In Vitro Hydrolysis and Transesterification of CDP323, an α4β1/α4β7 Integrin Antagonist Ester Prodrug

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Received August 7, 2013; accepted October 31, 2013

ABSTRACT

We identified the enzyme(s) involved in the hydrolysis of the ethyl ester prodrug CDP323 (C28H29BrN4O3), and characterized its transesterification in the presence of ethanol with special emphasis on the risks of drug-drug interaction. The hydrolysis of CDP323 was evaluated in vitro using human liver and intestinal microsomes and recombinant human carboxylesterases (hCES1 and 2) and was shown to be approximately 20-fold higher in human liver microsomes when compared with human intestinal microsomes and in hCES1 when compared with hCES2. Nonspecific inhibitors of carboxylesterases significantly inhibited the hydrolysis of CDP323 (>80% inhibition) while specific inhibitors of CES2, acetylcholine esterase, arylolesterase, and butyrylcholinesterase did not impair the hydrolysis reaction. The effect of ethanol on the kinetic parameters for hydrolysis was investigated, demonstrating that at high concentration (2%), Michaelis-Menten constant (Km), maximum velocity (Vmax), and intrinsic clearance (CLint) for the formation of the hydrolyzed product were decreased (~40%). The use of deuterated ethanol allowed more mechanistic investigations of the transesterification mechanism and showed that the intrinsic clearance based on parent loss was not impaired in the presence of alcohol. Overall, our data demonstrate that CDP323 is mainly hydrolyzed by hCES1 and is prone to transesterification in the presence of ethanol. Transesterification mechanisms compete with hydrolysis without impairing the overall clearance of the ester prodrug. Based on in vitro results, the risk of a clinically significant drug-drug interaction with ethanol is anticipated to be low.

Introduction

CDP323 (C28H29BrN4O3) is an ethyl ester prodrug of CT7758 (C26H25BrN4O3), a potent carboxylic acid antagonist of α4β1 (very late antigen-4, VLA-4) and, to a lesser extent, α4β7 integrins (Fig. 1). All leukocytes, except neutrophils, constitutively express α4β1. The endogenous ligand for α4β1 is vascular adhesion molecule-1 (VCAM-1), which is expressed on the vascular endothelium and is up-regulated in response to proinflammatory mediators. Interaction between α4β1 and VCAM-1 mediates the adhesion of leukocytes to vessel walls, their transendothelial migration into the inflamed tissues, and finally their activation. Blocking α4β1 inhibits leukocyte migration into the central nervous system, thereby preventing the uncontrolled accumulation of immune cells that release proinflammatory cytokines and tissue-damaging free radicals known to be an important component in demyelination and multiple sclerosis pathogenesis (Davenport and Munday, 2007).

CDP323 was developed as a VLA-4 antagonist prodrug for the treatment of multiple sclerosis (Bailey et al., 2013). Its development was discontinued in 2009 based on inadequate interim efficacy data in a phase II clinical trial (Wolf et al., 2013).

CDP323 was designed as an ester prodrug to circumvent the low oral bioavailability of the active carboxylic acid moiety CT7758 observed in most animal species (mice, rat, monkey), which was assumed to be due to poor intestinal permeability. The ester prodrug strategy is a popular approach for compounds with unfavorable physicochemical properties, limiting their intestinal permeability (e.g., highly hydrophilic and/or ionized drugs) (Beaumont et al., 2003). However, development of ester prodrugs as drug candidates is also associated with additional challenges, such as species differences in hydrolysis, potential instability in the gastrointestinal tract and in blood, and high intersubject and intrasubject variability in disposition, which all affect differently the level and tissue of bioactivation.

Ester-containing drugs are most frequently cleaved by carboxylic ester hydrolyses (E.C.3.1.1) with a prominent role of carboxylesterases (CES, E.C.3.1.1.1) compared with the other esterase subfamilies or cytochrome P450 (Liederer and Borchardt, 2006). Mammalian carboxylesterases (Hosokawa et al., 1990, 2008; Taketani et al., 2007; Williams et al., 2011) comprise a multigene family, and the isoenzymes are classified into five main groups with several subgroups (Satoh and Hosokawa, 2006). The major carboxylesterases involved in hydrolysis of xenobiotics are human carboxylesterase 1 (hCES1) and hCES2. Although both forms have high mRNA expression in the liver, hCES1 levels exceed those of hCES2 (Satoh et al., 2002). But most important, the extrahaepatic expression differs between hCES1 and hCES2 (Satoh et al., 2002; Hosokawa, 2008). The mRNA expression of hCES1 in decreasing order is the stomach, testis, kidney, spleen, and colon. It contrasts with...
hCES2 where it is the colon, small intestine, and heart. The differences in mRNA levels in the liver and small intestine parallel differences in functional activity (Imai, 2006). In addition, hCES1 and hCES2 differ in their substrate specificity. Although hCES1 and hCES2 have overlapping substrate recognition, clear evidence of ester-based substrate specificity has been observed (Satoh et al., 2002). In general, hCES1 accommodates substrates with a large acyl moiety whereas hCES2 favors substrates with a large alcohol substituent (Laizure et al., 2013).

Carboxylesterases efficiently catalyze the hydrolysis of a variety of ester and amide-containing compounds such as cocaine (Kamendulis et al., 1996; Pindel et al., 1997), heroin (Kamendulis et al., 1996; Pindel et al., 1997), irinotecan (CPT-11) (Humerrickhouse et al., 2000), temocapril (Imai et al., 2005), benzoic acid derivatives (Imai et al., 2006), clopidogrel (Tang et al., 2006), oseltamivir (Shi et al., 2006), and methylphenidate (Sun et al., 2004).

These enzymes are known to show interspecies variability in their activity and distribution, which complicates the preclinical pharmacologic and toxicologic evaluation of ester prodrugs. As an example, dog intestine expresses very limited carboxylesterase activity compared with other species (Taketani et al., 2007; Williams et al., 2011).

In addition, genetic polymorphism has been reported for CES genes which impacts on the bioactivation of ester prodrugs in human (Tarkkainen et al., 2012), especially in terms of interindividual variability in drug disposition (Zhu and Markowitz, 2013).

Our study identified the enzymes involved in the hydrolysis of CDP323 with a specific focus on CES isoforms to better predict the potential risk for drug-drug interaction as well as intersubject variability as a result of genetic polymorphism. Taking advantage of the specific tissue distribution of these two enzymes, experiments were performed with intestinal and liver microsomes. During the frame of preliminary assays using liver microsomes, it was discovered that CDP323 was prone to transesterification when methanol was present in the incubation medium. This observation triggered additional investigations to understand the potential impact of the transesterification on the disposition of CDP323, with a special emphasis on the risk of in vivo drug-drug interaction with ethanol.

Materials and Methods

Chemicals and Reagents. Loperamide, propranolol, BW284c51 [1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide], DTNB [5,5′-dithiobis(2-nitrobenzoic acid)], benzyl, BNPP [bis(4-nitrophenyl) phosphate], ethopropazine, and verapamil were purchased from Sigma-Aldrich (Bornem, Belgium). Paraxoxon was obtained from Chemservice (West Chester, PA). The NADPH-regenerating system [NADP (1.3 mM), glucose 6-phosphate (3.3 mM), MgCl2 (3.3 mM), and glucose 6-phosphate dehydrogenase (0.4 U/ml)] was obtained from Gentest (Woburn, MA). Ethanol and d6-ethanol were purchased from VWR (Leuven, Belgium) and Acros Organics (Geel, Belgium), respectively. Pooled human liver and intestinal microsomes (mixed gender) containing representative activities of CYP450 were obtained from Xenotech (Lenexa, KS). Bactosomes (recombinant human CES1 and CES2 expressed in Escherichia coli) were purchased from Cypex (Dundee, Scotland, United Kingdom).

Incubation with Human Liver Microsomes, Human Intestinal Microsomes, and Human Recombinant Carboxylesterases Enzymes. All the incubations of human liver microsomes (HLM), human intestinal microsomes (HIM), and bactosomes with CDP323 were performed in polypropylene containers at 37°C in a shaking water bath, with 50 mM potassium phosphate buffer (pH 7.4). Incubations were performed without NADPH as the objective of the experiments was to characterize CDP323 hydrolysis except on some occasions where a NADPH-regenerating system was used, as mentioned in the assay descriptions. After a 2-minute preincubation at 37°C of buffer and HLM, HIM, or bactosomes, the reactions were initiated with the addition of CDP323. The reactions were stopped by 1 volume of ice-cold acetonitrile containing verapamil, as the internal standard. The tubes were then thoroughly mixed using a vortex mixer and centrifuged (~10,000 g, 4°C) for 10 minutes. CDP323 and inhibitors were dissolved in dimethylsulfoxide (DMSO). The final solvent concentrations in the incubates was ±0.5%. Supernatants were used for quantification by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). A first set of incubations (time and protein linearity) were always performed to determine the initial rate conditions for the Km, Michaelis-Menten constant (Km), maximum velocity (Vmax) experiments.

Analytic Methods. The high-performance liquid chromatography (HPLC) system used was an Agilent 1100 Series (Agilent Technologies, Santa Clara, CA) coupled with a Quattro Micro mass spectrometer (Waters Corporation, Milford, MA). The analytical column was a Sunfire C18 (50 × 2.1 mm, 3.5 μm; Waters Corporation), operated at 30°C. Analyses were performed with a gradient method from 100% A to 10% A/90% B in 8 minutes. Eluent A was 0.1% formic acid/acetonitrile (90/10; v/v), and eluent B was 0.1% formic acid/ acetonitrile (10/90; v/v). For metabolite identification, full-scan and product ion scan monitoring using electrospray ionization was used; for analytes quantification, multiple reaction monitoring in positive-ion mode with electrospray ionization was used. Deuterated transester (d5-CDP323) was assumed to have a similar mass response as CDP323. Data acquisition and the analytic parameters including the selection of ions for each compound were performed by the application software MassLynx 4.1 (Waters Ltd., Hertfordshire, United Kingdom). The concentrations were determined by the peak area ratio method.

Data Analysis. Substrate (S) concentrations and corresponding rates (pmol/min/mg protein) from individual experiments were analyzed by nonlinear regression with Graftit software (version 6.0.4; Erithacus Software Ltd., Surrey, United Kingdom) fitting the data to the full nonlinear form of the two enzyme eq. 1 to obtain estimates of Km, Vmax, and intrinsic clearance of enzyme 2 (CLint2).
\[ v = \frac{V_{\text{max}}[S]}{K_m + [S]} + CL_{\text{int2}} \cdot S \]  

(1)

This equation describes a situation where there are two different enzymes involved in the investigated enzymatic reaction with enzyme 1 having a high affinity for the substrate (defined by \( K_m \) and \( V_{\text{max}} \)) and is saturable and with enzyme 2 having a lower affinity for substrate (defined by \( CL_{\text{int2}} \)) but not saturable in the conditions of the present assay.

The intrinsic clearance \( CL_{\text{int}} \) (\( \mu l/min/mg \) prot) was calculated as follows (depending on the data):

\[ CL_{\text{int}} = \frac{V_{\text{max}}}{K_m} \]  

(using results from eq. 1)  

(2)

or

\[ CL_{\text{int}} = \frac{v}{[S]} \]  

(for formation of CT7758 and transester \([d_9\text{-CDP323}]) \)  

(3)

or

\[ CL_{\text{int}} = \frac{0.693}{t_{1/2}} \cdot \frac{[\text{HLM}]}{([\text{HLM}])} \]  

(for CDP323 disappearance)  

(4)

The predicted hepatic clearance was calculated using the well-stirred model and \( fu \) (unbound fraction) estimated at 0.045:

\[ CL_H = \frac{Q_H \cdot CL_{\text{int}} \cdot fu_{\text{plasma}}}{Q_H + CL_{\text{int}} \cdot fu_{\text{plasma}}} \]  

(5)

where \( CL_{\text{int,vivo}} \) (ml/min/kg) was obtained from eq. 6, and a hepatic blood flow (\( Q_H \)) of 22 ml/min/kg was used:

\[ CL_{\text{int,vivo}} = CL_{\text{int,vitro}} \times \text{microsomal yield} \times \text{organ weight} \]  

(6)

where a microsomal protein yield value of 45 mg/g liver and liver weight value of 23g/kg body weight were used.

The predicted hepatic extraction coefficient (\( E_H \)) was calculated as:

\[ E_H = \frac{CL_H}{Q_H} \]  

(7)

**Statistical Analysis.** The \( t \) test and one-way analysis of variance (ANOVA) (with Dunnett’s post hoc test) were performed with Graphpad Prism Software v5.02 (La Jolla, CA).

**Results**

Initially, CDP323 (MH\(^+\) 549) was incubated at \( 1\mu M \) with HLM in the presence or absence of NADPH. In the presence of NADPH, four major metabolites were observed, one being the hydrolyzed product CT7758 (MH\(^+\) 521) coming from the cleavage of the ethyl ester bond (Fig. 1). Two hydroxylated metabolites were detected (hydroxylation on bromolactone moiety and hydroxylation of the naphthyridine ring) as well as a metabolite with a protonated molecular ion at \( m/z \) 535.

In NADPH-free conditions, only the hydrolyzed product (CT7758) and the metabolite \( m/z \) 535 were observed. These preliminary investigations demonstrated that other metabolites were observed in addition to the expected one (CT7758, hydrolyzed product). Based on mass difference, this later metabolite \( m/z \) 535 should correspond either to the loss of methylene from the parent or to the addition of methyl to CT7758. As shown in Fig. 2C, the structural elucidation based on fragmentation pattern unequivocally showed that this metabolite corresponded to the methyl ester, presumably coming from the transesterification of CDP323 in the presence of methanol (i.e., organic solvent to prepare CDP323 stock solutions; 1% v/v final concentration in the incubation medium). In tandem mass spectrometry (MS/MS) spectra, a similar fragment ion of \( m/z \) 395 was observed in the fragmentation pattern of both CDP323 and this unexpected metabolite (\( m/z \) 535). The fragment ion corresponds to the loss of a \( m/z \) 154 mass units from CDP323 (loss of HOCOC\(_2\)H\(_4\) and HBr, Fig. 2A). The fragment ion \( m/z \) 395 in the unknown metabolite corresponded to the loss of HBr (80/82 mass units) and HOCOCH\(_3\) (60...
mass units), confirming that the metabolite MH⁺ 535 was the methyl transester of CDP323.

To check this hypothesis, the incubations were repeated with methanol, acetonitrile, DMSO, and ethanol as the organic solvent to prepare a CDP323 stock solution. Figure 3 shows the ion extracted chromatogram for MH⁺ corresponding to CDP323, CT7758, and the methylester metabolite (MH⁺ 535) with a corresponding retention time of 9.90, 9.01, and 9.56, respectively. Confirming the transesterification hypothesis, the methylester metabolite was only observed when incubations were performed in the presence of methanol (Fig. 3A); the transester was not detected when acetonitrile (Fig. 3B), DMSO (Fig. 3C), or ethanol (Fig. 3D) was used as the organic solvent to prepare CDP323 stock solutions. To check that the methylester was not formed directly from the esterification of CT7758, we incubated CT7758 in the presence of methanol in HLM (NAPDH-free conditions). No methylester was detected in these conditions (data not shown). Finally, incubation of CDP323 with methanol (1%) in pH7.4 buffer-only (without HLM) did not lead to the formation of methylester. These two controls confirm that the methylester was formed from the direct transesterification of CDP323, which occurred during the hydrolysis process of the ester mediated by an enzymatic reaction as described by Imai (2006).

The observed transesterification of CDP323 in the presence of alcohol triggered additional investigations to determine the likelihood of pharmacokinetic interaction between CDP323 and ethanol. The kinetics of CDP323 hydrolysis (Kᵦ, Vₘₐₓ) were measured in the presence or absence of different concentrations of ethanol. The maximum ethanol concentration was limited to 2% to avoid any enzyme denaturation. This ethanol concentration (2%) did not produce sufficient inhibition to allow Kᵦ determination. Figure 4 showed the formation rate of CT7758 in HLM (0.025 mg/ml) incubated 20 minutes at 37°C. The incubations were performed without a NADPH-regenerating system and showed the effect of increasing the concentration of ethanol: 0 (Fig. 4A), 0.25% (Fig. 4B), 0.5% (Fig. 4C), 1% (Fig. 4D), and 2% (Fig. 4E). All plots showed biphasic kinetics with a high and low clearance component (Table 1). Only the high clearance component of the hydrolysis is discussed hereafter. In control conditions (without ethanol), the Kᵦ and Vₘₐₓ values for the formation of CT7758 were 11.3 µM and 6280 pmol/min/mg protein, respectively, translating into an intrinsic clearance as high as 556 µl/min/mg protein. Interestingly, these kinetic parameter values decreased in the presence of ethanol, reaching values of 3.11 µM, 876 pmol/min/mg protein, and 282 µl/min/mg protein at 2% ethanol for Kᵦ, Vₘₐₓ, and CLₐ₉, respectively. Assuming that hydrolysis occurred mainly in the liver, these in vitro intrinsic clearances were scaled up to the in vivo hepatic clearance using the well-stirred model and estimating a free fraction of CDP323 in human plasma (fu) at 0.045 (data in UCB files). As shown in Table 2, the predicted value of CLₐ₉ remained close to the hepatic blood flow. As a result of this blood-flow limited clearance, ethanol demonstrated a moderate effect on CLₐ₉ (30% reduction at 2% ethanol) (see Table 2).

To further characterize the observed transesterification of CDP323, incubations were performed in HLM and in the presence or absence of 0.5% ethanol or d₆-ethanol. Figure 5A shows the formation of CT7758 over time when CDP323 was incubated in the presence or absence of d₆-ethanol; Fig. 5B shows the formation of the transester (d₅-CDP323) resulting from the exchange of the ethyl function of CDP323 with d₆-ethanol added in the incubation medium (transesterification reaction). The in vitro intrinsic clearance values describing CDP323 consumption, CT7758 formation, and CDP323 transesterification from the same experiment presented in Fig. 5 (formation of transester, d₅-CDP323) are given in Table 3. In NAPDH-free conditions, the clearance of CDP323 is explained by its hydrolysis into CT7758, as shown by the similar intrinsic clearance calculated from the disappearance rate of CDP323 and the formation rate of CT7758. In the presence of 0.5% ethanol, both intrinsic clearance (i.e., CDP323 consumption and CT7758 formation) decreased in a similar way.

**Fig. 3.** Ion extracted chromatogram for MH⁺ corresponding to CDP323, CT7758, and methylester metabolite (MH⁺ 535) with corresponding retention times of 9.90, 9.01, and 9.56, respectively. For the stock solution, CDP323 was solubilized either in (A) methanol, (B) acetonitrile, (C) DMSO, or (D) ethanol and then incubated in HLM (NAPDH-free conditions) for 10 minutes. At the end of incubation time, reaction was stopped by the addition of 1 volume of cold acetonitrile. Supernatants were analyzed by liquid chromatography/mass spectrometry (LCMS).
This observed decrease clearance of CDP323 is likely the result of the formation of transester, which is in this case the parent drug and is not due to a “true” inhibition of the enzyme involved in CDP323 clearance. To demonstrate this hypothesis, d6-ethanol was used to allow the quantification of the formation of the transester (d5-CDP323). In the presence of 0.5% deuterated ethanol, the intrinsic clearance calculated on disappearance rate of CDP323 was roughly similar to the one measured in the absence of ethanol, suggesting that ethanol did not decrease the disappearance rate of CDP323. Moreover, the formation rate of CT7758 was similar to the one in the presence of 0.5% ethanol. Of interest, the sum of both formation rates (transester [d5-CDP323] and CT7758) quantitatively accounts for the disappearance rate of CDP323 and also the formation rate of CT7758 in ethanol-free conditions.

Also of note is that the CLint values obtained in Table 3 differ from those obtained in Table 1. This is mainly explained by the two different approaches used to determine CLint values. In Table 1, CLint values were calculated as \( \frac{V_{\text{max}}}{K_m} \) and thus are representative of the CLint value when the substrate concentration is well below the \( K_m \) value (assumption of eq. 2); in Table 3, CLint was determined by nonlinear regression of the slope (eq. 4) at a substrate concentration equaling the \( K_m \) value (10 \( \mu \)M). Because CLint values depend on the substrate concentration, this explains the apparent discrepancy between Tables 1 and 3.

Phenotyping assays were performed to determine the (iso)enzyme(s) involved in CDP323 hydrolysis. As shown in Fig. 6, the hydrolysis rate of CDP323 is approximately 20-fold lower in HIM than in HLM, suggesting the preferred involvement of hCES1. Isoenzyme assignment was further confirmed using recombinant hCES1 and hCES2, as
shown in Fig. 7. The rate of hydrolysis of CDP323 was approximately 20-fold higher using hCES1 compared with hCES2. Finally, the hydrolysis reaction was measured in HLM in the presence of various known chemical inhibitors of hydrolyzing enzymes. DTNB, BW284c51, and ethopropazine are specific inhibitors of arylesterase, acetylcholine esterase, and butyrylcholinesterase, respectively (Yamaori et al., 2006) and had no inhibitory effect on CDP323 hydrolysis (Fig. 8). Similarly, the hCES2 inhibitor loperamide did not impair the reaction. No specific hCES1 inhibitors are currently available, so nonspecific CES inhibitors were used instead—BNPP, paraoxon, and benzil (Wadkins et al., 2005; Jewell et al., 2007; Eng et al., 2010). They all inhibited CDP323 hydrolysis significantly (>80% inhibition).

Discussion

In 1958, Adrien Albert used for the first time the term prodrug to define a pharmacologically inactive compound that needs to be biotransformed in vivo to release its active moiety (Albert, 1958). Since that time, the prodrug strategy has been a powerful tool in drug development not only to improve the physicochemical, biopharmaceutic, and pharmacokinetic properties of pharmacologically active compounds but also for ensuring a more specific delivery of the active drug to the action site (Hsieh et al., 2009; Jana et al., 2010; Coughlin et al., 2012; Dalpiaz et al., 2012). The prodrug approach is still relevant, considering the number of papers recently published on the topic (~500 papers in 2012, using [prodrug] and [2012] and [synthesis] or [design] as filters).

The ethyl ester prodrug CDP323 is designed to improve the intestinal absorption of the active carboxylic acid containing the VLA4-antagonist CT7758. CDP323 was designed, characterized, and developed for the treatment of multiple sclerosis.

The present in vitro study aims to further explore the hydrolysis of CDP323 by investigating its kinetic properties and the enzymes involved in the process. The preliminary investigations on metabolite identification in HLM demonstrated, as expected, that the CDP323 prodrug was mainly hydrolyzed, releasing the carboxylic acid moiety CT7758. However, our study also points to the oxidation of the ester prodrug itself occurring, leading to two metabolites (hydroxylation on the bromolactone moiety and hydroxylation of the naphthyridine ring); more unexpectedly, CDP323 was prone to be transesterified. Transesterification occurred during the hydrolysis of the ester prodrug, where the alcohol moiety of the prodrug was replaced by another alcohol present in excess to generate another ester prodrug instead of the hydrolyzed product (Imai, 2006).

Although a huge number of publications exist on prodrugs, the transesterification mechanism has been so far largely overlooked. Among the scarce reports, the best-known and studied example of transesterification is cocaine. As described in vitro and in vivo, in the presence of ethanol, cocaine can be transesterified into cocaethylene, which is more toxic than cocaine itself (Boyer and Petersen, 1992; Bourland et al., 1998; Laizure et al., 2003). Other examples of transesterification observed in vitro include clopidrogel (Tang et al., 2006), meperidine (Bourland et al., 1997), and methylphenidate (Bourland et al., 1997; Markowitz et al., 2000). Interestingly, Patrick et al. (2007, 2013) showed in human volunteers that the transesterification of methylphenidate was

| TABLE 1 |
| Enzymatic kinetic parameters for the formation of CT7758 in HLM incubated with and without ethanol |
| Ethanol | $K_{m}$ $^a$ | $V_{max}$ $^a$ | $CL_{int}$ $^b$ | $CL_{tot}$ $^a$ |
| % | $\mu M$ | pmol/min/mg | $\mu l/min/mg$ |
| 0 | 11.3 | 6280 | 556 | 18.8 |
| 0.25 | 8.88 | 4520 | 509 | 7.64 |
| 0.5 | 5.75 | 2560 | 445 | 6.76 |
| 1 | 3.29 | 1130 | 343 | 38.2 |
| 2 | 3.11 | 876 | 282 | 32.6 |

$^a$ Determined by fitting of eq. 1 as described in Materials and Methods.

$^b$ Calculated as $V_{max}/K_{m}$.

| TABLE 2 |
| Scaling up of in vitro intrinsic clearance (from Table 1) to in vivo hepatic clearance |
| Ethanol | $CL_{int, vmo}$ $^a$ | $CL_{int, vvo}$ | $CL_{int}$ $^b$ | $E_H$ |
| % | $\mu l/min/kg$ | $ml/min/kg$ |
| 0 | 575 | 595 | 12.1 | 0.55 |
| 0.25 | 517 | 535 | 11.5 | 0.52 |
| 0.5 | 452 | 468 | 10.8 | 0.49 |
| 1 | 382 | 395 | 9.8 | 0.45 |
| 2 | 314 | 325 | 8.8 | 0.40 |

$^a$ $CL_{int, vmo}$ calculated as sum of $CL_{int}$ and $CL_{int2}$ (Table 1).

$^b$ Using $fu = 0.045$. 

Fig. 5. (A) CT7758 and (B) transester (d$_5$-CDP323) production, respectively. Incubations with no ethanol (□) and 0.5% of d$_6$-ethanol (●) are represented. HLM (25 µg/ml) were incubated as single replicates with CDP323 at 10 µM in the absence of a NADPH-regenerating system. Incubations were performed with and without 0.5% d$_6$-ethanol. Reactions were stopped with cold acetonitrile after 0, 5, 10, 20, 30, 45, and 60 minutes. Mean ± S.D. (n = 3) are represented.
enantioselective, where L-methylphenidate was transesterified into L-ethylphenidate without any d-ethylphenidate formed. As shown in our study, transesterification can occur quite easily in vitro, which brings into question the use of alcohols (such as ethanol and methanol) as organic solvents to solubilize ester-containing drugs. This could lead to the formation of an artifactual transester metabolite and could bias the hydrolysis rate determination. In our study, the observed transester metabolite corresponded to a methylester because the incubations were performed in the presence of methanol. Such transesterification with methanol should not occur in vivo, given the very low levels of methanol observed in the liver.

In human subjects, the only exogenous alcohol that could typically reach a high concentration is ethanol. High levels of ethanol could potentially trigger two kinds of drug-drug interaction with ester prodrugs. First, and probably the most critical, is the formation of a new transester metabolite that is different from the parent drug (which should not be a concern for ethylester prodrugs). Such a drug-drug interaction would be of major concern because patients could be exposed to a noncharacterized metabolite (ethylester prodrug) that could cause unpredicted pharmacologic activity or toxicity (as reported earlier with cocaine). However, as described in our study, this potential risk could be proactively addressed by measuring transesterification in vitro.

Because endogenous alcohols (e.g., glycerol, cholesterol, or glucose) are present at relatively high plasma concentrations (up to 1 mM) (Maggs et al., 1995), one might also question whether they could also produce the transesterification observed with ethanol if we assumed the liver concentrations could reach similar levels as those observed in plasma. Crow et al. (2010) recently showed that endogenous alcohol such as oxysterols had inhibitory properties on CES, highlighting that these endogenous alcohol are also able to interact with CES. However, the potential for transesterification was not investigated.

Second, interaction with ethanol could also result in a decrease of the formation rate of the hydrolyzed product and a concomitant apparent decrease of the ester prodrug clearance (in the case of ethylester prodrug). Ethanol plasma levels in human subjects can rise up to 0.1% to 0.2% v/v without major safety issues. Assuming that ethanol liver concentrations are in equilibrium with plasma levels, such a concentration showed in the present in vitro assays the marginal effects, if any, on CDP323 transformation. Accordingly, the risk that significant clinical drug-drug interaction could occur between ethanol and CDP323 is regarded as highly unlikely.

In the present in vitro study, to explore the mechanism behind the interaction between ethanol and CDP323, ethanol concentrations that are not clinically relevant (up to 2%) were tested. In these conditions, the $K_m$ and $V_{\text{max}}$ values describing CDP323 hydrolysis significantly and dose-dependently decreased in the presence of ethanol. However, these two parameters were not affected to the same extent, resulting in a decrease of intrinsic clearance. A parallel decrease of $K_m$ and $V_{\text{max}}$ values without an impact on intrinsic clearance is a typical signature of uncompetitive inhibition where the inhibitor binds to the enzyme-substrate complex (Shou et al., 2001). In the special case of transesterification, we believe that the interaction between alcohol and CES is of an uncompetitive nature, meaning that ethanol interacts with the enzyme substrate complex. Indeed, it is known that transesterification occurs during the second step of the hydrolysis process when alcohol is in abundance and can interact with the substrate-enzyme complex to release the transester instead of the carboxylic acid (for an in-depth description, see the review by Imai (2006).)

To understand the reason for this decreased formation rate of CTP7758 in the presence of ethanol, deuterated ethanol ($d_6$-ethanol) was used to allow the detection and quantification of the transester formed ($d_6$-CDP323) by exchange of the ethyl function with $d_6$-ethanol (Chen et al., 2004). This experiment was very useful for dissecting and understanding the transesterification mechanism. As expected, based on the $K_m$/$V_{\text{max}}$ experiment, the formation rate of CTP7758 was decreased to the same extent in both incubations with ethanol or $d_6$-ethanol. Also in both conditions, the formation of CTP7758 (incubation with ethanol) or the sum of formation rates of CTP7758 and $d_6$-CDP323 (incubation with $d_6$-ethanol) accounted for the disappearance rate of CDP323. The main difference between the incubation with ethanol or with $d_6$-ethanol was observed in the disappearance rate of CDP323. Indeed, compared with control, an apparent decrease of $\text{Cl}_{\text{int}}$ was observed in the presence of ethanol while no major difference in the control values was noticed in the presence of $d_6$-ethanol. This critical observation clearly demonstrates that 1) the apparent inhibition of CDP323 consumption observed in the presence of ethanol was due to the formation of the transester indistinguishable from parent drug CDP323 and 2) that transesterification reactions per se do not impact the overall clearance of the ester prodrug itself (based on data obtained with $d_6$-ethanol).

However, it is important to state that the latter conclusion will not always translate into a lack of effect of ethanol on the ester prodrug clearance. To make that happen, the transester formed in the presence of alcohol should be different than the original ester prodrug. In our case, for example, in clinical use of CDP323 with ethanol, an apparent decrease of CDP323 clearance should be observed because the transester formed will be the same as the parent drug. Overall, these data clearly demonstrate that ethanol did not really inhibit the enzyme involved in the hydrolysis but rather promoted the formation of an additional metabolite (transester) that is in competition with the hydrolysis reaction (formation of the hydrolysis product). Similar in

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<td>Ethanol</td>
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NA, not applicable.

**TABLE 3**

**Fig. 6.** CT7758 rate of production ($\mu$mol/min/mg of proteins) when CDP323 (10 $\mu$M) was incubated in triplicate for 20 minutes with HLM (dark bar) and HIM (white bar) at 0.025 mg/ml. Mean $\pm$ S.D. (n = 4) are represented. ***$P < 0.001$.
vitro observations have been reported for cocaethylene (ethylester of cocaine) incubated in S9 fractions in the presence or absence of ethanol (Bourland et al., 1998).

In addition, in vivo evidence with methylphenidate (substrate of hCES1) further confirmed our findings that transesterification can occur in the presence of ethanol and that this does not impact the clearance of the ester prodrug itself although formation of its hydrolyzed product (ritalinic acid) was impaired (Koehm et al., 2010). However, this finding (the lack of effect of ethanol on the ester prodrug clearance) is not supported by the data published on the interaction between cocaine and ethanol, which clearly highlights that ethanol inhibits the clearance of cocaine (Laizure et al., 2003; Parker and Laizure, 2010). One of the potential reasons is that cocaine is not only cleared by hCES1 but also by hCES2. Indeed, Crow et al. (2010) observed that ethanol is a potent inhibitor of CES2, meaning that the observed effect of ethanol could be due to inhibition of presystemic clearance mediated by hCES2 (intestine) rather than an effect on systemic clearance mainly mediated by hCES1. This hypothesis is corroborated by the observation that the terminal half-life of cocaine was not impacted by ethanol treatment (Parker and Laizure, 2010).

The last objective of the study was the identification of the enzyme involved in the hydrolysis of CDP323. The data collected clearly support hCES1 as the major enzyme involved in the hydrolysis of the CDP323 ethyl ester prodrug. Indeed, chemical inhibitors of carboxylesterases (specific to CES2 and nonspecific) (Hatfield and Potter, 2011) as well as the use of recombinant enzyme (hCES1 and hCES2) both demonstrated that hCES1 plays a major role in CDP323 hydrolysis. Specific inhibitors of arylesterase, acetylcholine esterase, and butyrylcholinesterase were also tested and rule out the involvement of other esterases. In addition, CDP323 was found to be more readily hydrolyzed in the liver than in the intestinal fraction, consistent with the reported tissue distribution of hCES1. Moreover, CDP323 was demonstrated to be prone to transesterification, a reaction only reported for hCES1 (Imai, 2006). Finally, the preferred hydrolysis of CDP323 by hCES1 is in line with the known structure-activity relationship of this isoenzyme (Satoh et al., 2002).

As a consequence of CES involvement in drug clearance, one might expect challenging drug development. Indeed, the marked species differences are well described, with a predominant role of CES in rodent plasma that is not described in dog or human plasma. This enzymatic instability makes bioanalysis in rodent plasma quite difficult. In addition, there is more and more evidence of CES polymorphism impairing the pharmacokinetics of the ester prodrug, thus requiring special attention in clinical trials (Lewis et al., 2013; Suzuki et al., 2013).

Overall, we have unequivocally shown the ability of CDP323, an ethyl ester prodrug, to be transesterified in the presence of alcohol. Mechanistic studies demonstrated that the transesterification reaction competes with the overall disappearance rate of the parent drug. In addition, the risk of clinically significant interactions between ethanol and CDP323 appears to be marginal. Finally, the hydrolysis of the CDP323 prodrug is mediated by the hCES1 enzyme.

Acknowledgments

The authors thank Céline Jacques-Hespel for technical assistance.

Authorship Contributions

Participated in research design: Chanteux, Prakash, Smith, Nicolas.

Conducted experiments: Rosa.

Contributed new reagents or analytic tools: Delatour.

Performed data analysis: Chanteux, Rosa, Delatour, Prakash, Smith, Nicolas.

Wrote or contributed to the writing of the manuscript: Chanteux, Nicolas.

References
