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Anti-Influenza Prodrug Oseltamivir Is Activated by Carboxylesterase Human Carboxylesterase 1, and the Activation Is Inhibited by Antiplatelet Agent Clopidogrel

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ABSTRACT

Oseltamivir is the main medicine recommended by the World Health Organization in anticipation of next influenza pandemic. This anti-influenza viral agent is an ester prodrug, and the antiviral activity is achieved by its hydrolytic metabolite: oseltamivir carboxylate. In this study, we report that the hydrolytic activation is catalyzed by carboxylesterase human carboxylesterase (HCE) 1. Liver microsomes rapidly hydrolyzed oseltamivir, but no hydrolysis was detected with intestinal microsomes or plasma. The overall rate of the hydrolysis varied among individual liver samples and was correlated well with the level of HCE1. Recombinant HCE1 but not HCE2 hydrolyzed this prodrug and produced similar kinetic parameters as the liver microsomes. Several HCE1 natural variants differed from the wild-type enzyme on the hydrolysis of oseltamivir. In the presence of antiplatelet agent clopidogrel, the hydrolysis of oseltamivir was inhibited by as much as 90% when the equal concentration was assayed. Given the fact that hydrolysis of oseltamivir is required for its therapeutic activity, concurrent use of both drugs would inhibit the activation of oseltamivir, thus making this antiviral agent therapeutically inactive. This is epidemiologically of significance because people who receive oseltamivir and clopidogrel simultaneously may maintain susceptibility to influenza infection or a source of spreading influenza virus if already infected.

Influenza, commonly called the flu, is a major cause of sickness and death around the world. Outbreaks of influenza occur seasonally or in a form of pandemic (Olsen et al., 2006). Seasonal outbreaks usually take place in the winter and early spring, and serious illness can develop in vulnerable populations such as children. Flu pandemic is rare but represents one of the most devastating natural disasters (Stiver, 2004). A flu pandemic could claim millions of lives and completely disrupt all social activities world-wide (Stiver, 2004; Suzuki, 2005). In the past century, flu pandemics occurred three times, including the 1918 Spanish, 1957 Asian, and 1968 Hong Kong flu pandemics (Stiver, 2004; Suzuki, 2005). The Spanish flu pandemic, based on recorded history, is the most devastating global outbreak among all infectious diseases. This pandemic claimed more than 20,000,000 people, although many historians believe that this number might

have died in India alone. Although it is difficult to predict the exact time when the next pandemic will occur, influenza virus H5N1, which originated in Southeast Asian in recent years, shows all characteristics leading to a flu pandemic (Liu, 2006).

Influenza viruses are divided into types A, B, and C based on the nucleocapsid and matrix protein; however, type A viruses cause the most infection (Suzuki, 2005). Among type A viruses, there are many subtypes based on the combination of hemagglutinin (H1–16) and neuraminidase (N1–10) (Bentz and Mittal, 2003; Suzuki, 2005). Hemagglutinin interacts with sialic acid receptors on host cells and thus determines the infection preference (e.g., human versus bird). Such a preference, however, is determined by only a very few amino acids, and limited passage is sufficient to switch the preference (e.g., from avian to human) (Ito et al., 1997; Suzuki, 2005). Neuraminidase, on the other hand, plays an important role in the release of viral progenies (Wagner et al., 2002; Nayak et al., 2004; Ohuchi et al., 2006), although it has recently been reported that this sialidase plays a role in the initiation of viral entry process as well (Ohuchi et al.,

ABBREVIATIONS: HCE, human carboxylesterase; MTT, 3-[4,5-dimethyl-thiazolyl-2]-2,5-diphenyl-tetrazolium bromide; IS, internal standard; LC-MS/MS, liquid chromatography-tandem mass spectrometry.

2006). In recent years, the neuraminidase has become an attractive target for the development of anti-influenza viral agents (Hurt et al., 2004; Normile, 2006).

Oseltamivir (i.e., Tamiflu) represents the prototype of neuraminidase inhibitors and is the main medicine recommended by the World Health Organization in anticipation of H5N1 pandemic (Normile, 2006). This anti-influenza agent is an ester prodrug, and the hydrolytic metabolite but not the parent drug exerts antiviral activity (Sweeny et al., 2000; Oxford et al., 2003; Ward et al., 2005). The hydrolytic biotransformation occurs primarily in the liver, presumably by carboxylesterases (Oo et al., 2003a). In support of the importance of carboxylesterases in the activation, hydrolysis of oseltamivir is significantly delayed (4.6 versus 1.7 h) in children (1–2 year) who usually express low levels of carboxylesterases (He et al., 1999; Massarella et al., 2000; Oo et al., 2003b; Pope et al., 2005). In the liver, there are two major carboxylesterases, designated HCE1 and HCE2, respectively (Kroetz et al., 1993; Pindel et al., 1997; Schwer et al., 1997; Xie et al., 2002). More importantly, these two enzymes differ markedly in the hydrolysis of certain drugs (Schwer et al., 1997; Humerickhouse et al., 2000). For example, HCE2 is ~30-fold as active as HCE1 in hydrolyzing irinotecan, a topoisomerase inhibitor that is used for a variety of malignancies (Schwer et al., 1997; Humerickhouse et al., 2000). For both HCE1 and HCE2, there exist many polymorphic variants, and some of the variants exhibit altered hydrolytic activity toward certain substrates (Marsh et al., 2004; Zhao et al., 2005).

The aim of this study was to establish the identity of oseltamivir hydrolase(s) and to ascertain the possible clinical consequences of altered activity of this hydrolase on oseltamivir therapy. Liver microsomes rapidly hydrolyzed oseltamivir, and the hydrolytic rate was correlated well with the level of carboxylesterase HCE1. Recombinant HCE1 but not HCE2 hydrolyzed oseltamivir and yielded similar kinetic parameters as the liver microsomes. Several HCE1 natural variants differed from the wild-type enzyme on the hydrolysis of oseltamivir. In the presence of antiplatelet agent clopidogrel, the hydrolysis of oseltamivir was decreased by as much as 90% when equal concentrations were assayed. Given the fact that hydrolytic activation is essential for the antiviral activity of oseltamivir, inhibited activation by clopidogrel provides an example on diminished efficacy of oseltamivir and leads to increased risk of spreading influenza virus.

Materials and Methods

Chemicals and Supplies. 3-[4,5-Dimethyl-thiazolyl-2]-2,5-diphenyl-tetrazolium bromide (MTT) and *para*-nitrophenylacetate were from Sigma (St. Louis, MO). Clopidogrel sulfate was purchased from ChemPacific (Baltimore, MD). Clopidogrel carboxylate, oseltamivir, and oseltamivir carboxylate were purchased from Toronto Research Chemicals (Toronto, ON, Canada). The goat anti-rabbit-IgG conjugated with alkaline phosphatase was from Pierce (Rockford, IL). Cell culture media, Lipofectamine, and Plus Reagent were purchased from Invitrogen (Carlsbad, CA). Human liver microsomes were from CellDirect (Austin, TX). Human intestinal microsomes were from BD Bioscience (San Diego, CA). Normal human plasma was from the Rhode Island Blood Bank (Providence, RI). Unless otherwise indicated, all other reagents were purchased from Fisher Scientific (Pittsburgh, PA).

Enzymatic Assays. The hydrolysis of oseltamivir was carried out at 37°C in a total volume of 100 μl. Samples (5–20 μg protein) were prepared in 50 μl of reaction buffer Tris-HCl (50 mM, pH 7.4) and then mixed with an equal volume of oseltamivir (usually 400 μM) in the same buffer. After incubation (typically 10 min), the reactions were terminated with 200 μl of acetonitrile containing clopidogrel carboxylate (50 ng/ml) as the internal standard (IS). The reaction mixtures were subjected to centrifugation for 15 min at 4°C (12,000g). As controls, the reactions were stopped at 0 min or carried out without protein. The metabolism was monitored by LC-MS/MS described below, and the hydrolytic rate was calculated as nanomoles per milligram of protein per minute. Hydrolysis of *para*-nitrophenylacetate was spectrophotometrically determined, and the hydrolytic rate was calculated as micromoles per milligram of protein per minute. Determination of *para*-nitrophenylacetate hydrolysis was detailed elsewhere (Xie et al., 2002).

LC-MS/MS Analysis. A turbo ion spray-liquid chromatography-tandem mass spectrometry technique was used. The LC-MS/MS system consists of a PerkinElmer 200 series micropump and autosampler (PerkinElmer Life and Analytical Sciences, Wellesley, MA) attached to a PE Sciex API2000 series tandem mass spectrometer (Applied Biosystems, Foster City, CA). High-purity nitrogen gas obtained from a 240-liter Liquid Nitrogen Dewar (Med-Tech, Medford, MA) was used as nebulizer (gas 1), auxiliary (gas 2), and collision gases. Unless otherwise stated, clopidogrel carboxylic acid was used as the IS, and elution of oseltamivir, oseltamivir carboxylate, and IS was carried out isocratically using a mobile phase composition of 70/30% v/v acetonitrile/0.05% v/v formic acid in deionized water maintained at a flow rate of 0.25 ml/min with a total run time of 6.0 min.

Various parameters were adjusted to obtain optimal conditions for the detection and quantification of oseltamivir, oseltamivir carboxylate, and IS in multiple reactant monitoring mode. Detection of the analytes was performed in positive ion mode using the mass transitions of m/z, 313.3 $\rightarrow$ 166.1 for oseltamivir; m/z, 285.2$\rightarrow$138.0 for oseltamivir carboxylate; and m/z, 308.2 $\rightarrow$ 152.0 for IS. Flow injection analysis was performed at a flow rate of 20 μl/min to obtain optimum source parameters. The following compound parameters were used for oseltamivir, oseltamivir carboxylate, and IS, respectively: declustering potential, +5, +5, and + 30 V; focusing potential, +360 V each; entrance potential, +8 V each; collision cell entrance potential, +20 V each; collision energy, +25, +25, and + 30 V; and collision cell exit potential, +7 V each. The optimal source parameters that gave the highest oseltamivir intensity were curtain gas, 10 psi; collision gas, 4 psi; ion spray voltage, +5500 V; temperature, 450°C; ion source gas 1, 25 psi; and ion source gas 2, 85 psi. Integration of the peaks was performed by manual baseline adjustment using the ANALYST SP version 1.2 software (Applied Biosystems).

The assay was linear from 1 to 250 ng/ml for oseltamivir and from 4 to 1000 ng/ml for oseltamivir carboxylate. All quantifications were performed using peak area ratios, and the calibration curves consisted of oseltamivir or oseltamivir carboxylate to clopidogrel carboxylic acid concentration ratios plotted against the oseltamivir or oseltamivir carboxylate to clopidogrel carboxylic acid peak area ratios. The calibration curves were constructed with $1/x^2$ weighting, and the regression coefficients were greater than 0.99. The lower limit of quantification based on a signal/noise ratio of 10 was 0.08 ng/ml for oseltamivir and 0.78 ng/ml for oseltamivir carboxylate. Three quality control standards representing low, medium, and high concentrations of each compound to be monitored were analyzed for every batch of samples analyzed. Interday imprecision was less than 15% for both compounds.

Plasmid Constructs and Site-Directed Mutagenesis. Expression constructs encoding human carboxylesterases HCE1 and HCE2 were described elsewhere (Xie et al., 2002). Natural variants of HCE1 were prepared by site-directed mutagenesis as described previously (Li et al., 2003). Complementary oligonucleotides were synthesized to introduce a substitution. The primers were annealed to the HCE1 expression construct and subjected to a thermocycler for a total of 15 cycles. The resultant PCR-amplified constructs were then

digested with DpnI to remove the nonmutated parent construct. The mutated PCR-amplified constructs were used to transform XL1-Blue bacteria. All mutated constructs were subjected to sequencing analysis to confirm the desired mutation being made without secondary mutations.

Transfection. Human embryonic kidney cells (293T) were plated at a density of 60% in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. After reaching 80% confluence, cells were transfected by Lipofectamine and Plus Reagent. A plasmid construct or the empty vector (4 μg/100-mm dish) was initially mixed with 20 μl of Plus Reagent diluted in 750 μl of serum-free medium for 15 min and then mixed with 30 μl of Lipofectamine reagent diluted in 5 ml of serum-free medium for 15 min. The final transfection complexes were added to a monolayer of 293T cells. After a 3-h incubation, the medium was replaced by normal culture medium and incubated for 48 h in a 37°C humidified incubator with 5.0% CO_2. Cells were rinsed and harvested in 1.5 ml of Tris-HCl buffer (50 mM, pH 7.4). The cell suspension was sonicated by a Branson Sonifier, and cell debris was removed by centrifugation at 12,000g for 10 min at 4°C. The supernatant was assayed for hydrolytic activity toward oseltamivir and *para*-nitrophenylacetate.

Cytotoxicity Assay. Cells (293T) were cultured in six-well plates and transfected with the vector or a construct encoding HCE1 or HCE2 as described above (1 μg/well). After a 12-h incubation, the cells were collected and seeded into 96-well plates at a density of 10,000/well. After an additional 12-h incubation, the medium was replaced with reduced serum medium (1%) containing oseltamivir at various concentrations (0–320 μM), and the treatment lasted for 36 h. MTT was then added to each well at a final concentration of 1 mg/ml. After 4-h incubation at 37°C, the medium was gently decanted, and dimethyl sulfoxide (150 μl/well) was added to dissolve formazan product. The optical density was determined at 570 nm, and the final optical density values were expressed by subtracting the background reading (no seeded cells). The cell viability was expressed as the percentage of vector-transfected cells.

Other Assays. Protein concentration was determined with Micro BCA Reagents (Pierce) as described by the manufacturer. Antibodies against distinct carboxylesterases were described elsewhere (Xie et al., 2002). Data are presented as mean ± S.D. of at least three separate experiments, except where results of blots are shown, in which case a representative experiment is depicted in the figures. Comparisons were made according to the analysis of variance method for the variations among polymorphic variants followed by least significant difference post hoc test for the variations ($p \leq 0.05$).

Results

Liver but Not Intestinal Microsomes Hydrolyze Oseltamivir. The hydrolysis of oseltamivir was first determined with human plasma, intestinal, and liver microsomes. The selection of these tissues was based on their differences on the abundance of HCE1 and HCE2, two major carboxylesterases in humans. Both HCE1 and HCE2 are abundantly expressed in the liver (Kroetz et al., 1993; Schwer et al., 1997), whereas the intestine expresses predominantly HCE2 (Schwer et al., 1997). Neither HCE1 nor HCE2 is present in the plasma (Li et al., 2005). Therefore, the relative activity on the hydrolysis of oseltamivir would provide information on the identity of carboxylesterase(s) responsible for the hydrolysis of this prodrug. The hydrolysis was conducted with pooled samples. The plasma and intestine microsomes were pooled from five individuals each, whereas the liver microsomes were pooled from 19 individual samples. The metabolism was monitored by LC-MS/MS, and Fig. 1A shows the representative chromatogram containing oseltamivir, the carboxylate metabolite, and the IS (clopidogrel carboxylate).
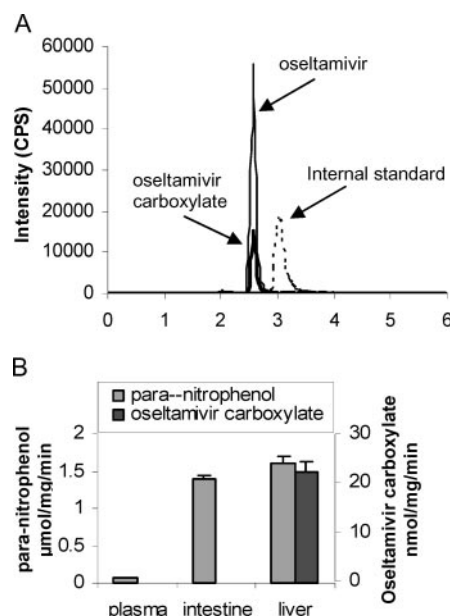

Fig. 1. Oseltamivir hydrolysis by plasma, intestine, and liver microsomes. A, representative chromatogram containing oseltamivir, oseltamivir carboxylate, and internal standard. The trace was generated by injecting a mixture (20 μl) of oseltamivir (8 ng/ml), oseltamivir carboxylate (31 ng/ml), and the internal standard (50 ng/ml). B, hydrolysis of oseltamivir by plasma, intestine, and liver microsomes. Samples (5–20 μg protein) were prepared in 50 μl of reaction buffer Tris-HCl (100 mM, pH 7.4) and then mixed with an equal volume of oseltamivir (400 μM). After 10 min of incubation, the reactions were terminated with 200 μl of acetonitrile containing clopidogrel carboxylate (final concentration = 50 ng/ml). The formation of oseltamivir carboxylate was detected by LC-MS/MS. Hydrolysis of *para*-nitrophenylacetate was spectrophotometrically determined as described previously (Morgan et al., 1994). All assays were performed in triplicate with three transfection experiments. The hydrolytic rates were expressed as the mean ± S.D. (nanomoles or micromoles per milligram per minute).

Results of the hydrolysis of oseltamivir by the plasma, liver, and intestinal microsomes are summarized in Fig. 1B. Oseltamivir was rapidly hydrolyzed by the liver microsomes. In contrast, no hydrolysis was detected with either the intestinal microsomes or plasma. All samples, however, hydrolyzed *para*-nitrophenylacetate. The hydrolytic rate of this standard substrate was comparable by the liver and intestine microsomes, but the hydrolysis was much lower by the plasma. These results suggest that the liver is the primary organ that catalyzes the hydrolysis of oseltamivir.

Individual Variation of Oseltamivir Hydrolysis. The predominance of hepatic hydrolysis suggests that the liver determines the activation of oseltamivir; thus, differences in the hepatic hydrolysis among population probably reflect individual variation in the activation of this prodrug. To shed light on the extent of individual variation regarding the activation of oseltamivir, we assayed 19 individual liver samples. In addition, the levels of HCE1 and HCE2 were determined by Western blots, and the correlation analysis was performed between the hydrolytic rate and the abundance of each carboxylesterase among individual samples. As shown in Fig. 2A, all samples hydrolyzed oseltamivir; however, the overall activity varied from sample to sample. The difference ranged by as many as 5-fold (7–34 nmol/mg/min).

We next performed the correlation analysis of the hydrolytic rate with the level of HCE1 or HCE2. The level of HCE2 was correlated poorly with the hydrolytic rate (data not
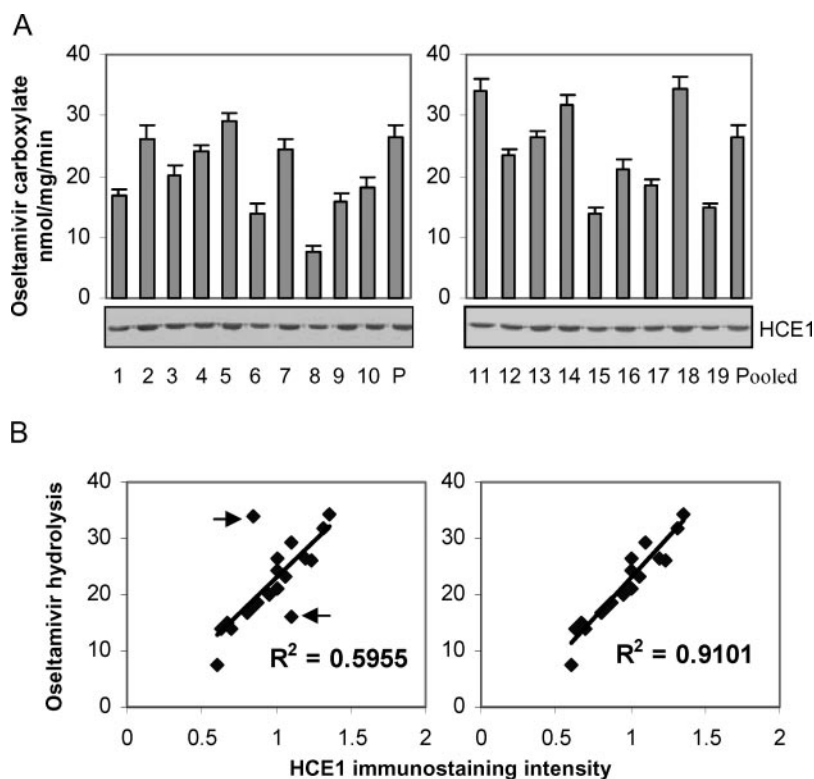
Fig. 2. Hydrolysis of oseltamivir by individual liver samples and correlation analysis of the hydrolysis with the abundance of HCE1. A, oseltamivir hydrolysis by liver microsomes. Individual liver samples (5 μg) were incubated with oseltamivir with a final concentration of 200 μM, and the formation of oseltamivir carboxylate was detected by LC-MS/MS. For Western blots, samples (1 μg) were subjected to SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to a Trans-Blot nitrocellulose membrane. The immunoblots were blocked in 5% nonfat dry milk, incubated with the antibody (10 μg/ml) against HCE1, and detected by alkaline phosphatase-conjugated anti-rabbit IgG. B, correlation between oseltamivir hydrolysis and the abundance of HCE1. The immunostaining intensity was quantified by densitometry and plotted with the hydrolytic rate of each sample toward oseltamivir. Left, total samples; right, total samples minus the samples marked with an arrow.

shown). In contrast, the rate was correlated fairly well with the level of HCE1. As shown in Fig. 2B, all data points were scattered closely along the regression line except two outliers (labeled with an arrow). The overall correlation coefficient was 0.5955 (Fig. 2B, left). However, the coefficient was markedly improved (up to 0.9101) when the two outliers (arrowed) were excluded (Fig. 2B, right). The samples with poor correlation (outliers) were probably from individuals who expressed HCE1 variants.

Carboxylesterase HCE1 but Not HCE2 Hydrolyzes Oseltamivir. The good correlation between oseltamivir hydrolysis and the level of HCE1 suggests that HCE1 is responsible for the hydrolysis of oseltamivir in the liver. We next used recombinant HCE1 and HCE2 to definitively establish this notion. Recombinant enzymes were prepared by transient transfection as described previously (Xie et al., 2002). Likewise, lysates were tested for the hydrolysis of oseltamivir and standard substrate para-nitrophenylacetate. As shown in Fig. 3A, lysates from HCE1-transfected cells were highly active toward oseltamivir. In contrast, lysates from HCE2-transfected cells showed no activity, although the HCE2 lysates were more active than the HCE1 lysates on hydrolyzing para-nitrophenylacetate. The lysates from vector-transfected cells (Fig. 3A, left) showed no activity toward either oseltamivir or para-nitrophenylacetate. It should be emphasized that Western analysis confirmed the expression of HCE1 and HCE2, respectively, in transfected cells (Fig. 3A, bottom).

To gain kinetic insight, the parameters K_m and V_{max} were determined with recombinant HCE1 and pooled liver microsomes. The hydrolytic rate of oseltamivir was determined as a function of substrate concentrations (0.001–2.00 mM). As shown in Fig. 3B, data from HCE1 yielded a linear Lineweaver-Burk plot. The apparent K_m value was 177 μM, and

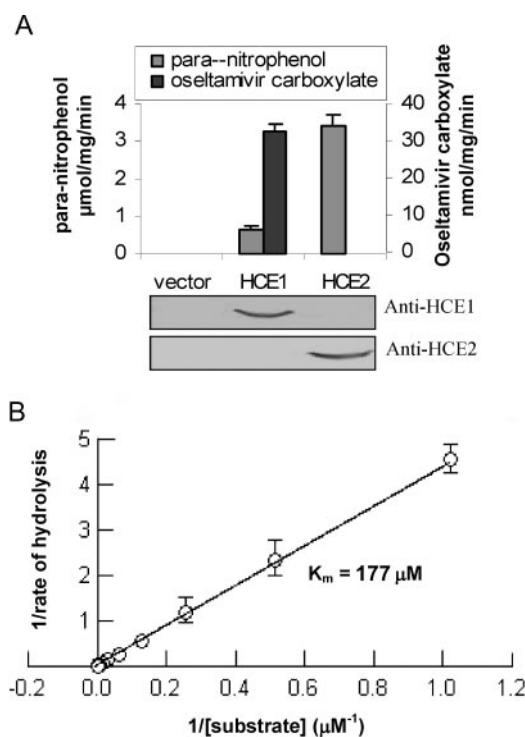Fig. 3. Hydrolysis of oseltamivir by recombinant HCE1 and HCE2 and determination of enzyme kinetics. A, hydrolytic rate. Lysates (5 μg) from cells transfected with the empty vector or a cDNA construct encoding HCE1 or HCE2 were assayed for their activity to hydrolyze oseltamivir (200 μM) and para-nitrophenylacetate (1 mM). The hydrolytic activity toward oseltamivir was monitored by LC-MS/MS, whereas the activity toward para-nitrophenylacetate was monitored spectrophotometrically. B, Lineweaver-Burk plot of oseltamivir hydrolysis by recombinant HCE1. The hydrolytic rate was determined as a function of oseltamivir (0.001–2 mM). K_m and V_{max} were calculated by VisualEnzymics.

the apparent V_{max} value was 102 nmol/mg/min. Likewise, data from pooled liver microsomes yielded a straight line. More importantly, the kinetic parameters from liver microsomes (K_m = 187 μM; V_{max} = 114 nmol/mg/min) were similar to those from recombinant HCE1, suggesting that HCE1 is the only enzyme that effectively hydrolyzes this prodrug in the liver.

Hydrolysis of Oseltamivir by Natural Variants. Molecular cloning studies from this and other laboratories have revealed several HCE1 polymorphic variants (Marsh et al., 2004; Zhao et al., 2005), and the database of single-nucleotide polymorphism from NCBI lists many natural variants of this enzyme as well. As an initial effort to establish the significance of HCE1 polymorphism on drug metabolism, we tested some of the variants for the altered hydrolysis toward oseltamivir. As shown in Fig. 4A, three variants differed from the wild-type enzyme on hydrolyzing oseltamivir. HCE1$_{S58N}$ slightly increased the hydrolysis (~25%), whereas variants HCE1$_{C70F}$ and HCE1$_{R128H}$ markedly decreased the hydrolysis. All variants were expressed to a comparable extent with the exception of HCE1$_{C70F}$, which was consistently expressed to a much lower level (Fig. 4A, bottom). It should be emphasized that increased amounts of the lysates from HCE1$_{C70F}$-transfected cells were assayed, and little hydrolysis was consistently observed, suggesting that this variant, in addition to decreased expression, is catalytically inefficient.

Oseltamivir Hydrolysis Is Inhibited by Antiplatelet Agent Clopidogrel. The predominance of HCE1 in oselta-

mivir hydrolysis suggests that this enzyme acts as a source for oseltamivir-drug interactions. Recently, we reported that HCE1 is also responsible for the hydrolysis of clopidogrel, an ester antiplatelet agent (Gachet, 2006; Gidwani and Body, 2006; Tang et al., 2006). More importantly, clopidogrel is widely used for long-term preventive therapy against atherosclerotic events; thus, it is likely that oseltamivir and clopidogrel are concomitantly administered. The hydrolysis of oseltamivir (50 μM) by HCE1 lysates was assayed in the presence of clopidogrel at various concentrations (0–50 μM). Likewise, the formation of oseltamivir carboxylate was monitored; however, the quantification was based on the relative areas from each incubation against calibration curves without internal standard-based normalization. The hydrolytic rate was calculated as the percentage of that in the absence of clopidogrel. As shown in Fig. 4B, the presence of clopidogrel caused proportional decreases on the hydrolysis of oseltamivir. At 5 μM (10% of the concentration of oseltamivir), clopidogrel inhibited oseltamivir hydrolysis by as much as 55%. At the equal concentration (50 μM), the hydrolysis of oseltamivir was inhibited by as much as 90%. It should be noted that comparable inhibition was detected with liver microsomes (data not shown), further establishing that HCE1 is the primary enzyme that hydrolyzes oseltamivir.

Hydrolysis of Oseltamivir Increases Toxicity. The decreased hydrolysis of oseltamivir by clopidogrel may have profound clinical consequence in terms of antiviral effect. Next, we examined whether hydrolysis of oseltamivir has any toxicological significance. Given the fact that oseltamivir carboxylate is negatively charged, the rate of uptake of this hydrolytic metabolite probably determines the cytotoxicity. To minimize the effect of uptake, we examined the toxicity in cells where the production of oseltamivir carboxylate occurred intracellularly and was regulated by transfection of HCE1 and HCE2. The transfected cells were seeded into 96-well plates and treated with oseltamivir at various concentrations (0–320 μM). The cytotoxicity was monitored for cell viability by MTT assay and microscopic examination (Li et al., 2002). The viability was expressed as percentage of optical density from the vector-transfected cells.

The results of the toxicity study are summarized in Fig. 5. Transfection of HCE1 markedly decreased the cell viability, and the decrease was significant even when oseltamivir was used as low as 10 μM (Fig. 5A). In contrast, the cells transfected with HCE2 showed no changes on the viability compared with the cells transfected with the vector. It should be noted that the vector-transfected cells had the same viability as nontransfected cells (data not shown). Under bright field, HCE1-transfected cells showed two different types of changes on the overall morphology (arrowed). Some cells were rounded and swollen, whereas some others shrank. However, both cases exhibited blebbing of plasma membrane and condensed nuclei (Fig. 5B, left). It is likely that they represent two different stages of cell death or cells that produced different levels of oseltamivir carboxylate (due to differential transfection). The changes occurred with ~60% of the cells, suggesting that these cells were effectively transfected. In contrast, no such changes were observed in the cells transfected with HCE2 (Fig. 5B, right). Likewise, the overall morphology of HCE2-transfected cells was similar as those of vector-transfected cells or nontransfected cells. It should be noted that Western analysis confirmed the expres-
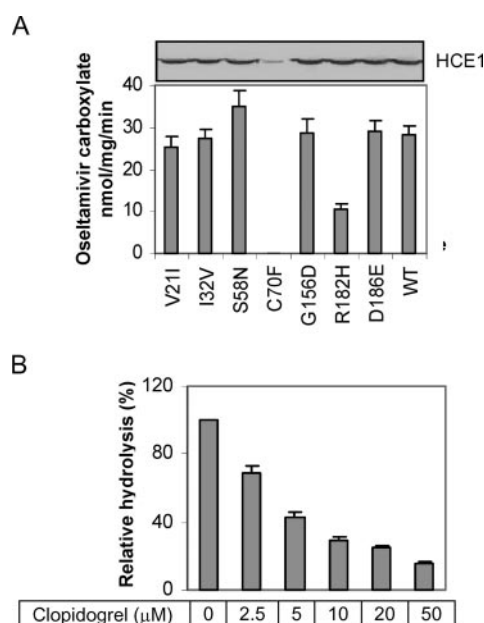

Fig. 4. Hydrolysis of oseltamivir by HCE1 variants and inhibition of the hydrolysis by clopidogrel. A, hydrolysis by HCE1 variants. Lysates (5 μg) from cells transfected with a cDNA construct encoding HCE1 or a variant were assayed for the activity to hydrolyze oseltamivir (200 μM). The hydrolysis was monitored by LC-MS/MS. The level of HCE1 or a variant was determined by Western blots with 1 μg of lysates. B, inhibition of oseltamivir hydrolysis by clopidogrel. Lysates (5 μg) from cells transfected with HCE1 construct were assayed for the hydrolysis of oseltamivir (50 μM) in the presence of clopidogrel at various concentrations (0–50 μM). Quantification on the formation of oseltamivir was based on the relative areas generated from each injection against the standard curve (without being normalized according to clopidogrel carboxylic acid). The hydrolytic activity was expressed as percentage of that in the absence of clopidogrel.
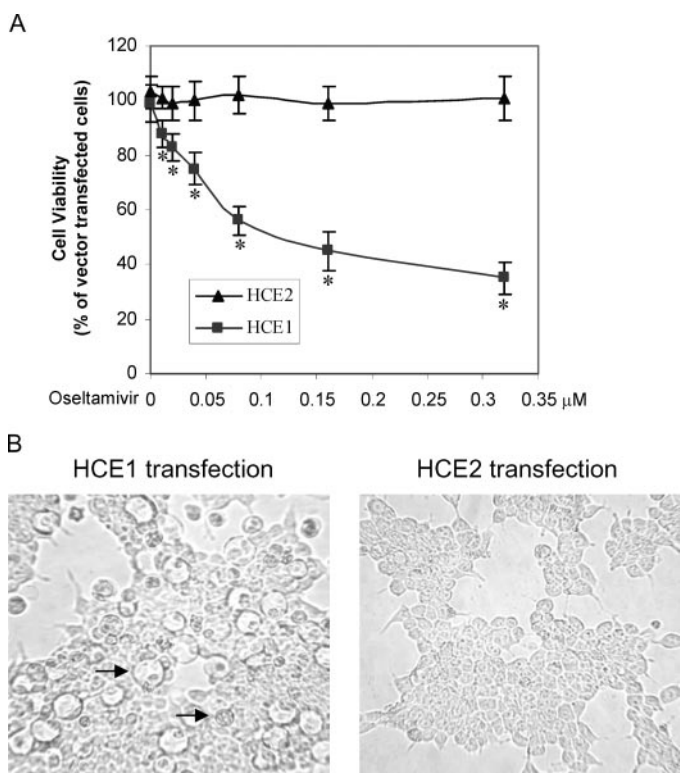
A

B

HCE1 transfection   HCE2 transfection

Fig. 5. Effect of oseltamivir hydrolysis on cell viability. A, cell viability. Cells (293T) were cultured in six-well plates and transfected with the vector or a construct encoding HCE1 or HCE2. After a 12-h incubation, the cells were collected and seeded into 96-well plates at a density of 10,000/well. After an additional 12-h incubation, cells were treated with oseltamivir at various concentrations (0–0.32 mM) for 36 h. Cell viability was determined with MTT assay as described under *Materials and Methods*. The cell viability was expressed as the percentage of vector-transfected cells. *, significantly different from HCE2-transfected cells according to the analysis of variance method followed by least significant difference post hoc test for the variations ($p \leq 0.05$). B, morphological analysis. The images were taken under bright field from cells treated with 0.32 mM oseltamivir (250×).

sion of HCE1 and HCE2, respectively, in transfected cells (data not shown).

Discussion

Flu pandemic represents one of the most devastating natural disasters (Olsen et al., 2006). Influenza virus H5N1, originated in Southeast Asian in recent years, shows all characteristics leading to a flu pandemic (Liu, 2006). Oseltamivir is an ethyl ester prodrug and the main medicine recommended by the World Health Organization in anticipation of next influenza pandemic (Normile, 2006). In this study, we report that HCE1 but not HCE2 is responsible for the hydrolytic activation of oseltamivir in the liver. Antiplatelet agent clopidogrel, a substrate of HCE1, inhibits the hydrolysis of oseltamivir by as much as 90% when assayed at equal concentrations. Several polymorphic variants of HCE1 differ markedly from the wild-type enzyme on the hydrolysis of oseltamivir.

HCE1 is likely the only enzyme that rapidly hydrolyzes oseltamivir; thus, it represents the major determinant on the efficacy of this prodrug. Several lines of evidence support this notion. First, there are two major carboxylesterases (HCE1 and HCE2) in the liver (Xie et al., 2002); however, only HCE1

but not HCE2 catalyzes the hydrolysis of oseltamivir (Fig. 3A). Second, the overall hydrolysis of oseltamivir by individual liver samples is correlated well with the abundance of HCE1, and recombinant HCE1 yields similar kinetic parameters as the liver microsomes (Fig. 3B). Third, both human plasma and intestinal microsomes are known to catalyze hydrolytic reaction but show no activity toward oseltamivir (Fig. 1B; Tang et al., 2006). Fourth, the hydrolysis of oseltamivir is significantly delayed in children (1–2 years) who usually express lower levels of HCE1 (He et al., 1999; Massarella et al., 2000; Oo et al., 2003b; Pope et al., 2005). Finally, the half-life of oseltamivir is markedly increased when administered through the colon (Oo et al., 2003a), a site from which the blood is not directly drawn to the liver (compared with other parts of the gastrointestinal tract such as the stomach). HCE1 is present in other tissues; however, the relative abundance is much lower than that in the liver (Xie et al., 2002).

The predominance of HCE1 in oseltamivir activation provides a critical source for oseltamivir-drug interaction. In this report, we have shown that clopidogrel effectively decreases the hydrolysis of oseltamivir (Fig. 4B). Clopidogrel is an antiplatelet agent and widely used for long-term preventive therapy against atherosclerotic events; thus, it is likely that oseltamivir and clopidogrel are concomitantly administered. Like oseltamivir, clopidogrel is an ester, and we have recently reported that HCE1 is also responsible for the hydrolysis of clopidogrel. Based on the kinetic parameters, clopidogrel is kinetically more favorable than oseltamivir (Fig. 3B; Tang et al., 2006). Given the fact that hydrolysis of oseltamivir is required for its therapeutic activity, concomitant use of both drugs would inhibit the activation of oseltamivir, thus making this antiviral agent therapeutically inactive. This is epidemiologically of significance because people who receive oseltamivir and clopidogrel simultaneously maintain susceptibility to influenza infection or a source of spreading influenza virus if already infected. This type of oseltamivir-drug interaction needs to be fully established in humans.

Slight decreases on the hydrolysis of oseltamivir, on the other hand, may enhance the efficacy of oseltamivir. Such an unexpected phenomenon is due to the unique role of hydrolysis in the pharmacokinetics of this antiviral agent. The elimination half-life of oseltamivir carboxylate following oral administration of oseltamivir is 6 to 10 h in comparison with approximately 2 h following i.v. administration of the metabolite (He et al., 1999). This discrepancy suggests that the oseltamivir carboxylate concentration-time profile undergoes flip-flop kinetics when administered orally because the rate of conversion to the active metabolite contributes to decreased rate of the elimination of this metabolite. As a result, slightly decreased hydrolysis of oseltamivir slows down the production of the active metabolite and extends the therapeutic effect of this antiviral agent. Prolonged presence of oseltamivir probably contributes to increased concentrations in the brain. In 7-day-old rats, the brain level of oseltamivir is reportedly 1500 times as much as that in adult rats, and such an increase is linked to animal death (Wooltorton, 2004). We have previously reported that 1- and 2-week-old rats express little hepatic carboxylesterases (Morgan et al., 1994), although it remains to be determined to which extent

the lack of hepatic carboxylesterases contributes to the increased level of oseltamivir in the brain.

Interestingly, transfection of HCE1 leads to marked increases on cell toxicity (Fig. 5), suggesting that the hydrolytic metabolite is more toxic than the parent compound. The precise mechanism on the increased toxicity remains to be determined. Given the fact that this metabolite is a potent inhibitor of influenza neuraminidase (Hurt et al., 2004; Normile, 2006), it may inhibit mammalian neuraminidases as well, particularly when it is significantly accumulated intracellularly. The accumulation probably occurs in the liver and kidney, where the formation and elimination of oseltamivir carboxylate take place, respectively. The efflux of the carboxylate in the liver and its elimination in the kidney are presumably achieved by different sets of transporters (Inui et al., 2000; Hill et al., 2002; Miyazaki et al., 2004). In this study, we have demonstrated that oseltamivir at as low as 10 μM significantly decreases the cell viability in HCE1-transfected cells (Fig. 5). Therefore, drugs sharing transporters with oseltamivir carboxylate may have both pharmacological and toxicological significance regarding the use of oseltamivir.

The activation of oseltamivir is likely altered in people who express polymorphic variants. In this study, we have tested several natural variants for the hydrolysis of oseltamivir, and some of them show altered activity toward this ester (Fig. 4A). For example, substitution of serine-58 with an asparagine slightly increases the hydrolysis, whereas substitution of arginine-182 with a histidine markedly decreases the hydrolysis. On the other hand, substitution of cysteine-70 with a phenylalanine profoundly decreases the expression (Fig. 4A). This cysteine is involved in the formation of intramolecular disulfide bond (Song et al., 2004). We have shown that substitution of this cysteine with an alanine in rat hydrolase B, although having little effect on the expression, causes complete loss of hydrolytic activity (Song et al., 2004). It remains to be determined whether the difference on the substitution residues (phenylalanine versus alanine) or the carboxylesterases involved (HCE1 versus hydrolase B) is responsible for decreased expression. Nevertheless, these findings underscore the importance of this cysteine in the biosynthesis and ultimately the catalysis of this carboxylesterase.

In summary, our work points to several important conclusions. First, oseltamivir is predominantly activated in the liver by carboxylesterase HCE1, and altered expression or polymorphic variants of this enzyme may profoundly affect the therapeutic effectiveness of this prodrug. Second, the activation of oseltamivir is probably inhibited by drugs such as clopidogrel that contain ester linkage, and the inhibition may severely diminish its therapeutic activity, particularly by those that are kinetically favorable substrates of HCE1. Third, the hydrolytic metabolite is more cytotoxic than the parent compound; thus, decreases on the hepatic efflux and/or renal secretion of this carboxylate have toxicological consequences. Given the fact that hydrolytic biotransformation is essential for oseltamivir to exert antiviral activity, identification of HCE1 as the primary enzyme for the hydrolysis provides a molecular basis for individual variation, drug-drug interaction, and toxicity regarding the use of this anti-influenza agent.

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