Drug Metabolism and Pharmacokinetics 38 (2021) 100391

Contents lists available at ScienceDirect

Drug Metabolism and Pharmacokinetics

journal homepage: http://www.journals.elsevier.com/drug-metabolism-and-pharmacokinetics

Regular Article

Effects of steric hindrance and electron density of ester prodrugs on controlling the metabolic activation by human carboxylesterase

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ARTICLE INFO

Article history:
Received 3 December 2020
Received in revised form
9 March 2021
Accepted 15 March 2021
Available online 25 March 2021

Keywords:
Carboxylesterase
Ester
Drug metabolism
Prodrug
Substrate specificity

ABSTRACT

Carboxylesterase (CES) plays an important role in the hydrolysis metabolism of ester−type drugs and prodrugs. In this study, we investigated the change in the hydrolysis rate of hCE1 by focusing on the steric hindrance of the ester structure and the electron density. For 26 kinds of synthesized indomethacin prodrugs, the hydrolytic rate was measured in the presence of human liver microsomes (HLM), human small intestine microsomes (HIM), hCE1 and hCE2. The synthesized prodrugs were classified into three types: an alkyl ester type that is specifically metabolized by hCE1, a phenyl ester type that is more easily metabolized by hCE1 than by hCE2, and a carbonate ester type that is easily metabolized by both hCE1 and hCE2. The hydrolytic rate of 1-methylpentyl (hexan−2−yl) ester was 10−times lower than that of 4−methylpentyl ester in hCE1 solution. hCE2 was susceptible to electron density of the substrate, and there was a difference in the hydrolysis rate of up to 3.5−times between *p*-bromophenyl ester and *p*-acetylphenyl ester. By changing the steric hindrance and electron density of the alkoxy group, the factors that change the hydrolysis rate by CES were elucidated.

1. Introduction

Carboxylesterase (CES) is an enzyme that catalyzes a hydrolysis reaction that belongs to the phase I reaction. CES catalyzes the hydrolysis reactions of carboxylic acid derivatives to produce metabolites such as carboxylic acid, alcohol, amine and thiol, which are targets of the phase II reaction. CES is known to have a wide range of substrate recognition capabilities and is involved in the detoxification of pesticides, environmental chemicals and foods as well as drugs [1,2]. In humans, human CES1 (hCE1), which is mainly expressed in the liver and lung [3], and human CES2 (hCE2), which is mainly expressed in the small intestine, liver and kidney [4], contribute significantly to drug metabolism. Studies on metabolism and metabolic activation of commercially available compounds have demonstrated that hCE1 and hCE2 recognize different substrates depending on the sizes of the acyl and alkoxy groups. Among the commercially available compounds, methylphenidate (a psychotropic drug) [5], meperidine (an analgesic drug) [6] and lidocaine (a local anesthetic) [7], which have relatively small alkoxy groups, are mainly metabolized by hCE1. On the other hand, heroin (a narcotic) [8] and procaine (a local anesthetic) [9], which have relatively small acyl groups, are mainly metabolized by hCE2. In a prodrug for which the parent compound is a carboxylic acid compound, the alkoxy group moiety as the modifying group is smaller than the acyl group. Such substrates with small alkoxy groups tend to be easily recognized by hCE1. Examples of hCE1 substrates are carboxylic acid-based prodrugs such as temocapril, imidapril, alacepril (ACE inhibitor) [10], oseltamivir (influenza drug) [11], indomethacin ester (anti-inflammatory agent) [12], atorvastatin ester [13] and clopidogrel (antiplatelet agent) [14]. On the other hand, in a prodrug for which the parent compound is an alcohol compound, the acyl group as a modifying group is smaller than the alkoxy group. Such substrates with small acyl groups tend to be easily recognized by hCE2. Examples of hCE2 substrates are alcohol (or phenol)-based prodrugs such as CPT-11 (anticancer drug) [15,16], prasugrel (antiplatelet drug) [17] and haloperidol ester (antipsychotic drug) [18]. Thus, the

Abbreviations: ACE, angiotensin-converting-enzyme; BPHB, butyl *p*-hydroxybenzoate; CES, carboxylesterase; DCC, dicyclohexylcarbodiimide; DMAP, dimethylaminopyridine; DMSO, dimethylsulfoxide; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; hCE, human carboxylesterase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HIM, human intestine microsomes; HLM, human liver microsomes; HPLC, high−performance liquid chromatography; IR, infrared spectroscopy; NMR, nuclear magnetic resonance; ND, not detected; PNPA, *p*-nitrophenyl acetate.

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https://doi.org/10.1016/j.dmpk.2021.100391
1347-4367/

substrate recognition ability of hCE1 and hCE2 is characterized in some substrates. However, it has not yet been investigated how much the hydrolysis rate changes due to the difference in more detailed structure and the difference in electron density of the ester. In this study, we investigated the change in the hydrolysis rate of hCE1 by focusing on the steric hindrance of the ester structure and the electron density. Using indomethacin as a substrate, we synthesized ester prodrugs with steric hindrance and alkoxy groups with different electron densities, and we clarified the relationship between these structures and the rate of hydrolysis.

2. Materials and methods

2.1. Reagents

Indomethacin was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Ester, amide and thioester derivatives were synthesized by a condensing reaction using indomethacin (**1**). Butyl *p*-hydroxybenzoate (BPHB) as the internal standard, alcohol reagents (**2a**: pentanol, **2b**: hexan-2-ol, **2c**: 2-methylpentanol, **2d**: 3-methylpentanol, **2e**: 4-methylpentanol, **2f**: phenol, **2g**: 2-methoxyphenol, **2h**: 3-methoxyphenol, **2i**: 4-methoxyphenol, **2j**: 2-bromophenol, **2k**: 3-bromophenol, **2l**: 4-bromophenol, **2m**: 2′-hydroxyacetphenone, **2n**: 3′-hydroxyacetphenone, **2o**: 4′-hydroxyacetphenone, **2p**: 2-fluorophenol, **2q**: 3-fluorophenol, **2r**: 4-fluorophenol, **2s**: ethanol, **3f**: aniline, **4f**: thiophenol), alkyl halide reagents (**2t**: chloromethyl pivalate, **2u**: 3-bromophthalide, **2v**: 1-chloroethyl isopropyl carbonate**, 2w**: 1-chloroethyl cyclohexyl carbonate, **2x**: 4-chloromethyl-1,5-dimethyl-1,3-dioxol-2-one), dicyclohexylcarbodiimide (DCC), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and dimethylaminopyridine (DMAP) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Human intestine microsomes (HIM) (7 doners, mixed gender, 24–69 years old, Caucasian and African American), human carboxylesterase 1b (hCES1b, hCE1) and hCE2 were purchased from Corning inc. (MA, USA).

2.2. Preparation of HLM samples

Human liver microsomes (HLM) (single donor, male, 51 years old, Caucasian) were prepared from human livers that were obtained from the Human and Animal Bridging Research Organization (HAB, Chiba, Japan), which is in partnership with the National Disease Research Interchange (NDRI, Philadelphia, PA, USA). The use of human livers was approved by the Ethics Committee of Chiba Institute of Sciences (No. 22-1). The livers were homogenized and centrifuged at 9,000 × G for 20 min at 4 °C, and the supernatant was ultra-centrifuged at 105,000 × G for 60 min at 4 °C. The microsome fraction was suspended in sucrose-EDTA-Tris (SET) buffer (pH 7.4).

2.3. Synthesis

2.3.1. General procedure for synthesis of ***2a*–*2f*, *2p*–*2r*** *and* ***2s***

A mixture of indomethacin (**1**) (0.5 mmol), alcohol reagent (0.75 mmol), DCC (0.75 mmol) and DMAP (0.05 mmol) in CH_2Cl_2 (5.0 mL) was stirred at room temperature under an argon atmosphere for 1 day. After addition of 10% citric acid solution (5.0 mL), the mixture was stirred for 5 min at the same temperature, and the mixture was extracted with CH_2Cl_2 (20 mL). The organic solution was dried over $MgSO_4$ and evaporated under reduced pressure. The residue was purified by column chromatography on SiO_2 (ethyl acetate/*n*–hexane) to give an ester derivative **2**.

2.3.2. General procedure for synthesis of ***2g*–*2o***, ***3f*** *and* ***4f***

A mixture of indomethacin (**1**) (0.5 mmol), alcohol reagent (0.75 mmol), EDC (0.75 mmol) and DMAP (0.05 mmol) in CH_2Cl_2 (5.0 mL) was stirred at room temperature under an argon atmosphere for 1 day. After addition of 10% citric acid solution (5.0 mL), the mixture was stirred for 5 min at the same temperature, and the mixture was extracted with CH_2Cl_2 (20 mL). The organic solution was dried over $MgSO_4$ and evaporated under reduced pressure. The residue was purified by column chromatography on SiO_2 (ethyl acetate/*n*–hexane) to give an ester derivative **2**.

2.3.3. General procedure for synthesis of ***2t*-*2x***

A mixture of indomethacin (**1**) (0.5 mmol), alkyl halide reagent (0.75 mmol), and Na_2CO_3 (1.5 mmol) in DMF (2.0 mL) was stirred at room temperature under an argon atmosphere for 1 day. After addition of H_2O (20 mL), the mixture was extracted with hexane/AcOEt (20 mL, 1/1). The organic solution was washed with H_2O (10 mL × 2) and brine (10 mL), dried over $MgSO_4$ and evaporated under reduced pressure. The residue was purified by column chromatography on SiO_2 (ethyl acetate/*n*–hexane) to give an ester derivative **2**.

2.4. Enzyme reaction

2.4.1. p-Nitrophenyl acetate hydrolase activity

A typical incubation mixture (final volume of 1.0 mL) contained 10 mM Na,K phosphate buffer (pH 7.4) and 1 mM *p*-nitrophenyl acetate (PNPA) (acetonitrile final concentration of 1.0% in the incubation mixture) at 30 °C. An enzyme source (0.05 mg/mL) was added, and the PNPA hydrolase activity was measured spectrophotometrically (detection at 405 nm) regarding the formation of 4-nitrophenol ($\varepsilon = 16400\ M^{-1}\ cm^{-1}$) at 30 °C for 3 min, with data obtained at 5-s intervals, using an Ultrospec 6300 pro (GE Healthcare, formerly Amersham Bioscience, NJ, USA) [19].

2.4.2. General procedure for hydrolysis reaction

Solutions of indomethacin prodrugs (0.3125, 0.625, 1.25, 2.5, 5.0, 10 and 50 mM) were prepared in DMSO. A typical incubation mixture (final volume of 100 μL) in 100 mM HEPES (pH 7.4) contained an enzyme (0.25 mg/mL) and 0.003125–0.5 mM of indomethacin prodrug (DMSO final concentration of 1.0% in the reaction mixture). After 30–min incubation at 37 °C, the reaction was terminated by the addition of 0.3 mM BPHB in acetonitrile solution (100 μL) on an ice bath at 10 min. The mixture was centrifuged at 21,600 × G for 15 min at 4 °C to precipitate the protein, and the supernatant was subjected to HPLC analysis to determine indomethacin.

2.5. Data analysis

2.5.1. Procedure for HPLC analysis

HPLC analyses were carried out using an LC solution (Shimadzu, Kyoto, Japan) chromatographic system equipped with an LC-10AT pump, SIL-20A auto sampler, CTO-10AS VP column oven, SPD-20A UV/VIS detector, and SCL-10A VP system controller. Separations were performed on a column of Mightysil RP-18 GP 150-4.6 (5 μm) (Cica-Reagent, Tokyo, Japan) at 30 °C with detection at 254 nm. Analytical HPLC was performed with elution at 1.0 mL/min using MeOH with 0.1% H_3PO_4 aqueous solution ramped up from 65% to 90% over 25 min and then returned to 65% over 10 min. Analytical HPLC was performed with BPHB as an internal standard (t_R = 7.2 min). The enzyme activity was calculated from the area ratio of indomethacin (t_R = 11.7 min) and BPHB. The K_m and V_{max} values were calculated by the Michaelis-Menten equation using nonlinear regression analysis with software (GraphPad Prism 7).

2.5.2. Statistical analysis

$P < 0.01$ was considered statistically significant in the Pearson correlation coefficient test.

3. Results

3.1. Synthesis

A total of 26 indomethacin prodrugs were synthesized. To confirm the effects of steric hindrance, pentyl ester **2a** and methyl group−substituted pentyl esters (**2b**−**2e**) were synthesized. To confirm the effect of electron density, phenyl ester **2f** and phenyl ester in which the methoxy group, bromo group, acetyl group and fluoro group were substituted at the *o*−, *m*−, and *p*−positions, respectively, were synthesized. Indomethacin prodrugs (**2s**−**2x**) were also synthesized with a modifying group frequently used in commercially available prodrugs. Alkyl esters (**2a**−**2e**, **2s**) and fluorophenyl esters (**2p**−**2r**) were synthesized using DCC, and aryl esters (**2f**−**2r**), amide **3f** and thioester **4f** were synthesized using EDC by reacting with the corresponding alcohol reagents (Fig. 1). Pivoxil ester **2t**, proxetyl ester **2v** and medoxomil ester **2x** were synthesized using alkyl halides by the previously reported method with slight modification [20] (Fig. 2). The other esters (**2u** and **2w**) were also synthesized using alkyl halides. The structures of the synthesized compounds were determined by ^{1}H-NMR, ^{13}C-NMR, and IR spectrum data.

3.2. Hydrolysis reaction in HLM and HIM

First, the hydrolytic rates of the synthesized prodrugs were investigated in HLM solution (Fig. 3A). Metabolic activation to indomethacin was observed in all of alkyl esters (**2a**−**2e**, **2s**−**2u**), aryl esters (**2f**−**2r**), and carbonate esters (**2v**−**2x**) in HLM solution. The hydrolytic rates of pivoxil ester **2t** and proxetyl ester **2v** were high: 23.6 and 23.4 nmol/mg protein/min, respectively. No hydrolysis was observed for the amide **3f**, and the hydrolysis rate of the thioester **4f** was 38−times lower than that of the ester **2f**. In the pentyl esters (**2a**−**2e**), the hydrolysis rate of 1−methylpentyl (hexan-2-yl) ester **2b** was 9.8−times lower than that of n−pentyl ester **2a**; however, the hydrolysis rate increased as the methyl group moved away from the ester, and a difference between the hydrolysis rates of n−pentyl ester **2a** and 4−methylpentyl ester **2e** was not found. Among the aryl esters (**2f**−**2r**), phenyl ester **2f** had the lowest hydrolytic rate. The hydrolytic rate of *m*-acetylphenyl ester **2n** was 3.4−times higher than that of phenyl ester **2f** and hydrolytic rate of *o*-fluorophenyl ester **2p** was 4.3−times higher than that of phenyl ester **2f**. Alkyl diesters (**2t** and **2u**) showed higher hydrolysis rates than those of alkyl monoesters (**2a**−**2e**, **2s**). Next, the hydrolytic rates of the prodrugs were investigated in HIM solution (Fig. 3B). Aryl esters (**2f**−**2r**), alkyl diesters (**2t** and **2u**) and carbonate esters (**2v**−**2x**) had relatively high hydrolytic rates, but alkyl esters (**2a**−**2e**, **2s**) were hardly hydrolyzed in HIM solution. Among the aryl esters (**2f**−**2r**), *p*-acetylphenyl ester **2o** had a hydrolytic rate about 2.8−times higher than that of phenyl ester **2f**. Based on the results shown in Fig. 3A and B, the HLM/HIM hydrolytic rate ratios were calculated (Fig. 3C). Alkyl esters (**2a**−**2e**, **2f**), which were hardly hydrolyzed in the HIM solution, had HLM/HIM hydrolytic rate ratios of 140−times or more, indicating that they were HLM−selective. Aryl esters (**2f**−**2r**) also had high HLM/HIM hydrolytic rate ratios as a whole, and only carbonate ester **2w** had an HLM/HIM ratio of less than 1.0.

3.3. Hydrolysis reaction in hCE1 and hCE2

Next, the hydrolytic rates of the indomethacin prodrugs were investigated in hCE1 solution (Fig. 4A). Similar to the hydrolysis reactions in HLM solution, all alkyl esters (**2a**−**2e**, **2s**−**2u**), aryl esters (**2f**−**2r**) and carbonate esters (**2v**−**2x**) showed metabolic activation to indomethacin. No hydrolysis was observed with amide **3f**, and the hydrolytic rate of thioester **4f** was low. The hydrolytic rates of alkyl diesters (**2t** and **2u**), aryl esters (**2f**−**2r**) and carbonate esters (**2v**−**2x**) were high, and the hydrolysis rate of carbonate ester **2v** was the highest of all synthesized prodrugs. The hydrolytic rate of 1-methylpentyl (hexan−2−yl) ester **2b** was 7.8−times lower than that of *n*-pentyl ester **2a**; however, that of 4-methylpentyl ester **2e** was 1.3−times higher than that of n-pentyl ester **2a**. In aryl esters (**2f**−**2r**), the hydrolytic rates of methoxyphenyl esters (**2g**−**2i**) and bromophenyl esters (**2j**−**2l**) were lower

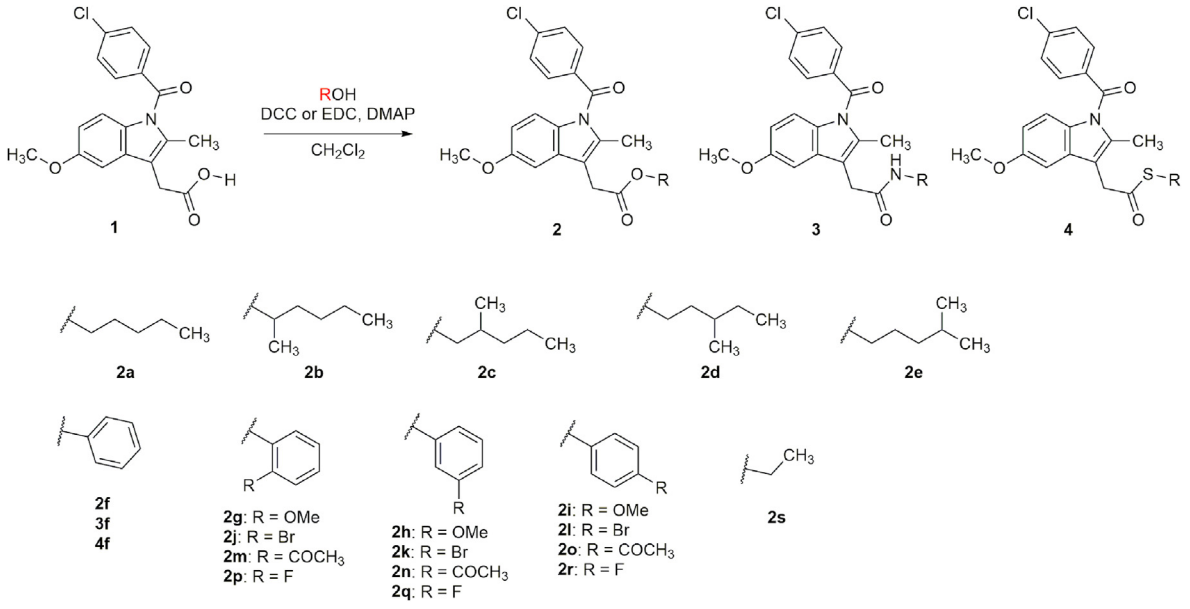


Fig. 1. Synthesis of indomethacin ester prodrugs (**2a**−**2s**, **3f** and **4f**) using alcohol reagents.

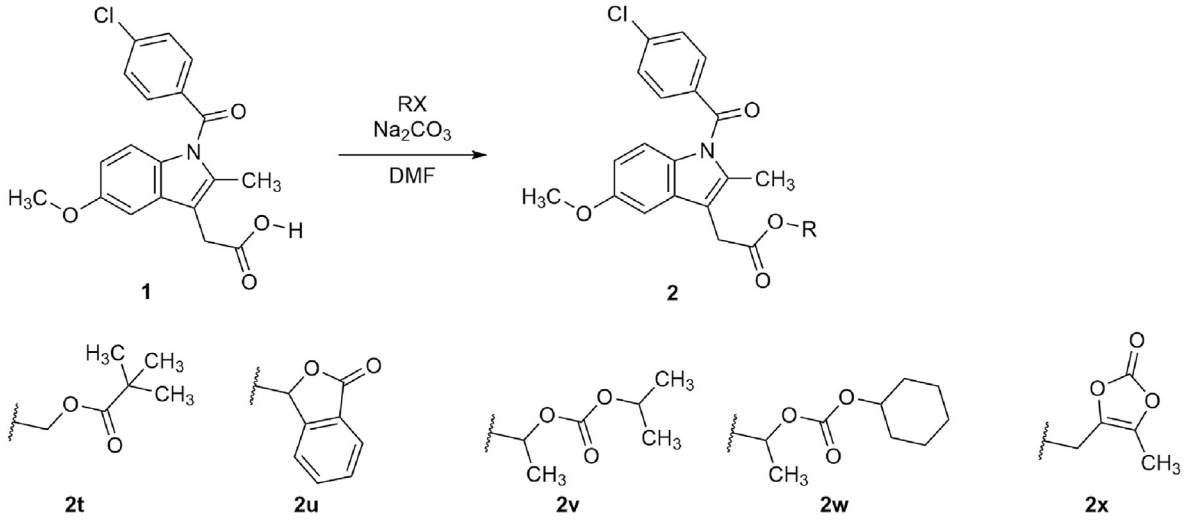
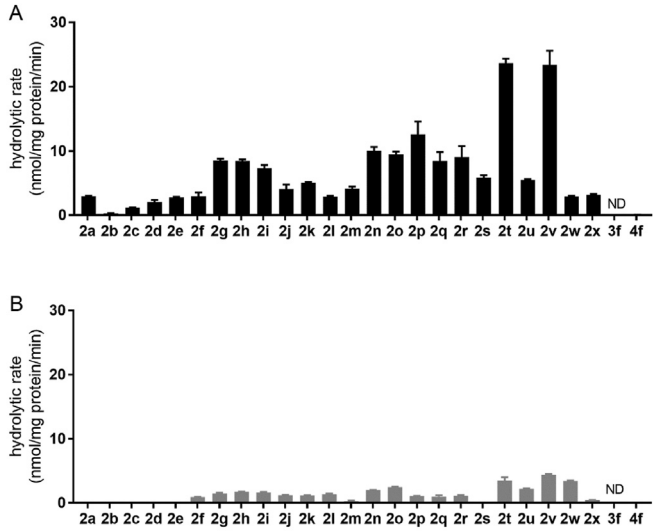


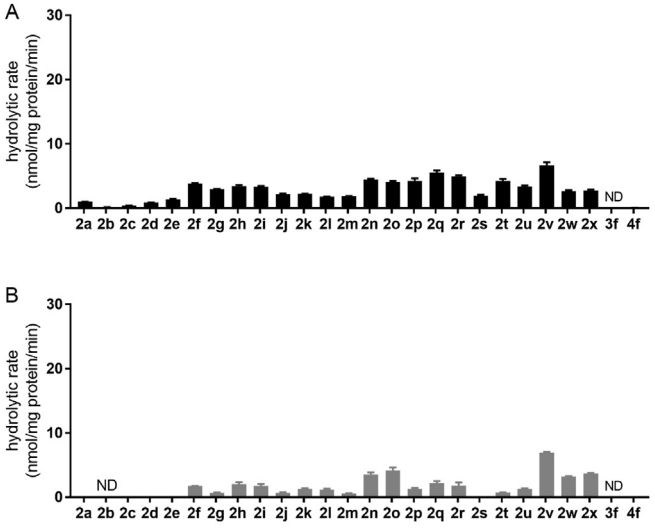
Fig. 2. Synthesis of indomethacin ester prodrugs (**2t**–**2x**) using alkyl halide reagents.



C

Compound	Ratio	Compound	Ratio	Compound	Ratio
2a	190.4	**2j**	3.4	**2s**	431.2
2b	243.8	**2k**	4.3	**2t**	6.8
2c	198.1	**2l**	2.1	**2u**	2.5
2d	148.3	**2m**	14.2	**2v**	5.4
2e	155.0	**2n**	5.1	**2w**	0.9
2f	3.3	**2o**	3.9	**2x**	7.4
2g	5.9	**2p**	11.7	**3f**	—
2h	4.9	**2q**	8.6	**4f**	1.4
2i	4.5	**2r**	8.2		

Fig. 3. Hydrolytic rates of indomethacin prodrugs (**2a**–**2x**, **3f**, **4f**) in the presence of HLM (A) and HIM (B), and their HLM/HIM ratios (C). Concentrations: prodrugs (0.5 mM), HLM (0.25 mg/mL), HIM (0.25 mg/mL); reaction time: 30 min; temperature: 37 °C; values are means ± S.D. (n = 3). ND: not detected.



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Compound	Ratio	Compound	Ratio	Compound	Ratio
2a	168.8	**2j**	3.2	**2s**	299.9
2b	—	**2k**	1.7	**2t**	5.7
2c	226.6	**2l**	1.5	**2u**	2.6
2d	199.6	**2m**	3.3	**2v**	1.0
2e	194.8	**2n**	1.3	**2w**	0.8
2f	2.2	**2o**	1.0	**2x**	0.7
2g	4.3	**2p**	3.2	**3f**	—
2h	1.7	**2q**	2.5	**4f**	2.3
2i	1.9	**2r**	2.7		

Fig. 4. Hydrolytic rates of indomethacin prodrugs (**2a**–**2x**, **3f**, **4f**) in the presence of hCE1 (A) and hCE2 (B), and their hCE1/hCE2 ratios (C). Concentrations: prodrugs (0.1 mM), hCE1 (0.25 mg/mL), hCE2 (0.25 mg/mL); reaction time: 30 min; temperature: 37 °C; values are means ± S.D. (n = 3). ND: not detected.

than that of the phenyl ester **2f**, and the hydrolysis rates of acetylphenyl esters (**2n**–**2o**) excluding the *o*-form **2m** and fluorophenyl esters (**2p**–**2r**) were higher than that of the phenyl ester **2f**. Next, the hydrolytic rates of the indomethacin prodrugs were investigated in hCE2 solution (Fig. 4B). As in HIM solution, the hydrolytic rate of the alkyl ester was low and the hydrolytic rates of the alkyl diesters (**2t** and **2u**), aryl esters (**2f**–**2r**), and carbonate esters (**2v**–**2x**) were relatively high. No hydrolysis was observed for 1-methylpentyl ester **2b** and amide **3f**, which had low hydrolysis rates in other enzyme solutions. In aryl esters (**2f**–**2r**), the hydrolytic rates of *m*– and *p*–methoxyphenyl esters (**2h** and **2i**) and *m*– and *p*–bromophenyl esters (**2k** and **2l**) were lower than that of the phenyl ester **2f**, and the hydrolytic rates of *m*– and *p*–acetylphenyl esters (**2n** and **2o**) and *m*– and *p*–fluorophenyl esters (**2q** and **2r**) were higher than that of the phenyl ester **2f**. However, all *o*–substituted aryl esters (**2g**, **2j**, **2m** and **2p**) had lower hydrolytic rates than those of the other positional isomers. The hydrolytic rate

of *o*−acetylphenyl ester **2m** was 7.3−times lower than that of *p*−acetylphenyl ester **2o**. Based on the results shown in Fig. 4A and B, the hCE1/hCE2 hydrolytic rate ratios were calculated (Fig. 4C). Monoalkyl esters (**2a**−**2e**, **2s**) were hydrolyzed with high hCE1 selectively. Alkyl diesters (**2t** and **2u**) and aryl esters (**2f**−**2r**) had hCE1/hCE2 ratios of 1.0 or higher. In addition, the *o*-substituted aryl esters (**2g**, **2j**, **2m** and **2p**) had higher hCE1 selectivity than the *m*-substituted and *p*-substituted aryl esters. Carbonate esters (**2v**−**2x**) had hCE1/hCE2 ratios of 1.0 or less.

3.4. Correlations among hydrolytic activities of the enzymes

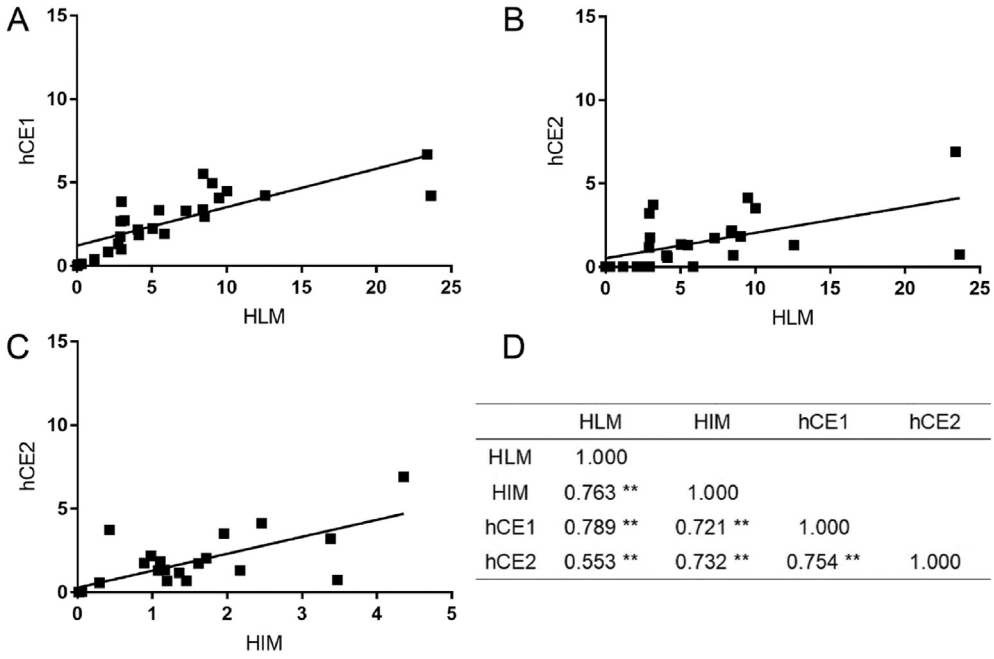
There are several hydrolases other than hCE1 and hCE2 in HLM and HIM [21]. In order to confirm whether the hydrolysis rate in HLM or HIM solution is related to the hydrolysis rate in hCE1 or hCE2 solution, the values shown in Figs. 3 and 4 were used for investigation (Fig. 5). The data for hCE1 and HLM, which are highly expressed in the human liver, were highly correlated (Fig. 5A), suggesting that the hydrolysis reaction of the indomethacin prodrug in HLM solution is mainly promoted by hCE1. The correlation between hCE2 and HLM was not higher than that of hCE1, because the expression level of hCE2 was low in the human liver (Fig. 5B). A correlation between HIM and hCE2 was also observed because hCE2 is the main hydrolase expressed in the human small intestine (Fig. 5C). Unfortunately, the results showed that there is a correlation between HIM and hCE1, which is not expressed in the small intestine (Fig. 5D). A correlation between hCE1 and hCE2 was also confirmed, and it is thought that this result was obtained because the overall reactivity of the synthesized indomethacin prodrugs was similar between hCE1 and hCE2.

3.5. Hydrolytic parameters

The hydrolytic parameters of hCE1 were investigated using alkyl esters (**2a**−**2e**, **2s**−**2u**), some aryl esters (**2f**−**2o**), and carbonate esters (**2v**−**2w**) (Table 1). The V_{max} and K_m values were calculated by the Michaelis−Menten equation using nonlinear regression analysis with GraphPad Prism 7. The hydrolytic rate measured in 0.1 mM solution (Fig. 4A) and the V_{max} value were correlated with most substrates, but the V_{max} values of **2f**, **2t** and **2v** with high K_m values were different. Substrates with a large V_{max} value also had a large K_m value. Next, the hydrolysis parameters of hCE2 were calculated using prodrugs (**2s**−**2x**) having a modifying group frequently used in commercially available prodrugs. The V_{max} values of alkyl ester **2s** and alkyl diesters (**2t** and **2u**) were low and those of carbonate esters (**2v**−**2x**) were as high as the V_{max} values of hCE1. Substrates with a large V_{max} value also had a large K_m value, but alkyl ester **2s** had a considerably large K_m value despite its low V_{max} value (see Table 2).

4. Discussion

As shown in the introduction, indomethacin is a carboxylic acid−containing drug, and indomethacin prodrugs have a structure in which the acyl group is larger than the alkoxy group and are easily hydrolyzed by hCE1. Therefore, it is thought that the reactivity with hCE1 was high for all of the synthesized prodrugs. The results showed that there are three types of synthesized prodrugs: alkyl esters (**2a**−**2e**, **2s**−**2u**) that are specifically metabolized by hCE1, aryl esters (**2f**−**2r**) that are more easily metabolized by hCE1 than by hCE2, and carbonate esters (**2v**−**2x**) that are easily metabolized by both hCE1 and hCE2. Alkyl esters are further divided into two types, monoalkyl esters (**2a**−**2e**, **2s**) and alkyl diesters (**2t** and **2u**), and it is the monoalkyl esters (**2a**−**2e**, **2s**) that are selectively hydrolyzed by hCE1. The electron densities of the carbonyl carbons of the monoalkyl esters (**2a**−**2e**, **2s**) are relatively high, and it is thought that the hydrolytic rate changes due to a simple difference in steric hindrance. It is thought that 1-methylpentyl (hexan-2-yl) ester **2b** is bulky near the ester and that it is difficult for **2b** to approach CESs. CESs are typical serine hydrolases, and hydrolysis proceeds by a ping−pong bibi−type reaction mechanism [22]. The first step is the formation of a CES−substrate complex by nucleophilic attack of serine on the ester, and the second step is hydrolysis of the CES−substrate



	HLM	HIM	hCE1	hCE2
HLM	1.000			
HIM	0.763 **	1.000		
hCE1	0.789 **	0.721 **	1.000	
hCE2	0.553 **	0.732 **	0.754 **	1.000

Fig. 5. Correlations among hydrolytic activities of the enzymes that were examined. A: HLM and hCE1, B: HLM and hCE2, C: HLM and hCE2, D: Pearson's correlation coefficients among the studied enzymes. $^{**}P < 0.01$.

Table 1
Kinetic parameters of indomethacin prodrugs (**2a**–**2x**) in hCE1 solutions. Prodrugs (**2p**–**2r**, **3f** and **4f**) were not tested. Concentrations: prodrugs (0.1, 0.05, 0.025, 0.0125, 0.00625, 0.003125 mM), hCE1 (0.25 mg/mL); reaction time: 30 min; temperature: 37 °C; values are means ± S.E. (n = 3).

Compound	V_{max} (nmol/mg protein/min)	K_m (μM)	Compound	V_{max} (nmol/mg protein/min)	K_m (μM)
2a	1.08 ± 0.0297	4.23 ± 0.526	**2l**	2.00 ± 0.0410	11.1 ± 0.754
2b	0.146 ± 0.0066	6.54 ± 1.15	**2m**	2.29 ± 0.125	13.9 ± 2.35
2c	0.408 ± 0.00607	4.42 ± 0.293	**2n**	6.10 ± 0.313	29.4 ± 3.73
2d	0.976 ± 0.0510	8.71 ± 1.62	**2o**	5.44 ± 0.363	24.2 ± 4.23
2e	1.38 ± 0.0540	7.29 ± 1.08	**2s**	2.37 ± 0.137	12.5 ± 2.32
2f	6.70 ± 0.469	70.9 ± 9.20	**2t**	6.65 ± 0.829	45.7 ± 12.2
2g	3.85 ± 0.248	20.2 ± 3.61	**2u**	4.42 ± 0.204	30.5 ± 3.44
2h	4.51 ± 0.363	20.6 ± 4.56	**2v**	12.8 ± 2.15	81.8 ± 24.4
2i	4.20 ± 0.212	18.9 ± 2.69	**2w**	3.76 ± 0.235	32.4 ± 4.85
2j	2.58 ± 0.121	13.3 ± 1.96	**2x**	3.75 ± 0.368	26.4 ± 6.60
2k	2.60 ± 0.100	12.7 ± 1.56			

Table 2
Kinetic parameters of indomethacin prodrugs (**2s**–**2x**) in hCE2 solutions. Prodrugs (**2a**-**2r**, **3f** and **4f**) were not tested. Concentrations: prodrugs (0.1, 0.05, 0.025, 0.0125, 0.00625, 0.003125 mM), hCE2 (0.25 mg/mL); reaction time: 30 min; temperature: 37 °C; values are means ± S.E. (n = 3).

Compound	V_{max} (nmol/mg protein/min)	K_m (μM)	Compound	V_{max} (nmol/mg protein/min)	K_m (μM)
2s	0.00955 ± 0.00180	22.3 ± 11.3	**2v**	14.3 ± 1.46	101 ± 17.1
2t	0.801 ± 0.0281	7.12 ± 0.947	**2w**	4.24 ± 0.239	25.8 ± 3.75
2u	1.55 ± 0.0670	12.4 ± 1.72	**2x**	4.87 ± 0.267	27.2 ± 3.77

complex by H_2O. It is thought that the reaction rate of an ester with a large steric hindrance is greatly reduced because the nucleophilic attack of serine in the first step is difficult to proceed. Since the hydrolysis rate increased as the methyl group moved away from the ester (**2b**–**2e**), it is possible that the steric hindrance caused by the substituent at the 4–position of the alkoxy group had almost no effect. Alkyl diesters (**2t** and **2u**) are known to have a different mechanism of metabolic activation than that of monoalkyl esters (**2a**–**2e**, **2s**) [23,24]. After the ester on the modifying group side is hydrolyzed, the resulting hemiacetal is autolyzed to produce an indomethacin, corresponding to aldehyde (formaldehyde or acetaldehyde) and carboxylic acid (see Supplementary Fig. S1). Therefore, it is thought that the selectivity of CES is controlled by the ester structure on the modifying group side. The pivoxyl ester **2t** having high selectivity has a structure that is easily recognized by hCE1 because the acyl group side is a bulky pivaloyl group and the alkoxy group is an oxymethylene group with less steric hindrance. However, the electron nucleophilicity of the oxygen atom near the pivaloyl ester enhances the nucleophilicity of the ester. As a result, it is thought that the reactivity of alkyl diesters (**2t** and **2u**) was higher than that of monoalkyl esters (**2a**–**2e**, **2s**), and the selectivity of hCE1/hCE2 was lowered. Therefore, it is thought that adding an electron withdrawing group to an alkyl ester has the effect of increasing the hydrolytic rate, while it may reduce the selectivity of hCE1/hCE2. Aryl esters (**2f**–**2r**) have been found to have lower nucleophilicity of phenoxide ions, which are hydrolysis products, than that of alkoxide ions, which are hydrolysis products of alkyl esters (**2a**–**2e**, **2s**–**2u**). This fact suggests that the reverse reaction of the first step in the hydrolysis mechanism of CES is less likely to occur. As a result, the hydrolysis rate of aryl esters (**2f**–**2r**) is increased in CES solution. It is thought that the overall reactivity was higher than that of alkyl esters (**2a**–**2e**, **2s**–**2u**) and the selectivity of hCE1 was lowered. The effects of steric hindrance on aryl esters have also been confirmed. In the hydrolysis reaction in hCE1 solution, the hydrolytic rate of *o*-acetylphenyl ester **2m** is more than 2.5–times lower than the hydrolytic rates of *m*–form **2n** and *p*–form **2o**, and the hydrolytic rates of all *o*–substituted esters (**2g**, **2j**, **2m** and **2p**) are lower than those of *m*-form and *p*-form in hCE2 solution. It is thought that hCE2 is susceptible to steric hindrance of the substituent at the *o*-position, but hCE1 is also thought to be affected when it becomes a large substituent such as an acetyl group. It was found that the electron density of the ester affects the hydrolysis rate of both hCE1 and hCE2. It is thought that hCE2 is susceptible to electronic influence because the hydrolysis rates of acetylphenyl esters (**2m**–**2o**), which have strong electron withdrawing properties, increased. Carbonate esters (**2v**–**2x**) are known to have the same metabolic activation mechanism as that of alkyl diesters [25] (see Supplementary Fig. S1). Since carbonate esters cannot be classified into acyl groups and alkoxy groups, it is thought that hCE1 and hCE2 cannot selectively recognize carbonate esters (**2v**–**2x**). In general, carbonate esters (**2v**–**2x**) are known to be less reactive for hydrolysis because they have a higher electron density than that of esters. Nonetheless, carbonate esters (**2v**–**2x**) had high hydrolytic rates in hCE1 and hCE2 solutions. It is thought that this is because the hydrolytic rate of CES is greatly affected by steric hindrance and the steric hindrance of carbonyl carbons of carbonate esters (**2v**–**2x**) are lower than that of ester. Since the hydrolysis rate of cyclic cilexetil **2w** having a bulky cyclohexyl group and medoxomil **2x** decreased, it is thought that the hydrolysis rate is likely to decrease due to steric factors.

Summarizing the above, the following three points are important new findings. First, even small alkyl groups such as methyl groups have been found to be effective means of limiting the rate of metabolism of hCE1 and hCE2. In particular, as is clear from the results of compound (**2b**–**2e**), it can be determined that it is possible to efficiently limit the metabolic rate by adjusting the steric hindrance near the ester. Second, the findings on the properties of alkyl and aryl esters have been clarified. For substrates with relatively large acyl groups, such as indomethacin esters, the use of small alkyl esters may be favorable as a prodrug that is not metabolized in human small intestine or hCE2 but metabolized in human liver or hCE1. Conversely, as long as the substrate has a large acyl group, it is considered difficult to form an hCE2–selective ester structure. Aryl esters reduce the selectivity of hCE1/hCE2 but increase the reactivity. Aryl esters metabolize faster than alkyl esters, but are unsuitable for maintaining hCE1/hCE2 selectivity. Third, it

has been shown that the metabolic reaction by CES is not significantly affected by the electron density of the ester. Carbonate esters are chemically less reactive than carboxylic acid esters. However, the metabolic rate of CES tends to be higher than that of carboxylic acid esters. In addition, electron-withdrawing substitution ester (**2m**–**2r**) has a slightly higher hydrolysis rate, and the ortho-substitution product is affected by steric hindrance and the metabolic rate decreases. In predicting the rate of metabolism by CES, it is necessary to consider the factor of steric hindrance rather than the factor of electron dencity.

In this study, 26 kinds of indomethacin prodrugs were used to elucidate the substrate recognition ability of carboxylesterases 1 and 2, which play the most important role in hydrolytic metabolism in humans. By changing the steric hindrance and electron density of the alkoxy group, the factors that change the hydrolysis rate by CES were elucidated. Based on the results of this study, it is hoped that a theoretical ester prodrug design will be carried out in consideration of the substrate recognition ability of the enzyme.

Author contributions

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Declaration of competing interest

The authors declare that there is no conflict of interest.

Acknowledgments

We thank the HAB Research Organization for providing human livers. This work was supported by JSPS KAKENHI Grant Number 19K16421.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dmpk.2021.100391.

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