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Carboxylesterase-2 is a highly sensitive target of the antiobesity agent orlistat with profound implications in the activation of anticancer prodrugs

Da Xiao [a], Deshi Shi [a], Dongfang Yang [a], Benjamin Barthel [b], Tad H. Koch [b], Bingfang Yan [a,*]

[a] Department of Biomedical Sciences, Center for Pharmacogenomics and Molecular Therapy, University of Rhode Island, Kingston, RI 02881, USA
[b] Department of Chemistry and Biochemistry University of Colorado, Boulder, CO 80309, USA

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ABSTRACT

Orlistat has been the most used anti-obesity drug and the mechanism of its action is to reduce lipid absorption by inhibiting gastrointestinal lipases. These enzymes, like carboxylesterases (CESs), structurally belong to the α/β hydrolase fold superfamily. Lipases and CESs are functionally related as well. Some CESs (e.g., human CES1) have been shown to hydrolyze lipids. This study was designed to test the hypothesis that orlistat inhibits CESs with higher potency toward CES1 than CES2, a carboxylesterase with little lipase activity. Liver microsomes and recombinant CESs were tested for the inhibition of the hydrolysis of standard substrates and the anticancer prodrugs pentyl carbamate of *p*-aminobenzyl carbamate of doxazolidine (PPD) and irinotecan. Contrary to the hypothesis, orlistat at 1 nM inhibited CES2 activity by 75% but no inhibition on CES1, placing CES2 one of the most sensitive targets of orlistat. The inhibition varied among some CES2 polymorphic variants. Pretreatment with orlistat reduced the cell killing activity of PPD. Certain mouse but not rat CESs were also highly sensitive. CES2 is responsible for the hydrolysis of many common drugs and abundantly expressed in the gastrointestinal track and liver. Inhibition of this carboxylesterase probably presents a major source for altered therapeutic activity of these medicines if co-administered with orlistat. In addition, orlistat has been linked to various types of organ toxicities, and this study provides an alternative target potentially involved in these toxicological responses.

1. Introduction

Obesity is probably the most important health issue and associated with a wide range of health conditions such as cardiovascular diseases, type II diabetes and certain types of cancers [1–3]. In Great Britain, obesity crisis has risen 10 times during the past decade [4]. In the United States (US), the direct cost of obesity is estimated as many as 10% of all medical cost [5]. Among the US population, more than 30% are considered obese and almost 70% are overweight. The prevalence of obesity is slightly lower in men than women, but the prevalence in men has shown a clear trend of increase during the past decade. Importantly, obesity is no longer an issue of adults only, and the prevalence has reached an all-time high in children and adolescents [6], and more than 10% of these populations are considered obese or overweight. Overweight children have much higher chance of becoming obese adults.

Obesity and overweight are caused by a combination of excessive food intake and lack of exercise. Clearly, changes in life styles probably represent the most effective approach to lose weight [7]. Intervention with medication has been increasingly used [8]. Orlistat is widely used for this purpose and marketed under prescription and over-the-counter [9]. The mechanism of action is to inhibit pancreatic and functionally related lipases. Pancreatic lipases are normally secreted into the lumen of the small intestine and hydrolyze triglycerides [11]. Lipids are absorbed upon hydrolysis, and inhibition of the lipases reduces the hydrolysis, thus decreasing the fat intake. In addition to weight loss, orlistat has been shown to improve conditions such as hypertension and type II diabetes [12]. On the other hand, orlistat has been implicated with severe liver toxicity [13].

Lipases, like carboxylesterases (CESs), structurally belong to the α/β fold hydrolase superfamily [10]. While CESs hydrolyze drugs and other xenobiotics, some CESs hydrolyze lipids as well [14,15]. In the human genome, seven CES genes exist with one being a pseudogene [16,17]. Nonetheless, only three human CESs are catalytically characterized: CES1, CES2 and CES3 [17]. CES3 is much weaker enzyme in terms of metabolizing common drugs. In a proteomic study, CES1 was identified as one of the top ten most abundant proteins in the adult liver [18]. More importantly, CES1

Abbreviations: CES, carboxylesterase; CHX, cycloheximide; DMEM, Dulbecco's modified eagle medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PPD, pentyl carbamate of *p*-aminobenzyl carbamate of doxazolidine.

* Corresponding author at: Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, Kingston, RI 02881, USA. Tel.: +1 401 874 5032; fax: +1 401 874 5048.
E-mail address: byan@uri.edu (B. Yan).

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but not CES2 has been shown to hydrolyze neutral lipids [15]. In contrast, CES2 preferably hydrolyzes bulky molecules such as the anticancer agents: irinotecan [19] and pentyl carbamate of *p*-aminobenzyl carbamate of doxazolidine (PPD) [20,21].

This study was performed to test the hypothesis that orlistat inhibits CESs with higher potency toward CES1 than CES2, a carboxylesterase with little lipase activity. To test this hypothesis, recombinant CESs from human, mouse and rat were incubated with orlistat at various concentrations (1–100 nM) and the hydrolytic activity was determined. While orlistat inhibited all CESs tested, the relative potency varied markedly. Contrary to the hypothesis, CES2 was much more sensitive than CES1. Among all CESs, human CES2 and mouse ces2c were inhibited to the most extent and represent two of the most sensitive targets of orlistat. The inhibition was irreversible and occurred intracellularly. Orlistat inhibited the activation of PPD and irinotecan, thus decreasing its cell killing activity.

2. Materials and methods

2.1. Chemicals and supplies

Cycloheximide (CHX), *p*-nitrophenylacetate, 1-naphthylacetate, orlistat and Hanks balanced salt solution were from Sigma (St. Louis, MO). Dulbecco's modified eagle medium (DMEM) and high fidelity Platinum *Taq* DNA polymerase were from Life Technologies (Carlsbad, CA). The antibody against glyceradehyde-3-phosphate dehydrogenase (GAPDH) was from Abcam (Cambridge, UK). The goat anti-rabbit IgG conjugated with horseradish peroxidase was from Pierce (Rockford, IL). Nitrocellulose membranes were from Bio-Rad (Hercules, CA). Expression constructs for mouse CESs were purchased from OriGene (Rockville, MD). Synthesis of PPD was described elsewhere [20,21]. Unless otherwise specified, all other reagents were purchased from Thermo-Fisher Scientific (Fair Lawn, NJ).

2.2. Enzymatic assays

Liver tissues were homogenized and microsomes were prepared by differential centrifugation as described previously [22–24]. Human liver tissues (n = 14, equal number of each gender) were from the University of Maryland Brain and Tissue Bank for Developmental Disorders (Baltimore, MD) [24]. Sprague-Dawley rats (male, n = 4) and CD-1 mice (male, n = 4) were from Charles River (Wilmington, MA). The use of the human samples and animals was approved by the Institutional Review Board and the Institutional Animal Care and Use Committee. The enzymatic activity was determined spectrophotometrically as described previously [19]. The activity was also determined by native gel electrophoresis stained for carboxylesterase activity [25]. Briefly, liver homogenates (10 μg) were solubilized with 0.2% Lubrol and subjected to electrophoresis through a 3% acrylamide stacking gel and a 7.5% acrylamide separating gel. After electrophoresis, the gels were washed for 1 h in 100 mM potassium phosphate buffer (pH 6.5), followed by incubating in the same buffer containing 1-naphthylacetate (5 mM) and 4-benzolamino-2,5-dimethoxybenzenediazonium chloride hemi (zinc chloride) salt, usually termed Fast Blue RR (0.4 mg/ml). Staining for carboxylesterases by this method is based on the formation of a black, insoluble complex between the hydrolytic products and Fast Blue RR. The images were captured by Carestream 2200 PRO Imager.

2.3. Western analysis

Samples were resolved by 7.5% SDS-PAGE in a mini-gel apparatus and transferred electrophoretically to nitrocellulose membranes. After non-specific binding sites were blocked with 5% non-fat milk, the blots were incubated with an antibody against CES1, CES2 or GAPDH. The antibodies against CES1 and CES2 were prepared with synthetic peptides and purified as described previously [24,26]. The specificity was established with the corresponding recombinant proteins. The primary antibodies were localized with goat anti-rabbit IgG conjugated with horseradish peroxidase. Horseradish peroxidase activity was detected with a chemiluminescent kit (SuperSignal West Pico). The chemiluminescent signal was captured by Carestream 2200 PRO Imager.

2.4. Site-directed mutagenesis and cell transfection

There are several polymorphic variants reported in the literature or in the database of the National Center for Biotechnology Information. To shed light on the sensitivity to orlistat, variants encoding single mutation were prepared by site-directed mutagenesis as described previously [27]. The parent CES2 plasmid was isolated with a cDNA trapping method and reported previously [26]. Complementary oligonucleotides (Table 1) were synthesized, annealed to the CES2 expression construct, and subjected to a thermocycler for a total of 15 cycles. The resultant PCR-amplified constructs were then digested with *Dpn*I to remove the non-mutated parent construct. The mutated PCR-amplified constructs were used to transform XL1-Blue. All mutated constructs were subjected to sequencing analysis to confirm the desired mutation being made without secondary mutations. To prepare the corresponding recombinant CES2 variant, 293T cells were transfected with the wild-type or a mutant construct with Genjet version II from SignaGen Lab (Rockville, MD) as described by the manufacturer. The transfected cells were harvested after a-24 incubation and cell lysates were prepared. Seven mutants were made including N85Q, A139T, A178V, R180H, A187P, G223R or N250Q (single amino acid substitution).

2.5. Cell viability assay

Cells (HepG2 and LS180) were seeded into 96-well plates (8,000/well). In some cases, cells were seeded in 6-well plates and transfected with CES2 or the vector. The transfected cells were then seeded in 96-well Plates 24 h after the transfection. The seeded cells, usually overnight after seeding, were treated with one or more chemicals and cultured in normal medium (chemicals kept the same) for 48 h. Thereafter, the medium was replaced with fresh medium containing MTT [(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] at a final concentration of 0.5 mg/ml. After a 2 h incubation at 37 °C, the medium was gently decanted, and DMSO (100 l/well) was added to dissolve formazan product. The optical density (OD) was determined at 570 nm, and the final OD values were calculated by subtracting the background reading (no seeded cells).

2.6. Molecular modeling

To gain molecular insight regarding orlistat-inhibition of CESs, we performed molecular modeling and docking studies. The

Table 1
Sequences of primers for site-directed mutagenesis.

Primer	Sequence
CES2-N85Q	5′-TTTCTTAGCCAGTTCCAGATGACCTTCCCTTCC-3′
CES2-A139T	5′-GATGGTTCCATGCTGACTGCCTTGGAGAACGTG-3′
CES2-A178V	5′-TACCTGGACCAAGTGGTTGCACTACGCTGGGTC-3′
CES2-R180H	5′-CAAGTGGCTGCACTACACTGGGTCCAGCAGAAT-3′
CES2-A187P	5′-GTCCAGCAGAATATCCCCCACTTTGGAGGCAAC-3′
CES2-G223R	5′-CAAGGACTCTTCCACAGAGCCATCATGGAGAGT-3′
CES2-N250Q	5′-TCCACGGTGGTGGCCCAACTGTCTGCCTGTGAC-3′