well established that PXR is a key regulator of xenobiotic-inducible CYP3A and CYP2B gene expression in the liver, as well as drug transporter proteins such as organic anion transporting polypeptide 2, multidrug resistance 1/P-glycoprotein, and multidrug resistance proteins (MRP) 2 and 3 in liver (Geick et al. 2001; Kast et al. 2002; Masuyama et al. 2001; Sonoda et al. 2002; Staudinger et al. 2003). It is well known that these two receptors form the molecular basis of an important class of drug-drug interactions through their actions in liver.

Although much is known regarding the identity and function of PXR target genes in the liver, little is known about their function in the intestine. The data from the study showed that the diminishing expressions of PXR and CAR were observed in both mRNA and protein level (Fig. 4, Fig. 5). It became evident that the expression profile of PXR and CAR in the entire intestine was in agreement with that of CES1 and CES2. Indeed, it was recognized that the decreasing expressions of CYP3A and CYP2B were observed in the whole rat intestine, as well as that of MRP2, which was similar to the expression of PXR and CAR. On these grounds, we hypothesized that the declining expression of CES1 and CES2 at lower intestinal regions may be explained at least in part by the PXR/CAR expression.

Many drugs with high hydrophilicity are synthesized into ester prodrugs to enhance their bioavailability. However, CESs isozymes located in the intestine can hydrolyze ester prodrugs. The active drugs were usually hydrolyzed from ester prodrugs by CES1 and CES2 when entering the systemic circulation. However, some metabolites of ester prodrugs can also cause substantial adverse effects. Thus, CESs are major determinants for pharmacokinetics and pharmacodynamics of ester prodrugs. Actually, it has been shown that the expression level of human CESs was correlated with the conversion ratio of CPT-11 to SN-38, the active metabolite, which is thought to be a key step for the chemotherapeutic action of this anti-tumor drug (Xu et al. 2002). Meanwhile, Khanna et al.(2000) suggested that gut toxicity from CPT-11 may be in part due to direct drug conversion by CESs present in the small intestine. Cecchin et al. (2005) reported that patients with CES2 mRNA expression above the median value presented a higher possibility of grade 3 to 4 neutropenia or diarrhea than those with CES2 mRNA below the median.

Besides, there are substrate specificities of CES1 and CES2. In the present study, the rat intestine showed a high expression of CES2, but little expression of CES1 in all segments. Therefore, the ester prodrugs, which were substrates of CES1, will not be hydrolyzed after oral administration. On the contrary, CES2 will rapidly hydrolyze ester prodrugs with a large alcohol group and small acyl group when passing the intestinal mucosa. Thus, the expression of CES1 and CES2 in rat intestine helps to predict the oral bioavailability of prodrugs. This study provides useful information for the study of metabolism and disposition of ester prodrugs as well as ester drugs.

In conclusion, this study proved the predominant role of CES2 isozymes in rat intestine. In contrast to a high expression level of CES2 in rat intestine, the expression of CES1 was too little to influence the metabolism of ester prodrugs. Moreover, the data clearly demonstrate a site-specific hydrolase activity of CES2 in the order duodenum > jejunum > ileum > colon. On the other hand, the expression of PXR and CAR may contribute to the expression of CES1 and CES2 in the intestine. These data will provide a basis for a better understanding of the extent of intestinal metabolism along the gastrointestinal tract and prediction of oral bioavailability of prodrugs.

4. Experimental

4.1. Materials

Imidapril and imidaprilat were purchased from Sigma (St. Louis, MO). CPT-11 and SN-38 were purchased from Toronto Research Chemicals Inc (North York, ON, Canada). Bis-p-nitrophenylphosphate (BNPP) was

purchased from Sigma (St. Louis, MO). Tissue culture medium TC 199 (10 × concentrated with Earle's salts) and glutamine were purchased from Sigma Aldrich Chimie (St Quentin Fallavier, France). Trizol was purchased from Invitrogen (Carlsbad, CA). Stratagene's first strand kit was purchased form Stratagene (La Jolla, CA). Peroxidase-conjugated goat anti-rabbit whole molecule IgG, and peroxidase-conjugated goat anti-mouse whole molecule IgG were purchased from Sigma-Aldrich (Munich, Germany). Protease inhibitor cocktail with EDTA was purchased from Roche (Basel, Switzerland), and EDTA and urea were purchased from VWR (Darmstadt, Germany). NuPAGE 2X LDS Sample Buffer, NuPAGE 2X Sample Reducing Agent, NuPAGE Novex 4∼12% Bis-Tris Gels were purchased from Invitrogen (Karlsruhe, Germany). All other chemicals and regents were of analytical grade.

4.2. Animals

Male Sprague Dawley rats (200∼250 g, 6–8 weeks of age) were obtained from the Experimental Animal Center, Tongji Medical College, Huazhong University of Science and Technology, and housed in an air-conditioned room with free access to commercial cow and tap water. The animals were maintained on a 12 h light/dark cycle (light on from 8:00 to 20:00) at ambient temperature (22∼24 °C) with 60% relative humidity. Rats were fasted for 12 h before the experiment. The animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

4.3. Hydrolysis of imidapril and CPT-11 in mucosal S9 fraction from the duodenum, jejunum, ileum and colon

Rats were anesthetized with urethane (120 mg/100 g) and duodenum, jejunum, ileum and colon were obtained from the rat whole intestine. The intestinal segments were rinsed with ice-cold physiological saline. Then, the mucous membrane was scraped gently with a slide following cut longitudinally. Afterwards, the mucosal scrapings were homogenized for 5∼10 min with 5 volumes (w/v) buffer (10 mM phosphate buffer containing 50 mM Tris-HCl, pH 7.4) to prepare 20% homogenates. The homogenates were centrifuged at 9000 g for 20 min at 0 °C to obtain the S9 fraction (van de Kerkhof et al. 2007). The protein concentrations of the S9 fractions were determined by the method of Bradford (1976). The intestinal S9 fractions were stored at −80 °C until use.

The intestinal segments S9 fractions were diluted to the appropriate concentration with HEPES buffer (50 mM, pH = 7.4). Hydrolysis of imidapril in intestinal segments S9 fractions was initiated by the addition of imidapril dissolved in acetonitrile after preincubated for 5 min. After incubated for 20 min, 100 μl ice-cold acetonitrile and 10 μl 2 μM cetirizine (internal standard) were added to terminate the hydrolysis. The final concentration of organic solvent in the reaction mixture was < 1.0%, which had no effect on hydrolase activity. The formation of imidaprilat was determined by HPLC-MS/MS.

After preincubation for 10 min, the hydrolysis of CPT-11 was initiated when adding CPT-11 dissolved in methanol. The reaction was terminated after addition of 100 μl ice-cold $HClO_4$ and 10 μL 1.5 μM camptothecin (CPT, internal standard) followed by incubation at 37 °C for 20 min. The final concentration of organic solvent in the reaction mixture was < 1.0%, which had no effect on hydrolase activity. After incubation, the formation of SN-38 hydrolyzed from CPT-11 was measured using methods reported previously (de Bruijn et al. 1997).

Kinetic parameters for the hydrolysis of imidapril and CPT-11 (final concentrations 2∼200 μM and 0.5∼200 μM, respectively), K_m and V_{max} were calculated according to Michaielis-Menten equation. The inhibition experiment of hydrolase activity in rat intestinal segments S9 fractions with BNPP (0.1 ∼ 100 μM), a specific CESs inhibitor, was carried out parallelly. The degree of inhibition was calculated as a percentage of control activity.

4.4. Quantitative real-time polymerase chain reaction

Total RNA was extracted from tissue using Trizol (Invitrogen, Carlsbad, CA) according to the manufacture's instruction. The quantity and quality of the RNA samples were assessed using Experion total RNA standard sensitive chips (Bio-Rad). Total RNA (1 μg) from selected intestinal segments was treated with RNase-Free (Promega) to prevent genomic DNA contamination and reverse transcribed by the Stratagene's first trand kit (La Jolla, CA) according to the manufacture's instruction. RT- PCR was conducted on an Applied Biosystem's (Foster City, CA) 7500 sequence detection system. Each reaction mixture was prepared using the iQTM SYBR Green Super mix (TOYOBO, Japan) in a total volume of 25 μl: 8 μl water, 2 μl of each primer (final concentration 0.3 um), 12.5 μl Super mix and 2.5 μl of template cDNA or diluted plasmid. Reaction was performed in 96 well plates by heating to 95 °C for 1 min, then cycled at 95 °C for 15 s, 58 °C for 15 s and 72 °C for 45 s for 40 cycles. β-Actin was used for all samples to normalize expression. RT-PCR was performed to amplify the segments of NM 001024365, AB010635, AY034877, PXR, CAR and β-actin. The forward and reverse primers are listed in Table 2.

4.5. Western blot analysis for PXR and CAR in rat intestine

The intestinal homogenate for the study of PXR and CAR protein expression in selected intestinal regions were prepared by a previously published method (Mitschke et al. 2008). For gel electrophoresis, 100 μg of total protein of each sample was precipitated with ice-cold acetone. Proteins were precipitated by 8 M urea (10 μl/100 μg of protein) and denatured for 30 min at 55 °C in "sample buffer" (NuPAGE 2X LDS Sample Buffer and NuPAGE 2X Sample Reducing Agent). Samples (100 μg of protein/lane) were separated by electrophoresis in NuPAGE Novex 4∼12% Bis-Tris Gradient Gel and transferred to a nitrocellulose membrane according to the manufacturer's instructions. Membranes were blocked with 5% nonfat dried milk in TBS-T buffer containing 10 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 0.1% (v/v) Tween 20. For detection of PXR and CAR proteins, blots were incubated with the respective primary antibodies diluted in 1% bovine serum albumin: goat monoclonal anti-PXR antibodies (catalog number 7737; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit polyclonal anti-CAR antibodies (LS-B2628 1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). β-Actin was used as a reference protein (mouse anti-β-actin monoclonal antibody 4AL403 1:2000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). To detect bound antibodies, membranes were incubated with goat anti-rabbit IgG (for PXR and CAR antibodies) and goat anti-rabbit IgG (for β-actin antibody) coupled to peroxidase (secondary antibody; Sigma) diluted 1:20000 for 1 h and then washed three times with TBS-T. The Western Lightning (PerkinElmer Life and Analytical Sciences, Waltham, MA) chemiluminescent detection system was used for band visualization in combination with the ChemiDoc XRS system (Bio-Rad Laboratories, Munich, Germany). The Western blots were determined densitometrically using Quantity One Software (BioRad, Laboratories) and normalized to β-actin.

4.6. Sample analysis

Imidapril, imidaprilat were determined by HPLC-MS/MS. The Diamonsil C_{18} (5 μm, 150 × 2.1 mm) was used as the analytical column with acetonitrile-0.1%(v/v) formic acid (1:2, v/v) as the mobile phase at the flow rate of 0.3 ml/min. The samples were ionized by electrospray ionization source in the triple quadruple tandem mass spectrometer, and imidapril, imidaprilat and internal standard determined with a multiple reaction monitoring mode of m/z406.2→234.2, m/z378.1→206.1 and m/z389.2→201.1, respectively.

CPT-11 and SN-38 were determined by HPLC (pump: waters 2690 autosample; MA, USA; fluorescence detector: waters 2475; MA, USA;). For simultaneous determination of CPT-11 and SN-38, a RP-C18-column (4.6 mm × 250 mm, 5 μm) was used with a mobile phase of methanol - 50 mM Na_2HPO_4 [1:1 (v/v)]v (adjusted pH = 3.0 with phosphate) at a flow rate of 1.0 ml/min. CPT-11 and SN-38 were both simultaneous detected at excitation and emission wavelengths of 380 nm and 550 nm, respectively.

4.7. Data analysis

Reaction velocity at saturated substrate concentration (V_{max}) and the Michaelis–Menten constant (K_m) were estimated by fitting velocity versus substrate concentration curves (over the range of 0.5 to 200 μm) to the equation:

$$V = \frac{V_{max}[S]}{K_m + [S]}$$

The computer program GraFit 3.0 (Erithacus Software LTD, Horley, Surrey, UK) was used to derive kinetic constants and perform statistical evaluations. One-way analysis of variance was performed to compare the site-specific degradation of imidapril and CPT-11 in rat; pairwise comparison of all segments was done by Tukey's multiple comparison test. In all cases, a *P*-value of < 0.05 was considered significant. Data are presented as mean ± SD, unless stated otherwise.

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