Evaluation of drug-drug interactions via inhibition of hydrolases by orlistat, an anti-obesity drug

Keiya Hirosawa, Tatsuki Fukami, Masataka Nakano, Miki Nakajima

Drug Metabolism and Toxicology, Faculty of Pharmaceutical Sciences, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan (K. H., T. F., Ma. N., Mi. N.); WPI Nano Life Science Institute, Kakuma-machi, Kanazawa 920-1192, Japan (T. F., Ma. N., Mi. N.)
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To whom all correspondence should be sent:

Tatsuki Fukami
Drug Metabolism and Toxicology
Faculty of Pharmaceutical Sciences
Kanazawa University
Kakuma-machi, Kanazawa 920-1192, Japan
Tel: +81-76-234-4438/Fax: +81-76-264-6282
E-mail: tatsuki@p.kanazawa-u.ac.jp

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**ABBREVIATIONS:** AADAC, arylacetamide deacetylase; AUC, area under the plasma concentration-time curve; CES, carboxylesterase; C\textsubscript{max}, maximum plasma concentration; DDI, drug-drug interactions; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EMA, European Medicines Agency; FDA, Food and Drug Administration; I\textsubscript{gut}, estimated intestinal luminal concentration of inhibitor; I\textsubscript{max,u}, maximum unbound plasma concentration of inhibitor; K\textsubscript{i}, inhibition constant; K\textsubscript{m}, Michaelis-Menten constant; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MBI, mechanism-based inhibition; MRM, multiple reaction monitoring; PMDA, Pharmaceuticals and Medical Devices Agency; P450, cytochrome P450; SD, standard deviation; TDI, time-dependent inhibition; Tris, tris(hydroxymethyl)aminomethane; V\textsubscript{max}, maximal velocity
Abstract

Drug-drug interactions (DDI) have a significant impact on drug efficacy and safety. It has been reported that orlistat, an anti-obesity drug, inhibits the hydrolysis of p-nitrophenol acetate, a common substrate of the major drug-metabolizing hydrolases, carboxylesterase (CES) 1, CES2, and arylacetamide deacetylase (AADAC), in vitro. The aim of this study was to examine whether orlistat affects the pharmacokinetics of drug(s) metabolized by hydrolases in vivo, after evaluating its inhibitory potencies against CES1, CES2, and AADAC in vitro. Orlistat potently inhibited the hydrolysis of acebutolol, a specific substrate of CES2, in a non-competitive manner (\(K_i = 2.95 \pm 0.16\) nM), whereas it slightly inhibited the hydrolysis of temocapril and eslicarbazepine acetate, specific substrates of CES1 and AADAC, respectively (IC\(_{50} > 100\) nM). The in vivo DDI potential was elucidated using mice, in which orlistat showed strong inhibition against acebutolol hydrolase activities in the liver and intestinal microsomes, similar to humans. The AUC of acebutolol was increased by 43\%, whereas the AUC of acetolol, a hydrolyzed metabolite of acebutolol, was decreased by 47\% by co-administration of orlistat. The ratio of the \(K_i\) value to the maximum unbound plasma concentration of orlistat (< 0.012) is lower than the risk criteria for DDI in the liver defined by the FDA guideline (> 0.02), whereas the ratio of the \(K_i\) value to the estimated intestinal luminal concentration (3.3 \(\times\) 10\(^5\)) is considerably higher than the risk criteria in the intestine (> 10). Therefore, this suggests that orlistat causes DDI by inhibiting hydrolases in the intestine.
Significance Statement

This study demonstrated that orlistat, an anti-obesity drug, causes drug-drug interactions \textit{in vivo} by potently inhibiting carboxylesterase 2 in the intestine. This is the first evidence that inhibition of hydrolases causes drug-drug interactions.
**Introduction**

Drug-drug interactions (DDI) are classified into pharmacokinetic interactions and pharmacodynamic interactions. Both types of DDI could affect drug efficacy and toxicity (Palleria *et al.*, 2013). Pharmacokinetic interactions account for approximately half of the DDI observed in clinics, 68% of which are related to drug metabolism (Tesfaye and Nedi, 2017). Cytochrome P450 (P450) is involved in the metabolism of 60% of clinical drugs (Fukami *et al.*, 2022), and most of the DDI associated with drug metabolism are caused by inhibition or induction of P450s (Palleria *et al.*, 2013). Pharmaceuticals and Medical Devices Agency (PMDA), European Medicines Agency (EMA), and Food and Drug Administration (FDA) have guidelines for assessment of the risk of DDI via inhibition or induction of P450s in the development of new chemical entities and require that clinical trials for DDI be conducted as needed.

Because P450s have large interindividual differences in enzymatic activity and the potential for DDI, an increasing number of drugs designed to avoid metabolism by P450s have been developed in recent years. Consequently, the contribution of non-P450 enzymes to drug metabolism tends to increase. Hydrolases are responsible for the metabolism of over 25% of clinical drugs, showing the highest contribution to drug metabolism among all non-P450s (Fukami *et al.*, 2022). Carboxylesterase (CES) 1, CES2, and arylacetamide deacetylase (AADAC) play major roles in the hydrolysis of drugs. For example, CES1 catalyzes the hydrolysis of temocapril (an anti-hypertensive drug) and oseltamivir (an anti-influenza drug) (Takai *et al.*, 1997; Shi *et al.*, 2006), CES2 catalyzes the hydrolysis of irinotecan (an anticancer drug) and acebutolol (an anti-hypertensive and anti-arrhythmic drug) (Sanghani *et al.*, 2004; Muta *et al.*, 2015), and AADAC catalyzes the hydrolysis of rifampicin (antibiotics) and eslicarbazepine acetate (an anti-epileptic drug) (Nakajima *et al.*, 2011; Hirosawa *et al.*, 2021). CES1 is predominantly expressed in the liver, whereas CES2 and AADAC are expressed in the small intestine as well as the liver (Watanabe *et al.*, 2009). Although several drugs that inhibit the enzymatic activities of these drug-metabolizing
hydrolases have been identified by in vitro studies (Quinney et al., 2005; Fukami et al., 2010; Shimizu et al., 2014), their effects on the pharmacokinetics of victim drugs in vivo have not been evaluated.

Patients with lifestyle diseases such as obesity and hypertension have an increased risk for DDI because they often take multiple medications. Orlistat is an anti-obesity drug that reduces lipid absorption by inhibiting pancreatic lipase in the gastrointestinal tract. It has been reported that orlistat inhibits the hydrolysis of p-nitrophenol acetate, a common substrate of the above drug-metabolizing hydrolases, in human liver microsomes (Xiao et al., 2013). In addition, our previous study clarified that orlistat inhibits the hydrolysis of abiraterone acetate (an anti-prostate cancer drug), a specific substrate of AADAC (Sakai et al., 2021). However, whether orlistat causes DDI at clinical doses has not been evaluated in vivo as with other inhibitors for hydrolases as described above.

The aim of this study was to examine whether orlistat affects the pharmacokinetics of drug(s) metabolized by hydrolases in vivo, after evaluating its inhibitory potencies against CES1, CES2, and AADAC in vitro.
Materials and Methods

Chemicals and Reagents.

Orlistat, cetilistat, and eslicarbazepine acetate were purchased from Tokyo Chemical Industry (Tokyo, Japan). Temocapril hydrochloride, ampiroxicam, and piroxicam were purchased from Fujifilm Wako Pure Chemical (Osaka, Japan). Temocaprilat, irinotecan hydrochloride trihydrate, and SN-38 were purchased from Toronto Research Chemicals (Toronto, Canada). Acebutolol hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO). Eslicarbazepine was purchased from Tocris Bioscience (Bristol, UK). Human liver (n = 50, mixed gender) and intestinal (n = 5, mixed gender) microsomes and monkey liver microsomes (cynomolgus, n = 6, male) were purchased from Corning (Corning, NY). Dog liver (beagle, n = 8, male) and intestinal (beagle, n = 3, male) microsomes were purchased from Xenotech (Lenexa, KS). Recombinant human and monkey CES1, CES2, and AADAC expressed in baculovirus-infected Sf21 cells were previously prepared (Fukami et al., 2010; Watanabe et al., 2010; Honda et al., 2021). All other reagents were of analytical grade or the highest quality available on the market.

Animals.

C57BL/6J mice and Wistar rats were housed in the institutional animal facility (12 hr light/dark cycle and temperature 25 ± 1°C) with ad libitum access to food and water. All procedures were approved by the Institutional Animal Care and Use Committee of Kanazawa University and performed in accordance with the National Institutes of Health Guide for Animal Welfare of Japan.

Preparation of Mouse and Rat Liver and Intestinal Microsomes.

Liver and intestinal microsomes were prepared from mice (9 weeks old, n = 6, male) and rats (liver: 10 weeks old, n = 3, male; intestine: 8 weeks old, n = 1, male) following our previous report (Sakai et al., 2021). Mouse and rat livers were homogenized using a Polytron...
PT1300D homogenizer (Kinematica AG, Lucerne, Switzerland) and a motor-driven Teflon-tipped pestle homogenizer (Yamato, Tokyo, Japan) in 3 volumes of ice-cold buffer A [100 mM Tris-HCl (pH 7.4), 100 mM KCl, and 1 mM EDTA], and the small intestines were similarly homogenized in 2 volumes of ice-cold buffer B [100 mM Tris-HCl (pH 7.4), 100 mM KCl, 1 mM EDTA, 0.04 unit/mL aprotinin, 10 μM leupeptin, 1 μM bestatin, 0.5 mg/mL trypsin inhibitor, and 100 μM dithiothreitol (DTT)]. The homogenates were centrifuged at 9,000 g for 15 min, and then the supernatants were centrifuged at 105,000 g for 90 min. The microsomal pellets were resuspended in 3 volumes of ice-cold buffer A (liver) or 2 volumes of ice-cold buffer B (intestine) and centrifuged again at 105,000 g for 60 min. The microsomal pellets were resuspended in 1 volume of ice-cold TGE buffer [10 mM Tris-HCl (pH 7.4), 20% (v/v) glycerol, and 1 mM EDTA] and homogenized. The microsomal samples were stored at -80°C until use.

**Temocapril Hydrolase Activity.**

The determination of the temocapril hydrolase activity was carried out following a previously reported method (Fukami et al., 2015) with slight modifications. Briefly, an incubation mixture (final volume, 0.2 mL) containing 100 mM Tris-HCl buffer (pH 7.4), 0.05 mg/mL homogenates of Sf21 cells expressing human CES1, and 0–100 nM orlistat dissolved in DMSO was preincubated at 37°C for 5 min, and then temocapril dissolved in DMSO was added to the incubation mixture, resulting in a final DMSO concentration of 2.0%. The concentration of temocapril was adjusted to 500 µM. After incubation at 37°C for 10 min, the reaction was stopped by adding 200 µL of ice-cold acetonitrile. After centrifugation at 20,380 g for 5 min to remove proteins, a 0.5-μL aliquot of the supernatant was injected into liquid chromatography-tandem mass spectrometry (LC-MS/MS). An LCMS-8040 liquid chromatograph mass spectrometer with an electrospray ionization source (Shimadzu, Kyoto, Japan) equipped with an LC-20AD HPLC system was used. The LC instrument consisted of a CBM-20A controller (Shimadzu), a SIL-20AC HT autosampler (Shimadzu), LC-20AD pumps (Shimadzu), an SPD-20A UV detector (Shimadzu), and a CTO-20AC column oven.
(Shimadzu) equipped with a Develosil ODS-UG-3 column (3 μm particle size, 4.6 mm i.d. × 150 mm; Nomura Chemical, Seto, Japan). The column temperature was set at 40°C, and the flow rate was 0.2 mL/min. The mobile phase was (A) 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The elution conditions were as follows: 30% B (0–1.5 min), 30–90% B (1.5–4 min), 90% B (4–9 min), and 30% B (9–12 min). Nitrogen was used as the nebulizing (flow rate: 3 L/min) and drying gas (15 L/min). The ions of interest were selected in the first quadrupole and dissociated in the collision cell using argon as the collision gas at a pressure of 230 kPa. Temocaprilat, a hydrolyzed metabolite of temocapril, \( m/z \) 448.80 > 270.00 was quantified in multiple reaction monitoring (MRM) positive mode. The collision energy was -16.0 eV. The acquired data were processed using LabSolutions software (version 5.97 SP1, Shimadzu).

**Preparation of Acetolol, a Hydrolyzed Metabolite of Acebutolol, by Non-enzymatic Hydrolysis of Acebutolol.**

Since acetolol is not commercially available, it was prepared according to a previously reported method (Muta et al., 2015). To 150 µL of 100 mM acebutolol, 150 µL of 1 M HCl was added, incubated at 100°C for 5 hr, and then neutralized by adding 150 µL of 1 M NaOH. The complete conversion of the parent compound to acetolol was confirmed by MS analysis (Fig. S1).

**Acebutolol Hydrolase Activity.**

The determination of the acebutolol hydrolase activity was carried out following a previously reported method (Muta et al., 2015) with slight modifications. A typical incubation mixture (final volume, 0.2 mL) contained 100 mM Tris-HCl buffer (pH 7.4), enzyme sources (0.2 mg/mL homogenates of Sf21 cells expressing human CES2 and human liver microsomes, 0.05 mg/mL human intestinal microsomes, 0.1 mg/mL mouse liver microsomes, monkey liver microsomes, and homogenates of Sf21 cells expressing monkey CES1, CES2, and AADAC, and 0.3 mg/mL mouse intestinal microsomes, rat and dog liver and intestinal microsomes),
and 0–100 nM orlistat or cetilistat dissolved in DMSO. The final DMSO concentration was 1.0%. After preincubation at 37°C for 5 min except for the IC50 shift assay (0- or 30-min), acebutolol dissolved in water was added to the incubation mixture. The concentration of acebutolol was set at 10 mM, 50 mM (IC50 shift assay), or 2–25 mM (kinetic analyses). After a 20-min (human liver and intestinal microsomes and mouse liver microsomes) or 30-min (recombinant human CES2, mouse intestinal microsomes, rat and dog liver and intestinal microsomes, monkey liver microsomes, and recombinant monkey hydrolases) incubation at 37°C, the reaction was stopped by adding 100 µL of ice-cold acetonitrile. After centrifugation at 20,380 g for 5 min to remove proteins, a 0.5-µL aliquot of the supernatant was injected into LC-MS/MS. The equipment and conditions were the same as described above. The mobile phase was (A) 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The elution conditions were as follows: 0% B (0–2 min), 15% B (2–4 min), 15–80% B (4–5 min), 80% B (5–8.5 min), 15% B (8.5–10.5 min), and 0% B (10.5–12.5 min). Acetolol, a hydrolyzed metabolite of acebutolol, ([m/z] 266.90 > 116.15) was quantified in MRM positive mode. The collision energy was -20.0 eV.

**Eslicarbazepine Acetate Hydrolase Activity.**

The determination of the eslicarbazepine acetate hydrolase activity was carried out following a previously reported method (Hirosawa et al., 2021) with slight modifications. Briefly, an incubation mixture (final volume, 0.2 mL) containing 100 mM Tris-HCl buffer (pH 7.4), 0.05 mg/mL homogenates of Sf21 cells expressing human AADAC, and 0–100 nM orlistat dissolved in DMSO was preincubated at 37°C for 5 min, and then eslicarbazepine acetate dissolved in DMSO was added to the incubation mixture, resulting in a final DMSO concentration of 2.0%. The eslicarbazepine acetate concentration was adjusted to 1 mM. After incubation at 37°C for 5 min, the reaction was stopped by adding 200 µL of ice-cold acetonitrile. After centrifugation at 20,380 g for 5 min to remove proteins, a 0.5-µL aliquot of the supernatant was injected into LC-MS/MS. The equipment and conditions were the same as described above. The mobile phase was (A) 0.1% formic acid and (B) acetonitrile with
0.1% formic acid. The elution conditions were as follows: 20% B (0–2 min), 20–80% B (2–6 min), 80% B (6–10 min), and 20% B (10–13 min). Eslicarbazepine, a hydrolyzed metabolite of eslicarbazepine acetate, \( (m/z \ 255.10 > 237.20) \) was quantified in MRM positive mode. The collision energy was -10.0 eV.

**Calculation of IC\textsubscript{50} and \( K_i \) Values.**

IC\textsubscript{50} values were determined from non-linear regression curves fitted to inhibitor concentration and hydrolase activity, according to the following Hill equation, where Max and Min are the maximum and minimum values of hydrolase activity in the sigmoidal regression, respectively.

\[
Y = Min + \frac{\text{Max} - \text{Min}}{1 + \left( \frac{X}{IC_{50}} \right)^\text{Hill coefficient}}
\]

IC\textsubscript{50} values were calculated using the least square fit of four parameter-sigmoidal curves executed.

The \( K_i \) value and inhibition type were determined using GraphPad Prism (GraphPad Software Inc., San Diego, CA) by fitting the kinetic data to a competitive, non-competitive, uncompetitive, or mixed inhibition model by non-linear regression analysis.

**Irinotecan Hydrolase Activity.**

The determination of the irinotecan hydrolase activity was carried out following a previously reported method (Takahashi et al., 2009) with slight modifications. Briefly, an incubation mixture (final volume, 0.2 mL) containing 100 mM Tris-HCl buffer (pH 7.4), 0.2 mg/mL homogenates of Sf21 cells expressing human CES2, and 0–100 nM orlistat dissolved in DMSO was preincubated at 37°C for 5 min, and then irinotecan dissolved in DMSO was added to the incubation mixture, resulting in a final DMSO concentration of 2.0%. The concentration of irinotecan was adjusted to 2 µM. After incubation at 37°C for 5 min, the reaction was stopped by adding 10 µL of 1 M HCl. After centrifugation at 20,380 g for 5 min
to remove proteins, a 50-μL aliquot of the supernatant was injected into LC-MS/MS. The equipment and conditions were the same as described above. The mobile phase was (A) 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The elution conditions were as follows: 30% B (0–2 min), 30–80% B (2–4 min), 80% B (4–7 min), and 30% B (7–10 min). SN-38, a hydrolyzed metabolite of irinotecan, \(m/z\) 392.90 > 349.20 was quantified in MRM positive mode. The collision energy was -26.0 eV.

**Ampiroxicam Hydrolase Activity.**

The determination of the ampiroxicam hydrolase activity was carried out as follows: Briefly, an incubation mixture (final volume, 0.2 mL) containing 100 mM Tris-HCl buffer (pH 7.4), 0.05 mg/mL homogenates of Sf21 cells expressing human CES2, and 0–100 nM orlistat dissolved in DMSO was preincubated at 37°C for 5 min, and then ampiroxicam dissolved in DMSO was added to the incubation mixture, resulting in a final DMSO concentration of 2.0%. The concentration of ampiroxicam was adjusted to 10 μM. After incubation at 37°C for 2 min, the reaction was stopped by adding 200 μL of ice-cold acetonitrile. After centrifugation at 20,380 g for 5 min to remove proteins, a 1-μL aliquot of the supernatant was injected into LC-MS/MS. The equipment and conditions were the same as described above. The mobile phase was (A) 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The elution conditions were as follows: 30% B (0–2 min), 30–80% B (2–5 min), 80% B (5–11 min), and 30% B (11–14 min). Piroxicam, a hydrolyzed metabolite of ampiroxicam, \(m/z\) 332.00 > 120.90 was monitored in MRM positive mode. The collision energy was -35.0 eV.

**Evaluation of Pharmacokinetics of Acebutolol in Mice.**

Mice (8–9 weeks old, n = 5–6, male, body weight: 25.0 ± 1.6 g) were preadministered vehicle or orlistat (25.0 mg/kg dissolved in 1% carboxymethylcellulose, p.o.). Four hours after pre-administration, mice were administered acebutolol (45.5 mg/kg dissolved in 1% carboxymethylcellulose, p.o.) alone or co-administered with orlistat (25.0 mg/kg) to the mice.
The doses of acebutolol and orlistat were derived from normalization of clinical doses with body surface area, a widely used method for determining animal and human doses in drug development (Nair and Jacob, 2016). Blood was collected from the tail vein at 0.5, 1, 1.5, 2, 4, and 6 hr after administration, and plasma concentrations of acebutolol and its metabolites were measured as follows: three microliters of plasma sample, 50 µL of acetonitrile, and 47 µL of water were mixed vigorously for 1 min. After centrifugation at 20,380 g for 5 min, a 3-µL aliquot of the supernatant was injected into the LC-MS/MS. The equipment and conditions were the same as those used for the measurement of acebutolol hydrolase activity. Acetolol levels were quantified as described above. Acebutolol levels ($m/z$ 337.00 $> 116.25$) were quantified in MRM positive mode and a collision energy of -22 eV. Levels of diacetolol, an acetylated metabolite of acetolol, ($m/z$ 308.90 $> 116.10$) were analyzed in MRM positive mode and a collision energy of -21 eV.

**Statistical Analysis.**

Statistical significance between two groups was evaluated by Student’s $t$-test. $P < 0.05$ was considered significant.
Results

Orlistat Potently Inhibits Human CES2.

It has been demonstrated that orlistat inhibits the hydrolysis of p-nitrophenyl acetate in human liver microsomes (Xiao et al., 2013). p-Nitrophenyl acetate is a common substrate for CES1, CES2, and AADAC, major drug-metabolizing hydrolases in humans. Thus, in this study, the inhibition potencies of orlistat against CES1, CES2, and AADAC were compared using specific substrates for each hydrolase. Temocapril (CES1 substrate; Takai et al., 1997), acebutolol (CES2 substrate; Muta et al., 2015), and eslicarbazepine acetate (AADAC substrate; Hirosawa et al., 2021) hydrolase activities by recombinant CES1, CES2, and AADAC, respectively, were measured (Fig. 1). The substrate concentrations were set at near $K_m$ value to relate the inhibitor’s IC$_{50}$ more closely to its $K_i$. As shown in Fig. 1, the acebutolol hydrolase activity by recombinant CES2 was potently inhibited by orlistat (IC$_{50}$ = 2.64 ± 0.45 nM), whereas the temocapril and eslicarbazepine acetate hydrolase activities by recombinant CES1 and AADAC, respectively, remained more than 50% even at 100 nM orlistat. These results suggest that orlistat potently inhibits human CES2.

Inhibition Mechanism of Orlistat against Human CES2.

The type of inhibition in which inhibitors irreversibly bind to an active site of enzymes through the metabolism of inhibitors is called mechanism-based inhibition (MBI). This type of inhibition is also called time-dependent inhibition (TDI) because the IC$_{50}$ value is decreased by the pre-incubation of enzymes and inhibitors. The IC$_{50}$ shift assay (Lee and Kim, 2013) was performed to determine whether the mechanism by which orlistat inhibits human CES2 is TDI. For evaluation of TDI, the substrate concentrations are recommended to be more than 4 times higher than the $K_m$ value because this condition minimizes reversible inhibition by inhibitors or their metabolites. Accordingly, the substrate concentration was set at 50 mM, which is considerably higher than the $K_m$ value. As shown in Fig. 2A, the IC$_{50}$ value of orlistat against acebutolol hydrolase activity by recombinant CES2 was not changed.
from 0-min to 30-min pre-incubation (from 2.18 ± 0.29 to 1.91 ± 0.12 nM), indicating that the mechanism by which orlistat inhibits human CES2 is direct inhibition, not TDI.

Then, to elucidate the inhibition mechanism, a kinetic analysis of acebutolol hydrolase activity in the presence of orlistat was performed (Figs. 2B and C). The $K_m$ value was almost unchanged by orlistat, whereas the $V_{\text{max}}$ value was decreased by orlistat in a concentration-dependent manner (Table 1). This result suggests that the mechanism by which orlistat inhibits human CES2 is non-competitive inhibition, and the $K_i$ value was calculated to be $2.95 \pm 0.16$ nM.

**Orlistat Inhibits the Hydrolysis of Irinotecan and Ampiroxicam.**

To investigate whether the inhibition of human CES2-catalyzing hydrolysis by orlistat is not acebutolol specific, the inhibition potencies of orlistat against the hydrolysis of irinotecan (Sanghani et al., 2004) and ampiroxicam (Hirosawa et al., in preparation), other CES2 substrates, were evaluated (Fig. 3). Similar to the results in acebutolol hydrolysis, the hydrolysis of both irinotecan and ampiroxicam by recombinant CES2 was potently inhibited by orlistat (IC$_{50}$ = 3.31 ± 0.95 and 0.75 ± 0.20 nM, respectively), indicating that orlistat inhibits hydrolysis by CES2 regardless of the substrate.

**Cetilistat Does Not Inhibit Human CES2.**

Cetilistat is another anti-obesity drug whose mechanism of action is also the inhibition of pancreatic lipase in the gastrointestinal tract. The inhibitory potency of cetilistat against pancreatic lipase (IC$_{50}$ = 6.0 nM) has been reported to be comparable to that of orlistat (7.5 nM) (Kumar and Chauhan, 2021). Their dosages (120 mg) are also the same. To clarify whether cetilistat also inhibits human CES2, the inhibitory effects of cetilistat on acebutolol hydrolase activity by recombinant CES2 were examined (Fig. 4). Interestingly, the acebutolol hydrolase activity was not inhibited by cetilistat at all, suggesting that inhibitory effects on human CES2 are specific characteristics of orlistat.
Species Differences in the Inhibitory Effects of Orlistat on Acebutolol Hydrolase Activities.

To determine the appropriate animal species for evaluating the DDI between acebutolol and orlistat in vivo, species differences in the inhibitory effects of orlistat were examined. The inhibitory effects of orlistat on acebutolol hydrolase activities in the liver and intestinal microsomes from mice, rats, dogs, and monkeys, which are frequently used for in vivo studies in drug development, were compared with those from humans (Fig. 5). The control activities in the liver and intestinal microsomes from each animal species are shown in Table 2. In humans, the acebutolol hydrolase activity in the intestinal microsomes was higher than that in the liver microsomes, while in mice, rats, and dogs, the activities in the intestinal microsomes were lower than those in the liver microsomes (Table 2). The activity in dog intestinal microsomes was below the limit of quantitation (< 3 pmol/min/mg protein). The acebutolol hydrolase activities in human liver (IC$_{50}$ = 19.73 ± 1.22 nM) and intestinal (IC$_{50}$ = 4.12 ± 0.07 nM) microsomes were potently inhibited by orlistat (Fig. 5A). Similarly, the activities in mouse liver (IC$_{50}$ = 3.61 ± 0.42 nM) and intestinal (IC$_{50}$ = 1.70 ± 0.15 nM) microsomes were potently inhibited (Fig. 5B). The inhibitory effects of orlistat on the activities in rat liver and intestinal microsomes were relatively weak, and the activities remained more than 50% even at 100 nM orlistat (Fig. 5C). The activity in dog (Fig. 5D) and monkey (Fig. 5E) liver microsomes was not inhibited by orlistat (Fig. 5D). Accordingly, the DDI between acebutolol and orlistat in vivo was evaluated using mice in a subsequent study.

Orlistat Increases the Exposure of Acebutolol in vivo.

Although several drugs including telmisartan and loperamide have been reported to inhibit human CES2 in vitro (Quinney et al., 2005; Shimizu et al., 2014), there are no reports that these inhibitors have altered the pharmacokinetics of CES2 substrates at clinical doses in vivo. It is possible that orlistat affects the pharmacokinetics of victims (CES2 substrates) in vivo because of its lower $K_i$ value than those of other CES2 inhibitors, which are on the order of $\mu$M. Potential DDI was evaluated using mice in which orlistat strongly inhibited acebutolol...
hydrolase activity \textit{in vitro}, as in humans (Fig. 6). Because acebutolol is hydrolyzed to acetolol and then acetylated to diacetolol (Fig. 6A), plasma concentrations of acebutolol and its two metabolites were measured. The plasma concentration of acebutolol was significantly increased by co-administration of orlistat, resulting in a 43% increase in the AUC (from 2.70 ± 1.01 to 3.85 ± 1.42 µg × hr/mL) (Fig. 6B; Table 3). The plasma concentrations of acetolol and diacetolol were significantly decreased by co-administration of orlistat, resulting in 47% (from 3.89 ± 0.70 to 2.04 ± 0.82 µg × hr/mL) and 28% (from 2.74 × 10^6 ± 0.46 × 10^6 to 1.96 × 10^6 ± 0.49 × 10^6 peak area × hr) decreases in the AUC, respectively (Figs. 6C and D; Table 3). These results suggest that inhibition of hydrolysis by orlistat affects the pharmacokinetics of acebutolol \textit{in vivo}. 
Discussion

DDI has a significant impact on the efficacy and safety of drugs. Although several compounds have been demonstrated to inhibit drug-metabolizing hydrolases by in vitro studies (Fukami et al., 2010; Shimizu et al., 2014), an in vivo risk assessment has not been conducted. Patients with lifestyle diseases such as obesity and hypertension have an increased risk for DDI because they often take multiple medications. It has been reported that orlistat inhibits the hydrolysis of p-nitrophenol acetate, a common substrate of CES1, CES2, and AADAC, in human liver microsomes with an IC_{50} value of approximately 50 nM (Xiao et al., 2013), and abiraterone acetate, a specific substrate of AADAC, in recombinant human AADAC with an IC_{50} value of 1.2 nM (Sakai et al., 2021). In this study, the inhibitory potencies of the anti-obesity drug orlistat on the major drug-metabolizing hydrolases, CES1, CES2, and AADAC, were compared in vitro, and the effects on the pharmacokinetics of a victim drug in vivo were evaluated.

Orlistat potently inhibited human CES2 among the major drug-metabolizing hydrolases, CES1, CES2, and AADAC (Fig. 1). Xiao et al. (2013) reported that the inhibition potency of orlistat on p-nitrophenyl acetate hydrolase activity by recombinant human CES2 was stronger than that by recombinant human CES1, which is consistent with the results of this study. Our previous study revealed that human AADAC-catalyzing hydrolase activity for abiraterone acetate is potently inhibited by orlistat (IC_{50} = 1.2 nM; Sakai et al., 2021). However, in this study, eslicarbazepine acetate hydrolase activity by recombinant human AADAC was hardly inhibited by 100 nM orlistat (Fig. 1). The hydrolase activities of other AADAC substrates, flutamide and ketoconazole, were also not inhibited by 100 nM orlistat (data not shown). Thus, the inhibitory effects of orlistat on human AADAC appear to be specific to abiraterone acetate among substrates, although further study is needed. It is conceivable that orlistat might reduce the accessibility of abiraterone acetate to AADAC by causing conformational changes in AADAC. Molecular docking of orlistat with AADAC and its substrates to examine changes in accessibility may demonstrate this hypothesis.
The inhibitory pattern of orlistat against human CES2-catalyzing acebutolol hydrolase activity was non-competitive inhibition ($K_i = 2.95 \pm 0.16 \text{ nM}$, Fig. 2), indicating that orlistat binds to sites other than the substrate pocket of the CES2 protein. Orlistat has an alanine structure as with sofosbuvir (an anti-hepatitis C virus drug) and remdesivir (an anti-SARS-CoV-2 drug), which are other potent inhibitors of CES2 with IC$_{50}$ values against $p$-nitrophenyl acetate and/or 4-methylumbelliferyl acetate hydrolase activity on the nM order (Shen and Yan, 2017; Shen et al., 2021); therefore, the structure may be key for the inhibition of CES2. This hypothesis is supported by the fact that cetilistat, which does not have the alanine structure, did not inhibit CES2 (Fig. 4). Upon developing new chemical entities containing alanine in the structures, the potential for CES2 inhibition may need to be evaluated.

In our previous study, acebutolol hydrolysis was clarified to be primarily catalyzed by CES2 in humans (Muta et al., 2015), but the responsible enzyme(s) in the other animal species are unknown. According to the NCBI database (https://www.ncbi.nlm.nih.gov/gene/), CES2 in humans, dogs, or monkeys is a unigene, whereas there are multiple isoforms in mice (Ces2a–c, Ces2e–h) and rats (Ces2a, Ces2c, Ces2e, Ces2g–j) (Holmes et al., 2010). In addition, tissue distributions of CES2 are different between animal species as follows: in humans, mice, and monkeys, CES2 protein is expressed in both the liver and intestine, whereas it is expressed only in the intestine in rats and hardly expressed in both the liver and intestine in dogs (Taketani et al., 2007; Satoh and Hosokawa, 2010; Yoshida et al., 2018). Like in humans, acebutolol hydrolase activities in mouse liver and intestinal microsomes were inhibited by orlistat (Figs. 5A and B). Mouse Ces2c is recognized as an ortholog of human CES2 (Eisner et al., 2022), but amino acid homology is not as high (71%), and whether Ces2c catalyzes acebutolol needs to be examined using the expression system. Although rat Ces2 is not expressed in the liver (Taketani et al., 2007; Satoh and Hosokawa, 2010), acebutolol hydrolase activity in rat liver microsomes was higher than that in intestinal microsomes (Table 2; Fig. 5C). Therefore, enzyme(s) other than Ces2 would be responsible for acebutolol hydrolysis in rats, which may explain the weak inhibition by orlistat. The acebutolol
hydrolase activity in dog liver microsomes was not inhibited by orlistat, and the activity in the
intestinal microsomes was marginal (Fig. 5D). Considering the lack of CES2 expression, it is
reasonable that acebutolol hydrolysis did not proceed in the dog intestine, and the activity
observed in the liver was probably due to enzyme(s) other than CES2, which may explain the
weak inhibition by orlistat. In the monkey liver, CES2 protein is expressed (Taketani et al.,
2007), and the amino acid homology between human and monkey CES2 proteins is as high as
89%, but the acebutolol hydrolase activity was not inhibited by orlistat (Fig. 5E). To
investigate the cause of the difference in the inhibitory effects of orlistat on acebutolol
hydrolase activity between humans and monkeys, acebutolol hydrolase activities were
measured using recombinant monkey CES1, CES2, and AADAC, which were prepared in our
previous study (Honda et al., 2021) (Fig. S2). Unexpectedly, acebutolol was hardly
hydrolyzed by monkey CES2 but was rather efficiently hydrolyzed by monkey AADAC, and
the inhibition potency of orlistat against acebutolol hydrolase activity by recombinant monkey
CES2 was much weaker than that in humans. Some specific residue(s) out of the 11% amino
acid residues, which are different between human and monkey CES2, would determine the
recognition by orlistat.

The in vivo DDI potential was elucidated using mice, in which orlistat showed strong
inhibition against acebutolol hydrolase activities in the liver and intestine, similar to humans.
The plasma concentration of acebutolol was significantly increased, and the plasma
concentration of acetolol, a hydrolyzed metabolite of acebutolol, was significantly decreased
by co-administration of orlistat (Fig. 6), indicating that concomitant use of orlistat altered the
pharmacokinetics of acebutolol. PMDA, EMA, and FDA guidelines define the risk criteria for
DDI in the liver or intestine using the ratio of the $K_i$ value to the maximum unbound plasma
concentration ($I_{\text{max,u}}$) or the estimated intestinal luminal concentration ($I_{\text{gut}}$) of the inhibitor,
respectively (liver: $I_{\text{max,u}} / K_i > 0.02$; intestine: $I_{\text{gut}} / K_i > 10$). According to the package insert
of XENICAL®, plasma orlistat was near the detection limit (< 10 nM) when 360 mg of
orlistat was orally administered to humans, with >99% binding to plasma proteins. Therefore,
the maximum unbound plasma concentration ($I_{\text{max,u}}$) of orlistat at the clinical dose of 120 mg
is estimated to be less than 0.03 nM, and $I_{\text{max, u}} / K_i$ was below 0.012, which is lower than the risk criteria for DDI in the liver. On the other hand, the estimated intestinal luminal concentration ($I_{\text{gut}}$) of orlistat was calculated to be 968 µM according to the formula in the PMDA, EMA, and FDA guidelines (dose/250 mL), and $I_{\text{gut}} / K_i$ was $3.3 \times 10^5$, which is considerably higher than the risk criteria for DDI in the intestine. Therefore, DDI via inhibition of hydrolases by orlistat would occur primarily in the intestine, indicating that orlistat could cause DDI with orally administered drugs that are substrates of CES2. Although the possibility of systemic or hepatic CES2 inhibition cannot be completely excluded, DDI by orlistat would occur primarily in the intestine because acebutolol is efficiently hydrolyzed in intestinal first-pass metabolism and orlistat is hardly absorbed and present at high concentrations in the intestine.

In conclusion, we demonstrated that orlistat causes DDI in vivo by potently inhibiting CES2. This is the first evidence that inhibition of hydrolases causes DDI. The establishment of risk assessment platforms for DDI mediated by hydrolases would be useful for the development of safe drugs.
Data Availability Statement

The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.
Authorship Contributions

Participated in research design: Hirosawa, Fukami, Nakano, Nakajima

Conducted experiments: Hirosawa

Contributed new reagents or analytical tools: Hirosawa

Performed data analysis: Hirosawa

Wrote or contributed to the writing of manuscript: Hirosawa, Fukami, Nakajima
References


Watanabe A, Fukami T, Takahashi S, Kobayashi Y, Nakagawa N, Nakajima M, and Yokoi T (2010) Arylacetamide deacetylase is a determinant enzyme for the difference in


Footnotes

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The authors declare that there are no conflicts of interest.

Send reprint requests to: Tatsuki Fukami, Ph.D. Faculty of Pharmaceutical Sciences, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan. E-mail: tatsuki@p.kanazawa-u.ac.jp
Figure legends

**Fig. 1.** Effects of orlistat on human CES1, CES2, and AADAC activities. The inhibitory potencies of orlistat against temocapril, acebutolol, and eslicarbazepine acetate hydrolase activities by recombinant CES1, CES2, and AADAC, respectively, were examined. The concentrations of temocapril, acebutolol, and eslicarbazepine acetate were 500 µM, 10 mM, and 1 mM, respectively. The control activities of temocapril, acebutolol, and eslicarbazepine acetate hydrolysis were 27.8 ± 0.6 nmol/min/mg protein, 445.6 ± 87.9 pmol/min/mg protein, and 63.2 ± 6.8 nmol/min/mg protein, respectively. Each data point represents the mean ± SD of triplicate determinations.

**Fig. 2.** Inhibition mechanism of orlistat against human CES2-catalyzing acebutolol hydrolase activity. (A) Effects of pre-incubation on acebutolol hydrolase activity by recombinant CES2. The reaction mixture was pre-incubated for 0 or 30 min before the addition of acebutolol at a final concentration of 50 mM. The control activities were 509.8 ± 12.6 (pre-incubation: 0 min) and 446.1 ± 5.0 pmol/min/mg protein (30 min). (B) S-V plot and (C) Dixon plot for kinetic analysis of acebutolol hydrolase activity by recombinant CES2 in the absence or presence of orlistat. Each data point represents the mean ± SD of triplicate determinations.

**Fig. 3.** Effects of orlistat on hydrolase activities for irinotecan and ampiroxicam, human CES2 substrates. The inhibitory potencies of orlistat against (A) irinotecan and (B) ampiroxicam hydrolase activities by recombinant CES2 were evaluated. The concentrations of irinotecan and ampiroxicam were 2 µM and 10 µM, respectively. The control activities of irinotecan and ampiroxicam hydrolysis were 0.53 ± 0.10 pmol/min/mg protein and 3.01 ± 0.15 nmol/min/mg protein, respectively. Each data point represents the mean ± SD of triplicate determinations.
Fig. 4. Effects of cetilistat on acebutolol hydrolase activity by recombinant CES2. The control activity was 374.8 ± 3.9 pmol/min/mg protein at a substrate concentration of 10 mM. Each data point represents the mean ± SD of triplicate determinations.

Fig. 5. Species differences in inhibitory effects of orlistat on acebutolol hydrolase activities. The effects of orlistat on acebutolol hydrolase activities in (A) human, (B) mouse, (C) rat, (D) dog, and (E) monkey liver and intestinal microsomes were evaluated. The substrate concentration was 10 mM. Each data point represents the mean ± SD of triplicate determinations.

Fig. 6. Effects of co-administration of orlistat on the pharmacokinetics of acebutolol in mice. (A) The metabolic pathways of acebutolol. The plasma concentrations of (B) acebutolol, (C) acetolol, and (D) diacetolol were measured at 0.5, 1, 1.5, 2, 4, and 6 hr after administration of acebutolol and orlistat (acebutolol: 45.5 mg/kg, orlistat: 25 mg/kg dissolved in 1% carboxymethyl cellulose, p.o.) following pre-administration of orlistat or vehicle. *P < 0.05 and **P < 0.01, compared with the concentrations of control mice using an unpaired t test. Each data point represents the mean ± SD (n = 5–6).
Table 1. Kinetic parameters of acebutolol hydrolase activities in the absence or presence of orlistat.

<table>
<thead>
<tr>
<th>Orlistat (nM)</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.70 ± 0.55</td>
<td>700.3 ± 31.7</td>
</tr>
<tr>
<td>2</td>
<td>3.00 ± 0.91</td>
<td>384.2 ± 32.8</td>
</tr>
<tr>
<td>5</td>
<td>2.82 ± 0.96</td>
<td>281.2 ± 26.0</td>
</tr>
<tr>
<td>10</td>
<td>3.18 ± 2.07</td>
<td>116.8 ± 21.9</td>
</tr>
</tbody>
</table>

All data are expressed as the mean ± SD.
Table 2. Acebutolol hydrolase activities in human, mouse, rat, dog, and monkey liver and intestinal microsomes.

<table>
<thead>
<tr>
<th></th>
<th>Human</th>
<th>Mouse</th>
<th>Rat</th>
<th>Dog</th>
<th>Monkey</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>418.7 ± 6.8</td>
<td>775.9 ± 24.4</td>
<td>86.8 ± 2.3</td>
<td>36.5 ± 1.2</td>
<td>2877.6 ± 13.5</td>
</tr>
<tr>
<td>Intestine</td>
<td>3363.5 ± 22.3</td>
<td>85.7 ± 2.6</td>
<td>51.7 ± 1.4</td>
<td>N.D.</td>
<td>-</td>
</tr>
</tbody>
</table>

The substrate concentration was 10 mM. All data are the mean ± SD of triplicate determinations. N.D.: Not detected. -: Not measured.
Table 3. Pharmacokinetic parameters of acebutolol, acetolol, and diacetolol in mice orally administered acebutolol and orlistat.

<table>
<thead>
<tr>
<th></th>
<th>Acebutolol</th>
<th></th>
<th>Acetolol</th>
<th></th>
<th>Diacetolol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µg/mL)</td>
<td>AUC (µg × hr/mL)</td>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µg/mL)</td>
<td>AUC (µg × hr/mL)</td>
<td>C&lt;sub&gt;max&lt;/sub&gt; (Peak area × 10&lt;sup&gt;5&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Control</td>
<td>1.02 ± 0.11</td>
<td>2.70 ± 1.01</td>
<td>1.34 ± 0.36</td>
<td>3.89 ± 0.70</td>
<td>7.44 ± 2.07</td>
</tr>
<tr>
<td>Orlistat</td>
<td>1.49 ± 0.22***</td>
<td>3.85 ± 1.42</td>
<td>0.74 ± 0.41*</td>
<td>2.04 ± 0.82**</td>
<td>5.46 ± 1.25*</td>
</tr>
</tbody>
</table>

All data are expressed as the mean ± SD (n = 5–6). *P < 0.05, **P < 0.01, and *** P < 0.001, compared with the control mice using an unpaired t test.
Fig. 1

IC₅₀
CES2: 2.64 ± 0.45 nM
Fig. 2

A

Residual activity (%)

0 min

0 min: 2.18 ± 0.29 nM

30 min: 1.91 ± 0.12 nM

B

Orlistat hydrolyase activity

(pmol/min/mg protein)

Orlistat

0 nM

2 nM

5 nM

10 nM

C

Acebutolol hydrolyase activity

(pmol/min/mg protein)

Acebutolol

2 mM

5 mM

10 mM

25 mM
Fig. 3

A. Irinotecan

IC$_{50}$: 3.31 ± 0.95 nM

B. Ampiroxicam

IC$_{50}$: 0.75 ± 0.20 nM
Fig. 4

![Graph showing residual activity vs. Cetilistat concentration](image-url)
Fig. 5

A. Human

- Liver
- Intestine

IC$_{50}$
Liver: 19.73 ± 1.22 nM
Intestine: 4.12 ± 0.07 nM

B. Mouse

- Liver
- Intestine

IC$_{50}$
Liver: 3.61 ± 0.42 nM
Intestine: 1.70 ± 0.15 nM

C. Rat

- Liver
- Intestine

D. Dog

- Liver

E. Monkey

- Liver
Fig. 6

A

\[
\text{Acebutolol} \xrightarrow{\text{Hydrolysis}} \text{Acetolol} \xrightarrow{\text{Acetylation}} \text{Diacetolol}
\]

B

\[
\text{Acebutolol (Control)} \quad \text{Acebutolol (Orlistat)}
\]

C

\[
\text{Acetolol (Control)} \quad \text{Acetolol (Orlistat)}
\]

D

\[
\text{Diacetolol (Control)} \quad \text{Diacetolol (Orlistat)}
\]