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Synthesis and evaluation of haloperidol ester prodrugs metabolically activated by human carboxylesterase

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ABSTRACT

Two types of haloperidol prodrugs in which a chemical modification was carried out on the hydroxyl group or carbonyl group were synthesized, and their metabolic activation abilities were evaluated in a human liver microsome (HLM) solution, a human small intestine microsome (HIM) solution and solutions of human recombinant carboxylesterases (hCESs). The metabolic activation rates of alcohol ester prodrugs in HLM solution were similar to those in hCES2 solution, and haloperidol pentanoate and haloperidol hexanoate showed high metabolic activation rates in the synthesized alcohol ester prodrugs. In addition, haloperidol acetate and haloperidol 2-methylbutanoate were hydrolyzed as slowly as haloperidol decanoate. The results suggested that haloperidol prodrugs with a small chain or a branched chain are useful as prodrugs for sustained release. The metabolic activation rate of the enol ester prodrug in HLM solution was similar to that in hCES1 solution, and the enol ester prodrug was found to behave differently from alcohol ester prodrugs, which were metabolically activated by hCES2.

1. Introduction

Haloperidol (**1**) is an antipsychotic drug with a butyrophenone skeleton that was developed in Belgium in 1958. Treatment of schizophrenia was advanced by the development of haloperidol (**1**) (Froemming et al., 1989); however, there was a problem of some patients forgetting to take the medicine. To solve this problem, haloperidol decanoate in which the hydroxyl group of haloperidol is replaced with a decanoate group was developed. Haloperidol decanoate, which is intramuscularly administered, is slowly metabolized and haloperidol (**1**) continues to be produced in the body for 4 weeks. Because of the long administration interval, it is thought to be helpful for some noncompliant patients (Beresford and Ward, 1987). In addition, many ester prodrugs for sustained release such as nalmefene prodrug (Gaekens et al., 2016), entecavir prodrug (Ho et al., 2018) and ibuprofen prodrug (Liu et al., 2017) have been studied recently.

Carboxylesterases (CESs) are hydrolytic enzymes that play an important role in metabolic activation of ester–type and amide–type prodrugs (Hosokawa, 2008). Haloperidol decanoate is also known to be metabolically activated by CESs (Nambu et al., 1987). The two dominant forms CES1 and CES2 are present in various mammals (Hosokawa et al., 2007; Satoh and Hosokawa, 1998; Satoh and Hosokawa, 2006), and CESs contribute to hydrolysis of drugs in many tissues including the liver, small intestine, lung and kidney. CES1 and a small amount of CES2 are expressed in the human liver, in which many drugs are metabolized (Hosokawa et al., 1995). Human CES1 (hCES1) catalyzes a hydrolysis reaction of ester compounds containing a small alkoxy group, such as temocapril (Takai et al., 1997), meperidine (Zhang et al., 1999), oseltamivir (Shi et al., 2006), atorvastatin prodrugs (Mizoi et al., 2016), indomethacin prodrugs (Takahashi et al., 2018) and methylphenidate (Sun et al., 2004). In contrast, CES2 catalyzes the hydrolysis of ester compounds containing a small acyl group, such as CPT–11 (Satoh et al., 1994), cocaine (Kamendulis et al., 1996) and heroin (Brzezinski et al., 1997). Therefore, an ester compound is metabolized by CES1 or CES2 depending on the structure of the ester. Furthermore, among the substrates hydrolyzed by CES1, it has been found that the rate of hydrolysis varies greatly depending on the size of the ester structure and electron density (Mizoi et al., 2016; Imai et al., 2006). Examination of the substrate recognizing ability of these CES isozymes is therefore important for understanding the metabolism of drugs and metabolic activation of prodrugs.

Studies on haloperidol prodrugs have been intensively conducted in recent years. Besides haloperidol decanoate, studies have been conducted on a haloperidol prodrug incorporating PEG (Heath et al., 2016)

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or linear acyl ester (Morris et al., 2008; Morris et al., 2009). There have also been studies on a prodrug of reduced haloperidol that was obtained by reducing the carbonyl group of haloperidol (**1**) (Olivieri et al., 2016; Sozio et al., 2015). Although haloperidol prodrugs having a linear acyl group have been reported, there has been no report on the metabolic ability of haloperidol prodrugs having a branched alkyl acyl group. In this study, we synthesized and evaluated the metabolic activation abilities of a linear acyl group–containing prodrug and branched chain acyl group–containing prodrug. Furthermore, focusing on the carbonyl group of haloperidol, enol ester–type prodrug **3** was newly synthesized and its metabolic activation abilities were evaluated.

2. Materials and methods

2.1. Materials

The ester derivatives were synthesized using the haloperidol (**1**), acid anhydride or acid chloride. Materials for the reaction, such as haloperidol (**1**), acid anhydride, dimethylaminopyridine (DMAP) were purchased from Wako pure chemical industries (Japan). Human intestine microsomes (HIM), hCES1 and hCES2 were purchased from Corning Inc. (Woburn, MA, USA). Human plasma sample (single donor, treated by EDTA–2Na) was purchased from KAC Co., Ltd. (Amagasaki, Hyogo, Japan). Human liver microsomes (HLM) were prepared from human livers which were obtained from the Human and Animal Bridging (HAB) Research Organization, which is in partnership with the National Disease Research Interchange (NDRI). The livers were homogenized and centrifuged at 9000 × 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).

2.2. Synthetic protocols

A mixture of haloperidol (**1**) (1.0 mmol), acid anhydride (or acid chloride) (2.0 mmol), DMAP (0.1 mmol) and Et_3N (5.0 mmol) in CH_3CN (10 mL) was stirred at 80 °C under argon atmosphere for 1 day. After addition of sat. $NaHCO_3$ solution (5 mL), and the whole was extracted with AcOEt (50 mL). The organic solution was washed with H_2O (5 mL × 2) and brine (5 mL × 2), and 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**. The synthesized compounds were confirmed by ^{1}H NMR, ^{13}C NMR and IR analyses.

2.2.1. Haloperidol acetate ***2a***

Isolated yield 29%; pale yellow solids; mp 101–102 °C; IR (KBr) 1737, 1695 cm^{-1}; ^{1}H NMR (400 MHz) δ 1.78–1.92 (2H, m), 1.94 (3H, s), 2.20–2.26 (4H, m), 2.23–2.33 (2H, m), 2.36 (2H, t, J = 7.2 Hz), 2.68–2.70 (2H, m), 2.89 (2H, t, J = 7.2 Hz), 7.00–7.05 (2H, m), 7.14–7.17 (2H, m), 7.18–7.19 (2H, m), 7.89–7.94 (2H, m); ^{13}C NMR (100 MHz) δ 21.9, 22.0, 35.4, 36.2, 49.2, 57.6, 79.9, 115.6 (d, J = 22.0 Hz), 126.0, 128.5, 130.6 (d, J = 9.0 Hz), 133.0, 133.7 (d, J = 3.0 Hz), 143.0, 165.6 (d, J = 253.0 Hz), 169.5, 198.3; HPLC retention time (method A) t_R = 21.5 min, (method B) t_R = 13.7 min.

2.2.2. Haloperidol butanoate ***2b***

Isolated yield 5.0%; pale yellow solids; mp 53–54 °C; IR (KBr) 1733, 1681 cm^{-1}; ^{1}H NMR (400 MHz) δ 0.85 (3H, t, J = 7.6 Hz), 0.86 (3H, t, J = 7.6 Hz), 1.5–1.59 (2H, m), 1.87–1.90 (4H, m), 2.18–2.25 (2H, m), 2.18–2.25 (4H, m), 2.21 (2H, t, J = 7.6 Hz), 2.38 (2H, t, J = 7.2 Hz), 2.70–2.73 (2H, m), 2.91 (2H, t, J = 6.8 Hz), 7.03–7.08 (2H, m), 7.20–7.22 (2H, m), 7.16–7.19 (2H, m), 7.91–7.96 (2H, m); ^{13}C NMR (100 MHz) δ 13.8, 18.4, 21.9, 35.4, 36.2, 37.2, 49.2, 57.6, 79.5, 115.6 (d, J = 21.0 Hz), 126.0, 128.5, 130.6 (d, J = 9.0 Hz), 133.0, 133.6 (d, J = 3.0 Hz), 143.1, 165.6 (d, J = 253.0 Hz), 172.0, 198.3; HPLC retention time (method B) t_R = 18.6 min.

2.2.3. Haloperidol hexanoate ***2c***

Isolated yield 46%; pale yellow oil; IR (KBr) 1735, 1685 cm^{-1}; ^{1}H NMR (400 MHz) 0.89 (3H, t, J = 7.2 Hz), 1.23–1.36 (4H, m), 1.55–1.62 (2H, m), 1.88–2.04 (4H, m), 2.24–2.35 (4H, m), 2.40–2.47 (4H, m), 2.77–2.80 (2H, m), 2.98 (2H, t, J = 6.8 Hz), 7.09–7.14 (2H, m), 7.29–7.31 (4H, m), 7.98–8.03 (2H, m); ^{13}C NMR (100 MHz) δ 13.9, 21.8, 22.3, 24.6, 31.3, 35.2, 35.4, 36.2, 49.2, 57.6, 79.5, 115.6 (d, J = 22.0 Hz), 126.1, 128.5, 130.6 (d, J = 9.0 Hz), 133.0, 133.6 (d, J = 3.0 Hz), 143.1, 165.6 (d, J = 253 Hz), 172.2, 198.3; HPLC retention time (method B) t_R = 24.3 min.

2.2.4. Haloperidol octanoate ***2d***

Isolated yield 28%; yellow oil; IR(KBr) 1735, 1685 cm^{-1}; ^{1}H NMR (400 MHz) δ 0.88 (3H, t, J = 6.8 Hz), 1.11–1.31 (8H, m), 1.56–1.61 (2H, m), 1.87–2.04 (4H, m), 2.27–2.34 (4H, m), 2.40–2.47 (4H, m), 2.76–2.79 (2H, m), 2.98 (2H, t, J = 7.2 Hz), 7.08–7.14 (2H, m), 7.23–7.29 (4H, m), 7.98–8.03 (2H, m); ^{13}C NMR (100 MHz) δ 14.1, 21.8, 22.6, 25.0, 28.9, 29.2, 31.7, 35.3, 35.4, 36.2, 49.2, 57.6, 79.5, 115.6 (d, J = 22.0 Hz), 126.1, 128.5, 130.6 (d, J = 9.0 Hz), 133.0, 133.6 (d, J = 3.0 Hz), 143.1, 165.6 (d, J = 253.0 Hz), 172.2, 198.3; HPLC retention time (method B) t_R = 28.8 min.

2.2.5. Haloperidol pentanoate ***2f***

Isolated yield 28%; pale yellow solids; mp 31–41 °C; IR (KBr) 1729, 1683 cm^{-1}; ^{1}H NMR (400 MHz) 0.90 (3H, t, J = 7.6 Hz), 1.28–1.37 (2H, m), 2.28–2.47 (8H, m), 2.77–2.79 (2H, m), 2.98 (2H, t, J = 6.8 Hz), 7.08–7.14 (2H, m), 7.23–7.29 (4H, m), 7.97–8.02 (2H, m); ^{13}C NMR (100 MHz) δ 13.7, 21.8, 22.3, 27.0, 35.0, 35.4, 36.2, 49.2, 57.6, 79.5, 115.6 (d, J = 21.0 Hz), 126.0, 128.5, 130.6 (d, J = 9.0 Hz), 133.0, 133.6 (d, J = 3.0 Hz), 143.1 165.6 (d, J = 253.0 Hz), 172.2, 198.3; HPLC retention time (method B) t_R = 22.1 min.

2.2.6. Haloperidol 3–methylbutanoate ***2g***

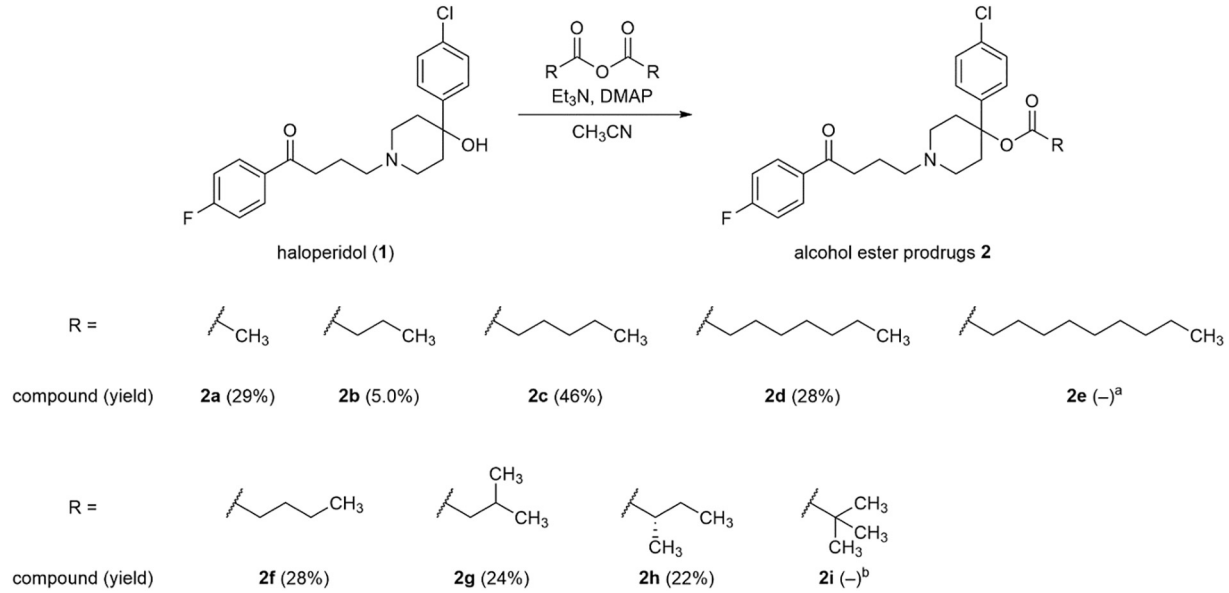
Isolated yield 24%; pale yellow solids; mp 74.5–77 °C; IR (KBr) 1733, 1683 cm^{-1}; ^{1}H NMR (400 MHz) 0.93 (6H, d, J = 6.8 Hz), 1.88–2.11 (5H, m), 2.16 (2H, d, J = 6.8 Hz), 2.32 (2H, t, J = 11.6 Hz), 2.40–2.47 (4H, m), 2.76–2.79 (2H, m), 2.98 (2H, t, J = 6.8 Hz), 7.08–7.12 (2H, m), 7.24–7.30 (4H, m), 7.97–8.02 (2H, m); ^{13}C NMR (100 MHz) δ 21.9, 22.5, 25.6, 35.4, 36.2, 44.4, 49.2, 57.6, 79.6, 115.6 (d, J = 21.0 Hz), 126.1, 128.5, 130.6 (d, J = 9.0 Hz), 133.0, 133.7 (d, J = 3.0 Hz), 143.1 165.6 (d, J = 253.0 Hz), 171.5, 198.3. HPLC retention time (method B) t_R = 22.0 min.

2.2.7. Haloperidol 2–methylbutanoate ***2h***

Isolated yield 22%; pale yellow solids; mp 61–70 °C; IR (KBr) 1733, 1685 cm^{-1}; ^{1}H NMR (400 MHz) δ 0.89 (3H, t, J = 7.2 Hz), 1.30–1.40 (2H, m) 1.49–1.56 (2H, m), 2.37 (3H, s), 2.86 (2H, t, J = 7.6 Hz), 3.84–3.85 (5H, m), 6.68 (1H, dd, J = 8.8, 2.4 Hz), 6.89 (1H, d, J = 8.8 Hz), 6.95 (1H, d, J = 2.4 Hz), 7.48 (2H, d, J = 6.8 Hz), 7.66 (2H, d, J = 6.8 Hz); ^{13}C NMR (100 MHz) δ 11.8, 16.7, 21.9, 26.6, 35.4, 36.2, 42.0, 49.2, 57.7, 79.2, 115.6 (d, J = 21.0 Hz), 126.1, 128.5, 130.6 (d, J = 9.0 Hz), 133.0, 133.7 (d, J = 4.0 Hz), 143.1, 165.6 (d, J = 254.0 Hz), 174.9, 198.3; HPLC retention time (method B) t_R = 21.7 min.

2.2.8. Haloperidol pivalate ***2i***

Isolated yield 3%; pale yellow oil; IR (KBr) 1731, 1685 cm^{-1}; ^{1}H NMR (400 MHz) δ 1.20 (9H, s), 1.88–2.00 (4H, m), 2.29–2.35 (2H, m), 2.41–2.47 (4H, m), 2.79–2.81 (2H, m), 2.98 (2H, t, J = 7.2 Hz), 7.10–7.14 (2H, m), 7.20–7.30 (4H, m), 7.98–8.02 (2H, m); ^{13}C NMR (100 MHz) δ 21.8, 27.3, 35.3, 36.2, 39.3, 49.2, 57.7, 79.0, 115.6 (d, J = 22.0 Hz), 125.8, 128.5, 130.6 (d, J = 9.0 Hz), 133.0, 133.6 (d, J = 3.0 Hz), 143.1, 165.6 (d, J = 253.0 Hz), 176.4, 198.3; HPLC retention time (method B) t_R = 21.1 min.



Scheme 1. Synthesis of haloperidol alcohol ester prodrugs (**2a–2d**, **2f–2h**). [a]**2e** was purchased from Wako Chemical Ind. [b]No product was obtained in this condition.

*2.2.9. Haloperidol enol pivalate **3***

Isolated yield 11%; pale yellow solid; mp 116–123 °C; IR (KBr) 1747, 1664 cm^{-1}, ^1H NMR (400 MHz) δ 1.37 (9H, s), 1.70–1.73 (2H, m), 2.07–2.15 (2H, m), 2.28–2.33 (2H, m), 2.44–2.56 (4H, m), 2.79–2.81 (2H, m), 5.74 (1H, t, J = 7.2 Hz), 6.98–7.02 (2H, m), 7.26–7.32 (2H, m), 7.34–7.37 (2H, m), 7.42–7.45 (2H, m); 23.7, 27.3, 38.4, 39.2, 49.2, 57.3, 71.1, 115.4 (d, J = 22.0 Hz), 126.1, 126.3 (d, J = 8.0 Hz), 128.4, 131.7 (d, J = 3.0 Hz), 132.8, 146.1, 146.4, 162.6 (d, J = 246.0 Hz), 176.1; HPLC retention time (method B) t_R = 17.8 min.

2.3. Hydrolysis reaction (HLM, HIM)

Enzyme solutions were diluted to 5 mg/mL solutions with 200 mM SET buffer (pH 7.4). 25 mM haloperidol ester (**2a–2i**, **3**) in dimethylsulfoxide (DMSO) solutions (1 μL, final concentration 0.25 mM) were warmed with 5 mg/mL enzyme solutions in SET buffer (5 μL, final concentration 0.25 mg/mL), 200 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (50 μL, final concentration 100 mM) and purified water (44 μL) at 37 °C for 24 h. After addition of 0.5 mM biphenyl in isopropanol (100 μL, 50 nmol) immediately, the whole was cooled on an ice bath at 10 min. The mixture was centrifuged at 21,600 × G for 15 min at 4 °C to precipitate protein, and then the supernatant was filtered and analyzed by high performance liquid chromatography (HPLC).

2.4. Plasma stability

10 mM haloperidol ester (**2a**, **2c**, **2e**, **2h** or **3**) in DMSO solutions (3 μL, final concentration 0.10 mM) were warmed with 200 mM HEPES buffer (147 μL), human plasma sample (150 μL) at 37 °C. The reaction solutions (30 μL) were collected at various time points (0, 1, 3, 6, 12, 24, 48 h). After addition of 0.5 mM biphenyl in isopropanol (100 μL) immediately, the whole was cooled on an ice bath at 10 min. The mixture was centrifuged at 21,600 × G for 15 min at 4 °C, and then the supernatant was filtered and analyzed by HPLC.

2.5. Hydrolysis reaction (hCES1, hCES2)

Enzyme solutions were diluted to 5 mg/mL solutions with 200 mM SET buffer (pH 7.4). 0.3125–20 mM haloperidol ester (**2a–2i**, **3**) in DMSO solutions (1 μL, final concentration 0.003125–0.10 mM) were warmed with 5 mg/mL enzyme solutions in SET buffer (5 μL, final concentration 0.25 mg/mL), 200 mM HEPES buffer (50 μL, final concentration 100 mM) and purified water (44 μL) at 37 °C for 24 h. After addition of 0.5 mM biphenyl in isopropanol (100 μL, 50 nmol) immediately, the whole was cooled on an ice bath at 10 min. The mixture was centrifuged at 21,600 × G for 15 min at 4 °C to precipitate protein, and then the supernatant was filtered and analyzed by HPLC.

2.6. HPLC analysis

*2.6.1. Method A for compound **2a***

The HPLC system was performed on a column of Mightysil RP–18 GP 150-4.6 (5 μm) at 30 °C with elution at 1.0 mL/min using the mixture of 0.5% ethylamine in MeOH/0.5% ethylamine in H_2O = 65/35 for 30 min. The haloperidol was monitored at 220 nm. Analytical HPLC was performed in biphenyl as an internal standard (t_R = 18.7 min). The hydrolysis rate was calculated from the detecting area ratio of haloperidol (t_R = 10.8 min) and biphenyl.

*2.6.2. Method B for compound (**2b–2i, 3**)*

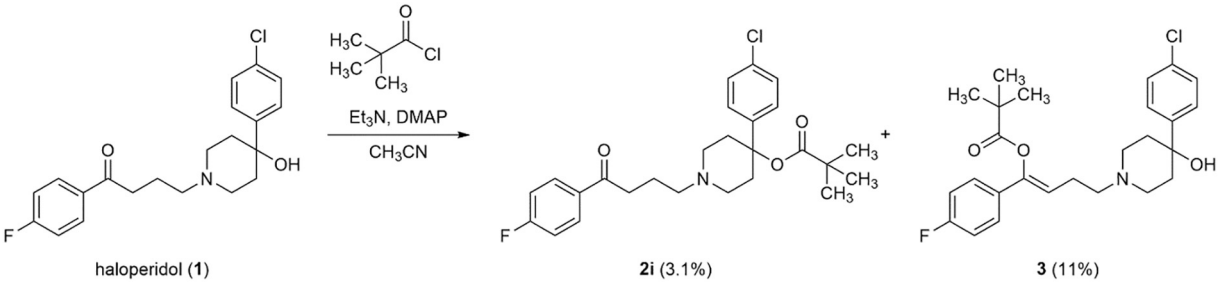
The HPLC system was performed on a column of Mightysil RP–18 GP 150-4.6 (5 μm) at 30 °C with elution at 1.0 mL/min using the gradient of 0.5% ethylamine in MeOH/0.5% ethylamine in H_2O = 65/35 being ramped up from 65/35 to 95/5 for 30 min and then returned to 65/35 in 10 min. The haloperidol was monitored at 220 nm. Analytical HPLC was performed in biphenyl as an internal standard (t_R = 13.7 min). The hydrolysis rate was calculated from the detecting area ratio of haloperidol (t_R = 9.3 min) and biphenyl.

3. Results and discussion

*3.1. Synthesis of prodrugs (**2a–2i, 3**) and these chemical properties*

Haloperidol alcohol ester prodrugs (**2a–2d**, **2f–2h**) were synthesized using haloperidol (**1**) and acid anhydrides (Scheme 1). Using acid chloride instead of acid anhydride, it was possible to synthesize both alcohol ester prodrug **2i** and enol ester prodrug **3** (Scheme 2). These compounds were purified by column chromatography, and the structure was confirmed by ^1H NMR, IR and ^{13}C NMR. The *E/Z* conformation of enol ester **3** was determined by NOESY. Haloperidol decanoate (**2e**) was purchased commercially and used for experiments.

Next, in order to evaluate the lipophilicity of the haloperidol



Scheme 2. Synthesis of pivalate prodrugs (**2i**, **3**) by haloperidol (**1**) and pivaloyl chloride.

Table 1
Chemical properties of haloperidol (**1**) and ester prodrugs (**2f–2i**, **3**). ClogP was calculated using Chem3D 15.0. HPLC retention time was measured under method B.

Compound	ClogP	HPLC retention Time (min)
1	3.85	9.3
2a	4.70	13.7
2b	5.76	18.6
2c	6.82	24.3
2d	7.88	28.8
2e	8.94	31.4
2f	6.29	22.1
2g	6.07	22.0
2h	6.16	21.7
2i	5.94	21.1
3	5.42	17.8

prodrugs (**2a–2i**, **3**), calculation of ClogP value and retention time of HPLC were measured (Table 1). Due to the increase in the carbon number of the modifying group, the ClogP value and retention time of HPLC were increased, it was confirmed that any prodrug was higher in lipophilicity than haloperidol (**1**).

3.2. Hydrolysis reaction in HLM and HIM

It was examined whether hydrolysis of the prodrugs (**2a–2i**, **3**) proceeds in a human liver microsome (HLM) solution (Fig. 1). Haloperidol hexanoate (**2c**) showed a higher metabolic activation rate than those of the prodrugs (**2a–2e**). Acetate **2a** was hydrolyzed as slowly as haloperidol decanoate (**2e**). A comparison of the C5 acyl ester prodrugs (**2f–2i**) showed that the hydrolysis rates of branched acyl esters (**2g–2i**) were slower than those of the linear acyl ester **2f**. Although 3-methylbutanoate **2g** and pivalate **2i** were not hydrolyzed in 24 h, 2-methylbutanoate **2h** was hydrolyzed. The results suggested that haloperidol prodrugs with a small chain or a branched chain are useful as prodrugs for sustained release.

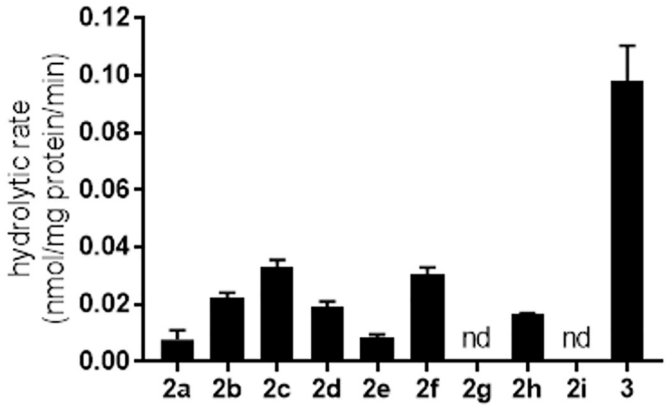


Fig. 1. Hydrolysis rates of haloperidol ester prodrugs (**2a–2i**, **3**) in HLM solution. Values are means ± S.D. (n = 3). nd: not detected.

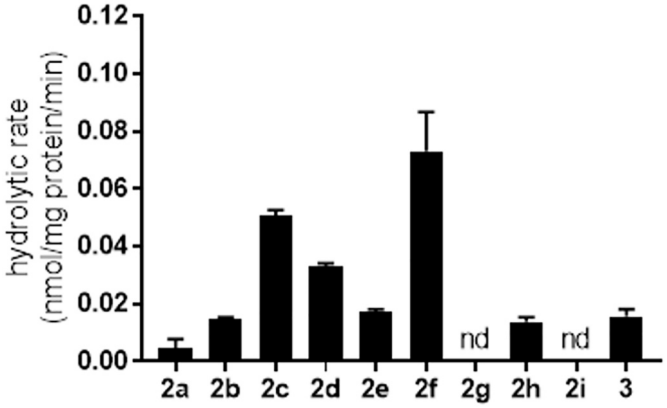


Fig. 2. Hydrolysis rates of haloperidol ester prodrugs (**2a–2i**, **3**) in HIM solution. Values are means ± S.D. (n = 3). nd: not detected.

The prodrugs were subjected to hydrolysis reaction in HIM solution and the stability of the prodrugs in HIM solution was investigated (Fig. 2). Although the data showed that the hydrolysis rates of alcohol ester prodrugs (**2a–2i**) in HIM solution are similar to those in HLM solution, the hydrolysis rate of **3** was significantly reduced compared with that in HLM solution. Therefore, it was thought that the kinds of hCES that catalyze the hydrolysis of **2** and the hydrolysis of **3** are different. hCES1 and small amount of hCES2 are also expressed in the liver and hCES2 is expressed in the small intestine. Since alcohol ester prodrugs (**2a–2i**) have a bulky alkoxy group, they are expected to be mainly catalyzed by CES2. On the other hand, the enoxy position of enol ester prodrug **3** is more vacant than the alkoxy position of alcohol ester prodrugs (**2a–2i**). For these reasons, it is thought that alcohol ester prodrugs are mainly catalyzed by hCES2 and enol ester prodrugs are mainly catalyzed by hCES1. The alcohol ester prodrugs (**2a–2i**), which are CES2–hydrolyzed types, may be hydrolyzed in both the liver and small intestine and are therefore to be inconvenient for oral administration. The enol ester prodrug **3**, which is a CES1–hydrolyzed type, is considered to be a more suitable prodrug for oral administration since it is hardly hydrolyzed in the small intestine. It is thought to be almost impossible to confirm the hydrolysis of alcohol ester prodrugs (**2a–2i**) with CES1 since the alkoxy group has a structurally very bulky carbon near the ester.

3.3. Plasma stability

The plasma stabilities of hexanoate **2c** and enol pivalate **3**, which have high hydrolysis rate in HLM solution and acetate **2a**, decanoate **2e** and 2–methylbutanoate **2h**, which have relatively low hydrolysis rate in HLM solution were evaluated. The amount of haloperidol prodrugs (**2a**, **2c**, **2e**, **2h** and **3**) when reaction was carried out for 0, 1, 3, 6, 12,

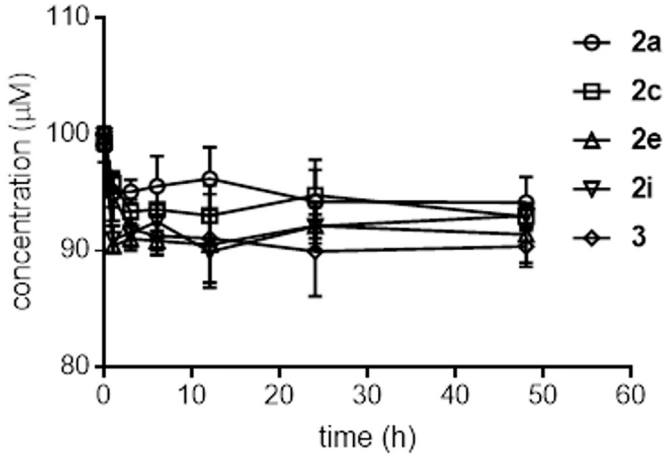


Fig. 3. Plasma Stabilities of haloperidol esters (**2a**, **2c**, **2e**, **2h**, **3**) in human plasma solution. Values are means ± S.D. (n = 3).

Table 2
Kinetic parameters of haloperidol ester prodrugs (**2a–2i**, **3**) in hCES2 solutions. Values are means ± S.E. (n = 3). nd: not detected.

Compound	Vmax (nmol/mg protein/min)	Km (μM)	CLint (mL/mg protein/min)
2a	23.0 ± 4.80	1730 ± 979	0.0133
2b	22.4 ± 1.76	261 ± 103	0.0858
2c	29.8 ± 1.66	25.6 ± 3.68	1.16
2d	23.4 ± 1.85	53.4 ± 8.58	0.438
2e	11.2 ± 1.42	52.6 ± 13.0	0.213
2f	27.0 ± 3.32	36.4 ± 10.3	0.742
2g	nd	nd	nd
2h	2.27 ± 0.276	25.5 ± 8.16	0.0889
2i	nd	nd	nd
3	2.97 ± 0.557	4.21 ± 3.59	0.705

Table 3
Kinetic parameters of haloperidol ester prodrugs (**2f–2i**, **3**) in hCES1 solutions. Values are means ± S.E. (n = 3). nd: not detected.

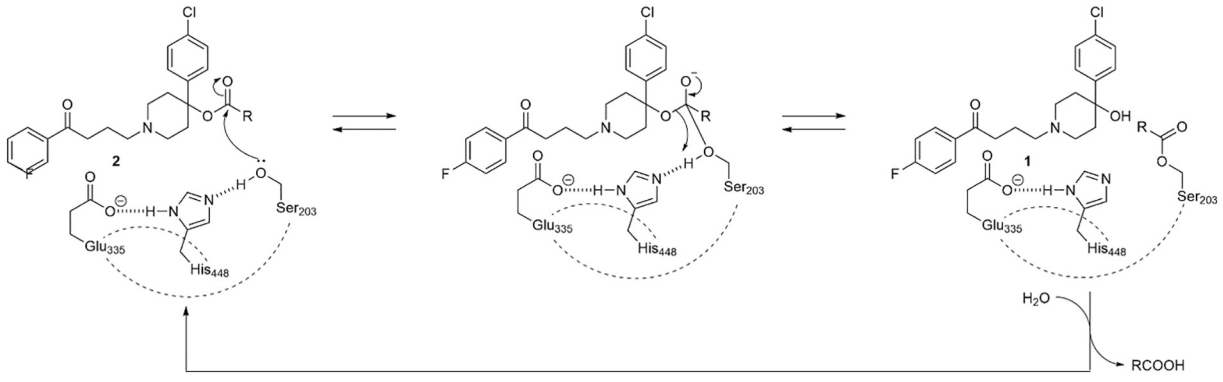
Compound	Vmax (nmol/mg protein/min)	Km (μM)	CLint (mL/mg protein/min)
2f	nd	nd	nd
2g	nd	nd	nd
2h	nd	nd	nd
2i	nd	nd	nd
3	16.8 ± 0.679	5.87 ± 0.960	10.9

24 and 48 h was calculated and the stabilities of these esters was evaluated (Fig. 3). About 5–10% disappearance of haloperidol prodrugs (**2a**, **2c**, **2e**, **2h** and **3**) was observed, however, haloperidol (**1**) was not detected even when reacted in the presence of human plasma for 48 h. Hydrolytic enzymes such as butyrylcholinesterase, paraoxonase and albumin are mainly present, but carboxylesterases are no contained in human plasma (Fukami and Yokoi, 2012). It is thought that the synthesized haloperidol prodrugs (**2a–2i**, **3**) are unlikely to be recognized by hydrolases in human plasma and can stably exist in human plasma.

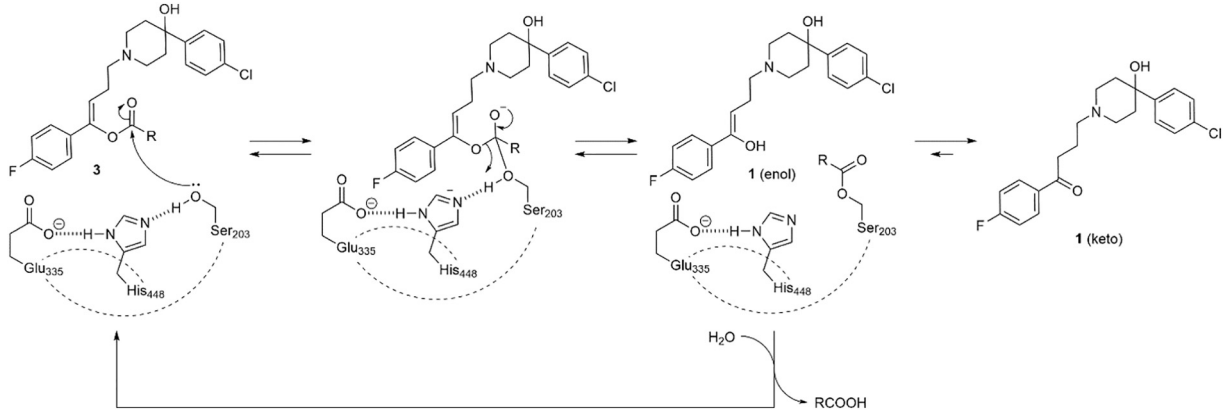
3.4. Kinetics analysis of hydrolysis reaction catalyzed by hCES1 or hCES2

To confirm that the haloperidol prodrugs (**2a–2i**, **3**) are metabolically activated by hCESs, these prodrugs were subjected to hydrolysis reaction in hCES1 or hCES2 solution. The V_{max} and K_m values were calculated by the Michaelis-Menten equation using nonlinear regression analysis with GraphPad Prism 7 software, and CL_{int} was calculated by the V_{max} and K_m values. In the reaction in hCES2 solution (Table 2), V_{max} values of CES2 for ester prodrugs (**2a–2i**, **3**) showed patterns similar to the hydrolysis rates in HIM solution: V_{max} values of pentanoate **2f** and hexanoate **2c** were relatively high, while those of acetate **2a**, decanoate **2e**, 2-methylbutanoate **2h** and pivalate **3** were low. The K_m values of hCES2 for the haloperidol prodrugs increased as the number of carbons of the prodrug became smaller or larger than the appropriate number. The K_m value of acetate **2a**, which has a relatively small steric hindrance, was larger than the K_m values of the other esters. It is thought that the affinity of the enzyme varies greatly depending on the structure of the ester, and hexanoate **2c** and its analogs were thought to be easily recognizable by hCES2. When designing a sustained–release prodrug that is metabolically activated by hCES2, it is important not to decrease the rate of metabolic activation by the construction of a bulky ester but to control the rate of metabolic activation in consideration of the affinity between an ester and a metabolic enzyme. The largest CL_{int} value was also for hexanoic acid ester. The CL_{int} values of acetate **2a**, decanoate **2e** and 3–methylbutanoate **2g** were relatively small among the alcohol esters (**2a–2i**). Therefore, the CL_{int} values of esters that are structurally different from hexanoate **2c** were found to be small. These results suggest that hCES2 may have catalyzed the hydrolysis of haloperidol prodrugs (**2a–2i**, **3**). Next, hydrolysis in hCES1 solution was examined (Table 3). Interestingly, although no hydrolysis reaction was observed for alcohol ester prodrugs (**2a–2i**), enol ester prodrug **3** was effectively hydrolyzed in hCES1 solution. Therefore, it is thought that the hydrolysis of alcohol ester prodrugs (**2a–2i**) is catalyzed by hCES2 rather than hCES1 and that the hydrolysis of enol ester prodrug **3** is catalyzed by hCES1 rather than hCES2.

A possible reason for the difference in hydrolysis of alcohol ester prodrugs (**2a–2i**) and hydrolysis of the enol ester prodrug **3** is the difference in bulkiness of the ester in these prodrugs. The alkoxy group of alcohol ester prodrugs (**2a–2i**) has a tertiary carbon neighboring the



Scheme 3. Proposed mechanism for the hydrolysis of an alcohol ester prodrug by CES.



Scheme 4. Proposed mechanism for the hydrolysis of an enol ester prodrug by CES.

ester and is very bulky, whereas the acyl group of these prodrugs (**2a–2i**) has relatively small chains. These esters that have a small acyl group and a large alkoxy group were recognized by hCES2. In contrast, the enoxy group of the enol ester prodrug **3** has a carbon neighboring the ester, which takes a planar structure and is not bulky, whereas the acyl group of prodrug **3** has a tertiary carbon neighboring the ester. This ester, which has a large acyl group and a small alkoxy group, is recognized by hCES1. Thus, by changing the modification site of the acyl group, it is thought that two kinds of prodrugs with different metabolic properties can be synthesized.

3.5. Hydrolysis mechanism

The hydrolysis mechanisms of the synthesized haloperidol prodrugs (**2a–2i**, **3**) are shown in Schemes 3 and 4. The hydrolysis reaction by CES is a typical ping–pong–bi–bi reaction (Satoh and Hosokawa, 1998). Nucleophilic attack to ester is occurred by the hydroxyl group of serine which the nucleophilicity of which is enhanced by the hydrogen bond with glutamine and histidine in CES. Next, an acyl–enzyme complex is formed by elimination of the haloperidol (**1**). Subsequently, a nucleophilic addition–elimination reaction in the acyl–enzyme complex by water occurs, the acyl moiety is eliminated from the acyl-enzyme complex to form CES. The eliminated acyl group is converted to the corresponding carboxylic acid by nucleophilic reaction of water. These carboxylic acids are highly safe as contained in foods and essential oils.

The first nucleophilic addition step affects the metabolic activation ability depending on the type of ester. From the results of X–ray structural analysis, the active center of hCES1 enzyme is composed of a rigid site and a flexible site and has a wide space (Bencharit et al., 2003). On the other hand, one of the loops constituting the flexible site is deleted in the amino acid sequence in hCES2, and it is thought that the flexibility of the active center of hCES2 is inferior to that of hCES1. The difference in substrate specificity between hCES1 and hCES2 is thought to be due to the difference in the site size and flexibility. It is thought that hCES2 does not catalyze the hydrolysis of a compound having a bulky acyl group because the steric hindrance of the active center prevents binding between the acyl groups which is the first nucleophilic addition step, it is thought that the only ester having an acyl groups of the appropriate size can be bonded.

4. Conclusions

In this study, two types of haloperidol prodrugs were synthesized, and the metabolic activation abilities were investigated. Based on the results, prodrug **2** of the alcohol ester type is thought to be metabolically activated mainely by hCES2, and it was found that haloperidol pentanoate and haloperidol hexanoate have relatively high metabolic activation rates. Also, besides the prodrug with a long acyl group such as decanoate, the prodrug with a short acyl group or a branched acyl group showed the possibility of being useful in prodrugs for sustained release. In addition, it was found that prodrug **3** of the enol ester type is more likely than an alcohol ester–type prodrug to be metabolized in the liver. The application of these findings is expected to further accelerate the study of prodrugs for alcohol–type and ketone–type pharmaceuticals.

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