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Xenobiotica
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ISSN: 0049-8254 (Print) 1366-5928 (Online) Journal homepage: http://www.tandfonline.com/loi/ixen20

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Jinfang Jiang, Xiaoyan Chen & Dafang Zhong

To cite this article: Jinfang Jiang, Xiaoyan Chen & Dafang Zhong (2017): Predominant contributions of carboxylesterase 1 and 2 in hydrolysis of anordrin in humans, Xenobiotica, DOI: 10.1080/00498254.2017.1333658

To link to this article: http://dx.doi.org/10.1080/00498254.2017.1333658

Accepted author version posted online: 22 May 2017.

Predominant contributions of carboxylesterase 1 and 2 in hydrolysis of anordrin in humans

Jinfang Jiang[1,2], Xiaoyan Chen[1,2], Dafang Zhong[1,2*]

Abstract

1. Anordrin (2*α*, 17*α*-diethynyl-A-nor-5*α*-androstane-2*β*, 17*β*-diol diproprionate) is post-coital contraceptive drug that is on the market in China for more than 30 years. This study aims to elucidate enzymes involved in anordrin hydrolysis, and to evaluate the significant role of carboxylesterases in anordrin hydrolysis in humans.

2. Human liver and intestinal microsomes, recombinant human carboxylesterase were selected as enzyme sources. In human liver microsomes, intrinsic clearance was 684 ± 83 μL/min/mg protein, which was considerably higher than the value of intestine microsomes (94.6 ± 13.3 μL/min/mg protein). Carboxylesterase (CES) 1 has more contribution than CES2 in human liver.

3. Inhibition studies were performed using representative esterase inhibitors to confirm esterase isoforms involved in anordrin hydrolysis. Simvastatin strongly inhibited hydrolytic process of anordrin in liver and intestine microsomes, with IC_{50} values of 10.9 ± 0.1 and 6.94 ± 0.03 μM, respectively.

4. The present study investigated for the first time hydrolytic enzyme phenotypes of anordrin. Anordrin is predominantly catalyzed by CES1 and CES2 to generate the main active metabolite, anordiol. Moreover, anordrin and its metabolite anordiol can be altered by esterase inhibitors, such as simvastatin, upon exposure *in vivo*.

Key words: **anordrin; anordiol; carboxylesterase; hydrolysis in humans; drug-drug interaction**

[1] State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 501 Haike Road, Shanghai 201203, China; [2]University of Chinese Academy of Sciences, No.19A Yuquan Road, Beijing 100049, China.

[*]To whom correspondence should be addressed.

Tel/Fax: 86-21-50800738, E-mail: dfzhong@simm.ac.cn

Introduction

Anordrin (2α, 17α-diethynyl-A-nor-5α-androstane-2β, 17β-diol diproprionate) is domestically synthetic, steroid-like estrogen that is used in China as a post-coital contraceptive for more than 30 years (Chih-ping et al., 1976; Gu et al., 1984). Anordrin is also marketed in combination with mifepristone (Sang et al., 1999). Anordrin is a selective estrogen receptor modulator, with both weak estrogenic and antiestrogenic properties (Mehta et al., 1981; Mehta et al., 1982; Chatterton et al., 1989). In cynomolgus monkeys, rapid hydrolysis of dipropionate esters is the main metabolic pathway, resulting in forming of unesterified anordriol (Chatterton et al., 1994) (Figure 1). Anordriol shows 36-fold higher affinity than anordrin for cytosol estrogen receptor of rat uterus *in vitro*, and is the main component responsible for resistance to pregnancy (Gu et al., 1984), thus anordrin can be called as a prodrug for the contraceptive indication. Moreover, α-anordrin shows inhibitory effect on multiple kinds of tumors (Bin et al., 1989; Ma et al., 2000), indicating that anordrin can be developed for variety of potential applications.

Esterases hydrolyze compounds containing ester, amide, and thioester bonds, which contribute to metabolism of more than 10% of therapeutic drugs. In humans, carboxylesterases (CES) are members of serine hydrolase superfamily. CES are mainly categorized into five families: namely CES1, CES2, CES3, CES4A, and CES5A (Holmes et al., 2010). Among these families, CES1 and CES2 reportedly catalyze various drugs, such as imidapril (Takahashi et al., 2008), oseltamivir (Zhu and Markowitz, 2013), and irinotecan (Khanna et al., 2000), and have different tissue distribution, substrate specificity, immunological properties, and gene regulation. CES1 is mainly expressed in liver, whereas CES2 is in intestine (Imai, 2006), however, both share considerable individual differences in expression (Ross et al., 2012). Arylacetamide deacetylase (AADAC) is another serine hydrolase that is highly expressed in livers; this enzyme is responsible for hydrolysis of flutamide (Watanabe et al., 2009), phenacetin (Watanabe et al., 2010), rifamycin (Nakajima et al., 2011)

and others. CES1 generally prefers substrates containing large acyl and small alcohol/amine moieties, whereas CES2 prefers substrates having large alcohol/amine and small acyl moieties, AADAC and CES2 have similar substrate specificity (Fukami and Yokoi, 2012; Fukami et al., 2015).

In previous study by Chatterton et al. on the pharmacokinetics of anordrin in cynomolgus monkeys, dipropionate esters of anordrin were rapidly hydrolyzed to dihydroxy metabolite anordriol after intravenous administration of 0.2 mg per kg anordrin, half-life was about 3.5 min (Chatterton et al., 1994), monoester metabolite was not detected and no other metabolites were found. Conversion also occurred in sample storage, hydrolysis was hypothesized to be mediated by butyrylcholinesterase in serum (Chatterton et al., 1994), however, this assumption was not confirmed, and no more information can be accessed to understand metabolic profile in humans. As shown in Figure 1, anordrin contains large alcohol group and small propionyl moiety, this chemical structure is in accord with substrate specificity of CES2. Therefore, we speculate that CES2 is involved in anordrin hydrolysis in humans. From our previous results, hydrolytic process can be effectively inhibited by simvastatin, suggesting that drug-drug interactions (DDI) may occur to cause efficacy reduction.

Although anordrin is used in China for more than 30 years, its metabolic enzyme phenotype remains to be clarified. Hence, this study aims to identify enzymes involved in anordrin hydrolysis and to evaluate role of CES in such process in humans.

Materials and methods

Chemicals and reagents Anordrin (98% purity) was provided by Shanghai Aoqi Pharmaceutical and Technology Co., Ltd. (Shanghai, China). Radioactive [^{3}H]-anordiol was provided by Shanghai Institute of Applied Physics. (Shanghai, China). Levonorgestrel (LNG) was purchased from TCI (Shanghai, China). Fenofibrate, phenacetin, procaine, loperamide, and bis-*p*-nitrophenyl phosphate (BNPP) were purchased from Sigma-Aldrich (St. Louis, MO). Digitonin and

simvastatin were purchased from Dalian Meilun Biology and Technology Co., Ltd. (Dalian, China). Human liver microsomes (HLM, from Caucasian), human liver cytosol (HLC), and human intestine microsomes (HIM) were purchased from BD Gentest (Woburn, MA). Recombinant human CES1 and CES2 were purchased from Cypex Ltd (10 mg/ml, Scotland, UK), and recombinant human AADAC was purchased from CUSABIO Biotech Co., Ltd. (Wuhan, China). Deionized water (18.2 mΩ, TOC ≤ 50 ppb) was purified using Millipore Milli-Q Gradient Water Purification System (Molsheim, France). All other chemicals were of analytical grade.

Anordrin hydrolase activity

Anordrin hydrolase activities in different enzyme sources were determined as follows: incubation mixture (final volume of 0.1 mL) contained 100 mM potassium phosphate buffer (containing 3.2 mM $MgCl_2$, pH 7.4) and enzyme sources (HLM: 0.1 mg/mL，HIM and HLC: 0.5 mg/mL; recombinant human CES1, CES2, and AADAC: 0.1 mg/mL, and fresh human blood and plasma). Recombinant enzymes were diluted with Tris-HCl buffer (50 mM Tris-HCl buffer, pH 7.4). After 5 min of preincubation at 37°C, reactions were initiated by addition of anordrin (final concentration of 1 μM). Final concentration of organic reagent was < 1% in incubation mixture. After 60 min (10 min for HLM, incubation extended to 4 h in plasma and 6 h in blood) of incubation, reactions were terminated by adding equal volume of ice-cold acetonitrile. Control samples were incubated with thermally inactivated enzymes.

Substrate concentrations of phenacetin, fenofibrate, and procaine were all set to 200 μM. We confirmed linear hydrolytic rates with respect to protein concentrations and incubation time. For phenacetin, enzyme source was 0.4 mg/mL, incubation time was 30 min; for fenofibrate, enzyme sources was 0.05 mg/mL, incubation time was 5 min; for procaine, enzyme sources was 0.1 mg/mL, incubation time was 30 min.

Kinetics of anordrin hydrolysis

The parent depletion method was used to determine kinetics of anordrin hydrolysis in different enzyme sources. In preliminary study, we confirmed linear

depletion rate of anordrin with respect to protein concentrations (<1.5 mg/mL HLM and HIM, <1.0 mg/mL CES1 and CES2) and incubation time (<60 min in HLM and HIM, <2 h in CES1 and CES2). For kinetic analysis, selected final conditions were as follows: HLM : 0.1 mg/mL, 10 min；HIM: 0.2 mg/mL, 40 min；CES1 and CES2: 0.2 mg/mL, 30 min. Anordrin concentration ranged from 0.1 μM to 20 μM. Incubation procedure was similar to method described for anordrin hydrolysis activity. Control samples were incubated with thermally inactivated enzymes. Data are presented as averages of triplicate experiments. Kinetic constants were obtained by fitting Michaelis-Menten equation shown in Equation 1 to experimental data by using nonlinear regression (Prism 5.0; GraphPad Software Inc.):

$$v = V_{max} \times S/(K_m + S) \qquad (1)$$

where v is reaction velocity, V_{max} is maximum velocity, K_m is Michaelis constant (substrate concentration at $0.5V_{max}$), and S is substrate concentration. *In vitro* CL_{int} was calculated as V_{max}/K_m.

Studies on inhibition of anordrin hydrolase activity

To confirm esterase isoform involved in anordrin hydrolysis in humans, we conducted inhibition studies on HIM, HLM, recombinant CES1 and CES2 using representative esterase inhibitors, including digitonin, loperamide, simvastatin, and BNPP. BNPP is nonselective inhibitor of esterases (Watanabe et al., 2009), and digitonin and loperamide specifically inhibit CES1 and CES2 (Shimizu et al., 2014), respectively. Simvastatin strongly inhibits CES1 and CES2 activities (Shimizu et al., 2014). Inhibitors (digitonin, loperamide, and simvastatin: 20 μM; BNPP: 1 mM) were preincubated with different enzyme sources for 5 min at 37°C, and reactions were initiated by adding anordrin solution (final 1 μM). After 30 min (10 min for HLM) of incubation, reactions were terminated by adding equal volume of ice-cold acetonitrile. Control samples were incubated with thermally inactivated enzymes.

Contribution of CES1 and CES2 to anordrin hydrolase activity in HLM

For relative contribution estimation, relative activity factor (RAF) method and

relative expression factor (REF) method are the two commonly used methods. For the former one, RAF was applied as the ratio of activity values to evaluate contribution of CES1 and CES2 to anordrin hydrolysis in HLM. As described previously by Watanabe et al.(Watanabe et al., 2010), RAF values for CES1 ($RAF_{CES1,\ HLM}$) were determined as ratios of probe substrate fenofibrate hydrolase activities in HLM to value of recombinant human CES1. Meanwhile, RAF values for CES2 ($RAF_{CES2,\ HLM}$) were determined as ratios of probe substrate procaine hydrolase activities in HLM to value of recombinant human CES2. The following formlas were used to predict anordrin hydrolase activities by CES1 ($V_{CES1,\ HLM}$), and CES2 ($V_{CES2,\ HLM}$) in HLM:

$$V_{CES1,\ HLM} = V_{rec\text{-}CES1} \times RAF_{CES1,\ HLM} \quad (2)$$

$$V_{CES2,\ HLM} = V_{rec\text{-}CES2} \times RAF_{CES2,\ HLM} \quad (3)$$

$V_{rec\text{-}CES1}$ and $V_{rec\text{-}CES2}$ are anordrin hydrolase activities by recombinant CES1 and CES2, respectively. The following equations were used to calculate contributions of CES1 and CES2 to anordrin hydrolase activities in HLM:

$$\text{CES1 in HLM}(\%) = (V_{CES1,\ HLM}/V_{HLM}) \times 100 \quad (4)$$

$$\text{CES2 in HLM}(\%) = (V_{CES2,\ HLM}/V_{HLM}) \times 100 \quad (5)$$

where V_{HLM} value is observed anordrin hydrolase activities in HLM.

Another method for estimation of esterases to the anordrin hydrolysis in HLM is REF. Instead of simple clearance values used in RAF method, enzyme contents, or protein levels, was taken into consideration. This method has been applied to liver uptake transporters (Hirano et al., 2006). The ratio of the expression level of each esterase in HLM to that in recombinant enzyme system (pmol/mg protein) was defined as R_{exp}. The hydrolytic clearance by each esterase in HLM was separately calculated by multiplying the clearance of anordrin in recombinant enzyme ($CL_{CES,test}$) by R_{exp} as described in the following equation:

$$CL_{HLM,CES1} = CL_{CES1,test} \times R_{exp,CES1} \quad (6)$$

$$CL_{HLM,CES2} = CL_{CES2,test} \times R_{exp,CES2} \quad (7)$$

The relative contribution (%) of each esterase to the hydrolysis in HLM was

defined by the ratio of $CL_{HLM,CES}$ for target esterase to that of the sum of $CL_{HLM,CES}$ for CES1 and CES2. The expression level of each esterase in HLM was calculated based on LC-MS/MS study described by Sato et al.(Sato et al., 2012).

LC-MS/MS bioanalytical method

Calibration curve concentration ranged from 0.500 nmol/L to 1000 nmol/L for anordrin. LNG was used as internal standard. Calibration curve was fitted with linear least-squares regression model ($y = 1/x^2$). Calibration samples were extracted alongside study samples. Aliquots (25 μL) of either calibrant or study samples were added to 1.5 mL polypropylene tube, a total of 25 μL of internal standard solution (LNG, 100 ng/mL) was then added, followed by 150 μL acetonitrile to precipitate proteins. System was vortexed and centrifuged at 11,000 × *g* for 5 min. Finally, the supernatant was injected into LC-MS/MS system.

LC-MS/MS data were acquired using LC-30AD liquid chromatographic system (Shimadzu, Kyoto, Japan) coupled to Triple Quad 5500 mass spectrometer (AB Sciex). Analyst V1.6.2 software (AB Sciex) was used for data processing. Chromatographic separation was conducted on Phenomenex Luna 5u PFP (2) (50 × 2.0 mm I.D., 5 μm), which was maintained at 40°C. Mobile phases used for gradient elution were 40% (solvent A) 5 mM ammonium acetate–formic acid (100/0.1, v/v) and 60% (solvent B) methanol. Initial mobile phase was 60%B for 0.7 min, followed by linear increase to 95% over 0.1 min and then maintained for 1.2 min. Column was equilibrated the with initial mobile phase. The total run time was 3.0 min, and flow rate was 0.50 mL/min. Anordrin and LNG exhibited peak retention times of 2.5 and 2.0 min (Figure 2), respectively. Mass spectrometer equipped with electrospray ionization source was operated in positive multiple reaction monitoring mode. Ion spray voltage and source temperature were set to 5,500 V and 500°C, respectively. Nebulizer gas, heater gas, curtain gas, and collision-activated dissociation gas were optimized at 50, 50, 30, and 9 psi, respectively. Samples were detected using multiple reaction monitoring, and parent-to-product transitions were as follows: *m/z*

456.3→291.2 for anordrin, collision energy (CE) 15 eV; LNG *m/z* 313.2→245.2, CE 18 eV; fenofibrate hydrolysis metabolite *m/z* 319.1→233.0, CE 30 eV; procaine hydrolysis metabolite *m/z* 138.1→120.0, CE 17 eV; phenacetin hydrolysis metabolite *p*-phenetidine *m/z* 138.1→110.1, CE 21 eV.

Results

Anordrin hydrolase activity

Chatterton et al. have found that after intravenous administration of radioactive [3-^{14}C]anordrin to monkeys, the majority of the radioactivity in plasma after 15 min was found as metabolite [^{14}C]anordiol (Chatterton et al., 1994), i.e., the propionate esters were cleaved. The monoesters were not present in detectable amounts (Chatterton et al., 1994). After 4 h, the radioactivity in plasma was only formed as [^{14}C]anordiol (Chatterton et al., 1994) in radiochromatogram. It suggested that the major metabolic pathway *in vivo* is hydrolysis to the main metabolite anordiol.

In addition, our laboratory has been testing a compound A (Figure 4), an analogue of anordrin, which only differs in ester bonds: the side chain of ester of compound A contains 4 carbons (Figure 4). After radioactive [^{3}H]A was orally given to ICR mice, the radioactivity in plasma was mainly formed as [^{3}H]A and [^{3}H]anordiol，the sum of them accounted for >90% of total radioactivity in plasma, while [^{3}H]anordiol accounted for >75% of total radioactivity. Besides, in HLM incubation system, [^{3}H]anordiol was no longer metabolized to other substances. These results suggested that the main metabolic pathway of the two compounds were hydrolysis to anordiol, and the hydrolytic metabolite anordiol was difficult to convert to other metabolites.

In the present study, anordiol was the only detected metabolite after incubation with different enzyme sources under our experimental conditions. The monopropionate ester of anordrin was not detectable under this experimental condition, it is probably due to the rapid conversion to the dihydroxy compound, anordiol. Figure 3 shows MS fragmentation profiles. *m/z* 456 and *m/z* 344 were parent

ions of anordrin and anordiol, respectively, adducted with NH_4^+, and *m/z* 291 was typical fragment of steroidal structure. Meanwhile, owing to low ionization efficiency of anordriol, detection sensitivity was not reached in LC-MS/MS (low limit of detection ~ 300 nM). Because the depletion of anordrin represents formation of metabolite anordiol, thus parent drug depletion method was used to determine hydrolase activity of anordrin in various enzyme sources.

Probe substrates, including fenofibrate, procaine, and phenacetin were used as markers to confirm hydrolase activities of these enzyme sources. Hydrolytic rates of three substrates were 92.8, 3.34, and 0.0504 nmol/min/mg protein in recombinant CES1, CES2, and AADAC, respectively, with values of 213, 4.01, and 0.102 nmol/min/mg protein in HLM, respectively (Table 2).

Anordrin is stable in human blood for 6 h and human plasma for 4 h (Figure 5). Therefore, cholinesterase, paraoxonase, and albumin esterase were not assumed to be involved in anordrin hydrolysis. Hydrolytic rate in HLM was approximately 500 pmol/min/mg protein, showing highest catalytic efficiency and considerably higher values in HIM and HLC (Figure 5). CES1 and CES2 had comparable hydrolytic rates (approximately 150 pmol/min/mg protein), whereas AADAC was proven not to be responsible for anordrin hydrolysis.

Kinetics of anordrin hydrolysis

Hydrolase activity was detected through parent drug depletion, and data for activities followed Michaelis-Menten equation (Figure 6). In HLM and HIM, the K_m values were 2.83 ± 0.52 and 2.24 ± 0.52 μM, respectively, indicating comparable enzyme affinity. Calculated *in vitro* intrinsic clearances were 684 ± 83 and 94.6 ± 13.3 μL/min/mg protein in HLM and HIM, respectively. This study is the first to report on kinetics of anordrin hydrolysis in HLM and HIM, and results showed that liver has higher contribution to anordrin hydrolysis than intestine. CES1 and CES2 possessed CL_{int} values of 117 ± 13 and 197 ± 17 μL/min/mg protein, respectively. Table 1 presents detailed Michaelis-Menten constants.

Inhibition studies on anordrin hydrolase activity

To evaluate contribution of each esterase to anordrin hydrolysis in human intestine and liver, we investigated effects of various chemical inhibitors on anordrin hydrolase activities (Figure 7). Nonspecific esterase inhibitor BNPP efficiently inhibited anordrin hydrolysis in different enzyme sources. Digitonin and loperamide are specific inhibitors of CES1 and CES2 (Shimizu et al., 2014), respectively, effectively inhibiting anordrin hydrolysis in recombinant enzymes. However, these drugs did not show inhibitory effects on HLM and HIM, it is probably because HLM is a mixture of CES1 and CES2, and the weak inhibitory potency of loperamide toward CES2 in HIM might be caused by the interference of some proteins. Simvastatin is an effective esterase inhibitor according to several studies (Shimizu et al., 2014; Wang et al., 2015). This finding is consistent with our study, which showed that simvastatin strongly inhibited anordrin hydrolysis both in HLM and HIM at IC_{50} values were 10.9 ± 0.1 and 6.94 ± 0.03 μM (Figure 8), respectively. Although plasma concentration of simvastatin was relatively low (Ahmed et al., 2013), liver concentration was much higher; for example, liver-to-plasma concentration ratio was approximately 10 to 20 in wild-type mice (Higgins et al., 2014), indicating that effect of exposure of anordrin can be changed by esterase inhibitors, such as simvastatin.

Contribution of CES1 and CES2 to anordrin hydrolase activity in HLM

To estimate contribution of CES1 and CES2 to anordrin hydrolase activity in HLM, RAF and REF values were calculated. For the RAF method, in HLM and CES1, values of fenofibrate hydrolase activity were 213 and 92.8 nmol/min/mg protein, respectively, and calculated $RAF_{CES1,\ HLM}$ was 2.30. In HLM and CES2, values of procaine hydrolase activity were 4.01 and 3.34 nmol/min/mg protein, respectively, and calculated $RAF_{CES2,\ HLM}$ was 1.20. Observed $V_{rec\text{-}CES1}$ and $V_{rec\text{-}CES2}$ were 123 and 137 pmol/min/mg protein, respectively. Thus, by using Equations (2) and (3), calculated $V_{CES1,\ HLM}$ and $V_{CES2,\ HLM}$ were 283 and 164 pmol/min/mg protein, respectively. Finally, the contribution ratios of CES1 and CES2 in HLM were

estimated according to Equations (4) and (5), resulting in CES1 and CES2 values of 59.4% and 34.6%, respectively (Table 2).

For the REF method, according to Sato's research, in pooled HLMs, the overall averages of quantified concentrations of CES1 and CES2 were 363 and 22.2 pmol/mg protein (Sato et al., 2012). In this study, concentration of pooled HLMs was 0.1 mg/mL, while concentration of CES1 or CES2 in the samples was 0.2 mg/mL. Molecular weights of CES1 and CES2 are about 60 kDa, 1 mg recombinant CES1 or CES2 protein contains 16670 pmol moleculs. $R_{exp,CES1}$ and $R_{exp,CES2}$ were calculated to be 0.01089 and 6.66×10^{-4}, respectively. $CL_{CES1,test}$ and $CL_{CES2,test}$ was the observed value of 117 and 197 μL/min/mg protein (Table 1), respectively. By the Equations (6) and (7), the final relative contribution of CES1 and CES2 in HLM was estimated to be 90.7% and 9.3%, respectively.

For relative contribution estimation, REF method based on enzyme contents (or protein levels) may be more accurate than the RAF method based on simple clearance values.

Discussion

As increasing number of drugs are designed to avoid metabolism by CYP450, we witnessed more in-depth research into esterases in humans, indicating their important role in drug ADME/T evaluation. In present study, human blood and plasma, liver microsomes, and intestine microsomes were selected as matrices, because they contain different esterase subtypes. Butyrylcholinesterase, paraoxonase, and albumin esterase are present in human plasma, whereas CES is absent (Li et al., 2005). Human liver and intestine are main tissues responsible for drug metabolism and contain abundant CES activities; human liver microsomes (HLM) is also standard matrix for investigation of drug hydrolytic properties in humans (Watanabe et al., 2009; Watanabe et al., 2010). In present study, after incubation with human primary hepatocytes (data not shown), HLM, and HIM, anordiol was the only detected metabolite (Figure 3). Anordrin was rapidly hydrolyzed in HLM (approximately 500

pmol/min/mg protein) but was stable in human blood and plasma (Figure 5). Hydrolytic rate in HIM was about 20% of value for HLM. We further observed that CES1 and CES2 were involved in anordrin hydrolysis, but not AADAC (Figure 5).

Chatterton et al.(Chatterton et al., 1994) and our experiments on the radioactive compounds have proved that the main metabolic pathway of anordrin was hydrolysis to anordiol. Thus the parent depletion method was used to determine kinetics of anordrin hydrolysis in different enzyme sources. We performed kinetic analyses of anordrin hydrolase activity by HLM, HIM, recombinant human CES1, and CES2 (Figure 6), showing that highest catalytic potency was detected in HLM (Table 1). Obtained K_m value in HLM was similar to that in HIM (2.83 ± 0.52 versus 2.24 ± 0.52 μM), with CL_{int} values of 684 ± 83 and 94.6 ± 13.3 μL/min/mg protein, respectively. These findings indicate that liver plays more important role than intestine in anordrin hydrolysis. CES1 and CES2 kinetics show relatively similar enzyme affinity (13.6 ± 2.8 versus 16.9 ± 3.0) and comparable intrinsic clearance (117 ± 13 versus 197 ± 17 μL/min/mg protein), suggesting that CES1 and CES2 play important role in anordrin hydrolysis both in human liver and intestine. By using equations for RAF values of each enzyme, contribution of CES1 and CES2 was 59.4% and 34.6%, respectively. While by the REF method, the estimated contribution of CES1 and CES2 was 90.7% and 9.3%, respectively. For relative contribution estimation, REF method based on enzyme contents (or protein levels) may be more accurate than the RAF method based on simple clearance values. These results suggest that CES1 and CES2 are the predominant enzymes responsible for anordrin hydrolysis in humans, with CES1 having more contribution than CES2 in liver.

Aside from liver, the small intestine is also an important organ involved in the first-pass metabolism after oral dosing. Although contribution of the liver to anordrin hydrolysis was estimated to be higher than that of intestine, small intestine is logically important for hydrolysis. Thus, direct conversion of anordrin to anordiol possibly occur within intestine because of high expression of CES2 in human intestine

epithelial cells. Remaining parent compound is probably hydrolyzed by hepatic esterases.

We performed inhibition studies by using several inhibitors to estimate contribution of CES to anordrin hydrolysis (Figure 7). CES1 specific inhibitor digitonin and CES2 specific inhibitor loperamide did not exert any inhibitory effect on anordrin hydrolysis in HLM and HIM, which could be explained as the compensation effect of CES1 and CES2 in HLM, while the weak inhibitory potency of loperamide toward CES2 in HIM might be caused by the interference of some proteins. The combination of specific inhibitors (digitonin and loperamide) in HLM and HIM did not produce certain degrees of inhibition. In recombinant CES1 and CES2, the two specific inhibitors effectively inhibited anordrin hydrolysis (approximately 60% inhibition, Figure 7). Moreover, simvastatin strongly inhibited anordrin hydrolysis both in HLM and HIM, with estimated IC_{50} values of 10.9 ± 0.1 and 6.94 ± 0.03 μM, respectively. Concentration in humans is relatively low (Ahmed et al., 2013), but simvastatin is possibly concentrated in human liver (Higgins et al., 2014), this finding suggests that DDI may occur *in vivo* between anordrin and esterase inhibitors, such as simvastatin.

The hydrolytic metabolite anordiol is the active compound for the termination of early pregnancy, thus the parent drug anordrin can be called as a prodrug. Hydrolysis is the bioactivation pathway in humans, and that this hydrolytic pathway is directly related with *in vivo* exposure of active metabolite anordiol and pharmacological efficacy. In this study, we found that some esterase inhibitors played some inhibition effects on anordrin hydrolysis. Not limited to simvastatin, if anordrin is coadministered with such inhibitors, the production of anordiol can be reduced, which could lead to contraceptive failure. While side effects are also associated with the varying exposures of parent drug and metabolite *in vivo*.

This study is the first to report on anordrin hydrolytic kinetics in HLM, HIM, and recombinant enzymes. Enzyme phenotype was further investigated by using

recombinant enzymes and specific esterase inhibitors. In conclusion, we discovered that anordrin is predominantly catalyzed by CES1 and CES2 in human liver and intestine to generate the main metabolite, anordiol. Kinetic analyses of anordrin hydrolase activity by HLM, HIM, recombinant human CES1 and CES2 were further performed. We hypothesized that DDI toward anordrin can be induced *in vivo* by esterase inhibitors, such as simvastatin. This finding should be considered in clinical applications.

ABBREVIATIONS

CES1, carboxylesterase 1; CES2, carboxylesterase 2; AADAC, arylacetamide deacetylase; HLM, human liver microsomes; HIM, human intestine microsomes; HLC, human liver cytosol; LC-MS/MS, liquid chromatography–tandem mass spectrometry; BNPP, bis-*p*-nitrophenyl phosphate；RAF, relative activity factor; LNG, levonorgestrel; DDI, drug-drug interactions.

Acknowledgments

The study was supported by the National Natural Science Foundation of China (grant number 81521005).

Author contribution

Jinfang Jiang and Dafang Zhong are responsible for the research design; Jinfang Jiang conducted experiments; Dafang Zhong and Xiaoyan Chen contributed new reagents or analytical tools; Jinfang Jiang and Dafang Zhong performed data analysis and wrote this manuscript.

Disclosure

The authors report no conflicts of interest in this work.

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Titles and legends to figures:

Figure 1. Chemical structures of anordrin and its hydrolysis metabolite anordiol.

Figure 2. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) of anordrin (upper trace) and internal standard LNG (lower trace) for the accurate quantification of anordrin in different matrics. LNG: levonorgestrel.

Figure 3. Product ions of anordrin and its hydrolysis metabolite anordriol. *m/z* 456 and *m/z* 344 were parent ions adducted with NH_4^+ of anordrin and anordriol, respectively.

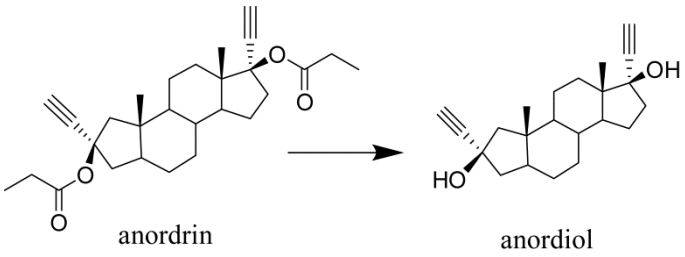
Figure 4. Chemical structure of compound A and [^{3}H]anordiol.

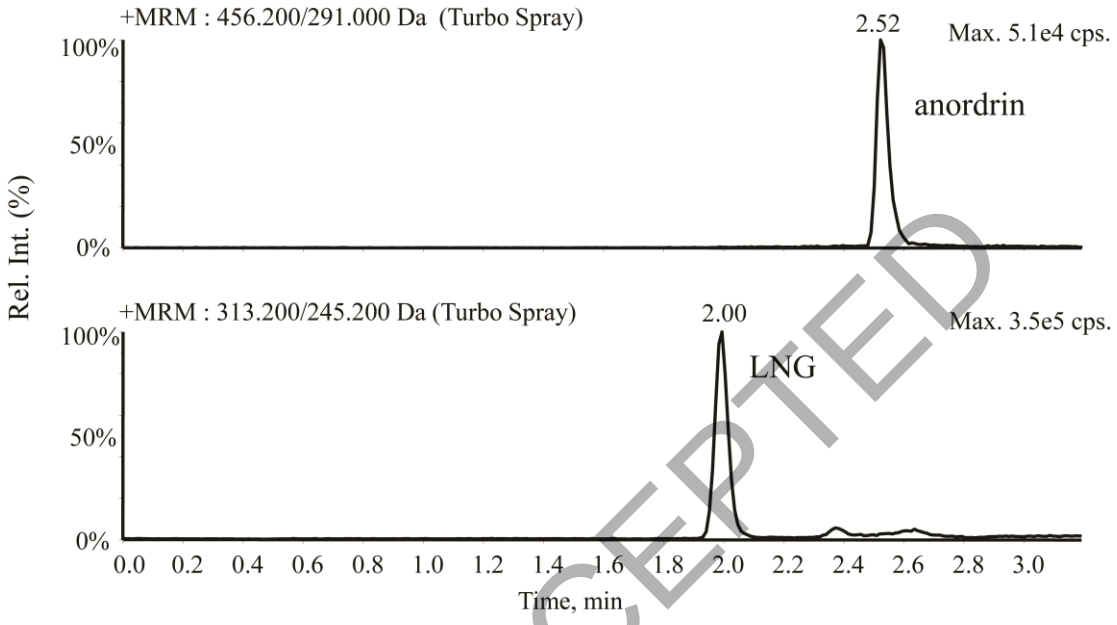
Figure 5. Hydrolase activity of anordrin in different enzyme sources. Each column represents the mean ± S.D. of triplicate determinations. HLM: human liver microsomes; HIM: human intestine microsomes; HLC: human liver cytosol; CES: carboxylesterase; AADAC: arylacetamide deacetylase.

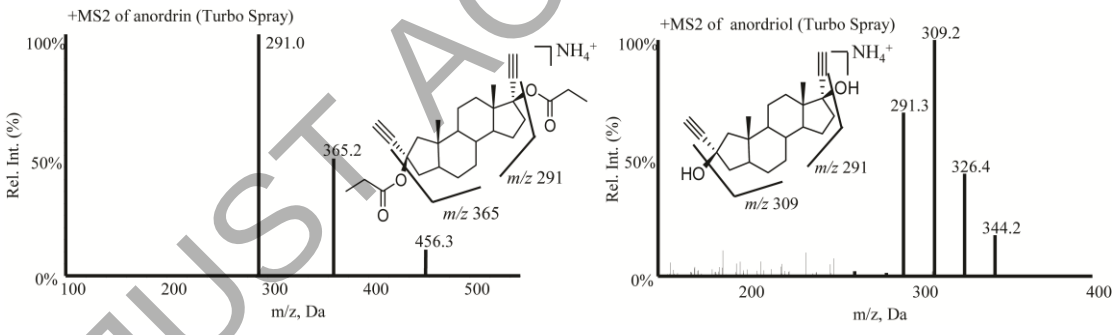
Figure 6. Kinetic profiles for the substrate depletion of anordrin in HLM and HIM (left), recombinant human CES1 and CES2 (right). The kinetic parameters were estimated from the fitted curve using the computer program GraphPad designed for nonlinear regression analysis. Each data point represents the mean ± S.D. of triplicate determinations.

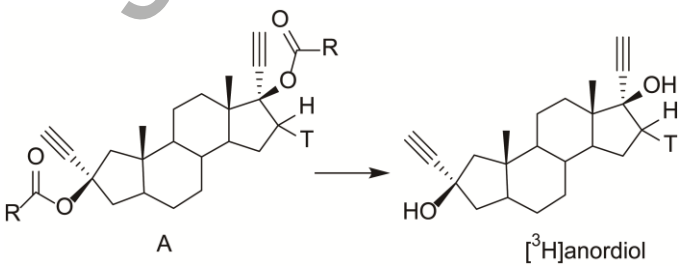
Figure 7. Inhibitory profile of chemical inhibitors against anordrin hydrolase activity in different enzyme sources. Each column represents the mean ± S.D. of triplicate determinations. $^{**}p < 0.01$.

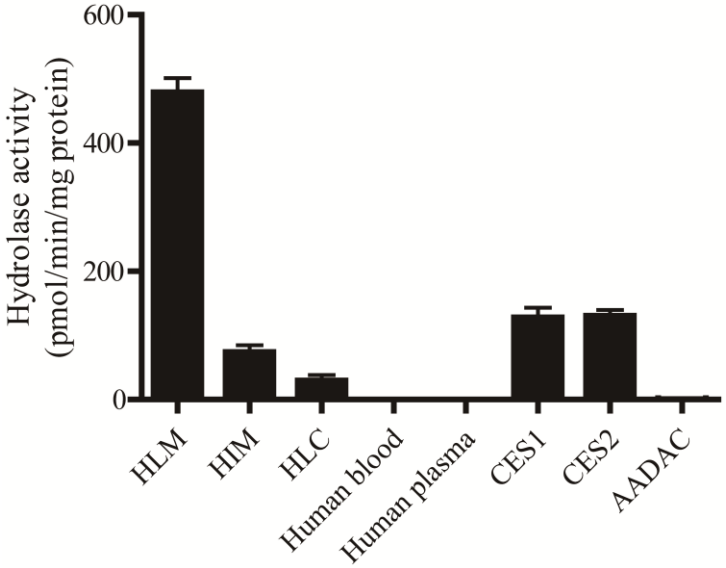
Figure 8. The inhibitory effect of simvastatin on anordrin hydrolysis catalyzed by carboxylesterase in HLM and HIM. Data are presented as the mean ± SD of triplicate determinations. The calculated IC_{50} were 10.9 ± 0.1 and 6.94 ± 0.03 μM in HLM and HIM, respectively.

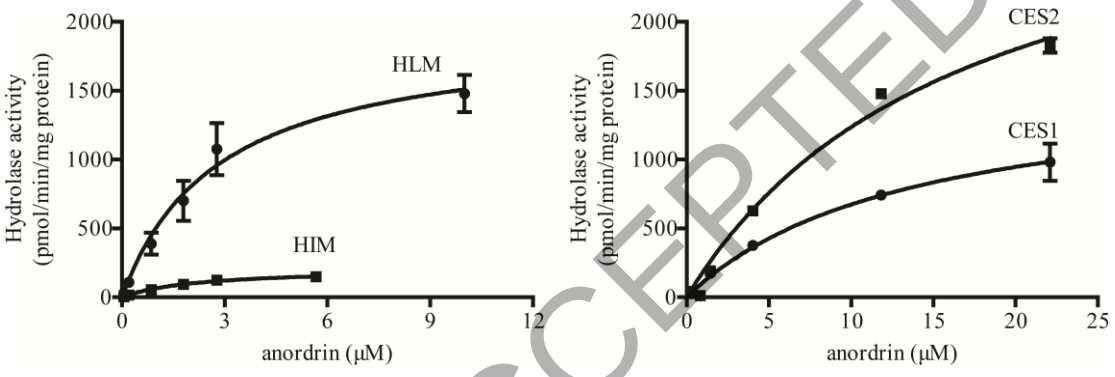


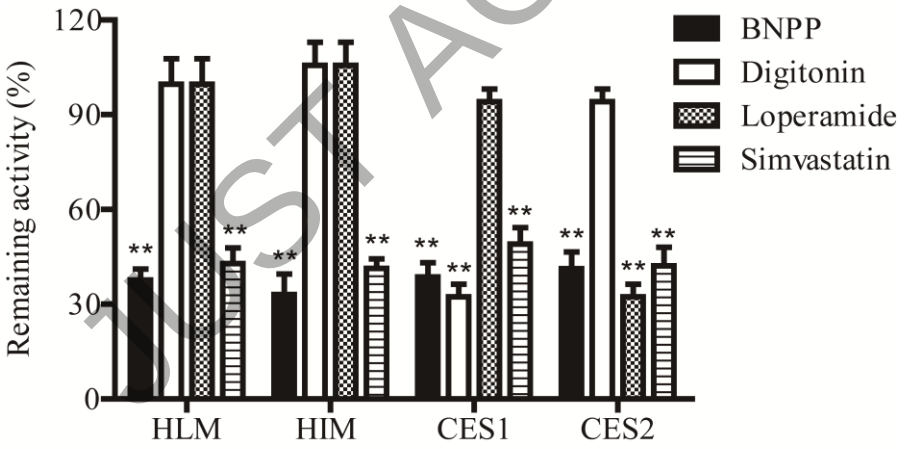












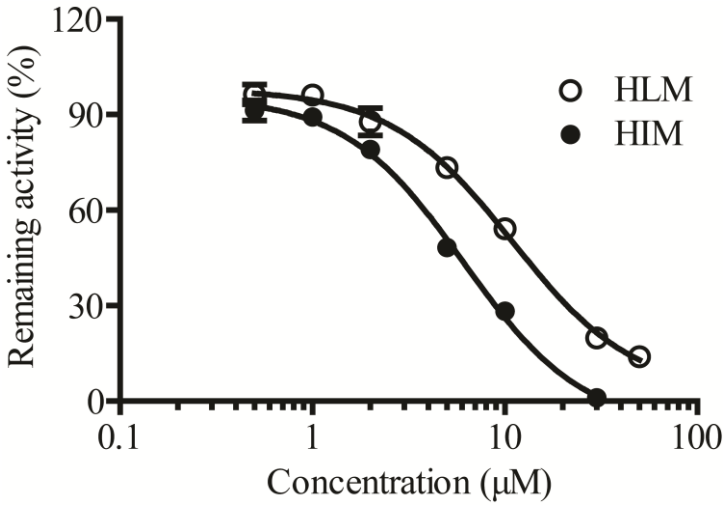


Table 1. Michaelis-Menten constants obtained in HLM, HIM, recombinant human CES1 and CES2. Data are represented as the mean ± S.D. of triplicate determinations.

Enzyme Source	K_m *(μM)*	V_{max} *(pmol/min/mg protein)*	CL_{int} *(μL/min/mg protein)*
HLM	2.83 ± 0.52	1936 ± 149	684 ± 83
HIM	2.24 ± 0.52	212 ± 21	94.6 ± 13.3
CES1	13.6 ± 2.8	1590 ± 163	117 ± 13
CES2	16.9 ± 3.0	3321 ± 312	197 ± 17

Table 2. RAF values calculated from the marker activity and the contributions of CES1 and CES2 to anordrin hydrolysis in HLM.

Enzyme Source	Hydrolase Activity				RAF		Contribution	
	Fenofibrate	Procaine	Phenacetin	Anordrin	CES1	CES2	CES1	CES2
	nmol/min/mg	*nmol/min/mg*	*pmol/min/mg*	*pmol/min/mg*	%	%	%	%
CES1	92.8			123				
CES2		3.34		137				
AADAC			50.4	0.721				
HLM	213	4.01	102	476	2.30	1.20	59.4	34.6
HIM				54.5				