Pharm Res (2014) 31:238–251
DOI 10.1007/s11095-013-1158-5

RESEARCH PAPER

Contributions of Intestine and Plasma to the Presystemic Bioconversion of Vicagrel, an Acetate of Clopidogrel

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Received: 12 April 2013 / Accepted: 22 July 2013 / Published online: 14 September 2013

ABSTRACT

Purpose To investigate the contributions of intestine and plasma to the presystemic bioconversion of vicagrel, and track its subsequent bioconversion to 2-oxo-clopidogrel *in vivo* and *in vitro* to rationalize the design of vicagrel, an acetate analogue of clopidogrel.

Methods The concentration-time profiles of 2-oxo-clopidogrel and active metabolite (AM) in presystem and circulation system was determined in the cannulated rats. Also, the rat intestinal S9 and human intestinal microsomes were conducted to examine the formation of 2-oxo-clopidogrel and AM. Meanwhile, the esterases in plasma and intestinal fractions responsible for the bioconversion of vicagrel to 2-oxo-clopidogrel were screened by the esterase inhibition and recombinant esterases.

Results The intestine was responsible for the formation of 2-oxo-clopidogrel and AM *in vivo* and *in vitro*, where carboxylesterases 2 (CE2) contributed greatly to the vicagrel cleavage during absorption. Other related esterases in plasma were paraoxonases (PON), carboxylesterases 1 (CE1) and butyrylcholine esterases (BChE).

Conclusion The findings rationalized the prodrug design hypothesis that vicagrel could overcome the extensive invalid hydrolysis of clopidogrel by the hepatic CE1 but experience the extensive hydrolysis to 2-oxo-clopidogrel and subsequent oxidation to AM in the intestine. This also supported the theory of improved pharmacological activity through facilitated formation of 2-oxo-clopidogrel, thus warranting much needed future clinical pharmacokinetic studies of vicagrel.

KEY WORDS 2-oxo-clopidogrel · esterases · presystemic hydrolysis · vicagrel

ABBREVIATIONS

AM	Active metabolite
AUC	Area under the plasma concentration *versus* time curve
BChE	Butyrylcholine esterases
CE	Carboxylesterases
CE1	Carboxylesterases 1
CE2	Carboxylesterases 2
$Cl_{in\ vitro}$	*In vitro* intrinsic clearance
CR	Clopidogrel resistance
PON	Paraoxonases
PON1	Paraoxonases 1

INTRODUCTION

Nowadays, clopidogrel is the most widely prescribed anti-platelet agent used either alone or alongside aspirin as dual therapy for the treatment of acute coronary syndromes, especially for stent replacement patients (1–3). However, the limitations of clopidogrel, such as frequent inter-individual variability in pharmacodynamic

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response and delayed onset have emerged, bringing into question its efficiency in clinical practice. Some 34% of patients prescribed clopidogrel exhibit less than optimal pharmacological responses to the drug; this low or non-response being classified as clopidogrel resistance (CR) is thought to arise from inefficient metabolism of the parent drug due to cytochromic polymorphisms/drug-drug interactions (4). Prasugrel, a novel thienopyridine prodrug with faster and greater response rate than clopidogrel, is considered as an alternative for the treatment of patients suffering from serious recurrent ischemic complications (5,6); however, FDA has already put the Black Box Warning to warn the patients and physicians about the fatal bleeding risk (sometimes) during prasugrel usage (7).

In consideration of the pros and cons of using clopidogrel and prasugrel, vicagrel, the acetate derivative of clopidogrel (structure shown in Fig. 1), is esterified on the main skeleton of clopidogrel, with identical ester acyl moiety and sharing similar hypothesis to that of prasugrel (8). Both of the acetate are established based on the hydrolysis preference of CE1 and CE2 that is allied to the molecular weights of the acyl moieties of esters (5,8,9). CE1 exhibits higher potency to the larger acetyl moiety, while CE2 shows higher affinity to the larger alcohol moiety (9,10). According to the research by Williams *et al.*, the biotransformation of prasugrel to R-95913(its thiolactone intermediate, chemical structure shown in Fig. 1) is mediated more efficiently by CE2 than CE1 (11,12). CE2 but not CE1 family is highly expressed in the rat and human small intestine, while the activity of the latter in the liver is far higher than the former (13,14). Theoretically, vicagrel would be mostly hydrolyzed to its intermediate directly and rapidly by the involving of CEs during the absorption before entering into the liver. Meanwhile, the possible contributions of intestinal paraoxonase (PON), butyrylcholinesterase (BChE) and acetylesterases should also be taken into consideration (15).

Besides of CEs (belonging to B-estesases), the aforementioned esterases, such as A-esterases (PON), B-esterases (BChE;AChE) and C-esterases (acetylesterases), are major types of esterases in mammals with varying distribution and activity in various species and tissues (16). Specifically, the PON family consists of three isoforms, PON1, PON2 and PON3. PON1, ubiquitously located in blood of all the mammalian, is in charge of the hydrolysis of a number of lactone-or cyclic-drugs, while a limited number of compounds are hydrolyzed by PON3, but little information about PON2 (17). BChE and AChE also contribute to the drug hydrolysis (18). The total hydrolysis activity of rat small intestine is similar to that of human small intestine (14). Moreover, CYP3a family is extensively located in the rat intestine, which reveals higher homology of human CYP3A4 (81%) (19). Herein, the rat intestine is a proper model for the rationalization of vicagrel and prediction in human.

During pharmacological screening, vicagrel exhibited improved anti-platelet aggregation effects over clopidogrel although it was less potent than prasugrel *in vitro* and *in vivo* (in rats) (8). By comparison to prasugrel, the lower pharmacological efficiency of vicagrel was probably due to formation of acid form from vicagrel, 2-oxo-clopidogrel as well as active metabolite (AM, chemical structure shown in Fig. 1), by losing methyl via hydrolysis with similar pattern to that of clopidogrel, since vicagrel was designed on the main skeleton of clopidogrel (8). However, our previously reported pharmacokinetic study showed a more rapid conversion efficiency of vicagrel to its intermediate metabolites 2-oxo-clopidogrel and its AM than clopidogrel, with about 5-fold improved system exposure than that following clopidogrel administration at

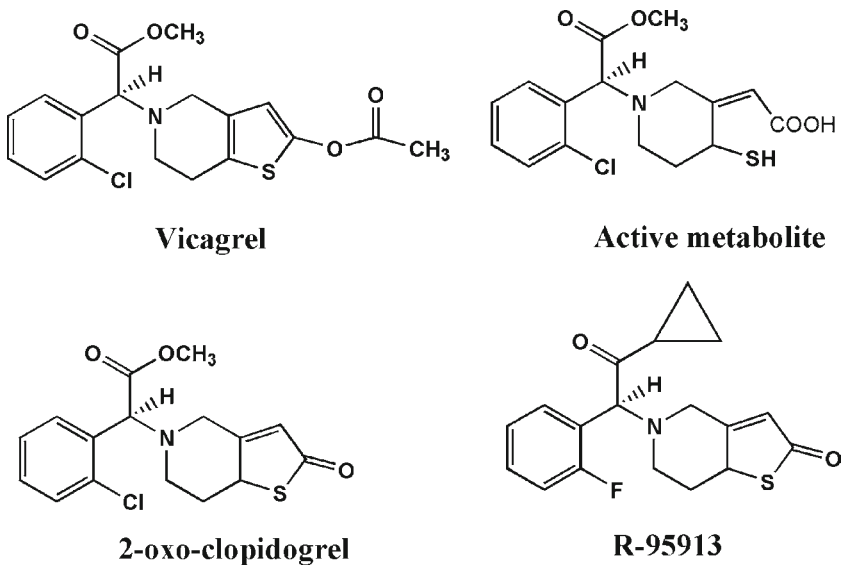

Fig. 1 Chemical structures of vicagrel, active metabolite, 2-oxo-clopidogrel and R-95913 (ISTD-thiolactone metabolite of prasugrel).

equivalent molar doses (20). The enhanced pharmacological efficiency was ascribed to the raised formation of AM from 2-oxo-clopidogrel. There were two hypotheses concerning the increased AM formation. Firstly, the formation of 2-oxo-clopidogrel may be raised before reaching the liver under the involving of intestinal esterases, then metabolized into AM via hepatic CYPs. Secondly, the formed intermediate in the intestine could be oxidized to AM subsequently by the intestinal CYPs. Hypothetically, the one-step CYP-dependent oxidation was simplified, which was identical to the second step of AM from clopidogrel involving CYP3A4, 2B6, 2C19, and 2C9 (4,21).

In order to clarify the above hypotheses and rationalize the pharmaceutical design, it is of great importance to investigate whether CE2 or other possible esterases could contribute to the improved formation of 2-oxo-clopidogrel and elevated pharmacological potency. Initially, portal vein and jugular vein catheterization rat model was introduced to determine the amount of 2-oxo-clopidogrel and AM in presystem and circulation system (22), then to elucidate the contribution of intestine to formation of 2-oxo-clopidogrel and consequent AM following intraduodental administration. Afterwards, the *in vitro* system was applied to examine the rationale by investigation of vicagrel metabolism pattern in the intestinal microsomes (human) or S9 (rat). After the *in vitro* and *in vivo* evaluation, the esterases responsible for the cleavage of vicagrel to 2-oxo-clopidogrel in plasma and intestinal fractions were identified by inhibitions of the hydrolysis with specific esterase inhibitors or incubation with recombinant esterases. Eventually, the theory of drug design was rationalized via *in vitro* and *in vivo* investigation models, warranting further clinical pharmacokinetic studies of vicagrel.

MATERIALS AND METHODS

Chemicals and Reagents

Vicagrel (free base, 98% purity), clopidogrel (99% purity) and 2-oxo-clopidogrel (99% purity) were obtained from the State Key Laboratory of Natural Medicines and Center of Drug Discovery, College of Pharmacy, China Pharmaceutical University. R-95913 (prasugrel thiolactone metabolite, purity 98%) used as an internal standard (ISTD) was kindly gifted from Chia-Tai Tian Qing Pharmaceutical Co. Ltd (Jiangsu, China). MP-AM (active metabolite derivatized with 3-methoxyphenacyl bromide, MPBr) was supplied by the Center of Drug Discovery, China Pharmaceutical University. The derivatizing agent 3-methoxyphenacyl bromide (MPBr) was supplied by TCI (Shanghai) Development Co. Ltd. Sodium bis-p-nitrophenyl phosphate (99%, BNPP), 5, 5′-dithiobis-2-nitrobenzoic acid (99%, DTNB) and phenylmethylsulfonyl fluoride (99%, PMSF) were purchased from J&K Scientific (Shanghai, China). Tetra isopropyl pyrophosphoramide (iso-OMPA) was purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile and methanol of HPLC grade were obtained from Tedia (Fairfield, OH, USA). Sodium carboxymethyl cellulose (CMC-Na) was purchased from Dai-Ichi Kogyo Seiyaku Co., Ltd (Shanghai). Nicotinamide adenine dinucleotide phosphate (NADPH) regenerating system was purchased from Sigma-Aldrich (St. Louis, MO). Ethylenediamine tetra acetic acid disodium salt (EDTA), sodium fluoride (NaF), formic acid and acetic acid were of analytical grade.

Rat Intestinal S9 and Human Intestinal Microsomes

Pooled intestinal microsomes from human were purchased from BD Gentest (Woburn, MA, USA). Rat intestinal S9 were prepared by centrifugation at 9,000 g at 37°C for 20 min in lab. The protein concentration was determined by Bradford commercial kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Plasma and Recombinant Esterases

Rat plasma was harvested and pooled from Sprague-Dawley (SD) rats. Pooled human plasma was obtained from Nanjing First Hospital (Nanjing, China). Plasma was stored at −80°C, thawed under 4°C before use. After centrifugation at 3,000 rpm for 10 min, the supernatant was transferred and acclimated to the optimized conditions (pH = 7.4).

Both rhCE1 and rhCE2 expressed in murine myeloma cell lines (NS0-derived) were purchased from R&D Systems (Minneapolis, USA). rhPON1 expressed in *E. Coli* and purified by propriety chromatographic techniques was supplied by ProSpec-Tany TechnoGene Ltd (Ness Ziona, Israel). The purity of the three recombinant esterases (>95%) was determined by SDS-PAGE under reducing conditions. BChE prepared from equine serum using ammonium sulfate fractionation was purchased from Sigma-Aldrich (St. Louis, MO), with ≈ 70% protein in lyophilized powder.

EXPERIMENTAL DESIGN

Contribution of Intestine to the Formation of Intermediate and AM

Sprague-Dawley rats (350 ± 20 g) of both genders were supplied by Shanghai SIPPR/BK Experimental Animal Co (Shanghai, China). The rats were kept under optimized conditions (temperature: 20 ± 2°C, relative humidity: 50 ± 20%) and acclimatized to the housing environment for 1 week prior to the study. The rats were fasted but with free access to water for overnight before the administration. The study was conducted according to the Animal Ethics Committee of China Pharmaceutical University.

The abdomen of rats was opened with about 2 cm incision along the median line under pentobarbital anesthesia. After the separation, a heparin-loaded polyethylene tubing cannula (I.D. 0.28 mm; O.D. 0.61 mm; Becton Dickinson) was inserted into the pyloric vein, and partly stretched into the hepatic vein to withdraw the blood in portal vein. The second heparinization cannula (I.D. 0.53 mm; O.D. 0.96 mm; Becton Dickinson) was inserted into the jugular vein. After surgery, about 0.1 ml of blood was extracted from the portal vein and jugular vein before dosing respectively. As our previously reported method, the dosing solution (vicagrel, clopidogrel and 2-oxo-clopidogrel) was dispersed in CMC-Na suspensions (20). The solution of vicagrel or clopidogrel or 2-oxo-clopidogrel was intraduodenally to the cannulated rats at a dose level of 20 μmol/kg. From the inserted port, an aliquot of 100 μL blood was collected by heparinized syringe at 5, 10, 20, 30, 60, 90, 120 min post dosing, from the jugular and portal vein respectively. To avoid the blood coagulation in the tube and in the vessel, the blood left in the cannula was pushed back to the vein by heparin saline. After sampling, the blood samples were centrifuged immediately at 12,000 rpm for 1 min at 4°C. An aliquot of 50 μL of harvested plasma was transferred into the double volume of cold acetonitrile containing 5% acetic acid and 50 ng/ml ISTD to prevent the instability of possible metabolites. After centrifugation at 16,000 rpm for 10 min at 4°C, the supernatant was pipetted into the 10 mM MPBr (final concentration) solution to derivatize the AM under optimized condition (20). The prepared samples were kept at −80°C till analysis by LC-MS/MS.

The pharmacokinetic parameters based on the non-compartmental method were calculated by the computer program WinNonlin Professional (Version 4.0; Pharsight Corp., Mountain View, CA). Area under the curve from the beginning to the last time point (AUC_{0-2h}) was estimated by the trapezoidal rule. The contribution of intestine was estimated by the difference between portal and system exposures of 2-oxo-clopidogrel and AM after dosing (22).

In order to evaluate the balance of AM formation in the intestine and its formation and extraction in the liver, the extraction fraction (E) has been taken into account. The relationship between extraction fraction (E) and hepatic availability ($F_{h(AM)}$) is depicted as $F_{h(AM)}=1-E$. The hepatic availability of AM $F_{h(AM)}$ is calculated as:

$$F_{h(AM)} = \frac{BA}{F_{clopidogrel\ to\ 2-oxo} \cdot F_{2-oxo\ to\ AM} \cdot F_a},$$

where BA represented the oral bioavailability of AM from clopidogrel in rats, $F_{clopidogrel\ to\ 2\text{-}oxo}$ was the fraction of 2-oxo-clopidogrel produced from clopidogrel, $F_{2\text{-}oxo\ to\ AM}$ was the conversion fraction of AM from 2-oxo-clopidogrel, F_a represented the fraction of absorbed clopidogrel from intestine. Referring to the published data, $F_{clopidogrel\ to\ 2\text{-}oxo}$, $F_{2\text{-}oxo\ to\ AM}$ and F_a were set at 0.15, 0.52, and 0.75 respectively (23,24). BA of AM from clopidogrel was the in-lab data, set as 5.4% (Calculated by $AUC_{(AM,\ clopidogrel-p.o.)}/AUC_{(AM,\ AM-i.v.)}$). For vicagrel, the AM in hepatic vein was divided into two parts. One was from the AM produced during absorption process but experienced extraction by liver. The second part was converted in the liver from the produced 2-oxo-clopidogrel during absorption.

$$F_a \cdot F_{g(vicagrel\ to\ AM)} = Q_p \cdot R_b \cdot \frac{AUC_{portal(AM)} - AUC_{system\ (AM)}}{Dose},$$

$$F_a \cdot F_{g(vicagrel-to\ 2-oxo)} = Q_p \cdot R_b \cdot \frac{AUC_{portal(2-oxo)} - AUC_{system(2-oxo)}}{Dose}.$$

Therefore, the oral BA of AM from vicagrel was calculated as follows:

$$BA = F_a \cdot F_{g(vicagrel\ to\ AM)} \cdot F_{h(AM)} + F_a \cdot F_{g(vicagrel\ to\ 2-oxo)} \cdot F_{2-oxo\ to\ AM.}$$

Where $F_{g(vicagrel\ to\ AM)}$ and $F_{g(vicagrel\ to\ 2\text{-}oxo)}$ represented the conversion fraction of AM and 2-oxo-clopidogrel from vicagrel during absorption, $AUC_{portal(AM)}$ and $AUC_{system(AM)}$ was the area under the time-plasma concentration profile of AM in the portal vein and circulation system (jugular vein) respectively, likewise, $AUC_{portal(2\text{-}oxo)}$ and $AUC_{system(2\text{-}oxo)}$ represented the area under the time-plasma concentration profile of 2-oxo-clopidogrel in the portal and jugular vein separately. Q_p was the hepatic blood flow rate, set at 3.31 L/h/kg. R_b was the blood/plasma ratio, set as 1 referring to the published data and in-lab data (20).

Formation of 2-oxo-clopidogrel in Rat Intestinal S9 and Human Intestinal Microsomes

Firstly, it was essential to assay the chemical stability of vicagrel in various pH conditions. The incubations were at 37°C in Tris buffer at pH 2.0, 6.0, 7.4 and 8.5. The reaction was initiated by adding an aliquot of vicagrel (in acetonitrile) with a final concentration at 1 μM. At different time intervals (0, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24 h), an aliquot of 100 μl mixture was mixed with twice the volume of cold acetonitrile containing ISTD in a tube placed on ice. All reactions were conducted in triplicate. The analyte/ISTD peak area ratios for remaining vicagrel or 2-oxo-clopidogrel were calculated with the ratio at the initial time set as 100%. The slope of the log percentage remaining *versus* the time point was calculated as the rate constant λ, which was converted to $t_{1/2}$ using the equation: $t_{1/2}$ (min)=0.693/λ

Secondly, the enzyme reaction system contained S9 or microsomes in Tris-HCl (pH=7.4) buffer solution with 1 ml of total incubation volume, containing rat intestinal S9 (final

concentration, 50 μg protein/ml) or human intestinal microsomes (final concentration, 20 μg protein/ml). The mixture was pre-incubated in buffer solution at 37°C for 5 min, then 100 μl of vicagrel was added to initiate the reaction with a final concentration at 1 μM. All reactions were performed in triplicate. In the preliminary experiment, the protein amount in the incubation system was optimized. The incubation was terminated by addition of double volume of cold acetonitrile containing ISTD at 0, 1, 2, 5, 10, 15, 30, 45, 60, 90 and 120 min and by placing the tubes on ice.

Formation of AM in Rat Intestinal S9 and Human Intestinal Microsomes

Based on the above investigation, another incubation system consisted of 5 mM NADPH, 10 mM $MgCl_2$, 5 mM glutathione and 2 mg/ml rat intestinal S9 or 1 mg/ml human intestinal microsomes (final concentration). The mixture was pre-incubated at 37°C for 5 min, then an aliquot of vicagrel (final concentration at 1 μM) was added into the system to initiate the incubation. After the incubation for the time intervals at 0, 5, 10, 15, 20, 30, 45, 60 and 90 min, the reaction was terminated by double volume of cold acetonitrile containing ISTD and 10 mM derivation agent (MPBr). The AM was derivatized as our proposed method previously (20).

Screening for Esterases Involved in the Hydrolysis of Vicagrel

In order to screen the related-esterases, the rat or human plasma was co-incubated with vicagrel in the incubation system, since the plasma contained esterases in great variety, such as CE (exclude human plasma), PON and BChE, etc. Initially, the temperature- & time- effect was evaluated under the optimized conditions. The plasma (rat or human) was pre-incubated in buffer solution at 4°C, 25°C and 37°C. The preferred plasma volume of rat and human was set at 0.1% and 10% of the total incubation volume. The hydrolysis was terminated by addition of double volume of cold acetonitrile (containing 50 ng/ml ISTD) at 0, 0.5, 1, 2, 5, 10, 20 and 30 min by placing the tubes on ice. Meanwhile, incubation with the buffer instead of inhibitor served as the vehicle control, which was applied to correct for the non-enzyme instability. Due to the different volume of plasma in the incubations, the *in vitro* clearance ($Cl_{in\ vitro}$) of vicagrel was calculated using equation below to assess the effect of temperature and NaF:

$$Cl_{in\ vitro}\left(_{ml\cdot \min^{-1}\cdot mg\ protien^{-1}}\right) = \lambda\left(\min^{-1}\right) \times \frac{V_{(ml)}}{P_{\ incubation\ (mg\ protein)}}$$

where $P_{incubation}$ was the protein amount of plasma in the incubation mixture and V represented the reaction volume, λ was the first-order hydrolysis rate constant.

Afterwards, rat and human plasma were pre-incubated with the recommended esterase inhibitor for 30 min at 37°C respectively. Likewise, in order to investigate the esterases in the intestine responsible for vicagrel cleavage, each recommended inhibitors were co-incubated with rat intestinal S9 fraction and human intestinal microsomes under preferred condition. BNPP inhibits the hydrolytic activity related to CE (25,26). iso-OMPA is the selective inhibitor of BChE (27). EDTA could chelate with calcium ion, which is essential for the hydrolysis process, then suppress the hydrolysis mediated by PON (28,29). PMSF is the inhibitor of serine-hydrolylase (30). DTNB is the specific inhibitor of ArE (31). NaF is the general esterase and esterase-like hydrolysis inhibitor (32,33). By adding an aliquot of vicagrel into rat or human plasma or intestinal fractions, the incubation was terminated by adding double volume of cold acetonitrile (containing 50 ng/ml ISTD) after incubation for 5 min. The inhibition ratio (R) was calculated as follows:

$$R(\%) = \left(1 - \frac{1 - Residual_{with\ inhibitor}}{1 - Residual_{without\ inhibitor}}\right)\ \%$$

where $Residual_{with\ inhibitor}$ and $Residual_{without\ inhibitor}$ represented the remaining of vicagrel (survived the hydrolysis) co-incubated with and without inhibitors in the incubation system respectively.

Moreover, recombinant enzyme (rhCE1, rhCE2, rhPON1 or BChE from equine serum) was added to identify the isoforms of esterase responsible for vicagrel hydrolysis. The enzyme concentration and incubation time were optimized via time-dependent depletion as described above. In order to guarantee the depletion within 20% of the total amount of vicagrel, the preferred protein concentration was set at 50, 25, 7.5 and 400 ng/ml for rhCE1, rhCE2, rhPON1 and BChE, respectively. Vicagrel was co-incubated with protein for 5 min with a final concentration at 10 μM and 100 μM, except for rhCE1 which was incubated for 10 min. The rate (pmol/min/μg protein) of 2-oxo-clopidogrel formation was compared to the contribution of specific esterase to the cleavage of vicagrel.

LC-MS/MS Assay

Vicagrel, 2-oxo-clopidogrel and ISTD (R-95913) were analyzed by a validated LC-MS/MS method (API 4000 and Shimadzu HPLC system) previously described. Briefly, the analytes were separated by gradient elution on Shim-pack VP-ODS column (Shimadzu, 150×2.0 mm), coupled with a Security Guard C18 guard column (4×3.0 mm, Phenomenex, Torrance, CA, USA). The LC and MS/MS conditions were optimized as mentioned previously. The

transitions of *m/z* 380.1 to *m/z* 212.1, *m/z* 338.2 to *m/z* 155.1, *m/z* 504.0 to *m/z* 212.1 and *m/z* 332.1 to *m/z* 149.1 were monitored in multiple reaction monitoring (MRM) mode, under the collision energy set at 35, 35, 25.4 and 35 eV, with declustering potential set at 45, 45, 36.5 and 45 V for vicagrel, AM, 2-oxo-clopidogrel, and ISTD, respectively. In order to guarantee the accuracy and precision of analysis, in each analysis batch, there were quality control samples, which were within the acceptable range of deviation. Moreover, for the determination of 2-oxo-clopidogrel produced in the recombinant system, the samples were concentrated to make certain that detection, along with the quality control samples which were processed in an identical manner.

RESULTS

Formation of 2-oxo-clopidogrel and AM in the Intestine of Cannulated Rats Model

Following intraduodenal administration of vicagrel to the cannulated rats, the amount of vicagrel was in trace amount in both the portal and jugular vein, failed to be determined by LC-MS/MS. The time-profile of 2-oxo-clopidogrel in portal and jugular vein was shown in Fig. 2. The $AUC_{0\text{-}2h}$ of 2-oxo-clopidogrel from vicagrel in portal vein was 186.0±41.9 μg·h/L, which was much higher than that in jugular vein with the value of 17.5±2.4 μg·h/L. The plasma concentration-time profile of 2-oxo-clopidogrel in the rats following 2-oxo-clopidogrel administration directly shared similar tendency, with the AUC_{0-2h} of 215.4±24.5 μg·h/L in portal vein and 23.0±4.8 μg·h/L in jugular vein respectively. But for the group following clopidogrel intraduodenally administration, both the AUC_{0-2h} of 2-oxo-clopidogrel in the portal vein (0.26±0.05 μg·h/L) and jugular vein (7.8±1.7 μg·h/L) were far lower than those received from vicagrel or 2-oxo-clopidogrel, as shown in Table I. The C_{max} of 2-oxo-clopidogrel exhibited similar difference to that of AUC_{0-2h} in the portal vein and jugular vein.

Meanwhile, as depicted in Fig. 3, the detected AMs from vicagrel, 2-oxo-clopidogrel in the portal vein were 47.4±4.2 μg·h/L and 55.7±4.3 μg·h/L higher than those from jugular vein, with the value of AUC_{0-2h} 26.8±2.9 μg·h/L and 26.1±2.1 μg·h/L respectively.

For clopidogrel, the AUC_{0-2h} of the formed AM in the portal vein was quite minor (0.04±0.01 μg·h/L), which was lower than that detected in the circulation system (4.3±0.2 μg·h/L). Therefore, the intestine availability was neglectable, set at 1 ($F_g = 1$), hereby, the hepatic availability was calculated as 0.92 ($F_{h(AM)} = 1\text{-}E$). But for vicagrel, it was converted to 2-oxo-clopidogrel by 4.8% ($F_a \cdot F_{g(\text{vicagrel to}2-\text{oxo})} + F_a \cdot F_{g(\text{vicagrel to AM})}$) and AM by 1.6% ($F_a \cdot F_{g(\text{vicagrel to AM})}$) during the absorption. Taking the $F_{h(AM)}$ into consideration, about 1.5% of the giving vicagrel entered the circulation system as AM after intestinal metabolism, while the fraction produced from 2-oxo-clopidogrel in the liver accounted for 1.7% of the administrated vicagrel.

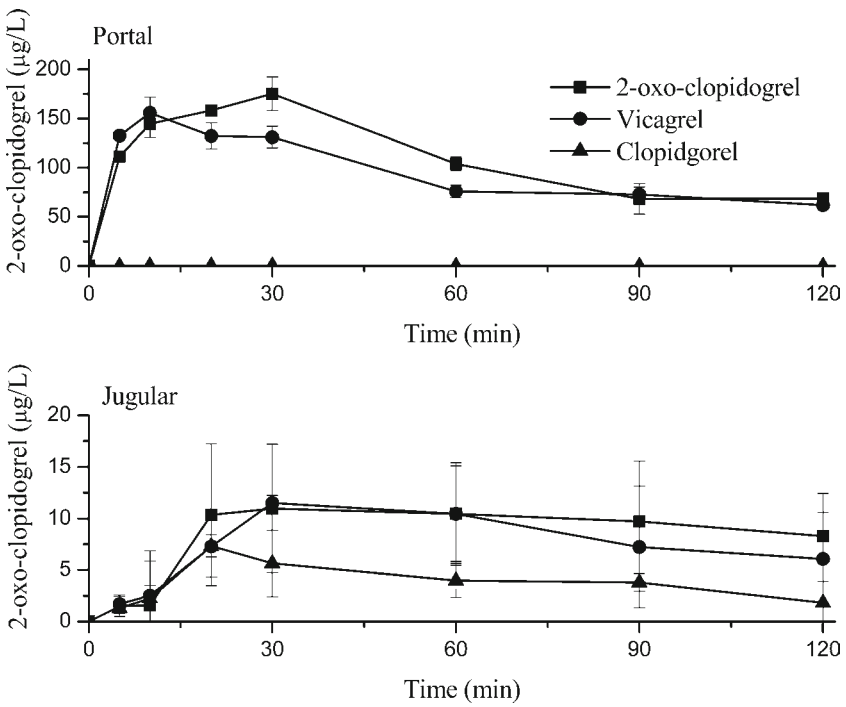

Fig. 2 Plasma concentrations of 2-oxo-clopidogrel in portal vein and jugular vein following intraduodenal administration of vicagrel, 2-oxo-clopidogrel and clopidogrel at 20 μmol/kg to rats.

Table I The AUC_{0-2h} and C_{max} of 2-oxo-clopidogrel and AM from Vicagrel, 2-oxo-clopidogrel and Clopidogrel in Portal Vein and Jugular Vein

		AUC_{0-2h} (μg·h/L)		C_{max} (μg/L)	
		Portal	Jugular	Portal	Jugular
2-oxo-clopidogrel	Vicagrel	186.0 ± 41.9	17.5 ± 2.4	156.1 ± 19.2	11.5 ± 0.7
	2-oxo-clopidogrel	215.4 ± 24.5	23.0 ± 4.8	175.1 ± 17.2	10.9 ± 6.2
	Clopidogrel	0.26 ± 0.05	7.8 ± 1.7	0.27 ± 0.12	7.3 ± 1.1
AM	Vicagrel	47.4 ± 4.2	26.1 ± 2.1	50.6 ± 19.1	18.6 ± 3.1
	2-oxo-clopidogrel	55.7 ± 4.3	26.8 ± 2.9	49.2 ± 17.3	20.2 ± 15.7
	Clopidogrel	0.04 ± 0.01	4.3 ± 0.2	0.05 ± 0.03	6.4 ± 1.8

Formation of 2-oxo-clopidogrel and AM in Rat Intestinal S9 and Human Intestinal Microsomes

Under various pH conditions, vicagrel remained stable at pH 2.0 with a $t_{1/2}$ exceeding 24 h, but was almost completely degraded under alkaline condition (pH 8.5) in 6 h. Under pH 7.4, vicagrel was cleaved with a $t_{1/2}$ at around 5 h. The formation of 2-oxo-clopidogrel from vicagrel increased gradually over 24 h. However, 2-oxo-clopidogrel formation peaked and decreased rapidly after 2 h (pH 8.5) and 6 h (pH 7.4) respectively, suggesting that alkaline conditions accelerate the hydrolysis rate of vicagrel and 2-oxo-clopidogrel.

In rat intestinal S9 and human intestinal microsomes, vicagrel was depleted quickly, along with the rapid formation of 2-oxo-clopidogrel. Except the hydrolysis of alkaline pH, the estesases in the intestine contributed greatly to the 2-oxo-clopidogrel formation, which could be inhibited by the addition of NaF (inhibition ratio, 97.1 ± 9.7% for rat and 89.9 ± 3.6% for human, shown in Table IV). After 120 min, vicagrel was almost totally converted to 2-oxo-clopiodgrel, as depicted in Fig. 4.

After the addition of NADPH and GSH, the formation of AM was increased gradually over the incubation time, and the amount of 2-oxo-clopidogrel produced immediately from vicagrel was decreased slowly, as shown in Fig. 5.

Influence of Esterases in Vicagrel Cleavage

At different temperatures, the logarithmic residual % of vicagrel *versus* time profile (in the absence or presence of NaF) was conducted in rat and human plasma, respectively. The hydrolysis rate declined with decreasing temperature and with the addition of NaF, ruling out the possibility that vicagrel hydrolysis was due to its chemical instability in buffer. Taking the incubation at 37°C for instance, the *in vitro* clearance of vicagrel in rat plasma and human plasma without inhibitors was 4.059 ± 0.576 $ml \cdot min^{-1} \cdot mg\ protein^{-1}$ and 0.072 ± 0.016 $ml \cdot min^{-1} \cdot mg\ protein^{-1}$, which could be reduced by the

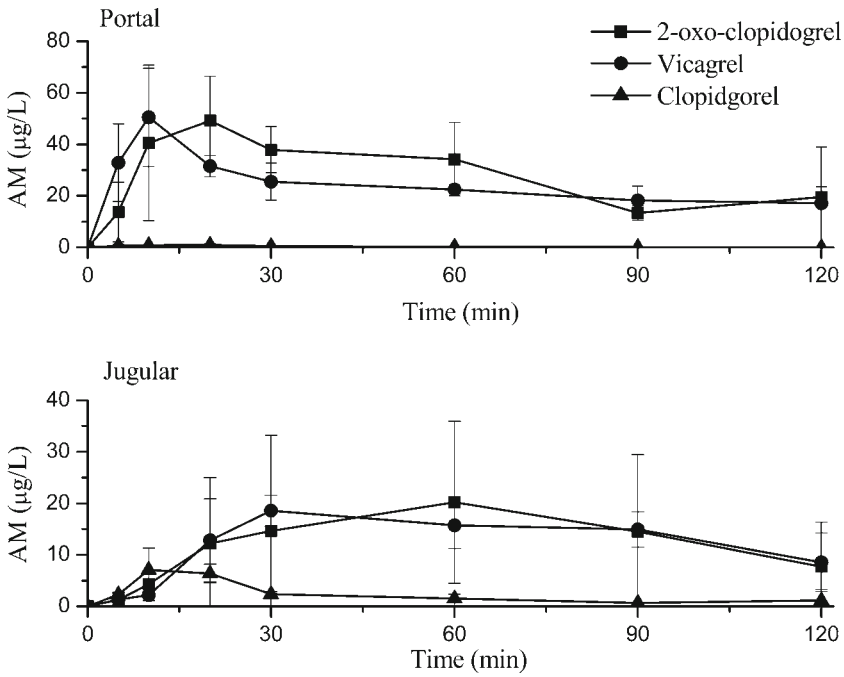

Fig. 3 Plasma concentrations of AM in portal vein and jugular vein following intraduodenal administration of vicagrel, 2-oxo-clopidogrel and clopidogrel at 20 μmol/kg to rats.

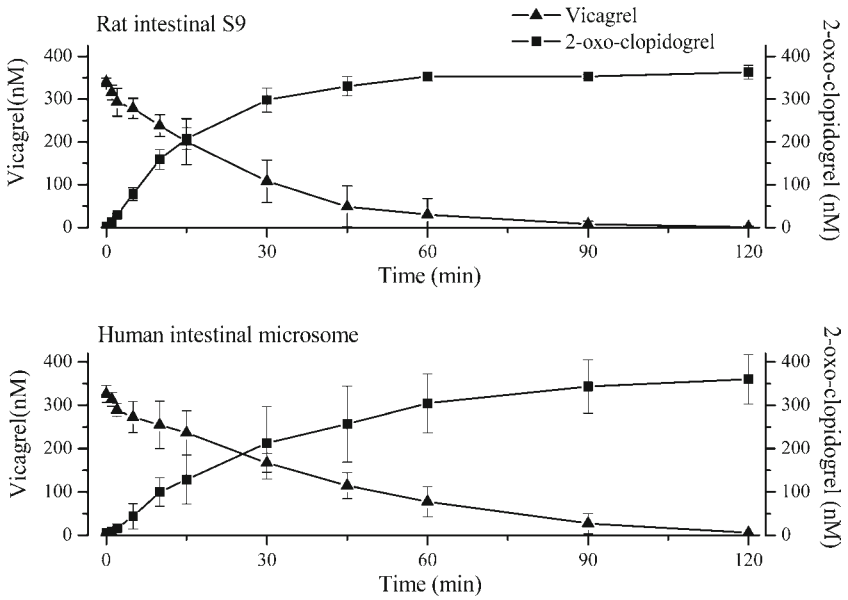

Fig. 4 Formation of 2-oxo-clopidogrel from vicagrel over incubation time in rat intestinal S9 and human intestinal microsomes.

inhibition of NaF, with the value of 1.325±0.336 and 0.010±-0.003 ml·min^{-1}·mg protein^{-1}, respectively. The clearance in rat plasma was higher at different temperatures than that conducted in human plasma, as exhibited in Table II.

Based on the above findings, the influence of various inhibitors on the hydrolysis of vicagrel in plasma was examined and summarized in Table III. In rat plasma co-incubated with BNPP (200 μM), the cleavage of vicagrel was most strongly inhibited (69.0±2.8%), being close to that observed with NaF (64.2±9.9%). The hydrolysis was also inhibited by the serine-enzyme inhibitor PMSF (62.3±1.6%). Moreover, the PON inhibitor EDTA and BChE inhibitor iso-OMPA inhibited vicagrel hydrolysis by 53% and 60%, respectively. However, in human plasma, BNPP and PMSF displayed only mild inhibition (<10%) of vicagrel bioconversion, while EDTA inhibited hydrolysis by 54.9±2.7%. The hydrolysis of vicagrel was only marginally (<10%) inhibited by the arylesterase inhibitor DTNB.

In the rat intestinal S9 fraction and human intestinal microsomes, the hydrolysis of vicagrel was severely inhibited by BNPP (at 200 μM) with the 89.9±14.5% and 83.7±18.5% inhibition for rat and human respectively. Likewise, PMSF (100 μM) exhibited similar inhibition on vicagrel cleavage in the intestinal materials (85.2±12.7% for rat and 46.0±14.4% for human). Except CE inhibitor BNPP and serine-esterase inhibitor PMSF, EDTA (PON), DTNB(Arylesterase) and iso-OMPA contributed to the vicagrel hydrolysis in a certain extent at higher concentrations but restrained slightly at lower

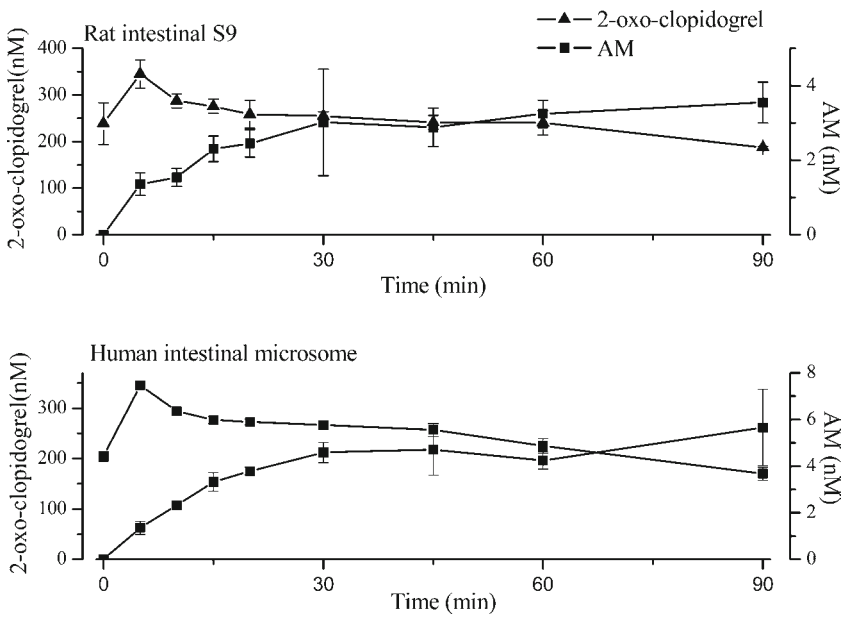

Fig. 5 Formation of AM from vicagrel over incubation time in rat intestinal S9 and human intestinal microsomes.

Table II Hydrolysis Rate Constant (λ) and $Cl_{in\ vitro}$ of Vicagrel in Rat and Human Plasma in the Absence and Presence of NaF at 4°C, 25°C, and 37°C

	Temperature(°C)	NaF (mg/ml)	λ (min^{-1})	$Cl_{in\ vitro}$ ($ml \cdot min^{-1} \cdot mg\ protein^{-1}$)
Rat	4	0	0.031 ± 0.006	0.391 ± 0.069
		20	0.002 ± 0.001	0.029 ± 0.008
	25	0	0.110 ± 0.020	1.371 ± 0.250
		20	0.01 ± 0.004	0.134 ± 0.039
	37	0	0.325 ± 0.046	4.059 ± 0.576
		20	0.106 ± 0.027	1.325 ± 0.336
Human	4	0	0.031 ± 0.002	0.016 ± 0.001
		20	0.003 ± 0.001	0.002 ± 0.001
	25	0	0.047 ± 0.007	0.024 ± 0.004
		20	0.011 ± 0.003	0.006 ± 0.002
	37	0	0.141 ± 0.032	0.072 ± 0.016
		20	0.019 ± 0.005	0.010 ± 0.003

concentrations of each inhibitors. The inhibition ratios of various inhibitors were summarized in Table IV.

In the recombinant enzyme incubation system, at 10 μM of vicagrel concentration, rhCE1, rhCE2, rhPON1 and BChE from equine serum exhibited hydrolytic activity with the rate of 2-oxo-clopidogrel formation calculated at 1.2, 20.0, 71.9 and 0.9 pmol/min/μg protein. All four recombinant esterases exhibited increased formation rate with 100 μM of vicagrel. PON1 showed the highest catalytic activity (1770.9 pmol/min/μg protein), CE2 exhibited the hydrolysis efficiency of 394.4 pmol/min/μg protein. While CE1 and BChE contributed to the hydrolysis of vicagrel to a minor extent at 45.1 pmol/min/μg protein and 22.2 pmol/min/μg protein, respectively, as summarized in Fig. 6.

Table III Effect of Esterase Inhibitors on the Cleavage of Vicagrel in the Rat and Human Plasma at 37°C

Inhibitors	Target esterase	Concentration (μM)	Inhibition ratio (%)	
			Rat	Human
BNPP	CE	200	69.0 ± 2.8	6.3 ± 2.1
		20	57.9 ± 3.9	3.0 ± 0.3
PMSF	Serine hydrolase	100	62.3 ± 1.6	8.9 ± 0.6
		20	34.1 ± 0.9	4.2 ± 0.5
EDTA	PON	1,000	53.0 ± 2.6	54.9 ± 2.7
		100	47.8 ± 1.2	16.2 ± 1.2
DTNB	Arylesterase	200	9.7 ± 1.9	1.6 ± 0..9
		10	1.0 ± 0.3	1.1 ± 0.4
ISO-OMPA	BchE	200	60.1 ± 4.0	10.8 ± 0.7
		10	49.0 ± 1.8	9.6 ± 0.3
NaF	General	20 mg/ml	64.2 ± 9.9	23.8 ± 1.3

Table IV Effect of Esterase Inhibitors on the Cleavage of Vicagrel in the Rat Intestinal S9 and Human Intestinal Microsomes at 37°C

Inhibitors	Target esterase	Concentration (μM)	Inhibition ratio (%)	
			Rat	Human
BNPP	CE	200	89.9 ± 14.5	83.7 ± 18.5
		20	49.6 ± 26.2	34.6 ± 10.1
PMSF	Serine hydrolase	100	85.2 ± 12.7	46.0 ± 14.4
		20	33.2 ± 15.3	14.5 ± 6.6
EDTA	PON	1,000	35.5 ± 7.6	16.7 ± 3.7
		100	3.3 ± 0.3	1.4 ± 0.1
DTNB	Arylesterase	200	19.4 ± 12.8	19.1 ± 15.4
		10	0.2 ± 0.1	2.2 ± 0.6
ISO-OMPA	BchE	200	34.1 ± 10.3	28.2 ± 11.4
		10	2.2 ± 0.7	1.9 ± 0.4
NaF	General	20 mg/ml	97.1 ± 9.7	89.9 ± 3.6

DISCUSSION

Traditionally, a prodrug is designed so as to improve the bioavailability thus enhancing the pharmacological potency, through release of the active metabolite or intermediate at an appropriate time post-administration and in an efficient manner. Vicagrel, the acetate-derivative of clopidogrel, was designed to further improve its anti-platelet aggregation efficiency and therapeutic reliability in a clinical setting. Through simple chemical modification of clopidogrel, vicagrel was aimed to steer the metabolic pathway predominantly towards the AM, while also ensuring increased rate of hydrolysis of the parent drug would go some way in circumventing CR, which continues to be of concern with clopidogrel use, due to CYP polymorphisms or drug-drug interactions (8,34).

The contribution of intestine to the presystem metabolism of vicagrel was evaluated by the detection of 2-oxo-clopidogrel and AM in the rat portal vein and jugular vein following intraduodenal administration of vicagrel. Encouragingly, the presystem exposures of 2-oxo-clopidogrel and AM from vicagrel (AUC_{0-2h}) in the portal vein was far greater than that from clopidogrel, which was only detected in quite a low amount. Meanwhile, the system exposures of 2-oxo-clopidogrel and AM from vicagrel in the jugular vein was also higher than that from clopidogrel. The comparison of system exposure was in accordance with our previous investigation conducted in rats (20). In terms of clopidogrel, it was difficult for clopidogrel to be metabolized to 2-oxo-clopidogrel in the rat intestine, due to the minimal expression or low activity of CYPs (35,36). The majority was initially absorbed as unchanged prodrug from the intestine, followed by rapid hydrolysis to the inactive acid metabolite catalyzed via the hepatic CE1, with a small portion (about 15%) entering the oxidation to the 2-oxo-clopidogrel by hepatic CYPs, minor of the produced 2-oxo-

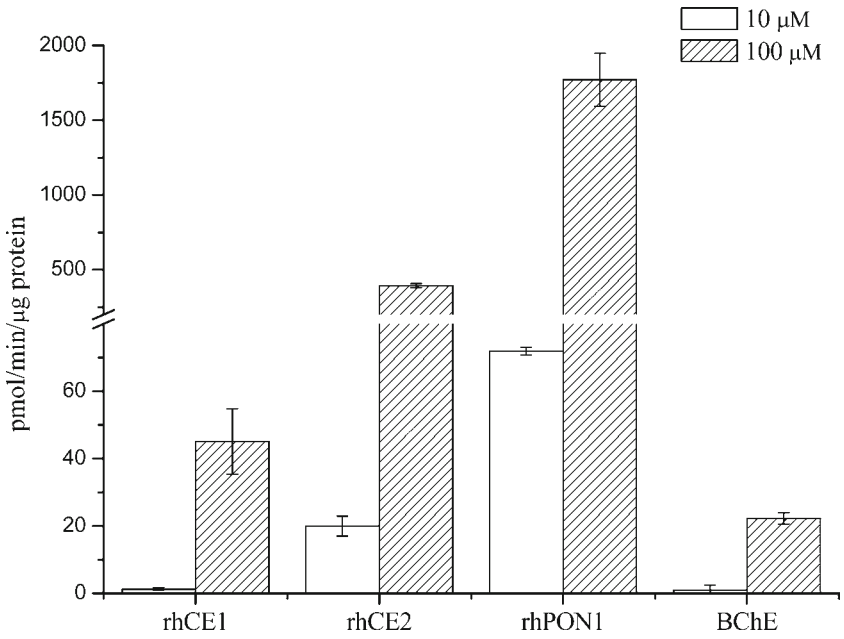

Fig. 6 Rate of formation for 2-oxo-clopidogrel from 10 μM and 100 μM vicagrel in a series of recombinant esterases.

clopidogrel (52%) subsequently undergone the further oxidation to AM by CYPs (37), but the major proportion was hydrolyzed to acid form by hepatic CE1 (36,38,39). Likewise, the cascading AM was partly deactivated by CE1 in the liver (39). All these factors contributed to the lower presystem and system exposure of 2-oxo-clopidogrel and AM following clopidogrel administration. On the contrary, the acetate of clopidogrel, vicagrel could be extensively hydrolyzed to 2-oxo-clopidogrel during intestinal absorption. The produced 2-oxo-clopidogrel was subsequently converted to AM via the oxidation of CYPs in the rat intestine (40). The plasma could possibly contribute to the improved 2-oxo-clopidogrel from the survived vicagrel during the absorption. The produced 2-oxo-clopidogrel in the intestine and plasma arrived in the liver and undergone further oxidation to AM, the same way as the second step of clopidogrel by hepatic CYPs (21,35), as summarized in Fig. 7. Since the hepatic availability of AM was 0.92, the contribution of intestinal production accounted for 46% (fraction of production in the intestine experienced hepatic extraction/total fraction of AM, 1.5%/3.2%) of the circulation exposure of AM. Therefore, the intestinal metabolism of vicagrel played an indispensible role in the AM production. The absorption fraction (F_a) probably contributed to the relative low total absorption availability, which was also partly ascribed to the presystem metabolism of AM with great possibilities. On the one hand, CE1 is highly located in the rat plasma (41), which could contribute to the hydrolysis of the produced AM. On the other hand, the formed AM transferred into the liver, along with the instant produced AM from 2-oxo-clopidogrel in the liver (36,39), could partly undergo further deactivation losing methyl group via hepatic CE1 (shown in Fig. 7). Actually, it did not exclude the possibility of efflux pump participation in the efflux of AM, such as P-gp, MRP2, etc. Considering that CE is not located in the human plasma (14,16,41), it was more predicable that the amount of AM from vicagrel in the human could be more considerable to some extent, in other words, the contribution of intestinal formation of AM would be probably more significant.

The *in vitro* investigation found that vicagrel was almost completely hydrolyzed to 2-oxo-clopidogrel in the rat intestinal S9 and human intestinal microsomes. In the phase I system containing NAPDH and GSH (Conjugate with the formed AM), 2-oxo-clopidogrel was formed immediately, as the initiation by adding of vicagrel, while AM was produced gradually with the increasing of incubation time. The produced AM in the intestinal S9/microsomes system was limited to a minor amount, far lower than the amount of 2-oxo-clopidogrel cleared. The disproportion between the elimination and production was probably ascribed to the formation of other metabolites. Other uncertain esterases and esterase-like enzyme (not restricted to CE and BChE) located in the intestine might be also involved in the hydrolysis of 2-oxo-clopidogrel. For instance, it is reported that 2-oxo-clopidogrel is cleared to endo-SH via PON (mainly PON1) (35).

On account of drug design hypothesis, together with the key contribution of intestine, it was of top importance to identify the specific esterase responsible for hydrolysis of vicagrel, especially in the intestine. The enzyme inhibition and recombinant enzymes are two acceptable and reliable methods to screen the responsible enzymes (42). The rat and human plasma were introduced to co-incubate with the recommended esterase inhibitors, since the plasma held esterases in great variety, such as CE, PON, BChE and ArE, etc. (41,43). Initially, stability of vicagrel was found pH-dependent, with decreased stability shown under alkaline conditions in the non-enzymatic buffer solution. The simulated pH conditions were close to physiological conditions found

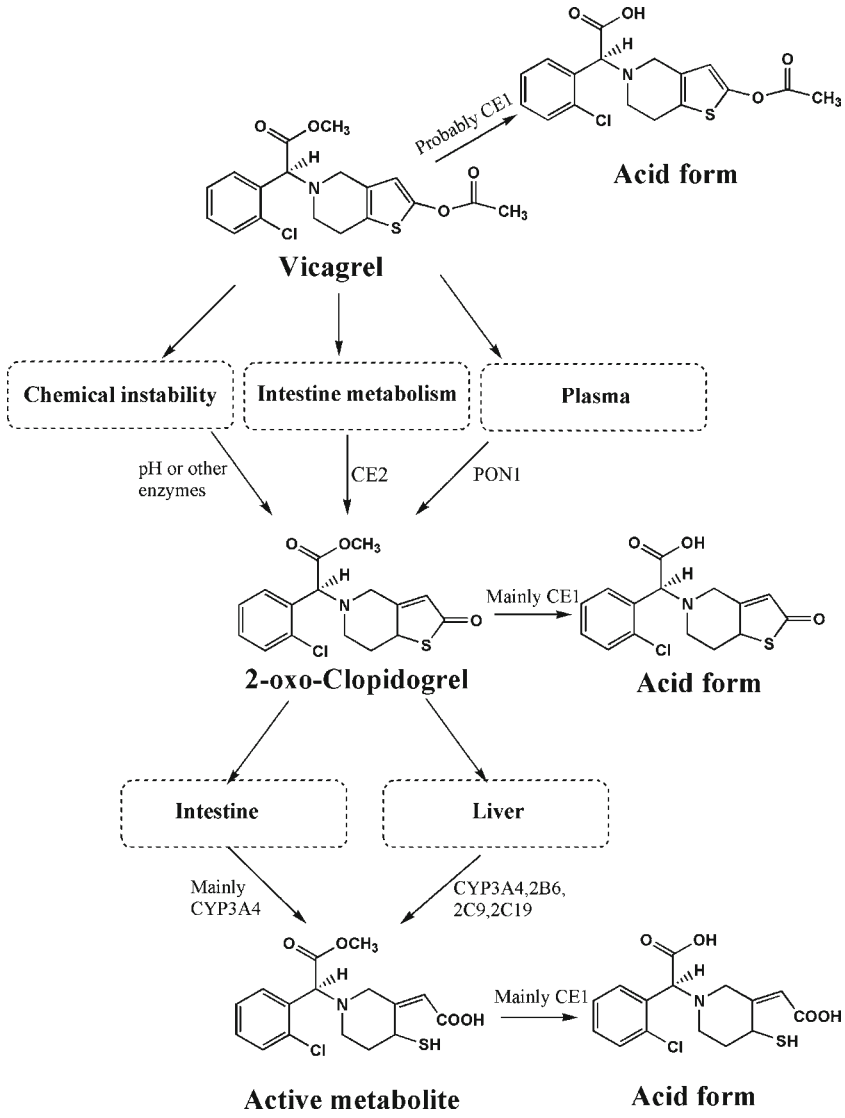

Fig. 7 Proposed cleavage pathways of vicagrel and responsible enzymes for its bioconversion.

in the stomach, intestine and plasma. The stability results suggested that vicagrel was expected to survive passing through the stomach, but was expected to be cleaved to 2-oxo-clopidogrel in the intestine to some extent. The hydrolysis of vicagrel was much faster in rat or human plasma than in buffer solution, typically inhibited by NaF. This finding indicated that the hydrolysis of vicagrel was largely mediated by plasma esterases, while also pH and temperature-dependent.

We went on to introduce a range of different esterase inhibitors to identify the specific esterase responsible for hydrolysis of vicagrel into 2-oxo-clopidogrel. In rat plasma, BNPP, PMSF, EDTA and iso-OMPA exhibited strong levels of inhibition, while DTNB had little impact, indicating that CE, PON and BChE (and excluding ArE) were primarily responsible for the bioconversion of vicagrel into 2-oxo-clopidogrel in rat. In human plasma, most extensive inhibition by EDTA was indicative of the role of PON in hydrolysis, while the other inhibitors (except NaF) showed only slight effects on the hydrolysis of vicagrel. Therefore, PON was found to be the major esterase responsible for hydrolysis of vicagrel while BChE contributed mildly to vicagrel cleavage in human plasma. The findings were in accordance with the fact that human plasma has no CE but relatively high PON-related hydrolytic activity (41,44). Both in human and rat intestinal fractions, BNPP and PMSF showed the great inhibitions. It was illustrated that CE extensively contributed to the cleavage of vicagrel, which was in parallel to the widely accepted fact that rat intestine shares similar CE expression with high homology to that in human intestine. Furthermore, BChE located in rat and human intestine, took the charge of vicagrel hydrolysis in a minor extent by comparison to the impact of CE. The inhibition by EDTA (35.5 ± 7.6% and 16.7 ± 3.7% for rat and human respectively) put forward the possibility of the existence of PON in the intestine.

Based on the results of screening experiments with inhibitors, the recombinant esterases, rhCE1, rhCE2, rhPON1 and

BChE, were applied to further assess the hydrolysis of vicagrel. CE1 and CE2 are the two most important isoforms of CEs, which are the two pivotal isoforms for the design theory of vicagrel (8). PON1 is the reported as a major bioactivating enzyme for several lactone drugs (17), BChE is the representative isoform of cholinesterase (16). The four representative recombinant esterases, covering the A-and B-type of esterase, all showed some activity for the formation of 2-oxo-clopidogrel from vicagrel, and could be inhibited by recommend inhibitors. rhPON1 exhibited the highest hydrolytic rate, while the 2-oxo-clopidogrel formation rate in rhCE2 ranked 2nd, with around 7-fold higher than that in rhCE1, and BChE ranked the lowest. Comprehensively, CE2 played an indispensible role in the 2-oxo-clopidogrel producing from vicagrel, and PON1 exhibited the greatest hydrolysis activity, guaranteeing the further hydrolysis in case of vicagrel probably escaping from hydrolysis during absorption. Moreover, the failed detection of vicagrel in the portal vein may be on the account of the cooperation of various esterases, not restricted to CE2 in the intestine but the predominate expression of PON1 in the plasma. While the contributions of CE1 and BChE remained within a minor content. Admittedly, the roles of other esterases and esterase-like enzymes should not be ruled out, especially in the intestine.

In combination the inhibition and recombinant experiments, beside the cleavage to 2-oxo-clopidogrel from vicagrel, there were still other two possible hydrolysis pathways. On the one hand, vicagrel and 2-oxo-clopidogrel could be hydrolyzed to the acid form via losing methyl group by CE1. On the other hand, vicagrel and 2-oxo-clopidogrel probably produced endo-SH via opening the thiolactone ring of 2-oxo-clopidogrel by PON1. In addition, some investigations have testified the existence of PON1 in the rat and human intestine but with relative lower content by comparison to that in the plasma (15). The present investigation mainly focused on the contribution of representative esterase (including PON1) to the 2-oxo-clopidogrel production from vicagrel. In preliminary experiment, the transformation was found to be so rapid in the plasma and S9/microsomes that the protein concentration had to be adjusted to a lesser extent. Under the optimized condition, vicagrel was almost completely converted to 2-oxo-clopidogrel with no significant further hydrolysis to endo-SH or acid form. The dilution process may further reduce the possibility of the subsequent hydrolysis of 2-oxo-clopidogrel to generate endo-SH or acid form. The two possible hydrolysis pathways would be obvious when the enzyme concentration in the incubation system was raised, which could probably explain the lower AM formation in the intestinal fractions. The possible formation of endo-SH and acid form should be taken into consideration since the amount of subsequent AM production was relevant to the conversion extent and rate of endo-SH and acid form from 2-oxo-clopidogrel, which were competitive pathways of AM formation from 2-oxo-clopidogrel via CYPs (45).

Consequently, it was reasonable that the high presystem exposure of 2-oxo-clopidogrel was ascribed to the abundance of CE2. Since CE2 is predominately located in rat intestine with high homology to that of hCE2, which is highly expressed in human intestine (13,14). With this in mind rat intestine was regarded as an appropriate model of human intestine for vicagrel evaluation studies. In our previous pharmacokinetic comparison between clopidogrel and vicagrel following intragastric administration (20), the improved formation efficiency of AM from vicagrel was well explained by the contribution of intestinal CE2 to the enhanced and simplified formation of 2-oxo-clopidogrel. The extent and rate of 2-oxo-clopidogrel generation facilitated the production of AM and its pharmacological potency, due to the fact that the amount of AM exclusively hinged on the efficiency and rate of 2-oxo-clopidogrel formation. In view of the rats' model, it was predictable that hCE2 would be probably indispensable when it came to gastrointestinal hydrolysis of vicagrel to 2-oxo-clopidogrel in the human intestine, as well as PON1, which possessed with highest cleavage activity of vicagrel.

CONCLUSION

In summarizing, a significant portion of 2-oxo-clopidogrel was converted to AM during the intestinal absorption in rats, as a result of the more direct and facilitated formation of 2-oxo-clopidogrel achieved by the extensive hydrolysis of CE2 in the intestine. The *in vivo* and *in vitro* investigations well rationalized the prodrug design hypothesis. It is hoped the improved formation of 2-oxo-clopidogrel and AM will translate into improved potency of the drug in a clinical setting.

ACKNOWLEDGMENTS AND DISCLOSURES

The authors thank the State Key Laboratory of Natural Medicines and Center of Drug Discovery (China Pharmaceutical University) and Chia-Tai Tian Qing Pharmaceutical Co. Ltd (Jiangsu, China) for their generous supply of investigated agents. The study was supported by Jiangsu Province Nanjing City Innovative Graduate Research Program (No.CXLL11_0814) and Jiangsu Province Promotion Foundation for the Key Lab of Drug Metabolism and Pharmacokinetics (No.BM2012012).

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