Human carboxylesterase 1A plays a predominant role in hydrolysis of the anti-dyslipidemia agent fenofibrate in humans

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Running Title Page

Running Title: The role of hCES1A in fenofibrate hydrolysis in humans

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**List of nonstandard abbreviations:**

BNPP: bis-(p-nitrophenyl) phosphate  
DME: D-luciferin methyl ester  
EDTA: ethylenediaminetetraacetic acid  
FDA: Food and Drug Administration  
FA: Fenofibric acid  
FF: Fenofibrate  
GA: galanthamine  
hAchE: human acetylcholinesterase  
hBchE: human butyrylcholinesterase  
hCES1A: human carboxylesterase 1A  
hCES2A: human carboxylesterase 2A  
hCTSA: human cathepsin A  
hCTSL: human cathepsin L  
hIAH1: human isoamyl acetate-hydrolyzing esterase 1  
HIMs: human Intestine microsomes  
HKM: human kidney microsomes  
HLMs: human liver microsomes  
HLuMs: human lung microsomes  
hMAGL: human monoacylglycerol lipase  
hSA: human serum albumin  
IC$_{50}$: half maximal inhibition concentration  
LPA: loperamide  
PPAR-α: peroxisome proliferator activated receptor-alpha  
SFN: sulforaphane
Abstract

Fenofibrate, a marketed peroxisome proliferator-activated receptor-α (PPARα) agonist, has been widely used for treating severe hypertriglyceridemia and mixed dyslipidemia. As a canonical prodrug, fenofibrate can be rapidly hydrolyzed to release the active metabolite (fenofibric acid) in vivo, but the crucial enzyme(s) responsible for fenofibrate hydrolysis and the related hydrolytic kinetics have not been well-investigated. This study aimed to assign the key organs and crucial enzymes involved in fenofibrate hydrolysis in humans, as well as reveal the impact of fenofibrate hydrolysis on its non-PPAR mediated biological activities. Our results demonstrated that fenofibrate could be rapidly hydrolyzed in the preparations from both human liver and lung to release fenofibric acid. Reaction phenotyping assays coupling with chemical inhibition assays showed that human carboxylesterase 1A (hCES1A) played a predominant role in fenofibrate hydrolysis in human liver and lung, while human carboxylesterase 2A (hCES2A) and human monoacylglycerol esterase (hMAGL) contributed to a very lesser extent. Kinetic analyses showed that fenofibrate could be rapidly hydrolyzed by hCES1A in human liver preparations, while the inherent clearance of hCES1A-catalyzed fenofibrate hydrolysis is much higher (>200-fold) than much higher than that of hCES2A or hMAGL. Biological assays demonstrated that both fenofibrate and fenofibric acid showed very closed Nrf2 agonist effects, but fenofibrate hydrolysis strongly weaken its inhibitory effects against both hCES2A and hNtoum. Collectively, our findings reveal that the liver is the major organ and hCES1A is the predominant enzyme catalyzing fenofibrate hydrolysis in humans, while fenofibrate hydrolysis significantly reduces inhibitory effects of fenofibrate against serine hydrolases.

Keywords: Fenofibrate; hCES1A; Fenofibric acid; Serine hydrolases; non-PPAR effects
Significance Statement

Fenofibrate can be completely converted to fenofibric acid in humans and subsequently exert its pharmacological effects, but the hydrolytic pathways of fenofibrate in humans have not been well-investigated. This study reported that the liver was the predominant organ and human carboxylesterase 1A was the crucial enzyme involved in fenofibrate hydrolysis in humans.
Introduction

Fenofibrate, one of the most widely prescribed fibric acid derivatives, was approved for the treatment of hyperlipidemic by the US Food and Drug Administration (FDA) in 1998 (Saurav et al., 2012). Following oral administration, fenofibrate can be quickly absorbed into the circulation system and then extensively hydrolyzed by esterases to release fenofibric acid (Guay, 1999, 2006; Filippatos et al., 2008). It is generally recognized that fenofibrate exerts its anti-hyperlipidemic effect mainly through its active hydrolytic metabolite, fenofibric acid (Arakawa et al., 2005; Tsimihodimos et al., 2005; Zhu et al., 2010; Malátková et al., 2016). Fenofibric acid exerts a significant impact on the modulation of lipoprotein and fatty acid metabolism through its selective targeting of peroxisome proliferator-activated receptor-α, leading to substantial reductions in total plasma cholesterol, low-density lipoprotein cholesterol, triglycerides, and very low-density lipoprotein levels. Additionally, it elicits an elevation in serum high-density lipoprotein cholesterol as well as lipoprotein AI and AII concentrations (Saurav et al., 2012; Malátková et al., 2016). Due to its excellent tolerability and high safety profiles, fenofibrate has been extensively used for the treatment of dyslipidemia all over the world in the past few decades, whether used alone or in combination with statins (Guay, 2006; McKeage and Keating, 2011; Saurav et al., 2012). In addition to the PPAR-α agonist effect, recent studies have reported that fenofibrate shows a wide range of biological activities, from anti-inflammatory effect to the inhibitory effects against a variety of enzymes (Gong et al., 2016; Dai et al., 2017; Prasad et al., 2018; Li et al., 2021; Chen et al., 2022; Spartalis et al., 2022).

Although fenofibrate has been used clinically for several decades, its hydrolytic pathways in humans have not been well investigated yet. Following oral administration, fenofibrate can be rapidly hydrolyzed in the system to release fenofibric acid, which then enhances the PPAR-α signaling pathway to exert its major pharmacologic effects in vivo (Chapman, 1987; Adkins and Faulds, 1997; Miller and Spence, 1998; Streel et al., 2000; Ramjattan et al., 2002; Filippatos and Milionis, 2008; Fukami et al., 2015). Previous studies have indicated that the key enzymes involved in this process as well as the kinetics of fenofibrate hydrolysis are still poorly understood despite evidence of its release as fenofibric acid in vivo (Chapman, 1987;
Kraja et al., 2010; Filippatos, 2012). It is well-known that the hydrolytic metabolism may strongly affect the in vivo pharmacological effects of ester-bearing agents including prodrugs (Walther et al., 2017; Wind et al., 2019; Li et al., 2022). Although fenofibric acid has been found to have a PPAR-α agonist effect and an anti-inflammatory effect (Willson et al., 2000; Seber et al., 2006; Prasad et al., 2018; Spartalis et al., 2022; Jin et al., 2023), the impact of fenofibrate hydrolysis on its non-PPAR effects (such as Nrf2 agonist, and enzyme inhibition) is poorly understood. Thus, it is imperative to ascertain the key hydrolases and primary metabolic organs involved in fenofibrate hydrolysis in humans, and evaluate its impact on its biological activities (Schelleman et al., 2014; Lee et al., 2020; Pristner and Warth, 2020; S et al., 2022).

This study aimed to address two key issues related to fenofibrate hydrolysis: 1) to identify the major organs and enzymes responsible for its hydrolysis in humans; 2) to investigate the impact of fenofibrate hydrolysis on non-PPAR effects (such as Nrf2 agonist effects and enzyme inhibition activities). To achieve these goals, the hydrolytic metabolite of fenofibrate in human plasma and tissue preparations was carefully characterized, while a suite of assays (including reaction phenotyping assays, chemical inhibition assays and hydrolytic kinetics assays) were conducted to identify the key contributing enzymes responsible for fenofibrate hydrolysis. Furthermore, the biological activities of fenofibrate and its hydrolytic metabolite (fenofibric acid) were tested to reveal the impact of fenofibrate hydrolysis on its non-PPAR effects.

**Materials and Methods**

**Materials and reagents**

Fenofibrate, sulforaphane (SFN), fenofibric acid and vildagliptin were provided by Meilun Biotechnology Co., Ltd. (Dalian, China). Ethylenediaminetetraacetic acid (EDTA) and galanthamine (GA) were produced by J&K Chemical Co., Ltd (Beijing, China). Loperamide (LPA) and bis(p-nitrophenyl) phosphate (BNPP) were purchased from TCI Development Co., Ltd (Shanghai, China). Luciferin detection reagent (LDR) was provided by
Promega Biotech (Madison, USA). Steady-Lumi™ II Firefly Luciferase Assay Kit was offered by Beyotime (Shanghai, China). HPLC-grade methanol, acetonitrile and formic acid were provided by Sigma (St. Louis, MO, USA).

**Enzymes and tissue preparations**

The human lung microsomes (HLuMs, Lot No. X040032), human kidney microsomes (HKMs, Lot No. X03801), human plasma, HS9 (human liver S9 fractions), human liver cytosol and 16 individual-donor HLM were provided by Research Institute for Liver Diseases (Shanghai, China). Pooled human liver microsomes from 50 donors (HLMs, Lot No. 2010065) and pooled human Intestine microsomes from 6 donors (HIMs, Lot No. 1410066) were provided by Sekisui Xenotech (USA). Human acetylcholinesterase (hAchE, Lot No. C1682), human butyrylcholinesterase (hBchE, Lot No. SLCG5158), α-Amylase and human serum albumin (hSA, Lot No. SLCC9268) were provided by Sigma-Aldrich (St. Louis, MO, USA). Human monoacylglycerol lipase (hMAGL, Lot No. 10007812) was purchased from Cayman Chemicals (USA) and human cathepsin A (hCTSA, Lot No. FXY0619021) was purchased from R&D systems Co., Ltd (USA). Human cathepsin L (hCTSL, Lot No. C401) was purchased from Novoprotein (Shanghai, China). Recombinant human carboxylesterase 1A (hCES1A), human Notum (hNotum), human carboxylesterase 2A (hCES2A) and human isoamyl acetate-hydrolysing esterase 1 (hIAH1) were expressed and purified according to previously described methods (Fukuda et al., 2000). All biological reagents were stored at -80 °C.

**Quantitative analysis of fenofibrate and its hydrolytic metabolite(s) by HPLC-UV**

An HPLC-UV system consisted of a DGU-405 degassing unit, an SPD-M40 photo diode array detection, a SIL-40CXR autosampler, an LC-40BXR pump, a CTO-40S column oven, a CBM-40 system controller (Kyoto, Japan) and a C18 column (5 µm, 150 mm × 2.1 mm, Shimadzu, Kyoto, Japan) was applied to isolate and quantify fenofibrate and the hydrolytic metabolite. The mobile phases consisted of 0.1% formic acid in water (A) and acetonitrile (B), with the following elution gradients: 0-1.5 min, 60% B; 1.5-4 min, 60%-95% B; 4-5.5 min, 95% B; 5.5-6 min, 95%-60% B; after then, the solvent B was adjusted to its starting concentration of 60%. The flow rate was 0.4 mL/min and the column temperature was set at
40 °C. The detection wavelength of fenofibrate and its hydrolytic metabolite(s) was set at 280 nm (the maximum absorbance wavelength of two analytes in UV).

**Phenotyping assays using human hydrolases**

The hydrolytic rates of fenofibrate in various recombinant enzymes (hCES1A, hCES2A, hMAGL, α-Amylase, hBchE, hAchE, hCTSA, hCTSL, thrombin, hSA, hNotum, hIAH1) were evaluated under identical conditions (pH 7.4 at 37 °C), utilizing the same final substrate concentration (10 μM). Each enzyme was set at a final concentration of 5 μg/mL except for thrombin (5 NIH/mL, final concentration). After 0.5 h incubation at 37°C, the reaction was quenched by adding ice-cold acetonitrile. Followed by centrifugation (20000 × g for 30 min) at 4°C, the supernate was obtained for LC-UV analysis.

**Chemical inhibition assays**

The involvement of esterases in the hydrolysis of fenofibrate was also investigated by employing various inhibitors targeting specific human hydrolases, including BNPP (a specific inhibitor of CES), LPA (a selective inhibitor for hCES2A), GA (a selective hAchE inhibitor), EDTA (a selective PONs inhibitor), and vildagliptin (a selective inhibitor for hDPPIV) (Warner et al., 1992; Quinney et al., 2005; Lauster et al., 2007; He et al., 2009; Zou et al., 2016, 2018; Buckler et al., 2017). The reaction system (200 μL) contained PBS (pH 7.4), HLMs or HLuMs, inhibitors and fenofibrate (10 μM). The incubation was carried out at 37°C for 0.5 h and terminated by adding 200 μL acetonitrile. After centrifugation (20000 × g for 30 min) at 4 °C, the supernatants were collected for LC-UV analysis. Fenofibrate and its hydrolytic metabolite were detected by LC-UV at 280 nm.

**Fenofibrate hydrolytic kinetics**

The enzymatic kinetics of fenofibrate hydrolysis were analyzed in various tissue preparations (HS9, live cytosol, HLMs, HLuMs) and human recombinant enzymes (hCES1A, hCES2A, hMAGL) through incubation with a range of concentrations of fenofibrate at 37°C in triplicate. After 0.5 h incubation at 37°C, the reaction was quenched by adding ice-cold acetonitrile. Followed by centrifugation (20000 × g for 30 min) at 4°C, the supernate was obtained for LC-UV analysis. The kinetic constants were calculated by fitting the data to the Michaelis-Menten equation using nonlinear least-squares regression as previously reported.
(Zhang et al., 2022).

**Inter-individual variability in CES1A activity and correlation study**

Based on the procedure mentioned above, fenofibrate (10 μM) was incubated with a panel of 16 individual human liver microsomes (HLMs) at 37 °C for 0.5 h. The reaction was terminated by adding 200 μL ice-cold acetonitrile. Followed by centrifugation (20000 × g for 30 min) at 4°C, the supernate was obtained for LC-UV analysis. To assess the hCES1A activities of the 16 individual HLMs, D-luciferin methyl ester (DME), a specific substrate of hCES1A as previously reported was used (Devineni et al., 2015; Wang et al., 2016, 2018). Hydrolytic rates of fenofibrate and DME in each HLM sample were determined and compared to validate the reliability of using fenofibrate as an indicator for monitoring hCES1A activity in HLMs. Linear regression analysis was performed to determine the correlation between the hydrolytic rates of fenofibrate and DME in each HLM sample.

**Nrf2 agonist activities of fenofibrate and fenofibric acid**

The Nrf2 stable transfection HEK293 cells (Genomeditech, Shanghai, China) were grown as a monolayer in DMEM supplemented with 10% FBS and incubated at 37 °C with 5% CO2. The cells were seeded at a density of 2 × 10^4 cells per well in a volume of 100 μL in a 96-well plate and incubated for 24 hours. After that, fenofibrate, fenofibric acid, and the positive control (SFN) were added and further incubated for another 24 hours. Subsequently, the Nrf2 agonist activity was assessed by measuring luciferase activity (Li et al., 2023).

**Results**

**Characterization of the hydrolytic metabolite(s) of fenofibrate**

Firstly, the hydrolytic metabolite(s) of fenofibrate in human plasma and different tissue preparations (such as HLMs, HLuMs, HIMs, and HKMs) were analyzed using both HPLC-UV and UHPLC-Q-TOF-MS/MS. Fenofibrate underwent rapid hydrolysis to yield a stable polar product in both HLMs and HLuMs (Figure 1, Figure 2A and Figure S1). By contrast, a little amount of fenofibrate was found to be hydrolyzed in HIMs and HKMs, while no metabolite was detected in human plasma. The molecular ion ([M+H]^+) of this polar metabolite in positive ion mode was m/z 319.0738 Da, which is 42 Da lower than the...
prototype fenofibrate (m/z 361.1208 Da, [M+H]^+), indicating that the polar metabolite resulted from ester bond cleavage of fenofibrate. The fragmentation ions and retention time of this hydrolytic product were identical to fenofibric acid (Figure S2-S3), suggesting rapid hydrolysis of fenofibrate in human liver and lung tissue preparations. This stimulated further investigation into the key enzymes involved in fenofibrate hydrolysis within these organs.

**Identifying the key enzymes involved in fenofibrate hydrolysis in humans**

To elucidate the key enzymes involved in the hydrolysis of fenofibrate in humans, hydrolytic reaction phenotyping assays and chemical inhibition assays were performed. Firstly, the formation rates of fenofibric acid (the hydrolytic metabolite of fenofibrate) were determined in various hydrolases distributed in humans. As depicted in Figure 2B, hCES1A exhibited a faster rate of hydrolysis towards fenofibrate, while hCES2A and hMAGL also catalyzed this reaction. Subsequently, a panel of specific inhibitors targeting human hydrolases was employed to identify the contributions made by different enzymes to fenofibrate hydrolysis in HLMs and HLuMs. As shown in Figure 2C, BNPP strongly inhibited the hydrolytic metabolism of fenofibrate in HLMs, whereas LPA only slightly suppressed its hydrolysis. Conversely, GA, EDTA, and vildagliptin exhibit negligible impacts on the hydrolysis of fenofibrate in HLMs. Similarly, BNPP almost completely inhibited fenofibrate hydrolysis in HLuMs (Figure 2D), whereas LPA, GA, EDTA and vildagliptin hardly blocked this reaction. These observations clearly suggest that hCES1A plays a crucial role in fenofibrate hydrolysis in both HLMs and HLuMs, while hCES2A and hMAGL have only minor contributions to this process in the human liver.

**Fenofibrate hydrolytic kinetics in various enzyme sources**

Next, the kinetic parameters (K_m and V_max) of fenofibrate hydrolysis were meticulously characterized in various enzyme sources, including tissue preparations (HS9, live cytosol, HLMs, HLuMs), as well as recombinant enzymes (hCES1A, hCES2A, and hMAGL). As shown in Figure 3 and Table 1, fenofibrate hydrolysis in different human tissue preparations followed classic Michaelis-Menten kinetics. The K_m values of fenofibrate hydrolysis in HS9 and live cytosol were 3.75 μM and 4.27 μM, respectively, while the V_max values of fenofibrate hydrolysis in HS9 and live cytosol were determined as 93.56 pmol/min/g protein, and 37.60
pmol/min/g protein, respectively. The $K_m$ values for fenofibrate hydrolysis in HLMs and HLuMs were determined as 8.63 μM and 4.69 μM, respectively, while the $V_{max}$ values of fenofibrate hydrolysis in HLMs and HLuMs were 278.10 pmol/min/g protein, and 10.83 pmol/min/g protein, respectively. As a result, the inherent clearance ($C_{Lin}$) values of fenofibrate hydrolysis in HS9, live cytosol, HLMs and HLuMs, were calculated as 25.3 μL/min/μg protein, 8.81 μL/min/μg protein, 32.24 μL/min/μg protein and 2.31 μL/min/μg protein, respectively. These results unequivocally prove that the liver is the primary organ responsible for fenofibrate hydrolysis in humans.

As depicted in Figure 4, Figure S4 as well as Table 1, fenofibrate hydrolysis in hCES1A, hCES2A and hMAGL also followed classic Michaelis-Menten kinetics. The $K_m$ values of fenofibrate hydrolysis in hCES1A, hCES2A and hMAGL were determined as 9.27 μM, 2.29 μM and 2.25 μM, respectively. The $V_{max}$ values of fenofibrate hydrolysis were 2165 pmol/min/μg protein, 1.95 pmol/min/μg protein and 1.53 pmol/min/μg protein in hCES1A, hCES2A and hMAGL, respectively. Consequently, the $C_{Lin}$ value of fenofibrate hydrolysis in hCES1A was very high (233.55 μL/min/μg protein), while the $C_{Lin}$ values of fenofibrate hydrolysis in hCES2A and hMAGL were determined to be as low as 0.85 μL/min/μg protein and 0.68 μL/min/μg protein, respectively. It was obvious from Table 1 that the inherent clearance of hCES1A-catalyzed fenofibrate hydrolysis is 275-fold higher than that of hCES2A, and 338-fold higher than that of hMAGL. These results clearly demonstrate that hCES1A contributes significantly to the hydrolytic metabolism of fenofibrate in humans, while hCES2A and hMAGL contribute this reaction to a very lesser extent.

**Fenofibrate hydrolytic rates in HLMs agreed well with hCES1A activities**

Next, the metabolic rates of fenofibrate in 16 individual HLMs samples were assessed. As shown in Figure 5A, HLMs samples from 16 individual donors showed different hydrolytic rates towards fenofibrate, with approximately 10-fold variation in the hydrolytic rate. Notably, the metabolic rates of fenofibrate in 16 HLM samples showed a substantial association with the hydrolytic rates of DME (Figure 5B). This finding further confirms that hCES1A plays a crucial role in fenofibrate hydrolysis and suggests that it affects the activation and *in vivo* therapeutic efficacy of fenofibrate in individuals.
Docking simulations

Docking simulations were performed to investigate the interaction mechanisms between fenofibrate and hCES1A, hCES2A, hMAGL, hAchE and hBchE. The results in Figure 6 and Table 2 show that fenofibrate exhibited favorable docking into the catalytic cavities of hCES1A, hCES2A and hMAGL, with low predicted binding affinities of -9.211 kcal/mol, -8.595 kcal/mol and -8.494 kcal/mol, respectively. These results agreed well with the high binding affinities of fenofibrate towards these three enzymes (Table 2). It was also clear from Figure 6 that the distances between the carbonyl carbon of the isopropyl ester on fenofibrate and the Ser in the catalytic triad of hCES1A, hCES2A, and hMAGL were 3.60 Å, 3.79 Å, and 3.73 Å, respectively. These observations show that fenofibrate is a favorable substrate for hCES1A, while it can also undergo hydrolysis by both hCES2A and hMAGL.

It was also found that fenofibrate could be docked into hAchE and hBchE, but the distance between the carbonyl carbon of the methyl propanoic acid isopropyl ester on fenofibrate and the O⁶ atom of Ser in the catalytic triad of either hAchE or hBchE was significantly greater than the catalytic distance (< 5.5 Å), indicating that fenofibrate was difficult to be hydrolyzed in either hBchE or hAchE. These observations align well with experimental results, which demonstrate that two carboxylesterases (hCES1A and hCES2A) and hMAGL are capable of hydrolyzing fenofibrate, while neither hBchE nor hAchE can catalyze its hydrolysis.

Fenofibrate hydrolysis strongly weakens its inhibitory effects against two serine hydrolases

It has been reported that fenofibrate could inhibit some important serine hydrolases including hNotum and hCES2A. In these cases, we tested the inhibitory effects of both fenofibrate and fenofibric acid against both hCES2A and hNotum, while LPA and LP922056 were used as positive inhibitors. As illustrated in Figure 7 and Figure 8, fenofibrate could dose-dependently inhibit both hCES2A and hNotum, with the IC₅₀ values of 0.50 μM (for hCES2A) and 18.4 μM (for hNotum), respectively. By contrast, fenofibric acid exhibited weak inhibitory effects with IC₅₀ values greater than 100 μM for both serine hydrolases (Figure S5). These results clearly suggest that fenofibrate hydrolysis will significantly reduce the inhibitory effects of this agent on serine hydrolases.

Fenofibrate and fenofibric acid showed similar Nrf2 agonist effects
It has also been reported that fenofibrate can resist cellular oxidative stress and attenuate diabetic nephropathy via enhancing the Nrf2 signaling pathway (Liu et al., 2018; Cheng et al., 2020; Li et al., 2021). Herein, the agonist effects of both fenofibrate and fenofibric acid on Nrf2 were tested at cellular levels. As shown in Figure 9, both fenofibrate and its hydrolytic metabolite fenofibric acid showed similar Nrf2 agonist effects at three different doses. These findings provide clear evidence that both fenofibrate and fenofibric acid exhibit similar Nrf2 agonist effects, indicating that the hydrolysis of fenofibrate does not impact its Nrf2 agonist effect in vivo.

Discussion

Fenofibrate is a marketed fibric acid ester derivative that has been used globally to treat dyslipidemia for several decades. Previous investigations have shown that rapid hydrolysis of this agent to release fenofibric acid in humans, but the hydrolytic pathways and involved human enzymes have not been revealed yet. Herein, this research provides a detailed description of the hydrolytic pathway(s) of fenofibrate and key enzymes involved in its hydrolysis in humans for the first time. Our results clearly showed that fenofibrate could be readily hydrolyzed to release fenofibric acid in human liver and lung tissue preparations. In view of the fact that the weight of the human liver is larger than that of the lung and that the yield of microsomal protein obtained from the liver is 10–20 fold greater than that from the lung (Pacifici et al., 1988; Mutch et al., 2007; Doerksen et al., 2021), it is conceivable that the liver is the most important metabolic organ responsible for fenofibrate hydrolysis in humans. Meanwhile, our findings also suggested that hCES1A, hCES2A and hMAGL were involved in the hydrolysis of fenofibrate. Considering that hCES1A is a highly abundant esterase distributed in the liver, the protein level of hCES1A in this organ is about 9.6-fold greater than that of hCES2A (Sato et al., 2012; Boberg et al., 2017). Meanwhile, as listed in Table 1, the inherent clearance of fenofibrate in hCES1A is much higher than that in hCES2A (~275 folds) and in hMAGL (~343 folds). These findings well-explain why LPA (a specific hCES2A inhibitor) shows a very weak inhibitory effect on fenofibrate hydrolysis in both HLMs and HLuMs, and also suggest that hCES1A is a main enzyme involved in fenofibrate
hydrolysis in both HLMs and HLuMs.

One of the most significant hydrolases in the human liver, hCES1A is primarily distributed in the endoplasmic reticulum of hepatocytes and is involved in the hydrolytic metabolism of a wide range of medications that contain ester or amide bond(s) (Mentlein and Heymann, 1984; Redinbo and Potter, 2005; Landowski et al., 2006; Jin, Song, et al., 2022). Previous studies have demonstrated that hCES1A prefers to hydrolyze the esters with a small alcohol group and a large carboxyl group, whereas hCES2A tends to hydrolyze the esters with a large carboxyl group and a small alcohol group (Satoh and Hosokawa, 2006; Hosokawa et al., 2007; Sanghani et al., 2009; Lian et al., 2018). Structurally, fenofibrate is a carboxylic ester bearing a relatively large carboxyl group and a small alcohol group, which aligns with the substrate specificity exhibited by hCES1A. It is well-known that hCES1A predominantly localizes in the human liver, whereas hCES2A primarily resides in the small intestine. In this study, our results unequivocally demonstrated that the liver served as the primary metabolic organ and that hCES1A was the principal enzyme responsible for hydrolysis of hCES1A in humans. Our findings agreed well with the tissue distribution and substrate specificity of hCES1A, suggesting that hCES1A-catalyzed fenofibrate hydrolysis is the predominant metabolic pathway of fenofibrate in humans.

Many previous studies have demonstrated that fenofibrate exerts its anti-dyslipidemia effect mainly by activating PPAR-α. However, fenofibrate could be rapidly hydrolyzed to release its hydrolytic metabolite in vivo and this conversion is complete (Chapman, 1987; Guay, 1999; McKeage and Keating, 2011). Several studies have reported that fenofibric acid (EC₅₀ = 18-20 μM) showed a more potent agonist effect on PPAR-α when compared to fenofibrate (EC₅₀ = ~30 μM) (Willson et al., 2000; Kuwabara et al., 2004; Dietz et al., 2012; Honda et al., 2022). These data suggested that fenofibric acid rather than fenofibrate was the key bioactive substance for the treatment of dyslipidemia effect in vivo. It should also be noted that fenofibrate is not an ideal prodrug for treating dyslipidemia owing to the poor water solubility and low oral availability in humans (McKeage and Keating, 2011; Saurav et al., 2012). In these cases, more water-soluble fenofibrate derivatives, including water-soluble prodrugs should be designed and developed, aiming to find novel and ideal fenofibrate
prodrugs for translational applications. The ideal oral-administrated fenofibrate prodrugs should be well absorbed into the circulatory system, while they can be slowly hydrolyzed by esterases in the human body and then release the active hydrolytic product (fenofibric acid). In view of the fact that the esters of fenofibric acid also displayed a potent PPAR-α agonist effect, more esters of fenofibric acid can be designed and synthesized by medicinal chemists to study the structure-activity (PPAR-α agonist effect) relationships of these derivatives. Meanwhile, the oral bioavailability and hydrolytic kinetics of the esters of fenofibric acids with a potent PPAR-α agonist effect should also be investigated.

The agonistic effects of fenofibrate and its metabolite fenofibric acid on PPAR-α have been reported previously (Willson et al., 2000; Kuwabara et al., 2004; Dietz et al., 2012; Honda et al., 2022). Meanwhile, some non-PPAR mediated biological activities of fenofibrate and fenofibric acid have also been reported (Blanco-Rivero et al., 2007; Chen et al., 2007; Cheng et al., 2020; Li et al., 2021). Notably, it has been reported that fenofibrate could regulate the expression and activity levels of some important hydrolases, such as PON1 in HuH7 cells and MMP9 in macrophages (Ronsein et al., 2016; Gouédard et al., 2003). Fenofibrate could also down-regulate MMP9 in macrophages and its hydrolytic metabolite fenofibric acid also down-regulated MMP9 and inhibited MMP9 enzymatic activity in macrophages (Shu et al., 2000; Rival et al., 2004; Delayre-Orthez et al., 2005). These results have prompted us to further explore the non-PPAR effects of both fenofibrate and its hydrolytic metabolite fenofibric acid in humans. In this study, our findings indicated that fenofibrate was a potent inhibitor of hCES2A (IC$_{50}$ = 0.51 μM) and acted as a moderate inhibitor of hNotum (IC$_{50}$ = 18.4 μM), but its hydrolytic metabolite fenofibric acid displayed very weak inhibitory effects on these two serine hydrolases (IC$_{50}$ > 100 μM). These findings clearly suggest that fenofibrate hydrolysis will strongly reduce its inhibitory effects on both hCES2A and hNotum in vivo.

In summary, our investigation identified the primary metabolic organs and important hydrolases involved in fenofibrate hydrolysis in humans and revealed the differential impact of fenofibrate hydrolysis on its biological activities. According to our research, hCES1A (a predominant serine hydrolase distributed in human liver) was the crucial enzyme responsible
for fenofibrate hydrolysis in humans, whereas hCES2A and hMAGL contributed slightly to this reaction. Further investigations suggested that fenofibrate hydrolysis would significantly reduce the inhibitory effects of this agent on two serine hydrolases (hNotum and hCES2A), while such biotransformation did not affect its Nrf2 agonist effect. All these findings offer a deeper understanding of the hydrolytic pathways of the anti-dyslipidemia agent fenofibrate in humans, which is also helpful for medicinal chemists to develop novel ester prodrugs of fenofibrate with desirable pharmacokinetic behaviors.

Data Availability Statement.

The authors declare that all the data supporting the findings presented in this study are contained within the paper or its supplementary file.

Authorship Contributions

Participated in research design: Ge, Li, Sun, Zhang F, Song YQ and Hou.

Conducted experiments: Li and Zhang Y.

Contributed new reagents or analytic tools: Song LL.

Performed data analysis: Li, Zhang Y and Zhang F

Wrote or contributed to the writing of the manuscript: Ge and Li.
References


Footnotes.

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Figure legend

Figure 1. The hydrolytic pathways of fenofibrate in human liver preparations and human lung preparations.

Figure 2. (A) Reaction phenotyping assays of fenofibrate hydrolysis (10 μM) in the microsomes from human tissues (liver, lung, intestine and kidney, the final concentration used was 50 μg/mL) and human plasma (25% dilution) for 30 min at 37 °C. (B) Reaction phenotyping assays of fenofibrate hydrolysis (10 μM) using various human hydrolases (5 μg/mL, except that of thrombin was 5 NIH/mL) for 30 min at 37 °C. (C) (D) Inhibitory effects of the esterase inhibitors toward fenofibrate hydrolysis in HLMs and HLuMs. All assays were conducted in triplicate, and the data were expressed as mean ± SD.

Figure 3. Michaelis–Menten kinetic plots and the corresponding Eadie-Hofstee plots (as the insert) of fenofibrate hydrolysis in HS9 (A), live cytosol (B), HLMs (C) and HLuMs (D). All assays were conducted in triplicate, while the data were expressed as mean ± SD.

Figure 4. Michaelis–Menten kinetic plots and the corresponding Eadie-Hofstee plots (as the insert) of fenofibrate hydrolysis in hCES1A (A), hCES2A (B) and hMAGL (C). All assays were conducted in triplicate, while the data were expressed as mean ± SD.

Figure 5. (A) The hydrolytic rates of fenofibrate (10 μM) in human liver specimens from 16 individual donors (1 μg/mL). (B) Correlation analysis between the hydrolytic rates of fenofibrate and the hydrolytic rates of DME in individual human liver specimens (n = 16). All assays were conducted in triplicate, and the data were expressed as mean ± SD.

Figure 6. The binding modes and enzyme-substrate interactions of fenofibrate (blue) in hCES1A (A), hCES2A (B) and hMAGL (C).

Figure 7. (A) The dose-inhibition curves of LPA against hCES2A-catalyzed FD hydrolysis. (B) The dose-inhibition curves of fenofibrate against hCES2A-catalyzed FD hydrolysis. Data points were shown as mean ± SD (n = 3).

Figure 8. (A) The dose-inhibition curves of LP-922056 against hNotum-catalyzed OPTS hydrolysis. (B) The dose-inhibition curves of fenofibrate against hNotum-catalyzed OPTS hydrolysis. Data points were shown as mean ± SD (n = 3).
Figure 9. The agonist effects of fenofibrate (FF) and fenofibric acid (FA) on Nrf2. (A) The cells were treated with the Nrf2 agonist SFN (10 μM) or fenofibrate or fenofibric acid (12.5, 25, 50 μM) for 24 hours. Compared with the control group (DMSO), ****P<0.0001, ***P<0.001, **P<0.01, *P<0.1

Table 1. Kinetic parameters of fenofibrate hydrolysis in HLMs, HLuMs, hCES1A, hCES2A and hMAGL.

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (pmol/min/μg protein)</th>
<th>$CL_{int}$ (μL/min/μg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS9</td>
<td>3.75±0.27</td>
<td>93.56±1.93</td>
<td>25.0</td>
</tr>
<tr>
<td>Live cytosol</td>
<td>4.27±0.28</td>
<td>37.6±0.75</td>
<td>8.81</td>
</tr>
<tr>
<td>HLMs</td>
<td>8.63 ± 1.07</td>
<td>278.10 ± 15.05</td>
<td>32.24</td>
</tr>
<tr>
<td>HLuMs</td>
<td>4.69 ± 0.22</td>
<td>10.83 ± 0.15</td>
<td>2.31</td>
</tr>
<tr>
<td>hCES1A</td>
<td>9.27 ± 0.92</td>
<td>2165.00± 94.16</td>
<td>233.55</td>
</tr>
<tr>
<td>hCES2A</td>
<td>2.29 ± 0.11</td>
<td>1.95 ± 0.022</td>
<td>0.85</td>
</tr>
<tr>
<td>hMAGL</td>
<td>2.25 ± 0.19</td>
<td>1.53 ± 0.031</td>
<td>0.68</td>
</tr>
</tbody>
</table>

Table 2. Docking simulations of fenofibrate into the catalytic cavity hCES1A, hCES2A, hMAGL, hAchE and hBchE.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Crystal structure</th>
<th>Affinity (kcal/mol)</th>
<th>Distance (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCES1A</td>
<td>2dr0</td>
<td>-9.211</td>
<td>3.60</td>
</tr>
<tr>
<td>hCES2A</td>
<td>homology modeling</td>
<td>-8.595</td>
<td>3.79</td>
</tr>
<tr>
<td>hMAGL</td>
<td>3pe6</td>
<td>-8.494</td>
<td>3.73</td>
</tr>
<tr>
<td>hAchE</td>
<td>4ey7</td>
<td>-7.846</td>
<td>10.20</td>
</tr>
<tr>
<td>hBchE</td>
<td>1p0i</td>
<td>-7.771</td>
<td>11.20</td>
</tr>
</tbody>
</table>
Fig. 1
Fig. 2

A

B

C

D

V (pmol/min/µg protein)

V (pmol/min/µg protein)

% of control activity

% of control activity

HLMs

HLuMs

Control    BNPP    LPA    GA    EDTA    Vildagliptin

Control    BNPP    LPA    GA    EDTA    Vildagliptin

250 fold

At 250 fold

Human plasma

hCES1A    hCES2A    hMAGL    α-Amylase    nAchE    hBchE    CTLS1    HSA    hAH1    hMotum    Thrombin

(% of control activity)
Fig. 5

A: Hydrolytic rates of Fenofibrate (nmol/min/µg protein) for different HLM samples.

B: Hydrolytic rates of Fenofibrate (nmol/min/µg protein) compared to DME.

R² = 0.92
P < 0.0001
Fig. 7

(A) IC$_{50}$ = 0.40 ± 0.06 µM

IC$_{50}$ = 0.51 ± 0.11 µM
Fig. 8

A

\[ \text{IC}_{50} = 0.49 \pm 0.03 \mu M \]

B

\[ \text{IC}_{50} = 18.4 \pm 2.13 \mu M \]