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Covalent Inhibition of Recombinant Human Carboxylesterase 1 and 2 and Monoacylglycerol Lipase by the Carbamates JZL184 and URB597

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Abstract

Carboxylesterase type 1 (CES1) and CES2 are serine hydrolases located in the liver and small intestine. CES1 and CES2 actively participate in the metabolism of several pharmaceuticals. Recently, carbamate compounds were developed to inhibit members of the serine hydrolase family via covalent modification of the active site serine. URB597 and JZL184 inhibit fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively; however, carboxylesterases in liver have been identified as a major off-target. We report the kinetic rate constants for inhibition of human recombinant CES1 and CES2 by URB597 and JZL184. Bimolecular rate constants (k_{inact}/K_i) for inhibition of CES1 by JZL184 and URB597 were similar [$3.9 (\pm 0.2) \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ and $4.5 (\pm 1.3) \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$, respectively]. However, k_{inact}/K_i for inhibition of CES2 by JZL184 and URB597 were significantly different [$2.3 (\pm 1.3) \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$ and $3.9 (\pm 1.0) \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$, respectively]. Rates of inhibition of CES1 and CES2 by URB597 were similar; however, CES1 and MAGL were more potently inhibited by JZL184 than CES2. We also determined kinetic constants for spontaneous reactivation of CES1 carbamoylated by either JZL184 or URB597 and CES1 diethylphosphorylated by paraoxon. The reactivation rate was significantly slower (4.5x) for CES1 inhibited by JZL184 than CES1 inhibited by URB597. Half life of reactivation for CES1 carbamoylated by JZL184 was $49 \pm 15 \text{ h}$, which is faster than carboxylesterase turnover in HepG2 cells. Together, the results define the kinetics of inhibition for a class of drugs that target hydrolytic enzymes involved in drug and lipid metabolism.

Keywords

carboxylesterase; monoacylglycerol lipase; carbamate; bimolecular rate constant; reactivation; drug-drug interactions

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Conflict of interest
None declared.

1. Introduction

Serine hydrolases catalyze the hydrolysis of ester or amide bonds by utilizing a base activated serine nucleophile. Currently, there are approximately 240 predicted serine hydrolases in mammals [1]. Serine hydrolases are broadly characterized as being either metabolic serine hydrolases or serine proteases/peptidases [2]. The substrates for metabolic serine hydrolases are typically small molecules, such as neurotransmitters (e.g., acetylcholine and endocannabinoids) or neutral lipids (e.g., triacylglycerols and cholesteryl esters). Serine hydrolases participate in a wide variety of physiological and pathophysiological processes, including signal transduction in neural tissue [3], digestion [4], immune response [5], and the clotting cascade [6]. Enzymes are one of the major domains of the proteome that are targeted by pharmaceutical agents [7]. Not surprisingly, there has been an active interest in the development of specific inhibitors for individual serine hydrolases as potential pharmaceutical agents. The N-methylcarbamates have been used commercially in the United States and Europe as pesticides since the 1950's [8] and exert their toxicity via the inhibition of acetylcholinesterase [9]. Inhibition of acetylcholinesterase in the synapse increases the levels of synaptic acetylcholine resulting in continuous activation of the post synaptic neuron. Inhibition of acetylcholinesterase by N-methylcarbamates occurs as a result of carbamoylation of the active site serine [10]. The carbamoylated enzyme will undergo spontaneous hydrolysis thus regenerating active enzyme [3]; however, hydrolysis of the carbamoyl-enzyme intermediate is much slower than hydrolysis of the acetyl-enzyme intermediate formed during the catalytic turnover of acetylcholine. Thus, carbamates are essentially substrates for acetylcholinesterase but with very low turnover rates and are often termed pseudo-substrate inhibitors.

Organophosphates (OPs) also exert their toxicity through the inhibition of synaptic acetylcholinesterase. Oxons are generated primarily in the liver by the metabolism of OPs by cytochrome P450 and are the active metabolites of OPs that inhibit acetylcholinesterase via phosphorylation of the active site serine. OPs have been utilized extensively as insecticides since the 1940s and also been developed for use as nerve gas agents. As a result, the metabolism of OPs in humans has been thoroughly studied and described in some detail utilizing PBPK/PD modeling [11]. Humans are protected from some of the toxic effects of OPs by the stoichiometric reaction of oxons with CES1, a serine hydrolase that is abundantly expressed in the liver. In contrast, carbamate metabolism in humans has not been as thoroughly studied. Knaak et al. [12] recently developed an initial PBPK/PD model describing the metabolism of carbamate pesticides in humans. The model includes the inhibition of hepatic CES1 such that hepatic CES1 serves as a stoichiometric scavenger analogous to its function in OP metabolism. Reactivation of hepatic carbamoylated CES1 is also included in the model, although no experimentally determined kinetic constant was available.

Several compounds containing the carbamate moiety have recently been developed as pharmaceutical agents with the goal of specifically targeting individual members of the serine hydrolase family (see Fig. 1A for the specific compounds used in this study). For example, JZL184 inhibits the 2-arachidonoylglycerol hydrolyzing enzyme MAGL in the mouse brain membrane proteome with an IC_{50} of 8 nM [13] and URB597 inhibits the anandamide hydrolyzing enzyme FAAH in rat brain membranes with an IC_{50} of 4.6 nM [14]. These inhibitors typically act by covalently modifying the active site serine residue of the enzyme. However, the specificity of URB597, JZL184 and other carbamates have been called into question with the identification of 60 kD major off-target proteins that were subsequently identified as carboxylesterases in rat liver [15] and CES1 in cultured human THP1 monocytes/macrophages [16]. In humans, the two predominant carboxylesterases are CES1 and CES2. CES1 is present in large amounts in the liver [17, 18], whereas CES2 is

located predominantly in the intestine [19]. CES1 is involved in the metabolism of numerous ester drugs and prodrugs [20]. Therefore, sustained covalent inhibition of CES1 by a putative pharmaceutical agent could increase the risk for drug-drug interactions (DDI). However, the potential for DDI would be reduced if CES1 were rapidly reactivated to its active state following carbamoylation. CES2, by virtue of its location in the small intestine, may also participate in first pass metabolism of pharmaceutical agents that are absorbed from the intestine.

In order to compare the reactivity of JZL184 with its intended target MAGL [13] versus the human carboxylesterases CES1 and CES2, we have determined the bimolecular rate constants for inhibition of JZL184 with recombinant human MAGL, CES1 and CES2. We also compared the reactivity of CES1 and CES2 with JZL184 to the reactivity of CES1 and CES2 with URB597. Finally, we determined the rates of spontaneous reactivation of inhibited/carbamoylated CES1 following reaction with either JZL184 or URB597 and the rate of spontaneous reactivation of inhibited/phosphorylated CES1 following reaction with paraoxon, the active metabolite of the prototypical OP insecticide parathion.

2. Materials and Methods

2.1 Chemicals and Reagents

Human recombinant CES1 and CES2 proteins were expressed in baculovirus-infected *Spodoptera frugiperda* cells and purified as previously described [21]. Human recombinant MGL, URB597 and JZL184 were purchased from Cayman Chemical (Ann Arbor, MI). Zeba spin desalting columns were purchased from Pierce Biotechnology (Rockford, IL). Paraoxon was a gift from Dr. Howard Chambers, Department of Entomology, Mississippi State University. Paraoxon was of greater than 99% purity when assessed by thin-layer chromatography [22]. para-Nitrophenyl valerate (pNPV) and all other reagents and buffers were purchased from Sigma (St. Louis, MO). HepG2 cells were purchased from ATCC (Manassas, VA) and cultured using their recommendations. Thieno[3,2-e][1]benzothiophene-4,5-dione (S-3030; CES1 reversible inhibitor) was synthesized as previously described [23].

2.2 Enzyme Assays

Hydrolysis reactions were performed at 37°C in a 96-well plate format in a total volume of 300 µL in 50 mM Tris-HCl, which had been adjusted to pH 7.4 at room temperature. CES1 and CES2 were diluted to final concentrations between 0.65-1.1 nM and MAGL was diluted to a final concentration of 5.5 nM in the reaction mixtures used to determine inhibition constants. JZL184 and URB597 were dissolved in dimethylsulfoxide (DMSO) and added to the reaction mixture to give the desired concentrations. The final volume of DMSO in the wells was 1.0% (v/v) and the final volume of ethanol was 0.5% (v/v). pNPV was added to a final concentration of 500 µM. All reactions were corrected for the nonenzymatic hydrolysis of pNPV. Nonenzymatic hydrolysis of pNPV was typically < 5% of enzymatic activity. The reaction progress was monitored by measuring the absorbance at 405 nm for either 15 minutes or 45 minutes (depending on the enzyme-inhibitor pair being evaluated) to estimate the rate of formation of para-nitrophenol produced from the hydrolysis of pNPV.

CES1 was diluted to a final concentration of 4.4 nM in the reaction mixtures used to determine the reactivation constants. JZL184 and URB597 were dissolved in DMSO and added to the Paraoxon was diluted in ethanol and added to the reaction mixture to give the desired concentrations. The final volume of ethanol in the wells was 1.5% (v/v). pNPV was added to a final concentration of 500 µM. All reactions were corrected for the nonenzymatic hydrolysis of pNPV. Nonenzymatic hydrolysis of pNPV was typically < 5% of enzymatic

activity. The reaction progress was monitored by measuring the absorbance at 405 nm for 5 minutes to estimate the rate of formation of para-nitrophenol produced from the hydrolysis of pNPV. The slopes were determined and used to calculate the enzymatic activity. The curves were linear during the 5 minute reaction period.

2.3 Kinetic Studies of Inhibition

The competitive kinetic scheme describing the covalent inhibition of serine hydrolases (E) by carbamates (I) in the presence of an ester substrate (S) is shown in Figure 1B. We assumed that there was no significant degree of enzyme reactivation following inhibition for each of the enzyme inhibitor pairs during the time the reaction was monitored. This was confirmed by determination of the k_{react} for CES1 with JZL184 and URB597 (*vide infra*). The method to determine the bimolecular rate constant of enzyme inactivation has been previously described in Crow et al. [24]. To determine these rates either JZL184 or URB597 (various concentrations) and pNPV (500 μM) were added to the reaction buffer and brought to 37°C (5 min). The enzyme was then added to initiate the reaction. The progress of the reaction was followed by measuring the absorbance at 405 nm for either 15 minutes or 45 minutes, depending on the combination of the enzyme and carbamate used. The reaction curves were fit to the equation:

$$A_t = A_0 + (A_\infty - A_0)(1 - e^{-k_{\text{obs}} t}) \quad (1)$$

using SigmaPlot 8.0, and a value for the apparent first-order rate constant of enzyme inactivation (k_{obs}) was determined at each carbamate concentration. A_0 is absorbance at time 0, A_t is absorbance at time t , A_∞ is absorbance at time infinity, t is time in s, and k_{obs} is the observed rate constant in s^{-1} . k_{obs} corrected ($k_{\text{obs}}(\text{corr})$) for each inhibitor concentration was obtained by subtracting k_{obs} with no inhibitor present from the k_{obs} at that inhibitor concentration. $k_{\text{obs}}(\text{corr})$ is related to k_{inact} and K_i by the following equation:

$$k_{\text{obs}}(\text{corr}) = (k_{\text{inact}}) [I] / [K_i (1 + [S]/K_m) + [I]] \quad (2)$$

where k_{inact} is the rate constant for the inactivation (carbamoylation) of the enzyme by the carbamate, K_i is the dissociation constant for EI (enzyme inhibitor complex; i.e., the enzyme carbamate complex), $[I]$ is the inhibitor (carbamate) concentration, $[S]$ is the pNPV concentration, and K_m is the Michaelis constant for pNPV. If one substitutes K_i' (the apparent dissociation constant for the EI complex) for $K_i(1 + [S]/K_m)$, Equation (2) simplifies to:

$$k_{\text{obs}}(\text{corr}) = (k_{\text{inact}}) [I] / [K_i' + [I]] \quad (3)$$

which is a hyperbolic function.

If one assumes that $K_i' \gg [I]$, Equation (3) simplifies to:

$$k_{\text{obs}}(\text{corr}) = (k_{\text{inact}}) [I] / K_i' \quad (4)$$

which is a linear function, where the slope is the apparent bimolecular rate constant $k_i' = k_{\text{inact}} / K_i'$. The plots of $k_{\text{obs}}(\text{corr})$ vs $[I]$ for each reaction were fitted to both the equation for a hyperbola and the equation for a line. For each enzyme and JZL184 pair studied, the data fit the equation of a hyperbola better than that of a line as judged by r^2 values. The k_{inact} and K_i' were determined by fitting a hyperbola to the plot of $k_{\text{obs}}(\text{corr})$ vs $[I]$ using SigmaPlot 8.0. K_i was derived from K_i' using the relationship $K_i = K_i'/(1 + [S]/K_m)$. For each enzyme and URB597 pair studied, the data fit the equation of a line better as judged by r^2 values.

The apparent bimolecular rate constants were determined from the slopes of the lines and then the true bimolecular rate constant was calculated as follows:

$$k_i = k_i' (1 + [S] / K_m) \quad (5)$$

where k_i represents the true bimolecular rate constant, as defined by Main and Dauterman [25]. K_m values used were as follows: CES1 $K_m = 136 \pm 40 \mu\text{M}$, CES2 $K_m = 90 \pm 12 \mu\text{M}$, MGL $K_m = 158 \pm 37$ [24].

2.4 Kinetic Studies of Reactivation

CES1 was incubated with 2.5 μM JZL184, 2.5 μM paraoxon or 10 μM URB597 in 50 mM Tris-HCl buffer (described above) for 15 min at 37°C. These concentrations of inhibitor represent either a 10-fold or a 40-fold molar excess of enzyme present. The final concentration of DMSO in the reaction mixtures containing either JZL184 or URB597 was 1.0% and the final concentration of ethanol in the reaction mixture containing paraoxon was 1.0%. Vehicle controls were run for each inhibition reaction. The inhibition reaction was stopped and excess inhibitor removed by passing each reaction mixture through Zebco desalting columns that had been equilibrated in 50 mM Tris-HCl per the manufacturer's instructions 3 times. The mixtures containing inhibited enzyme with the inhibitor removed were then incubated at 37°C. An aliquot was removed at various times and assayed for enzymatic activity. The final concentration of CES1 in each reaction mixture was 4.4 nM. pNPV was added to a final concentration of 500 μM . The reaction progress was monitored by measuring the absorbance at 405 nm for 5 minutes to estimate the rate of formation of para-nitrophenol produced from the hydrolysis of pNPV. All reactions were corrected for the nonenzymatic hydrolysis of pNPV. Nonenzymatic hydrolysis of pNPV was typically < 5% of enzymatic activity. The slopes were determined and used to calculate the enzymatic activity. The curves were linear during the 5 minute reaction period. Vehicle controls were used to determine total enzyme activity at each time point. Data was expressed as % inhibition at each time with time zero defined as 100% inhibition.

Assuming a first order process of reactivation, the inhibition at time t (Inh_t) is related to time by the equation:

$$\ln(\text{Inh}_t) = \ln(\text{Inh}_0) - k_{\text{react}} t \quad (6)$$

Thus $\ln(\text{Inh}_t)$ was plotted versus t and $-k_{\text{react}}$ was the slope of the line. The half life for the inhibited enzyme ($t_{1/2}$) was determined by the relationship $t_{1/2} = \ln 2 / k_{\text{react}}$. Each reactivation reaction was monitored for greater than one half-life.

2.5 Inhibition of esterase activity in intact HepG2 cells

HepG2 cells were seeded into 12-well plates and grown to 60% confluence. Cells were washed and pre-treated with 10 μM each of JZL184 or URB597 for 30 min in serum-free Modified Eagle's medium or with ethanol vehicle (0.1% v/v). After 30 min, the medium containing drug was removed and cells washed 3x with serum-free medium. Complete growth medium containing 10% FBS was then added to the cells, which were incubated at 37°C in a humidified incubator containing 5% CO_2 . At 0, 12, 24, and 48 hr after drug treatment, the culture medium was removed and cells washed three times with cold $1 \times \text{PBS}$. Cells were then scraped into cold 50 mM Tris-HCl (pH 7.4) buffer and lysed by sonication. Cell lysate proteins were assayed using the BCA reagent (Pierce, Rockford, IL) and the esterase activity of the lysate was assayed using the pNPV substrate.

2.6 Statistical Analysis

Values for bimolecular rate constants and reactivation rate constants in each row and/or each column of Tables 1 and 2 were compared using a one-way analysis of variance with post hoc Tukey analysis for three groups of data and a Student's t-test for two groups of data. Values which did not pass the normality test were $\log_{10}$ transformed and then re-analyzed. Results of statistical comparisons are noted in the footnotes for Tables 1 and 2.

3. Results

3.1 Determination of the bimolecular rate constants

The bimolecular rate constants for the reactions of CES1, CES2 and MAGL with JZL184 and URB597 were determined as described in the Material and Methods section. The determination of k_{inact}/K_i for CES1 and JZL184 is shown as a representative example (Fig. 2). Figure 2B shows the hydrolysis of pNPV by CES1 as a function of time in the presence of various concentrations of JZL184. The first order rate constant of inactivation k_{obs} for each concentration of JZL184 was calculated by fitting each curve to equation (1). $k_{\text{obs}}(\text{corr})$ was calculated as described and plotted against the concentration of JZL184. k_{inact} and K_i were determined by fitting the curve, shown in Figure 2C, to equation (3). For this determination, $k_{\text{inact}} = 1.64 \times 10^{-3} \text{ s}^{-1}$, $K_i = 4.16 \times 10^{-7} \text{ M}^{-1}$, and the bimolecular rate constant ($k_i = k_{\text{inact}}/K_i = 3.94 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$). The results for the determination of the bimolecular rate constant of inhibition of CES1, CES2, or MAGL by JZL184 are shown in Table 1. The bimolecular rate constant for the reaction of CES1 with JZL184 and for the reaction of MAGL with JZL184 were not significantly different. However, the bimolecular rate constants for these reactions were both significantly greater (17-38-fold) than the bimolecular rate constant determined for the reaction of CES2 with JZL184.

The individual components of the bimolecular rate constants could not be determined for the reaction of CES1, CES2 or MAGL with URB597. Instead, the bimolecular rate constants for the reactions of CES1 and CES2 with URB597 were determined using equation (4). URB597, which was developed as a FAAH inhibitor, did not inhibit (i.e. react with) MAGL to any appreciable extent. The bimolecular rate constant of inhibition of CES1 by URB597 was not significantly greater than the bimolecular rate constant of inhibition of CES2 by URB597. In addition, URB597 was more reactive with CES2 than was JZL184.

3.2 Determination of the k_{react} for CES1 following inhibition by URB597, JZL184 and paraoxon

The determination of the reactivation rate constant k_{react} for CES1 following its inhibition (i.e. covalent modification) by the carbamates URB597 and JZL184 was determined as described in detail in the Materials and Methods section. The reactivation of enzyme activity (i.e., decay of inhibition) is shown in Figure 3A. The data was fit to equation (6) where k_{react} is the negative of the slope of the line in each plot (Fig. 3B; Table 2). The k_{react} for CES1 inhibited by paraoxon is also included for comparison. CES1 inhibited by URB597 reactivates significantly faster than CES1 inhibited by either JZL184 or paraoxon. Reactivation rates for CES1 following inhibition by JZL184 or paraoxon were not significantly different. The half life ($t_{1/2}$) for enzyme activity reactivation following reactions with excess URB597, JZL184 or paraoxon was calculated and presented in Table 2. The half life for reactivation of CES1 inhibited by URB597 was significantly shorter than the half life for reactivation of CES1 that had been inhibited by either JZL184 or paraoxon. We were unable to calculate the half life of reactivation of CES2 inhibited by URB597 or JZL184 because recombinant CES2 was not stable during extended incubations at 37°C. The half life of reactivation of CES1 inhibited by either URB597 (11 h) or JZL 184 (49 h)

was long enough that our assumption of no significant enzyme reactivation during the 15 to 45 min reactions to determine the bimolecular rate constants (see Fig. 2) was valid.

3.3 Reactivation of esterase activity in cultured HepG2 cells following treatment with JZL184 and URB597

Next, we determined the persistence of esterase inhibition in intact HepG2 cells following 10 μ M (30 min) treatment using the two carbamate drugs, JZL184 and URB597. Both drugs inhibited 90% of the esterase activity at time 0 (Fig. 5). The extent of esterase activity recovery was subsequently evaluated at 12, 24, and 48 h. Although esterase activity remained depressed (~90%) over 48 hr after treatment with JZL184, it had returned to 50% of the control activity following treatment with URB597 (Fig. 5). Using a selective reversible inhibitor for CES1 (S-3030, [16]), it was determined that CES1 accounted for $71.5 \pm 2.1\%$ of the esterase activity in untreated HepG2 cells when using pNPV as substrate (Fig. 5, *inset*). Therefore, the result presented in Fig. 5 is in line with faster turnover of the URB597-carbamoylated CES1 protein relative to JZL184-carbamoylated CES1. Furthermore, 74% of the esterase activity recovered by 48 hr following URB597 treatment was found to be sensitive to S-3030-mediated inhibition (data not shown), suggesting that a substantial fraction of the esterase activity recovered in the intact cells was due to reactivation of URB597-modified CES1.

4. Discussion

The N-methyl carbamates have been used commercially as insecticides since the 1950s [8]. Recently, newer carbamate compounds have been synthesized in an effort to develop specific inhibitors for various members of the serine hydrolase family of enzymes, which could be used for therapeutic purposes. However, several of these carbamate inhibitors have been shown to react with off-target enzymes [15, 16]. Not surprisingly, one group of off-target enzymes identified is the carboxylesterases, which are themselves serine hydrolases and are highly promiscuous enzymes [26]. In humans, there are two predominant carboxylesterases, CES1 and CES2. CES1 is the major carboxylesterase in liver [27] and may constitute as much as 1% of total hepatic protein [18]. CES2 is present in smaller amounts in the liver but is the predominant carboxylesterase in the intestine with highest expression in the jejunum [19]. As a result of their location, CES1 and CES2 may participate in first pass metabolism of oral pharmaceutical agents absorbed from the intestine. Drugs and prodrugs that are esters of carboxylic acids are in general more lipophilic and better absorbed than the corresponding carboxylic acid [20], making them popular for clinical use. CES1 and CES2 are known to metabolize these ester drugs [20]. Thus, covalent interaction of pharmaceutical agents with CES1 and CES2 leading to enzyme inhibition may affect the metabolism and bioavailability of many ester drugs that are already in use. Indeed, prolonged covalent inhibition of hepatic CES1 or intestinal CES2 by a potential pharmaceutical agent may greatly increase the risk of DDI.

Recently developed PBPK/PD models of carbamate insecticides include the inactivation of CES1 by the carbamate as one of the mechanisms of detoxication, followed by an enzyme reactivation step. One of the limitations of the PBPK/PD models is the paucity of data for the reactions of carbamates with CES1 and CES2. In addition, although human acetylcholinesterase reactivates following inhibition by carbamate insecticides, which partially accounts for their low human toxicity, very little is known about the reactivation kinetics of CES1 following carbamoylation. To our knowledge, no data has been published on the reactivation of human carboxylesterases following their inhibition by pharmaceutical agents containing the carbamate moiety, such as JZL184 and URB597.

On the basis of k_i (k_{inact}/K_i) values, we found that JZL184 is as potent an inhibitor of CES1 as it is of MAGL, its target enzyme (Table 1). In addition, URB597 and JZL184 are similarly reactive toward CES1. However, JZL184 and URB597 are not nearly as potent at inhibiting CES1 as are the OP oxons chlorpyrifos oxon, paraoxon and methyl paraoxon [24]. In the case of chlorpyrifos oxon and CES1, this oxon is 500x more reactive toward CES1 than JZL184. When examining k_i values [24], the 3 OP oxons exhibited bimolecular rate constants toward CES1 that were two orders of magnitude greater than both JZL184 and URB597. However, JZL184 and URB597 were more reactive toward CES1 than carbaryl, an N-methylcarbamate insecticide, was toward a rat ortholog of CES1 ($k_i = 8.56 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$; [28]). In addition, JZL184 was not as reactive with CES2 as it was with CES1, which mirrors the reactivity profile seen previously with OPs and the 2 human CES isoforms [24]. However, no differences in the rates of inactivation of CES1 and CES2 by URB597 were noted. It can be concluded that the marked reactivity of JZL184 and URB597 with CES1 and CES2 suggests the possibility of significant first pass metabolism following the oral administration of these compounds.

The inhibition constants (K_i) of JZL184 for recombinant CES1 and CES2 are in the submicromolar range (Table 1), which is higher than the nanomolar affinities that reversible inhibitors possessing the diphenylethane-1,2-dione scaffold exhibit toward these proteins [29]. For example, K_i values for benzil, the prototypical compound of this class of carboxylesterase inhibitor, and CES1 and CES2 are 45 nM and 15 nM, respectively. However, despite the lower affinity of JZL184 for CES1 relative to benzil, DDI caused by inhibition of CES1 by JZL184 will be dramatically more significant. For example, on the basis of DDI risk estimated for reversible enzyme inhibitors using the term $[I]/K_i$ ($[I]$, inhibitor concentration) and for irreversible enzyme inhibitors using the term λ/k_{deg} ($\lambda = k_{\text{inact}}*[I]/(K_i + [I])$; k_{deg} , turnover rate constant for enzyme or *k of degradation*) [30], benzil and JZL184 would enhance the area-under-plasma concentration curve (AUC) for an ideal CES1 specific substrate by 23-fold and 640-fold, respectively, assuming that the systemic concentration of each inhibitor $[I]$ equaled 1 μM and $\text{AUC}_i/\text{AUC} = 1 + [I]/K_i$ for reversible inhibitors and $\text{AUC}_i/\text{AUC} = 1 + \lambda/k_{\text{deg}}$ for irreversible inhibitors. This quantitative prediction of DDI highlights the potential for irreversible inhibitors to exert a more profound effect on in vivo DDI relative to reversible inhibitors when the target enzyme turnover rate (k_{deg}) is slow.

The stability of recombinant CES1 enzyme at 37°C allowed us to measure the rate of its reactivation following either carbamoylation or phosphorylation. The reactivation rate of CES1 following inhibition with either URB597 or JZL184 was the same order of magnitude as the reactivation rate of CES1 following inhibition with paraoxon. CES1 is a scavenger for oxons and it is assumed to reactivate so slowly that the generation of active CES1 enzyme following oxon mediated inhibition requires the biosynthesis of new protein. However, the reactivation half-life of diethylphosphorylated CES1 was 31 h (Table 2); thus, our data show that paraoxon does not irreversibly inactivate CES1. The carbamoylated CES1 enzyme obtained following reaction with URB597 can reactivate significantly faster ($t_{1/2}=11 \text{ h}$) than the carbamoylated enzyme obtained following reaction with JZL184 ($t_{1/2} = 49 \text{ h}$). The reason for this difference might be due to the smaller carbamoyl adduct rendered by URB597 compared to JZL184 (Fig. 4). The carbonyl carbon of the smaller carbamoyl adduct might be more accessible to the activated water molecule, thus accelerating the hydrolysis rate (k_{react}). Consistent with this notion, the reactivation half lives of the two carbamoylated forms of CES1 (Table 2) are substantially longer than that measured for a rat CES1 enzyme carbamoylated by carbaryl ($t_{1/2}=0.0175 \text{ h}$; [28]), which yields a much smaller N-methyl carbamoyl adduct. Therefore, the steric bulk of the carbamoyl adducts seem to dictate the rates of enzyme reactivation; the smaller the carbamoyl adduct the more rapidly it is removed. With regard to carbamate hydrolyzing enzymes, *O*-alkyl and *O*-aryl carbamate

derivatives can be hydrolyzed by butyrylcholinesterase in human plasma and carboxylesterases in rodent liver [31]. Although FAAH, MAGL, and KIAA1363 are known to be inhibited by carbamates, it is unclear that they catalytically turn these substrates over in a kinetically meaningful way. A detailed structure-activity study that described the relative ease of metabolic hydrolysis of carbamate drugs and prodrugs was given in Vacondio et al. [32], but descriptions of the relevant hydrolytic enzymes for the carbamates were not presented. It will be of future interest to determine whether arylacetamide deacetylase (AADAC), which has been shown to hydrolyze amide-containing chemicals and is expressed in high amounts in liver [33], can metabolize carbamates.

Mass spectrometric detection of the covalently modified serine residue (Ser221) in the active-site peptide of human CES1, with the goal of developing a unique biomarker of OP or carbamate exposure, has proven to be challenging. For example, MALDI-TOF mass spectrometric analysis of tryptic peptides derived from diethylphosphorylated CES1 did not yield a detectable OP-modified active site peptide (M.K. Ross, unpublished observations), which might be related to the length of the active-site peptide (38 amino acids) generated following tryptic digestion. Similar difficulties with MALDI-TOF mass spectrometry and tryptic digests of OP-modified CES1 have also been reported by Hemmert et al. [34]. We have previously shown that LC-MS/MS analysis of peptides derived from chymotrypsin digests of diethylphosphorylated CES1 could detect a singly-charged peptide fingerprint of a 12 aa active-site peptide containing Ser221 with the expected mass addition of 136 amu for the diethylphosphoryl adduct [35]; however, fragmentation of this peptide into daughter ions in the collision cell of the mass spectrometer proved difficult. Therefore, routine analysis of OP- and carbamate-modified CES1 proteins by mass spectrometry appears non-trivial at this stage, although detection of the covalent modification of recombinant MAGL by carbamate JZL184 has been demonstrated [13].

The half life of carboxylesterase protein turnover in HepG2 cells was estimated to be 96 hours [17], implying a k_{deg} of $7.2 \times 10^{-3} \text{ h}^{-1}$. If it is assumed that CES1 protein levels remain constant in cells, i.e. the rate of its degradation equals the rate of its synthesis ($k_{\text{deg}} = k_{\text{synthesis}}$), then the percentage of CES1 activity recovered after complete inhibition by either URB597 or JZL184 can be calculated because the k_{react} is known for each (Table 2). For example, following complete inhibition of CES1 by URB597, CES1 activity will be 64% and 97% recovered by 12 h and 24 h, respectively. Likewise, following complete inhibition of CES1 by JZL184, CES1 activity will be 25% and 46% recovered by 12 h and 24 h, respectively. This analysis suggests the possibility of significant DDI for URB597 and especially JZL184 if administered orally once or twice a day. The inhibitory behavior of the two carbamates in cultured HepG2 cells (Fig. 5) was consistent with the faster reactivation of CES1 protein covalently inhibited by URB597. Obviously, a more rapid reactivation of carbamoylated CES1 would be advantageous for future carbamates that are being considered for use as pharmaceuticals (less chance of DDI) or being used as insecticides (less human toxicity). Alternatively, carbamates that exhibit greater selectivity for their target enzyme, i.e. are less reactive with CES1 and CES2, would also be better candidates and are currently in development [36].

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References

1. Bachovchin DA, Cravatt BF. The pharmacological landscape and therapeutic potential of serine hydrolases. *Nat Rev Drug Discov.* 2012; 11:52–68. [PubMed: 22212679]
2. Long JZ, Cravatt BF. The metabolic serine hydrolases and their functions in mammalian physiology and disease. *Chem Rev.* 2011; 111:6022–6063. [PubMed: 21696217]
3. Fukuto TR. Mechanism of action of organophosphorus and carbamate insecticides. *Environ Health Perspect.* 1990; 87:245–254. [PubMed: 2176588]
4. Whitcomb DC, Lowe ME. Human pancreatic digestive enzymes. *Dig Dis Sci.* 2007; 52:1–17. [PubMed: 17205399]
5. Heutinck KM, ten Berge IJ, Hack CE, Hamann J, Rowshani AT. Serine proteases of the human immune system in health and disease. *Mol Immunol.* 2010; 47:1943–1955. [PubMed: 20537709]
6. Butenas S, Mann KG. Blood coagulation. *Biochemistry (Mosc).* 2002; 67:3–12. [PubMed: 11841335]
7. Robertson JG. Mechanistic basis of enzyme-targeted drugs. *Biochemistry.* 2005; 44:5561–5571. [PubMed: 15823014]
8. Kuhr, RJ.; Dorough, HW. Development and use. In: Zweig, G., editor. *Carbamate Insecticides: Chemistry, Biochemistry and Toxicology.* CRC Press; 1976. p. 1-14.
9. Metcalf RL. Structure-activity relationships for insecticidal carbamates. *Bull World Health Organ.* 1971; 44:43–78. [PubMed: 5315358]
10. Fukuto TR, Fahmy MAH, Metcalf RL. Alkaline hydrolysis, anticholinesterase, and insecticidal properties of some nitro-substituted phenyl carbamates. *J. Agric. Food Chem.* 1967; 15:273–281.
11. Timchalk C, Nolan RJ, Mendrala AL, Dittenber DA, Brzak KA, Mattsson JL. A Physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model for the organophosphate insecticide chlorpyrifos in rats and humans. *Toxicol Sci.* 2002; 66:34–53. [PubMed: 11861971]
12. Knaak JB, Dary CC, Okino MS, Power FW, Zhang X, Thompson CB, Tornero-Velez R, Blancato JN. Parameters for Carbamate Pesticide QSAR and PBPK/PD Models for Human Risk Assessment. *Rev Environ Contam Toxicol.* 2008; 193:53–212. [PubMed: 20614344]
13. Long JZ, Li W, Booker L, Burston JJ, Kinsey SG, Schlosburg JE, Pavon FJ, Serrano AM, Selley DE, Parsons LH, Lichtman AH, Cravatt BF. Selective blockade of 2-arachidonoylglycerol hydrolysis produces cannabinoid behavioral effects. *Nat Chem Biol.* 2009; 5:37–44. [PubMed: 19029917]
14. Kathuria S, Gaetani S, Fegley D, Valino F, Duranti A, Tontini A, Mor M, Tarzia G, La Rana G, Calignano A, Giustino A, Tattoli M, Palmery M, Cuomo V, Piomelli D. Modulation of anxiety through blockade of anandamide hydrolysis. *Nat Med.* 2003; 9:76–81. [PubMed: 12461523]
15. Zhang D, Saraf A, Kolasa T, Bhatia P, Zheng GZ, Patel M, Lannoye GS, Richardson P, Stewart A, Rogers JC, Brioni JD, Surowy CS. Fatty acid amide hydrolase inhibitors display broad selectivity and inhibit multiple carboxylesterases as off-targets. *Neuropharmacology.* 2007; 52:1095–1105. [PubMed: 17217969]
16. Xie S, Borazjani A, Hatfield MJ, Edwards CC, Potter PM, Ross MK. Inactivation of lipid glyceryl ester metabolism in human THP1 monocytes/macrophages by activated organophosphorus insecticides: role of carboxylesterases 1 and 2. *Chem Res Toxicol.* 2010; 23:1890–1904. [PubMed: 21049984]
17. Ross MK, Borazjani A, Wang R, Allen Crow J, Xie S. Examination of the carboxylesterase phenotype in human liver. *Arch Biochem Biophys.* 2012; 522:44–56. [PubMed: 22525521]
18. Sun A, Jiang Y, Wang X, Liu Q, Zhong F, He Q, Guan W, Li H, Sun Y, Shi L, Yu H, Yang D, Xu Y, Song Y, Tong W, Li D, Lin C, Hao Y, Geng C, Yun D, Zhang X, Yuan X, Chen P, Zhu Y, Li Y, Liang S, Zhao X, Liu S, He F. Liverbase: a comprehensive view of human liver biology. *J Proteome Res.* 2010; 9:50–58. [PubMed: 19670857]
19. Schwer H, Langmann T, Daig R, Becker A, Aslanidis C, Schmitz G. Molecular cloning and characterization of a novel putative carboxylesterase, present in human intestine and liver. *Biochem Biophys Res Commun.* 1997; 233:117–120. [PubMed: 9144407]

20. Sun H. Capture hydrolysis signals in the microsomal stability assay: molecular mechanisms of the alkyl ester drug and prodrug metabolism. *Bioorg Med Chem Lett*. 2012; 22:989–995. [PubMed: 22197392]
21. Morton CL, Potter PM. Comparison of *Escherichia coli*, *Saccharomyces cerevisiae*, *Pichia pastoris*, *Spodoptera frugiperda*, and COS7 cells for recombinant gene expression. Application to a rabbit liver carboxylesterase. *Mol Biotechnol*. 2000; 16:193–202. [PubMed: 11252804]
22. Chambers H, Brown B, Chambers JE. Nuncatalytic Detoxication of Six Organophosphorus Compounds by Rat Liver Homogenates. *Pesticide Biochemistry and Physiology*. 1990; 36:308–315.
23. Hyatt JL, Wadkins RM, Tsurkan L, Hicks LD, Hatfield MJ, Edwards CC, Ross CR 2nd, Cantalupo SA, Crundwell G, Danks MK, Guy RK, Potter PM. Planarity and constraint of the carbonyl groups in 1,2-diones are determinants for selective inhibition of human carboxylesterase 1. *J Med Chem*. 2007; 50:5727–5734. [PubMed: 17941623]
24. Crow JA, Bittles V, Herring KL, Borazjani A, Potter PM, Ross MK. Inhibition of recombinant human carboxylesterase 1 and 2 and monoacylglycerol lipase by chlorpyrifos oxon, paraoxon and methyl paraoxon. *Toxicol Appl Pharmacol*. 2012; 258:145–150. [PubMed: 22100607]
25. Main AR, Dauterman WC. Determination of the bimolecular rate constant for the reaction between organophosphorus inhibitors and esterases in the presence of substrate. *Nature*. 1963; 198:551–553.
26. Redinbo MR, Potter PM. Mammalian carboxylesterases: from drug targets to protein therapeutics. *Drug Discov Today*. 2005; 10:313–325. [PubMed: 15749280]
27. Godin SJ, Crow JA, Scollon EJ, Hughes MF, DeVito MJ, Ross MK. Identification of rat and human cytochrome p450 isoforms and a rat serum esterase that metabolize the pyrethroid insecticides deltamethrin and esfenvalerate. *Drug Metab Dispos*. 2007; 35:1664–1671. [PubMed: 17576809]
28. Stok JE, Goloshchapov A, Song C, Wheelock CE, Derbel MB, Morisseau C, Hammock BD. Investigation of the role of a second conserved serine in carboxylesterases via site-directed mutagenesis. *Arch Biochem Biophys*. 2004; 430:247–255. [PubMed: 15369824]
29. Wadkins RM, Hyatt JL, Wei X, Yoon KJ, Wierdl M, Edwards CC, Morton CL, Obenauer JC, Damodaran K, Beroza P, Danks MK, Potter PM. Identification and characterization of novel benzil (diphenylethane-1,2-dione) analogues as inhibitors of mammalian carboxylesterases. *J Med Chem*. 2005; 48:2906–2915. [PubMed: 15828829]
30. Fujioka Y, Kunze KL, Isoherranen N. Risk Assessment of Mechanism-based Inactivation in Drug-Drug Interactions. *Drug Metab Dispos*. 2012
31. Mata G, do Rosario VE, Iley J, Constantino L, Moreira R. A carbamate-based approach to primaquine prodrugs: antimalarial activity, chemical stability and enzymatic activation. *Bioorg Med Chem*. 2012; 20:886–892. [PubMed: 22189276]
32. Vacondio F, Silva C, Mor M, Testa B. Qualitative structure-metabolism relationships in the hydrolysis of carbamates. *Drug Metab Rev*. 2010; 42:551–589. [PubMed: 20441444]
33. Fukami T, Yokoi T. The Emerging Role of Human Esterases. *Drug Metab Pharmacokinet*. 2012
34. Hemmert AC, Otto TC, Wierdl M, Edwards CC, Fleming CD, MacDonald M, Cashman JR, Potter PM, Cerasoli DM, Redinbo MR. Human carboxylesterase 1 stereoselectively binds the nerve agent cyclosarin and spontaneously hydrolyzes the nerve agent sarin. *Mol Pharmacol*. 2010; 77:508–516. [PubMed: 20051531]
35. M.K.a.E. Ross, MJ. Carboxylesterases: A Multifunctional Enzyme Involved in Pesticide and Lipid Metabolism. American Chemical Society (ACS) Books; 2012. 2012 In press. Symposium Series In press
36. Chang JW, Niphakis MJ, Lum KM, Cognetta AB 3rd, Wang C, Matthews ML, Niessen S, Buczynski MW, Parsons LH, Cravatt BF. Highly Selective Inhibitors of Monoacylglycerol Lipase Bearing a Reactive Group that Is Bioisosteric with Endocannabinoid Substrates. *Chem Biol*. 2012; 19:579–588. [PubMed: 22542104]

A **JZL184** **URB597****B**

Figure 1. (A) Chemical structures of JZL184 and URB597. (B) Kinetic scheme describing inhibition of serine hydrolases by carbamates

The kinetic scheme used to describe the inhibition of a serine hydrolase (E) by a carbamate (I) in the presence of an ester substrate (S). E·I is carbamate non-covalently bound to enzyme, E-I* is carbamoylated enzyme, I* is the carbamate moiety remaining after the leaving group has been removed.

Figure 2. Determination of k_i for the inhibition of CES1 by JZL184

(A) Schematic of the reaction of JZL184 with CES1. pNP indicates liberation of *para*-nitrophenol. (B) The inhibition of CES1 by varying concentrations of JZL184 is shown. The progress of the hydrolysis of pNPV was followed by monitoring the absorbance of the reaction mixture at 405 nm. (C) The values of k_{obs} (corr) were plotted against each concentration of JZL184. k_{inact} , K_i , and k_i were determined as described in the Materials and Methods section.

Figure 3. Reactivation of CES1 inhibited by either JZL184, URB597, or Paraoxon

(A) The % of inhibition of CES1 following inhibition with JZL184, URB597, or paraoxon was plotted versus time following the removal of the inhibitor. (B) The ln of the % inhibition was plotted versus the time following the removal of the inhibitor. The k_{react} was determined as described in the Materials and Methods.

Reactivation time for CES1 following covalent modification

Figure 4. Structures of carbamoyl and phosphoryl adducts of CES1 and their half lives of reactivation

The $t_{1/2}$ for CES1-N-methylcarbamoyl reactivation is from Stok et al. (2004).

Figure 5. Reactivation of esterase activity in HepG2 cells after JZL184 and URB597-mediated inhibition

Cultured HepG2 cells were treated with carbamates (10 μ M) for 30 min, followed by removal of drugs. The esterase activity was subsequently monitored for the indicated times. Control esterase activity represents vehicle (ethanol)-treated cells determined at each time point and activities in the drug-treated cells were normalized to the control activity, expressed as % of control. Values represent the average ($\pm$ SD) of two independent experiments. *Inset*, Esterase activity of untreated HepG2 cell lysate was determined in the absence or presence of 10 μ M S-3030.

Table 1Rate constants for the inactivation of CES1, CES2, and MAGL by JZL184 and URB597^{*}

	JZL184			URB597
	k_{inact} (s ⁻¹)	K_i (M)	k_{inact}/K_i (M ⁻¹ s ⁻¹)	k_{inact}/K_i (M ⁻¹ s ⁻¹)
CES1	$1.9 (\pm 0.2) \times 10^{-3}$	$4.8 (\pm 0.5) \times 10^{-7}$	$3.9 (\pm 0.2) \times 10^3$ ^a	$4.5 (\pm 1.3) \times 10^3$ ^a
CES2	$1.2 (\pm 0.4) \times 10^{-3}$	$6.3 (\pm 2.6) \times 10^{-7}$	$2.3 (\pm 1.3) \times 10^2$ ^b	$3.9 (\pm 1.0) \times 10^3$ ^a
MAGL	$4.7 (\pm 2.0) \times 10^{-3}$	$7.7 (\pm 5.4) \times 10^{-7}$	$8.7 (\pm 5.0) \times 10^3$ ^a	no inhibition

^{*} Values are expressed as mean $\pm$ standard deviation of at least 4 experiments. All values in the same row or column with different alphabetic superscripts are significantly different ($p < 0.05$, ANOVA and Tukey's post hoc test or student's t test).

Table 2Reactivation of CES1 following inhibition with paraoxon, URB597, and JZL184^{*}

	$k_{\text{react}} \text{ (s}^{-1}\text{)}$	$t_{1/2} \text{ (h)}$
URB597	$1.9 (\pm 0.6) \times 10^{-5} \text{ }^{\text{a}}$	$11 \pm 4^{\text{a}}$
JZL184	$4.2 (\pm 1.0) \times 10^{-6} \text{ }^{\text{b}}$	$49 \pm 15^{\text{b}}$
Paraoxon	$6.3 (\pm 1.0) \times 10^{-6} \text{ }^{\text{b}}$	$31 \pm 4^{\text{b}}$

^{*} Values are expressed as mean $\pm$ standard deviation of 4 experiments. All values in the same column with different alphabetic superscripts are significantly different ($p < 0.05$, ANOVA and Tukey's post hoc test).