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Species Differences in Carboxylesterases Among Humans, Cynomolgus Monkeys, and Mice in the Hydrolysis of Atorvastatin Derivatives

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ABSTRACT

Nonclinical trials are crucial for assessing pharmaceutical efficacy and safety prior to clinical trials. However, disparities in drug metabolism between humans and animals complicate extrapolating animal data to humans. Variability in drug-metabolizing enzymes, such as carboxylesterases (CESs), contributes to differences in drug kinetics. This study aimed to explore species disparities in CES substrate specificity among humans (hCES1), mice (mCES1), and cynomolgus monkeys (mfCES1) using diverse atorvastatin ester derivatives. This study measured hydrolysis rates of 30 atorvastatin derivatives. Metabolites were identified via HPLC with an internal standard, measuring rates per unit time and enzyme amount. Enzyme metabolic activity was compared using hydrolysis rates. The structure of the alkoxy group resulted in differences ranging from approximately half to 8.97-fold between hCES1 and mCES1 and differences ranging from similar to 15.82-fold between hCES1 and mfCES1. Caution is warranted when extrapolating animal data to humans, especially for esters with diverse structures. Our focus on the alkoxy group structure highlights its impact on hydrolysis rates. Further investigation into species differences among CES enzymes is essential for accurate translational research.

1 | Introduction

Pharmaceuticals undergo clinical trials after confirming their efficacy and safety in nonclinical tests conducted on animals. However, there are species differences in pharmacokinetics between humans and experimental animals. Extrapolating data obtained from experimental animals to humans may be challenging. There are significant species differences in drug-metabolizing enzymes, leading to substantial variations in pharmacokinetic parameters.

Mammalian carboxylesterases (CESs, EC 3.1.1.1), comprising a multigene family, catalyze the hydrolysis of compounds containing ester, amide, and thioester bonds (Hosokawa 2008; Imai et al. 2006). CESs play a crucial role in the detoxification of pharmaceuticals, environmental chemicals, and endogenous compounds, as well as in the activation of metabolism (Hosokawa et al. 1990; Wang et al. 2018). CESs are categorized into CES1–CES7 based on amino acid sequence homology, with most being CES1 or CES2 in mammals (Ni et al. 2011).

Species differences in CESs have been identified (Table 1) (Hosokawa 2008). The organ distribution shows that human CES1 (hCES1) is predominantly expressed in the liver and lungs, whereas human CES2 (hCES2) is predominantly expressed in the small intestine and kidneys (Satoh and Hosokawa 1998). In mice, mouse CES1 (mCES1) is predominantly expressed in the liver, lungs, and kidneys, whereas mouse CES2

TABLE 1 | Tissue-specific expression profiles of CES1 isozymes in mammals (Hosokawa 2008).

Species	Liver	Small intestine	Kidney	Lung
Mouse	+++	−	+++	+++
Monkey	+++	++	−	NT
Human	+++	−	+	+++

Abbreviations: −, undetectable; +, weakly expressed; ++, moderately expressed; +++, strongly expressed; NT, not tested.

(mCES2) is predominantly expressed in the small intestine, kidneys, and liver (Hosokawa et al. 2007). In the cynomolgus monkey *Macaca fascicularis*, *Macaca fascicularis* CES1 (mfCES1) is predominantly expressed in the liver, and mfCES2v1 is predominantly expressed in the jejunum and kidneys (Uno et al. 2014; Williams et al. 2011). CESs contribute to hydrolytic activity in each organ, with distinct substrate specificities between CES1 and CES2. The substrate specificity of hCES has been investigated using pharmaceuticals and environmental compounds. Substrates easily hydrolyzed by hCES1 include oseltamivir (Shi et al. 2006), cocaine (methyl ester moiety) (Pindel et al. 1997), temocapril (Geshi et al. 2005), meperidine (Zhang et al. 1999), and methylphenidate (Sun et al. 2004). Conversely, substrates easily hydrolyzed by hCES2 include cocaine (benzoyl ester moiety) (Pindel et al. 1997), CPT-11 (Kojima et al. 1998), and heroin (Pindel et al. 1997). These findings suggest that hCES1 recognizes structures with acyl groups that are bulkier than alkoxy groups, whereas hCES2 is more likely to recognize substrates with acyl groups that are smaller than alkoxy groups. Furthermore, it has been reported that the rates of hydrolysis by CES1 and CES2 are influenced by the steric hindrance and electronic density of synthesized propranolol esters (Imai et al. 2006), atorvastatin esters (Mizoi et al. 2020), and indomethacin esters (Takahashi et al. 2021), not only commercial products.

Despite the elucidation of substrate specificity differences between hCES1 and hCES2, disparities in substrate specificity among animals in CES1 remain incompletely understood. Differences in substrate specificity from human carboxylesterase have been reported in some experimental animals (Bahar et al. 2012; Honda et al. 2021; Hosokawa et al. 2007; Uno et al. 2014; Yoshida et al. 2018). Commercially available compounds were used as substrates in previous studies, and more detailed substrate specificity investigations have been limited (Di 2019; Jin et al. 2022). The CESs hCES1, mCES1, and mfCES1 have different organ distributions, but these CESs are all expressed in the liver. In this study, we selected mCES1 (GenBank: AB023631) from mice and mfCES1v1 (GenBank: KJ922597) from cynomolgus monkeys based on their biological relevance. mCES1 is a major CES1 isozyme in the mouse liver and is known to be induced by bis(2-ethylhexyl) phthalate (DEHP), making it a suitable model for studying CES1 regulation (Furihata et al. 2004). Furthermore, mCES1 shares 99.84% amino acid sequence identity with the previously identified Ces1d (GenBank: BC019198), suggesting that the two enzymes are nearly identical (Dolinsky et al. 2001). Similarly, genome analysis has identified three CES1 (v1–v3) and three CES2 (v1–v3) genes in cynomolgus monkeys, with CES1v1 being the primary isozyme expressed in the liver (Uno et al. 2014). Given its similarity to human CES1, mCES1 and CES1v1 were considered the most appropriate isoforms for comparison. Mice are commonly used as model animals for drug metabolism research, providing foundational data for understanding human metabolism. Cynomolgus monkeys have biological characteristics closer to humans and serve as valuable models for mimicking human metabolic processes. Elucidating the differences in substrate specificity of enzymes responsible for liver metabolism is crucial for predicting the metabolic rates of drugs and first-pass effects. Therefore, we attempted to clarify the species differences in CESs in drug metabolism.

To develop prodrugs with higher membrane permeability, we chose atorvastatin as the substrate due to its moderate half-life of 14 h and bioavailability of 14%. We synthesized atorvastatin prodrugs, evaluated their metabolic activation using human liver and small intestine microsomes, and reported an efficiently hydrolyzed prodrug model in a liver microsome solution (Mizoi et al. 2020, 2016). Using atorvastatin prodrug models with various structural and electronic features that were synthesized during this research, we investigated substrate specificity in CES1 for humans, mice, and cynomolgus monkeys with a focus on its significant contribution to hepatic hydrolysis. To explore species differences, we conducted a comparative study of hydrolytic abilities using CESs.

2 | Materials and Methods

2.1 | Reagents

Thirty types of atorvastatin esters synthesized by our group were used. These atorvastatin esters were confirmed for purity (> 95%) using ^{1}H-NMR. Hydrolysis data for hCES1 were obtained from a previous report (Mizoi et al. 2020). Butyl *p*-hydroxybenzoate (BPHB), dimethyl sulfoxide (DMSO), and ECOS Competent E. coli JM109 were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Plasmid DNA was cloned in our laboratory. Human embryonic kidney 293 cells (HEK293 cells) were purchased from the Human Science Foundation (Tokyo, Japan). A QIAGEN Plasmid Midi Kit was purchased from QIAGEN N.V. (Venlo, Netherlands). Opti-MEM was purchased from Thermo Fisher Scientific (MA, USA). TransIT-293 was purchased from Mirus Bio LLC (Madison, USA).

2.2 | Preparation of Recombinant CES

Mouse mCES1 cDNA (GenBank: AB023631) (Furihata et al. 2004) and cynomolgus mfCES1 cDNA (GenBank: KJ922597) (Uno et al. 2014) were inserted into pTARGET mammalian expression vectors (mCES1/pTARGET and mfCES1/pTARGET). The parental HEK293 cells were cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin in an atmosphere of 5% CO_2 and 95% air at 37°C. The HEK293 cells were

cultured at 5×10^5 cells/well in a 6-well plate. The following day, the medium was removed from the 6-well plate and the cells were washed with PBS(−). Then, 2.5 mL/well of DMEM (FBS(+), Pen/St(−)) medium was added. Next, 2.5 μL (100 ng/μL) of purified plasmid DNA and 7.5 μL of TransIT-293 were added to 250 μL of Opti-MEM (serum-free medium), mixed, and incubated for 30 min at room temperature. TE buffer was added to control cells instead of plasmid DNA. A mixture of plasmid DNA (mCES1/pTARGET or mfCES1/pTARGET) and TransIT-293 was dropped into a 6-well plate and incubated for 48–72 h at 37°C in a 5% CO_2 incubator. Cells were then collected and stored at −80°C until used in experiments. Cells were diluted with sucrose (250 mM), EDTA-2Na (10 mM), and Tris (10 mM) (SET) buffer (pH 7.4). The obtained cell lysate was measured for protein concentration using the Lowry method.

2.3 | PNPA Activity

The standard hydrolytic activity of the cell lysate was measured using *p*-nitrophenyl acetate (PNPA) as a substrate. A substrate stock solution of PNPA (111 mM) was prepared in acetonitrile. The incubation mixture (final volume of 1.0 mL) consisted of 10 mM Na/K phosphate buffer (pH 7.4) and 1 mM PNPA (resulting in a final acetonitrile concentration of 1.0%) at 30°C. An enzyme source (0.05 mg/mL) was added to the mixture. The PNPA hydrolase activity was determined spectrophotometrically by measuring the formation of *p*-nitrophenol (ε = 16,400 M^{-1} cm^{-1}) at 405 nm and 30°C over 3 min. Data were recorded every 5 s using an Ultrospec 6300 pro.

2.4 | Staining for Esterase Activity

Staining for esterase activity was performed according to native polyacrylamide gel electrophoresis (PAGE) as previously described (Taketani et al. 2007). Briefly, the enzyme solution was loaded onto a 7.5% polyacrylamide gel. After electrophoresis at 100 mA for 150 min, the bands containing esterase were stained with a complex mixture of the dye fast blue RR salt and β-naphthol, generated by hydrolysis of β-naphthyl acetate, a substrate of esterase.

2.5 | Hydrolysis Reaction in CES

The atorvastatin ester dissolved in DMSO was combined with a solution of HEPES and CES. The final concentrations based on Vmax data were 0.3 mM for the ester, 188.8 mM for HEPES, and 0.25–0.5 mg/mL for CES. Preliminary experiments confirmed that the enzyme concentration and reaction time within these ranges produced a linear response. The mixture underwent a reaction at 37°C for 30–120 min. Then, 0.3 mM BPHB (100 μL) in acetonitrile was added to the reaction mixture, and the reaction mixture was chilled on ice for 10 min. The reaction mixture underwent centrifugation at 4°C, 21,600×g for 15 min, followed by filtration and analysis using HPLC.

2.6 | HPLC Conditions

HPLC analysis was performed using a Mightysil RP-18 GP 150–4.6 (5 μm) column at 25°C with a flow rate of 1.00 mL/min, and detection was carried out at 254 nm using a UV detector. Two different mobile phases were employed: citrate buffer (pH 4.0)/THF/acetonitrile = 53/20/27 (Condition A) and citrate buffer (pH 6.0)/THF/acetonitrile = 58/15/27 (Condition B). The acetonitrile concentration was increased from 27% to 78% over a period of 60 min, and after the compound was detected, the concentration was returned to 27% over a period of 10 min. BPHB was used as an internal standard, and the hydrolysis rate was calculated based on the area ratio of atorvastatin to BPHB obtained by HPLC.

2.7 | Statistical Analysis

A two-sided *t*-test with a significance level of 5% was used to assess the difference in PNPA activities among control cells, mCES1-expressing cells, and mfCES1-expressing cells. A significance level of 0.05 was set, and if the resulting *p* value was below this threshold, it was interpreted as indicative of a statistically significant difference. Hydrolysis data for hCES1, mCES1, and mfCES1 were analyzed by one-way ANOVA with Greenhouse–Geisser correction applied.

3 | Results

3.1 | *p*-Nitrophenyl Acetate Activity

Hydrolysis reactions were performed using *p*-nitrophenyl acetate (PNPA) to measure CES activity (Figure 1). Hydrolysis rates were calculated as substrate hydrolysis per enzyme protein per minute (μmol/min/mg protein). Both mCES1 and mfCES1 showed higher metabolic activity when compared to control activity, with mCES1 showing higher activity than that of mfCES1. The *t*-test results between control and mCES1 revealed a significant difference, $P < 0.05$, indicating a significant difference in PNPA hydrolysis activity per minute. Similarly, the *t*-test results between control and mfCES1 showed a significant difference, $P < 0.05$, highlighting the significant difference in

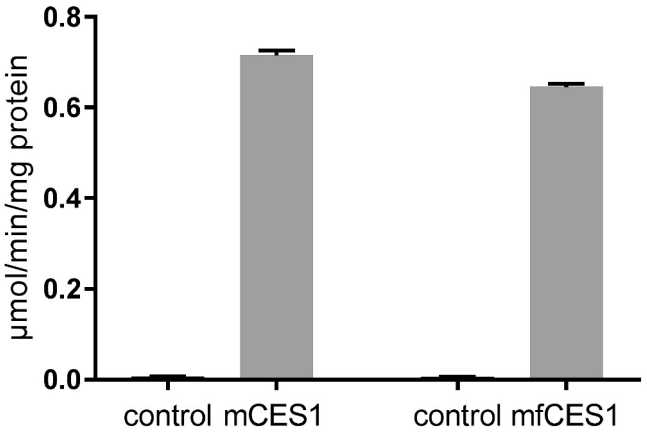

FIGURE 1 | Hydrolysis activity of mCES1 and mfCES1 using *p*-nitrophenyl acetate. Values represent means ± S.D. (n = 3).

PNPA hydrolysis activity between control and mfCES1. It was confirmed that CES-expressing cells were appropriately created.

3.2 | Native PAGE Analysis of CES Isozymes

CES isozymes were analyzed by staining with β-naphthyl acetate hydrolysis products and fast blue RR salt. As shown in Figure 2, one strong band was detected in all samples at approximately the same position as hCES1. This band was assigned to CES1 based on the similarity of its molecular weight as a trimer and its pI value. Although mfCES1 and mCES1 were analyzed at the same protein amount, the band of mCES1 was seen to be more intense. This may be due to differences in protein levels, substrate specificity, or catalytic efficiency. Further quantitative analysis is required to clarify this point. Nonetheless, because no bands other than those corresponding to CES1 were detected, the influence of other esterases is considered to be minimal.

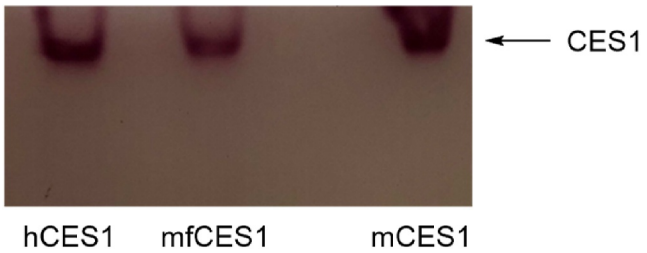

FIGURE 2 | Staining of CESs with β-naphthyl acetate. Recombinant hCES1, mfCES1, and mCES1 proteins were analyzed by native PAGE, followed by esterase activity staining with β-naphthyl acetate. The protein amounts of hCES1, mfCES1, and mCES1 loaded in each lane were 50, 100, and 100 μg, respectively. Arrows indicate the bands corresponding to each CES1 isozyme.

3.3 | Hydrolysis Reaction of Linear Alkyl Esters

This study initially involved the measurement of the hydrolysis rates of straight-chain alkyl esters (Figure 3). Previous research indicated that hCES1 exhibits high hydrolysis rates for methyl ester **1** and ethyl ester **2**, whereas a substantial decrease in hydrolysis rates is observed for esters with larger sizes such as propyl ester **3** (Mizoi et al. 2020). The hydrolysis rates gradually decrease with an increase in carbon chain length, and this phenomenon is attributed to factors such as steric hindrance between the active site of hCES1 and the ester substrates, decreased substrate specificity due to bulky alkoxy groups, altered molecular interactions, and instability of the transition state during hydrolysis.

In contrast, the high hydrolysis rates observed for methyl ester **1** and ethyl ester **2** in hCES1 were not replicated in mCES1. Instead, mCES1 displayed higher hydrolysis rates for propyl ester **3** and butyl ester **4**. Esters with more carbon atoms (**5**–**10**) showed a gradual decrease in hydrolysis rates with an increase in carbon chain length, resembling the trend observed in hCES1.

In the case of mfCES1, the hydrolysis rate for methyl ester **1** was high, but a significant reduction in hydrolysis rate was observed for ethyl ester **2**. As in the case of mCES1, propyl ester **3** and butyl ester **4** exhibited higher hydrolysis rates. However, esters with more carbon atoms (**5**–**10**) showed a pronounced decrease in hydrolysis rates compared to those for hCES1 and mCES1.

The hCES1/mCES1 and hCES1/mfCES1 ratios were calculated (Table 2). In hCES1/mCES1, methyl ester showed a 7.97-fold difference and ethyl ester exhibited a 4.08-fold difference, indicating a substantial disparity. Other ratios fell within the range of 0.90–1.88. In hCES1/mfCES1, ethyl ester showed a 7.97-fold difference and the ratio of hexyl ester to decyl ester was 4.08–6.88, demonstrating relatively large differences.

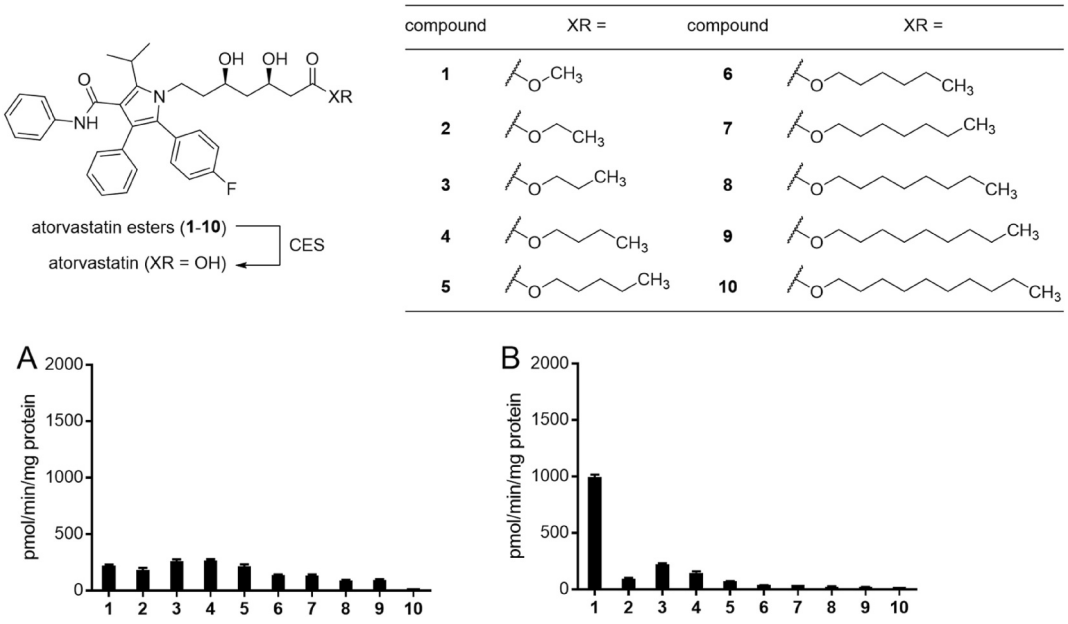

FIGURE 3 | Hydrolysis rates of atorvastatin esters (**1**–**10**) in a CES solution. (A) mCES1, (B) mfCES1. Values represent means ± S.D. (n = 3).

TABLE 2 | Hydrolytic rate ratios of hCES1/mCES1 and hCES1/mfCES1 for compounds (**1**–**30**). The ratios in C were calculated from existing hCES1 data (Mizoi et al. 2020).

Compound	hCES1/mCES1	hCES1/mfCES1	Compound	hCES1/mCES1	hCES1/mfCES1
1	7.97	1.77	**16**	1.30	2.86
2	4.08	7.97	**17**	0.53	7.48
3	1.17	1.38	**18**	6.01	8.31
4	1.05	1.92	**19**	1.36	1.85
5	1.10	3.36	**20**	8.97	9.93
6	1.78	6.88	**21**	3.68	3.83
7	1.88	6.77	**22**	2.95	5.41
8	1.15	4.08	**23**	3.56	4.79
9	0.94	4.96	**24**	2.03	1.82
10	1.57	6.49	**25**	1.17	1.72
11	2.81	2.23	**26**	4.15	6.19
12	0.87	1.27	**27**	0.84	5.28
13	3.70	3.45	**28**	—	—
14	0.60	15.82	**29**	—	—
15	0.61	0.92	**30**	0.78	2.26

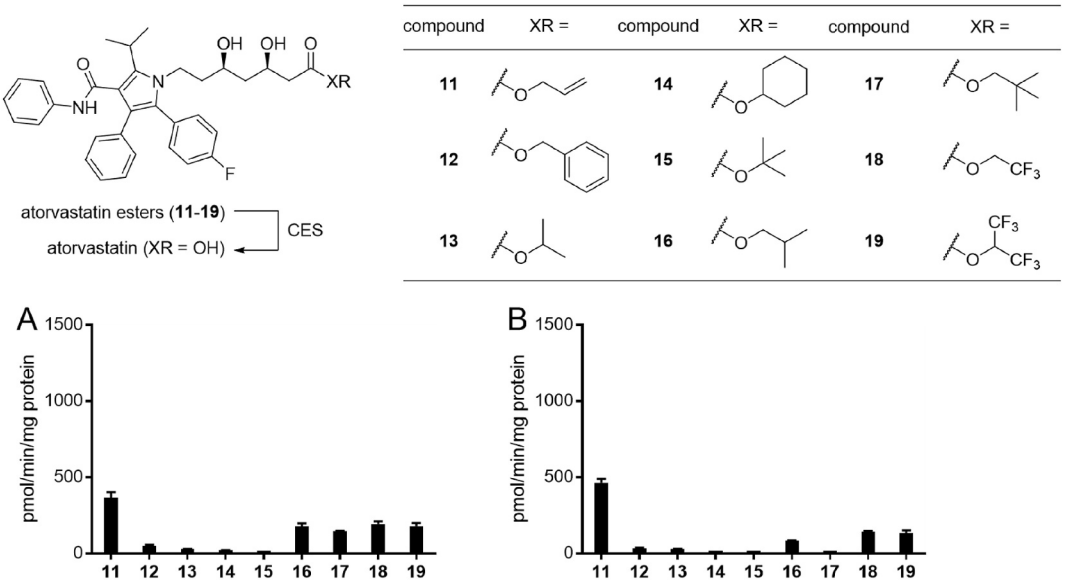

FIGURE 4 | Hydrolysis rates of atorvastatin esters (**11**–**19**) in a CES solution. (A) mCES1, (B) mfCES1. Values represent means ± S.D. ($n = 3$).

3.4 | Hydrolysis Reactions of Other Alkyl Esters

Next, the hydrolysis rates of esters containing branched, double-bond, or fluorine groups were measured (Figure 4). Previous research indicated that hCES1 exhibited high hydrolysis rates for esters with small steric hindrance, such as allyl ester **11**, but the hydrolysis rates largely decreased for esters with bulky substituents in proximity to the ester moiety, such as isopropyl ester **13** and *t*-butyl ester **15**. Moreover, esters with fluorine groups showed higher hydrolysis rates than those of their corresponding alkyl esters (ethyl ester **2** for trifluoroethyl ester **18**). Based on these findings, it was concluded that hCES1 is sensitive to the hydrolysis rate due to steric hindrance near the ester, the influence of electron-withdrawing groups, and intermolecular interactions.

Similarly, mCES1 exhibited high hydrolysis rates for allyl ester **11**. Esters with adjacent carbon atoms being secondary or tertiary (**13**–**15**) showed a significant decrease in hydrolysis rates. Although esters with fluorine groups (**18**, **19**) showed high hydrolysis rates, the hydrolysis rate of trifluoroethyl ester **18** was lower than that of hCES1.

mfCES1 also showed high hydrolysis rates for allyl ester **11**. For esters with adjacent carbon atoms being secondary or tertiary (**13**–**15**) and esters with branching at a slightly distant position

(**16**, **17**), hydrolysis rates decreased. The results suggest that mfCES1 is susceptible to the influence of steric hindrance, resulting in a relatively pronounced decrease in hydrolysis rates. Similar to mCES1, esters with fluorine groups (**18**, **19**) exhibited high hydrolysis rates, although the hydrolysis rates were lower than those for hCES1.

The hCES1/mCES1 and hCES1/mfCES1 ratios were calculated for esters (**11**–**19**) (Table 2). For hCES1/mCES1, allyl ester **11** showed a 2.81-fold difference, isopropyl ester **13** showed a 3.70-fold difference, and trifluoroethyl ester **18** showed a 6.01-fold difference. These esters have relatively short alkoxy groups, and it is thought that hCES1 hydrolyzes shorter esters more efficiently than mCES1 does. For other esters, the ratios were within the range of 0.60–1.36. For hCES1/mfCES1, allyl ester **11** showed a 2.23-fold difference, isopropyl ester **13** showed a 3.45-fold difference, and trifluoroethyl ester **18** showed an 8.31-fold difference. Moreover, cyclohexyl ester **14** showed a 15.82-fold difference, and neopentyl ester **17** showed a 7.48-fold difference. Similar to mCES1, shorter esters such as allyl ester **11** and trifluoroethyl ester **18** showed large differences, and for bulky esters such as neopentyl and cyclohexyl esters (**14**, **17**) with more carbon atoms, the difference from hCES1 was more pronounced.

3.5 | Hydrolysis Reactions of Aryl Esters, Thioesters, and Amides

Next, the hydrolysis rates of aromatic esters, thioesters, and amides were measured (Figure 5). Previous research indicated that phenyl ester **22** exhibited the highest hydrolysis rate of all aryl esters (**20**–**25**) in hCES1. It was concluded that the hydrolysis rate decreased regardless of the substituent type at the 4-position, suggesting that steric hindrance played a more significant role than the electronic effects of the substituents. Additionally, thioesters (**26**,**27**) exhibited lower reactivity than their corresponding esters (**2**,**13**), and amides (**28**–**30**) showed minimal hydrolysis.

mCES1 exhibited higher hydrolysis rates for phenyl ester **22**, as in the case of hCES1, and 4-position-substituted phenyl esters (**20**,**21**,**23**–**25**) showed a decrease in hydrolysis rates. Additionally, the average hydrolysis rate for these 4-position-substituted esters (**20**,**21**,**23**–**25**) was 152.8 nmol/min/mg protein, and the individual ester hydrolysis rates fell within the range of 0.6–1.4 times higher than the average rates. These results indicate that substituents led to a decrease in hydrolysis rates regardless of the substituent type. Similar to hCES1, amides (**28**–**30**) in mCES1 showed minimal hydrolysis.

Similarly, mfCES1 showed higher hydrolysis rates for phenyl ester **22**, as observed in hCES1 and mCES1. 4-position-substituted esters (**20**,**21**,**23**–**25**) displayed reduced hydrolysis rates. Unlike hCES1, thioesters (**26**,**27**) in mfCES1 exhibited similar or slightly increased reactivity compared to their corresponding esters (**2**,**13**). Amides (**28**–**30**) in mfCES1 showed minimal hydrolysis similar to those in hCES1 and mCES1.

The hCES1/mCES1 and hCES1/mfCES1 ratios were calculated for esters (**20**–**30**) (Table 2). For hCES1/mCES1, methoxyphenyl ester **20** showed an 8.97-fold difference, methylphenyl ester **21** showed a 3.68-fold difference, fluorophenyl ester **23** showed a 3.56-fold difference, and ethyl thioester **26** showed a 4.15-fold difference. For hCES1/mfCES1, methoxyphenyl ester **20** showed a 9.93-fold difference, methylphenyl ester **21** showed a 3.83-fold difference, phenyl ester **22** showed a 5.41-fold difference, fluorophenyl ester **23** showed a 4.79-fold difference, ethyl thioester **26** showed a 6.19-fold difference, and isopropyl thioester **27**

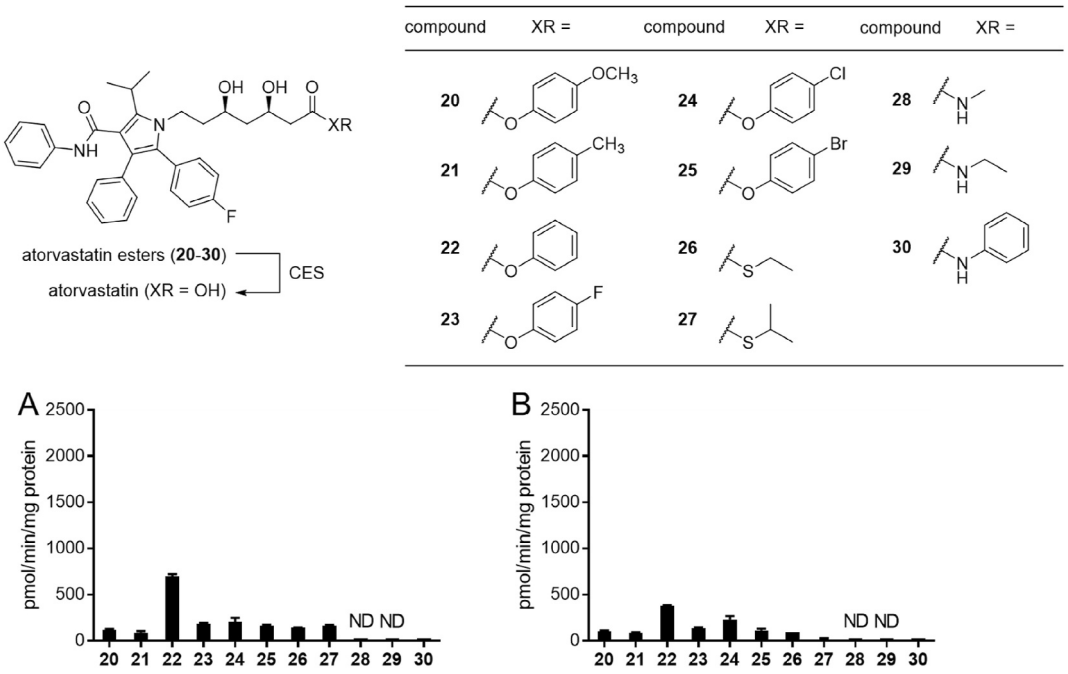

FIGURE 5 | Hydrolysis rates of atorvastatin esters (**20**–**25**), thioesters (**26**, **27**), and amides (**28**–**30**) in a CES solution. (A) mCES1, (B) mfCES1. Values represent means ± S.D. ($n = 3$).

showed a 5.28-fold difference. Compared to hCES, the ratio of the hydrolysis rates was larger for phenyl esters (**20**–**25**) with relatively large methyl groups and methoxy groups. The ratio of hydrolysis rates was also large for small thioesters such as ethyl thioester **26**, and the results are comparable to those for ethyl ester **2**.

4 | Discussion

In this study, we aimed to clarify the relationship between the structure of alkoxy groups and the rate of hydrolysis by CESs (carboxylesterases) using ester compounds with an acyl group side chain identical to atorvastatin and various alkoxy groups with different sizes and electron densities. CES1 contributes to hydrolysis in the liver of humans, mice, and cynomolgus monkeys, and investigating the differences in their reactivities is useful for predicting species differences in hepatic metabolism. The structure of the alkoxy group resulted in differences ranging from approximately half to 8.97-fold between hCES1 and mCES1, and differences ranging from similar to 15.82-fold between hCES1 and mfCES1. In addition, one-way analysis of variance (ANOVA) was performed using hydrolysis data (esters **1**–**30**). The ANOVA showed a statistically significant difference with an F value of 12.62 and a p value of 0.0005. These results suggest that differences in drug pharmacokinetics may make results obtained from animal models insufficient for predicting the effects and safety in humans.

Notable differences in metabolic rates between hCES1 and mCES1 were observed for methyl ester **1**, ethyl ester **2**, trifluoroethyl ester **18**, methoxyphenyl ester **20**, and ethyl thioester **26**. These are mostly short-chain alcohol esters with C1 or C2 carbons, suggesting that hCES1 hydrolyzes shorter alkyl esters more efficiently than mCES1 does. Conversely, mCES1 exhibited higher metabolic rates for cyclohexyl ester **14**, *t*-butyl ester **15**, and neopentyl ester **17**, indicating that mCES1 is less affected by steric hindrance near the ester. Hence, caution is needed when extrapolating data from mice to humans for alkyl esters with significant differences in alkoxy group structures or steric hindrance near the ester.

Large differences in metabolic rates between hCES1 and mfCES1 were observed for C2 esters such as ethyl ester **2**, trifluoroethyl ester **18**, and ethyl thioester **26** and for bulkier esters such as hexyl–decyl esters (**6**–**10**), cyclohexyl ester **14**, neopentyl ester **17**, and methoxyphenyl ester **20**. mfCES1 also showed lower capabilities than hCES1 for short-chain esters, suggesting significant differences in metabolic rates between hCES1 and mfCES1 even for short-chain esters. Additionally, differences in metabolic rates were observed for relatively bulkier esters.

The deduced amino acid sequences of hCES1, mfCES1, and mCES1 inferred from the cDNA sequences are shown in Figure 6 (Holmes et al. 2010; Silva et al. 2015; Uno et al. 2014). The similarities of the amino acid sequence of hCES1 were 93.0% for mfCES1 and 77.6% for mCES1, suggesting that the more evolved cynomolgus monkeys are more similar to humans. On the other hand, among the amino acid sequences of hCES1, mfCES1, and mCES1, many differences were found in the amino acid sequence from Ser 221 to His 468, which are the amino acid residues forming the catalytic triad. Although the steric structures of mCES1 and mfCES1 are unknown, the differences in activity compared with hCES1 indicate that the differences in the amino acid sequence between Ser 221 and His 468 likely affect the overall activity, including the binding site to the substrate.

Ser 221, Glu 354, and His 468 are in the same plane and constitute the active site for hydrolysis. The proposed mechanism is illustrated in Figure 7. In the first step, the hydroxyl group of Ser 221 activated by Glu 354 and His 468 undergoes nucleophilic addition to the ester. Then, through the transition state of the oxyanion stabilized by Gly142 and Gly143, the corresponding alcohol (HXR) is eliminated and an enzyme-acyl intermediate is formed. An exchange reaction between alcohol and water occurs, and the corresponding carboxylic acid (atorvastatin) is generated through nucleophilic addition and elimination reactions from water and the enzyme (CES1) is

FIGURE 6 | Amino acid sequence homology among hCES1A1, mCES1, and mfCES1v1 (Holmes et al. 2010; Silva et al. 2015; Uno et al. 2014). hCES1: 100%, mCES1: 77.6%, mfCES1v1: 93.0%.

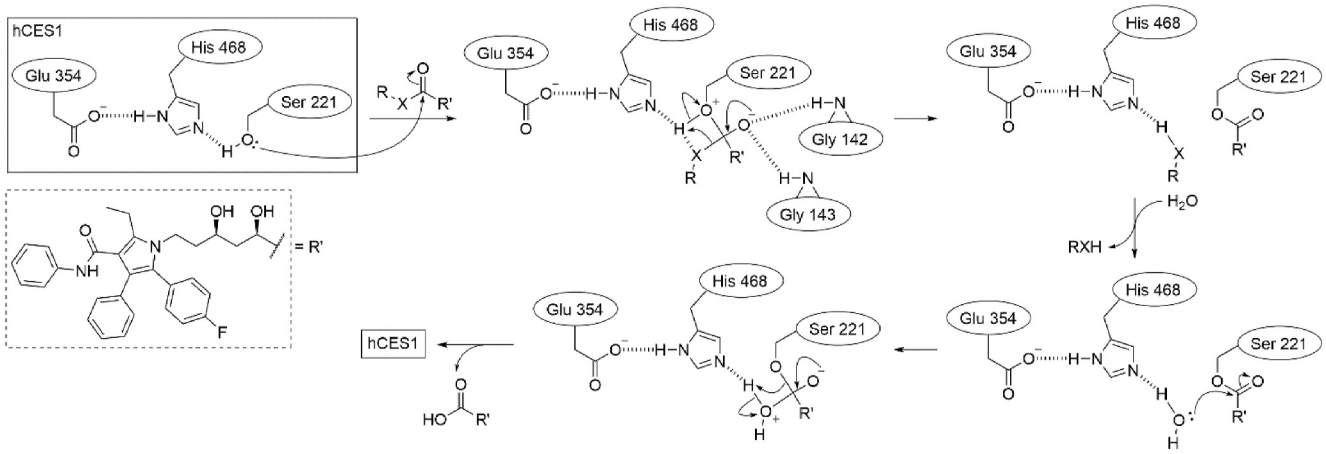

FIGURE 7 | Proposed mechanism of hydrolysis of atorvastatin esters by hCES1.

regenerated. Species differences were observed for alkoxy groups (-XR) with extremely small (esters **1**, **2**, **18**, **26**) or large (esters **14**, **15**, **17**) steric hindrance, indicating the need for consideration of species differences for these esters.

5 | Conclusion

In this study, using atorvastatin esters, the differences in substrate specificities among human (hCES1), mouse (mCES1), and cynomolgus monkey (mfCES1) carboxylesterases were elucidated. Our focus was on the structure of the alkoxy group, allowing us to investigate its impact on hydrolysis rates. With the accumulation of more hydrolysis data in the future, it will be essential to uncover species differences among various CES enzymes.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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