Design, synthesis and biological evaluation of water-soluble phenytoin prodrugs considering the substrate recognition ability of human carboxylesterase 1

Masato Takahashi , Yeon Joo Lee , Teruhiko Kanayama , Yusuke Kondo , Kazuki Nishio , Kota Mukai , Masami Haba , Masakiyo Hosokawa

PII: S0928-0987(20)30244-X
DOI: https://doi.org/10.1016/j.ejps.2020.105455
Reference: PHASCI 105455

To appear in: *European Journal of Pharmaceutical Sciences*

Received date: 4 March 2020
Revised date: 29 June 2020
Accepted date: 2 July 2020

Please cite this article as: Masato Takahashi , Yeon Joo Lee , Teruhiko Kanayama , Yusuke Kondo , Kazuki Nishio , Kota Mukai , Masami Haba , Masakiyo Hosokawa , Design, synthesis and biological evaluation of water-soluble phenytoin prodrugs considering the substrate recognition ability of human carboxylesterase 1, *European Journal of Pharmaceutical Sciences* (2020), doi: https://doi.org/10.1016/j.ejps.2020.105455

Highlights

・Ten phenytoin prodrugs were designed and synthesized.

・All prodrugs are efficiently converted to phenytoin in human liver microsomes.

・Glutarate ester–type prodrugs showed good solubility in buffers with pH values from 8.3 to 10.1.

・The cyclopropanedicarboxylate and 3,3-dimethylglutarate prodrugs were more stable in aqueous solution than the other synthetic prodrugs.

Design, synthesis and biological evaluation of water-soluble phenytoin prodrugs considering the substrate recognition ability of human carboxylesterase 1

Masato Takahashi*, Yeon Joo Lee, Teruhiko Kanayama, Yusuke Kondo, Kazuki Nishio, Kota Mukai, Masami Haba, Masakiyo Hosokawa

Faculty of Pharmacy, Chiba Institute of Science, 15-8. Shiomi-cho, Choshi, Chiba 288-0025, Japan

*corresponding author e-mail address matakahashi@cis.ac.jp

Keywords: prodrug; water-soluble; carboxylesterase; phenytoin; substrate; specificity

Abstract

Human carboxylesterase 1 (hCES1) is a hydrolase that is mainly expressed in the liver and lung and plays the most important role in the metabolic activation of ester–type prodrugs. In this study, design, synthesis and evaluation of water–soluble phenytoin prodrugs were performed with consideration of the substrate recognition ability of hCES1. The phenytoin prodrugs were synthesized in two steps without column chromatography. It was confirmed that all prodrugs are efficiently converted to phenytoin in a human liver microsome (HLM) solution (up to 54.6 nmol/mg protein/min). Although some of the prodrugs were degraded in strongly basic solution, the solubility of all prodrugs was greater than that of phenytoin in buffer solutions at pH 7.4 and 8.3. Among the synthesized phenytoin prodrugs, the 3,3-dimethylglutarate prodrug was superior in terms of solubility and stability, and it showed solubility of 10 mg/mL or more (phenytoin: <0.1 mg/mL) in a solution of pH 8.3. It was also found that the 3,3-dimethylglutarate prodrug was selectively activated by hCES1 but not hCES2 or arylacetamidodeacetylase.

Abbreviations

AADAC: arylacetamide deacetylase, BPHB: butyl *para*-hydroxybenzoate, CES: carboxylesterase, DMSO: dimethylsulfoxide, EDTA: ethylenediaminetetraacetic acid, HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, HIM: human intestine microsome, HLM: human liver microsome, HPLC: high-performance liquid chromatography, IR: infrared absorption spectrometry, mp: melting point, NMR: nuclear magnetic resonance, PCR: polymerase chain reaction, Tris: tris(hydroxymethyl)aminomethane

1. Introduction

Carboxylesterases (CESs) are known as hydrolases that play an important role in metabolic activation of ester–type and amide–type prodrugs (Hosokawa, 2008; Wang et al., 2018). CESs are classified into CES1 to CES5 based on the difference in amino acid sequence, and hCES1 and hCES2 are mainly expressed in humans (Hosokawa et al., 1995; Taketani et al., 2007). hCES1 is mainly expressed in the liver and lung and hCES2 is mainly expressed in the small intestine and kidney (Hosokawa et al., 2007). It is thought that the chemical structure of the prodrug needs to be easily recognized by hCES1 in order to be metabolized in the liver. The substrate recognition ability of hCES1 has been investigated, and it was found that it is easy for hCES1 to recognize a substrate with a large acyl group and a small alkoxy group near the ester. This substrate recognition ability of hCES1 was found by examining the metabolic abilities of an angiotensin–converting enzyme inhibitor (Takai et al., 1997), narcotics (Kamendulis et al., 1996; Zhang et al., 1999), an anti-tumor drug (Miwa et al., 1998; Satoh et al., 1994), a non-steroidal anti-inflammatory drug (Takahashi et al., 2018; Takahashi et al., 2020), an HMG-CoA reductase inhibitor (Mizoi et al., 2016), antipsychotics (Takahashi et al., 2019), and antiplatelet drug (Shi et al., 2006).

Phenytoin (**1**) is a type of antiepileptic drug with a hydantoin skeleton, and it was first synthesized in 1908. Although phenytoin (**1**) is a widely prescribed drug, intravenous administration of phenytoin (**1**) is known to cause vascular pain and inflammation (Jamerson et al., 1994). Phenytoin (**1**) has a pKa value of 8.3 for the hydantoin moiety, and it needs to be dissolved in a strongly basic aqueous solution (standard pH of 12.0) in order to be used for an aqueous solution (Agarwal and Blake, 1968; Jansod et al., 2016). Furthermore, it is known that when phenytoin solution is mixed with other drug solutions or diluted with a large volume of infusion, phenytoin precipitates due to a change in pH value of the solution (Gupta et al., 2018). Fosphenytoin (**2**) has been developed to solve this problem (Fig. 1). Fosphenytoin (**2**) can be dissolved in neutral aqueous solution by substituting phenytoin with a (phosphonooxy)methoxy group, and it is metabolically activated by alkaline phosphatase in

the body. Although fosphenytoin also has the disadvantages of being expensive and should be stored refrigerated, the water solubility of fosphenytoin is greatly improved compared to that of phenytoin (Stella, 1996; Patel et al., 2015). In addition to fosphenytoin, other prodrugs including oral absorption–type prodrugs (Haque et al., 1986; Stella et al., 1998; Tanino et al., 1998), carboxylate–type prodrugs (Bosch et al., 1999), amine–type prodrugs (Müller et al., 1994), guanidine–type prodrugs (Hamada et al., 2016) and sulfonic acid–type prodrugs have been synthesized (Varia et al., 1984).

Phosphate–type and methyloxyphosphate–type prodrugs have been developed to improve water solubility (Jornada et al., 2015; Sanches and Ferreira, 2019; Stella and Nti-Addae, 2007; Zhang et al., 2019), and these prodrugs are metabolically activated by alkaline phosphatase. However, there has been no report of a water–soluble prodrug that is metabolically activated by hCES1. hCES1 is abundantly expressed in the liver, and it can be expected that ester prodrugs would be metabolically activated efficiently in the liver. As described above, hCES1 is known to have substrate recognition abilities; however, a water–soluble prodrug has not been designed and synthesized on the basis of that information. In this study, we designed, synthesized, and evaluated a novel water-soluble phenytoin prodrug that is metabolically activated by hCES1 as an intravenous infusion agent that dissolves in neutral aqueous solutions.

2. Materials and Methods

2.1. Materials

The ester prodrugs **3** were synthesized using the phenytoin (**1**) in two steps. Materials for the reaction, such as phenytoin (**1**), formaldehyde, potassium carbonate and acid anhydride were purchased from Wako pure chemical industries (Japan). Buffer (pH 7.4) was prepared in Na_2HPO_4 solution with pH adjusted to 7.4 with KH_2PO_4 solution. Buffer (pH 8.3) was prepared in tris(hydroxymethyl)aminomethane (Tris) solution with pH adjusted to 8.3 with 5 M HCl solution. Buffers (pH 9.2 or 10.1) were prepared in Na_2CO_3 solution with pH adjusted to 9.2 or 10.1 with $NaHCO_3$ solution. Buffers (pH 11.0 or 11.9) were prepared with Na_2HPO_4 solution with adjusted to 11.0 or 11.9 with 1 M NaOH solution. ^{1}H-NMR spectra were recorded in CD_3OD on a 400 MHz spectrometer using TMS (0.00 ppm) as an internal standard. ^{13}C-NMR spectra were recorded in CD_3OD on a 100 MHz spectrometer using a center peak of CD_3OD (49.0 ppm) as an internal standard.

2.2. Synthetic protocols

2.2.1. Synthesis of hydroxymethyl phenytoin **4**

A mixture of phenytoin (**1**) (8.0 mmol), 37% HCHO (107.0 mmol) solution, and K_2CO_3 (0.7 mmol) in H_2O (72 mL) was stirred at room temperature under air for 1 day. The mixture was filtered and washed with H_2O to give hydroxymethyl phenytoin **4**.

Hydroxymethyl phenytoin **4**

mp 185–187°C; IR (KBr) 3394, 3338, 1768, 1706 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 4.82 (2H, d, *J*=8.2 Hz), 6.42 (1H, t, *J*=8.0 Hz), 7.34–7.43 (10H, m), 9.64 (1H, s).

2.2.2. Synthesis of phenytoin prodrug (**3a**–**3j**)

A mixture of hydroxymethyl phenytoin **2** (1.8 mmol) and anhydride (2.5 mmol) in pyridine (or acetone, acetonitrile) (10 mL) was stirred at room temperature under air for 1 day. After addition of hexane/AcOEt (1/1, 10 mL), The mixture was extracted with 5% Na_2CO_3 solution (20 mL). The aqueous solution was acidified by the addition of 5% HCl solution (20 mL). The mixture was extracted with $CHCl_3$ (10 mL×2). The organic solution was washed with H_2O (5 mL) and brine (5 mL), dried over $MgSO_4$, and evaporated, which was recrystallized from hexane/ether (1:1) to give prodrug **3a** (81%).

4-((2,5-dioxo-4,4-diphenylimidazolidin-1-yl)methoxy)-4-oxobutanoic acid **3a**

mp 41–43°C; IR (KBr) 2935, 1781, 1749, 1716 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 2.54–2.62 (4H, m), 5.58 (2H, s), 7.33-7.42 (10H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 28.1, 28.3, 61.7, 70.1, 126.7, 128.2, 128.3, 139.2, 154.6, 171.5, 173.0, 174.2; HPLC retention time t_R = 11.2 min.

4-((2,5-dioxo-4,4-diphenylimidazolidin-1-yl)methoxy)-3,3-dimethyl-4-oxobutanoic acid and 4-((2,5-dioxo-4,4-diphenylimidazolidin-1-yl)methoxy)-2,2-dimethyl-4-oxobutanoic acid **3b**

mp 70–75°C; IR (KBr) 2932, 1789, 1725, 1716 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) major product δ 1.21 (6H, s), 2.59 (2H, s), 5.55 (2H, s), 7.32–7.43 (10H, m), 7.88 (1H, s); minor product δ 1.19 (6H, s), 2.54 (2H, s), 5.58 (2H, s), 7.32–7.43 (10H, m), 7.88 (1H, s); ^{13}C NMR (100 MHz, CD_3OD) major product δ 24.3, 39.9, 43.0, 61.4, 78.0, 126.7, 128.2, 128.33, 139.1, 154.6, 170.3, 173.0, 175.8, 179.0; minor product δ 24.1, 40.2, 43.3, 61.7, 70.1, 126.8, 128.2, 128.30, 139.2, 154.6, 170.3, 173.0, 175.8, 179.0; HPLC retention time major product t_R = 16.6 min, minor product t_R = 17.5 min.

rac-(1*R*,2*S*)-2-(((2,5-dioxo-4,4-diphenylimidazolidin-1-yl)methoxy)carbonyl)cyclopropane-1-carboxylic acid **3c**

mp 64–66°C; IR (KBr) 1784, 1726 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 1.22–1.29 (1H, m), 1.49–1.54 (1H, m), 2.06–2.16 (2H, m), 5.51 (1H, d, *J*=10.4 Hz), 5.60 (1H, d, *J*=10.4 Hz), 7.33–7.42 (10H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 11.0, 20.8, 21.3, 48.5, 62.0, 70.1, 126.7, 126.8, 128.2, 128.30, 128.33, 139.15, 139.20, 154.6, 169.2, 172.1, 173.0; HPLC retention t_R = 10.4 min.

rac-(1*R*,2*S*)-2-(((2,5-dioxo-4,4-diphenylimidazolidin-1-yl)methoxy)carbonyl)cyclopentane-1-carboxylic acid **3d**

mp 72–74°C; IR (KBr) 2965, 1779, 1716 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 1.63–1.72 (1H, m), 1.80–1.89 (1H, m), 1.92–2.04 (4H, m), 3.01–3.07 (2H, m), 5.01 (2H, s), 7.33–7.41 (10H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 23.3, 28.4, 46.8, 61.3, 69.9, 74.7, 126.8, 128.1, 128.2, 128.6, 139.5, 155.8, 169.5, 173.8, 176.6; HPLC retention time t_R = 7.9 min.

rac-(1*R*,2*S*)-2-(((2,5-dioxo-4,4-diphenylimidazolidin-1-yl)methoxy)carbonyl)cyclohexane-1-carboxylic acid **3e**

mp 77–83°C; IR (KBr) 1784, 1721 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 1.27–1.54 (4H, m), 1.66–1.82 (2H, m), 1.83–2.06 (2H, m), 2.73–2.90 (2H, m), 5.50 (1H, d, *J*=10.4 Hz), 5.64 (1H, d, *J*=10.4 Hz), 7.33–7.43 (10H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 23.1, 23.6, 25.6, 26.0, 42.0, 42.2, 61.4, 70.1, 126.75, 126.78, 128.2, 128.3, 139.2, 154.6, 172.8, 173.0, 175.7; HPLC retention time t_R = 21.6 min.

(*Z*)-4-((2,5-dioxo-4,4-diphenylimidazolidin-1-yl)methoxy)-4-oxobut-2-enoic acid **3f**

mp 62–64°C; IR (KBr) 1780, 1724 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 5.64 (2H, s), 6.26 (1H, d, *J*=12.0 Hz), 6.36 (1H, d, *J*=12.0 Hz), 7.30–7.41 (10H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 62.0, 70.2, 126.8, 127.5, 128.2, 128.4, 131.7, 139.1, 154.5, 164.3, 166.9, 173.0; HPLC retention time t_R = 7.7 min.

2-(((2,5-dioxo-4,4-diphenylimidazolidin-1-yl)methoxy)carbonyl)benzoic acid **3g**

mp 185–188°C; IR (KBr) 3244, 1788, 1726 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 5.78 (2H, s), 7.30–7.45 (10H, m), 7.53–7.64 (3H, m), 7.75–7.85 (1H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 62.5, 70.2, 126.8, 128.2, 128.3, 129.0, 130.6, 131.0, 131.1, 131.8, 132.0, 139.1, 154.6, 167.1, 168.6, 173.1; HPLC retention time t_R = 14.6 min.

5-((2,5-dioxo-4,4-diphenylimidazolidin-1-yl)methoxy)-5-oxopentanoic acid **3h**
mp 140–145℃; IR (KBr) 3259, 1780, 1716 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ 1.81–1.88 (2H, m), 2.31 (2H, t, *J*=7.2 Hz), 2.39 (2H, t, *J*=7.2 Hz), 5.58 (2H, s), 7.33–7.43 (10H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 19.7, 32.3, 61.5, 70.1, 126.7, 128.2, 128.4, 139.1, 154.6, 172.0, 173.1, 175.2; HPLC retention time t_R = 12.9 min.

5-((2,5-dioxo-4,4-diphenylimidazolidin-1-yl)methoxy)-3,3-dimethyl-5-oxopentanoic acid **3i**
mp 141–145℃; IR (KBr) 3259, 1780, 1716 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ 1.03 (6H, s), 2.30 (2H, s), 2.45 (2H, s), 5.57 (2H, s), 7.32–7.44 (10H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 26.4, 31.9, 44.3, 44.6, 44.6, 61.1, 70.1, 126.7, 128.2, 128.3, 139.1, 154.6, 173.0, 173.9, 174.3; HPLC retention time t_R = 18.0 min.

6-((2,5-dioxo-4,4-diphenylimidazolidin-1-yl)methoxy)-6-oxohexanoic acid **3j**
mp 185–188℃; IR (KBr) 3244, 1788, 1726 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ 1.48–1.75 (4H, m), 2.18–2.42 (4H, m), 5.57 (2H, s), 7.25–7.50 (10H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 23.9, 32.9, 33.1, 61.5, 70.1, 126.7, 128.3, 128.4, 139.1, 154.6, 172.3, 173.0, 175.8; HPLC retention time t_R = 15.0 min.

2.3. Preparation of enzyme Samples

Human intestine microsomes (HIM), hCES1, and hCES2 were purchased from Corning inc. (Woburn, MA, USA). Human plasma sample (treated by EDTA–2Na) was purchased from KAC Co., Ltd. (Amagasaki, Hyogo, Japan). PCR amplification was carried out to determine the coding region of arylamide deacetylase (AADAC) cDNA using human liver cDNA. The amplified DNA fragments were subcloned into the pCR-Blunt II TOPO vector (Invitrogen, MA, USA). Expression of AADAC by using a BAC-TO-BAC Baculovirus Expression System (Invitrogen, MA, USA) was carried out in accordance with the directions of the manufacturer. Human liver microsomes (HLM) were prepared from human liver (single donor) which were obtained from the Human and Animal Bridging Research Organization, which is in partnership with the National Disease Research Interchange. The use of human livers was approved by the Ethics Committee of Chiba Institute of Sciences (No. 22-1), Japan, based on the Helsinki declaration. The livers were homogenized and centrifuged at 9,000 × G

for 20 min at 4 ℃, and the supernatant was ultra–centrifuged at 105,000 × G for 60 min at 4 ℃. The microsome fraction was suspended in sucrose–EDTA–Tris buffer (pH 7.4).

2.4. Hydrolysis reactions (HLM, HIM, hCES1, hCES2, and AADAC)

Enzyme solutions were diluted to 5 mg/mL solutions with 200 mM sucrose–EDTA–Tris buffer (pH7.4). 25 mM phenytoin prodrugs (**3a**–**3j**), temocapril or methylprednisolone hemisuccinate in dimethylsulfoxide (DMSO) solutions (1 µL, final concentration 0.25 mM) was warmed with 5 mg/mL enzyme solutions in sucrose–EDTA–Tris 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 ℃ for 0.5 h (HLM, hCES1, hCES2 or AADAC) or 3 h (HIM). After addition of 0.5 mM BPHB in acetonitrile (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 ℃ to precipitate protein, and then the supernatant was filtered and the phenytoin (**1**), temocaprilat or methylprednisolone was measured by high performance liquid chromatography (HPLC).

2.5. Hydrolysis reaction (human plasma)

25 mM phenytoin prodrugs (**3a**–**3j**) in dimethylsulfoxide (DMSO) solutions (1 µL, final concentration 0.25 mM) were warmed with 76.3 mg/mL human plasma (5 µL, final concentration 3.82 mg/mL), 200 mM HEPES buffer (50 µL, final concentration 100 mM) and purified water (44 µL) at 37 ℃ for 3 h. After addition of 0.3 mM BPHB in acetonitrile (100 µL, 30 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 ℃ to precipitate protein, and then the supernatant was filtered and the phenytoin (**1**) in the solution was measured by HPLC.

2.6. Aqueous solubility and stability

Buffer solutions at pH 7.4, 8.3, 9.2, 10.1, 11.0, and 11.9 were used so that the pH of the solution was not changed by dissolution of the prodrugs. 10 mg of phenytoin (**1**) or prodrugs (**3a**–**3j**) were prepared in a 2.0 mL Eppendorf tube, and 1 mL of buffer was added to the tube. After preparing a solution so that the solubility of prodrugs (**3a**–**3j**) and phenytoin (**1**) is 10 mg/mL, the solution was subjected to ultra–sonication for 30 minutes at 25–30℃ and then shaken for 1 hour at same

temperature. This solution was immediately filtered using Millipore Millex-LG (0.20 µm/4 mm), and the obtained filtrate was diluted 100 times with methanol. 100 µL of the diluted solution was mixed with 100 µL of 0.3 mM BPHB in acetonitrile, and phenytoin (**1**) and prodrugs (**3a**–**3j**) in the resulting solution was measured by HPLC.

2.7. HPLC analysis

The HPLC analyses were done using an LC system (Shimadzu, Kyoto, Japan) equipped with a LC-10AT pump, SIL-20A auto sampler, CTO-10AS VP column oven, SPD-20A UV/VIS detector, and SCL-10A VP system controller.

For phenytoin prodrugs (**3a**–**3j**), analytical HPLC was performed on a column of Mightysil RP-18 GP 150-4.6 (5 µm) at 25 °C with elution at 1.0 mL/min using the gradient of phosphate buffer (pH 3.5) with methanol being ramped up from 50% to 75% for 25 min and then returned to 50% in 10 min. The phenytoin prodrugs and phenytoin were monitored at 258 nm. Analytical HPLC was performed in butyl *p*-hydroxybenzoate (BPHB) as an internal standard (t_R=18.6 min). The metabolic rate was calculated from the detecting area ratio of prodrugs (**3a**–**3j**), phenytoin (t_R=8.4 min) and BPHB.

For temocapril, analytical HPLC was performed on a column of Mightysil RP-18 GP 150-4.6 (5 µm) at 40 °C with elution at 1.0 mL/min using the 0.1% phosphoric acid with acetonitrile being ramped up from 75% to 50% for 25 min and then returned to 75% in 10 min. The temocaprilat was monitored at 234 nm. Analytical HPLC was performed in butyl *p*-hydroxybenzoate (BPHB) as an internal standard (t_R=23.5 min). The metabolic rate was calculated from the detecting area ratio of temocaprilat (t_R=6.8 min) and BPHB.

For methylprednisolone hemisuccinate, analytical HPLC was performed on a column of Mightysil RP-18 GP 150-4.6 (5 µm) at 25 °C with elution at 0.9 mL/min using the acetonitrile and 0.05 M phosphate buffer (pH 7.0) (0.315 : 0.585) for 20 min. The methylprednisolone was monitored at 254 nm. Analytical HPLC was performed in dexamethasone as an internal standard (t_R=7.7 min). The metabolic rate was calculated from the detecting area ratio of methylprednisolone (t_R=6.5 min) and dexamethasone.

2.8. Data analysis

The amount of phenytoin produced by the hydrolysis reaction was calculated by the internal standard method by the molar ratio of metabolite/BPHB to the peak area ratio of metabolite/BPHB in

HPLC. The metabolic activation rate was calculated by dividing the amount of metabolite obtained by the amount of protein used in the reaction and the reaction time. The amount of prodrug in the solubility test was also calculated by the internal standard method using BPHB. Each experiment was measured three times. The average value and standard deviation were calculated using the statistical analysis software GraphPad Prism 7.

3. Results and Discussion

3.1. Chemistry

The designed prodrug has the chemical structure of a carboxyalkyl (or aryl, alkenyl) carbonyloxy methylene group. A carboxyl group is present to enhance water solubility, and 10 types of alkyl chains with a large acyl group and a small alkoxy group were selected in order to make the alkyl chain easy to be recognized by hCES1. In the metabolic activation mechanism, the ester moiety of the prodrug is firstly hydrolyzed by hCES1. Next, the generated hemiaminal moiety is spontaneously decomposed to form formaldehyde and the corresponding dicarboxylic acid together with the phenytoin (**1**) (Fig. 2).

All of the prodrugs **3** were synthesized in two steps. Hydroxymethyl phenytoin **4** was obtained from phenytoin (**1**) using the known method in the first step (Varia et al., 1984). The target prodrugs (**3a–3j**) were synthesized by reacting hydroxymethyl phenytoin **4** with the corresponding acid anhydride (Table 1). Purification was performed by recrystallization in the second step without column chromatography. The prodrug **3b** was obtained as a mixture of 3,3-dimethyl– and 2,2-dimethyl–isomer. Since these isomers **3b** are both thought to be converted to phenytoin (**1**) after metabolic activation, the prodrugs **3b** were examined for solubility and metabolic activation rate without separation. The pKa values for synthesized prodrugs with shorter alkyl chains (**3a**–**3g**) are lower than those for synthesized prodrugs with longer alkyl chains (**3h**–**3j**), and prodrugs with shorter alkyl chains (**3a**–**3g**) are considered to be soluble in an aqueous solution at lower pH. Prodrugs with an aryl or alkenyl chain have lower pKa values.

3.2. Hydrolysis reaction in HLM or HIM solutions

The hydrolysis rate of these synthetic prodrugs (**3a**–**3j**) was investigated (Figure 3). As positive controls, we also measured the rate of hydrolysis of temocapril (**5**) and methylprednisolone hemisuccinate (**6**). All phenytoin prodrugs were found to be efficiently metabolically activated in HLM solution. Temocapril (**5**) is an ester prodrug that is specifically metabolically activated by hCES1 and is known to have a fast metabolic activation rate among ester prodrugs (Yoshida et al., 2017). Methylprednisolone hemisuccinate (**6**) has been shown to be specifically metabolically activated by hCES2 (Furihata et al., 2005; Casey Laizure et al., 2013). Since hCES2 is expressed in both liver and small intestine, prodrugs that are metabolically activated by hCES2 are metabolically activated in both HLM and HIM solutions. Phenytoin prodrugs were metabolically activated faster in HLM solution and may be metabolically activated by hCES1. Furthermore, compared with the metabolic activation rate of temocapril, the metabolic activation rates of the synthesized prodrugs were comparable or slightly slower, suggesting the possibility of rapid metabolic activation in human liver. As expected, the metabolic activation rate of adipic acid ester **3j** with a long alkyl chain was the largest, and as the carbon number became shorter, the hydrolysis rate decreased in the order of glutaric acid ester **3h** and succinic acid ester **3a**. Unexpectedly, hydrolysis rates of esters (**3c**–**3e**) that were substituted with cyclic alkanes were unchanged or decreased compared with those of the corresponding linear alkyl chain esters (**3a**, **3b**). From these results, it is considered that it is necessary to increase the length of the acyl group in order to increase the metabolic activation rate and that it is necessary to increase the steric hindrance of the acyl group near the ester in order to reduce the metabolic activation rate. For all of the prodrugs (**3a**–**3j**), the hydrolysis rates in the HIM solution were very small compared to those in the HLM solution. The prodrugs substituted with the cyclic dicarboxylic acid (**3c**–**3e**) showed a difference of more than 17 times between hydrolysis rates in the HLM solution and those in the HIM solution. hCES2 is mainly expressed in the small intestine, and it is known that hCES2 can easily recognize the ester with a large alkoxy group and a small alkoxy group. Prodrugs substituted with a cyclic dicarboxylic acid have a very large acyl group and are difficult to be recognized by hCES2, and this is thought to be the reason why the hydrolysis rate in the HIM solution was small. Since all prodrugs had a higher hydrolysis rate in the HLM solution, it is thought that these prodrugs are metabolically activated efficiently by hCES1.

3.3. Plasma stability

Next, the metabolic activation rates of prodrugs (**3a**–**3j**) were also examined in human plasma solution (Fig. 4). The metabolic activation rates of all prodrugs (**3a**–**3j**) in human plasma solution

were much lower than those in HLM solution. Butylylcholinesterase and paraoxonase are mainly expressed in plasma, but CESs and arylamidedeacetylase (AADAC) are expressed in the liver in addition to butylylcholinesterase and paraoxonase (Fukami and Yokoi, 2012). Since the synthesized prodrugs (**3a**–**3j**) were metabolically activated efficiently in the HLM solution, it is thought that they were metabolically activated by CESs or AADAC. The substrates of hCES2 and AADAC are very similar and, in contrast to hCES1, it is known that substrates with small acyl groups and large alkoxy groups are easily recognized. The synthesized prodrugs are thought to be metabolically activated mainly by hCES1 since the metabolic activation rate in HLM solution was increased by lengthening the carbon chain of the acyl group.

3.4. Solubility and stability in buffers

The solubility of compounds (**1**, **3a**–**3j**) were examined. The solubility of compounds was calculated using HPLC (Table 2). As a result, all prodrugs (**3a**–**3j**) showed higher solubility than that of phenytoin (**1**) in the solutions at pH 7.4 and 8.3. In particular, the results showed that the five prodrugs (**3a**–**3c**, **3h**, **3i**) had relatively high solubilities even in aqueous solution at pH 8.3 to 10.1. Next, the stability of these five prodrugs (**3a**–**3c**, **3h**, **3i**) in each solution was evaluated. Unfortunately, the prodrugs (**3a**–**3c**, **3h**, **3i**) gradually hydrolyzed in a high pH solution (Fig 5), confirming the formation of phenytoin (**1**). The decrease in the solubility of the prodrugs in the pH 11.0 and 11.9 solutions may be due to phenytoin (**1**) produced by hydrolysis of the phenytoin prodrug. Therefore, although it is difficult to use these prodrugs in a strongly basic solution, it is thought that they can be used in neutral to weakly basic solutions. Among the prodrugs (**3a**–**3j**), the cyclopropane dicarboxylic acid ester **3c** and 3,3-dimethylglutaric acid ester **3i** were highly stable in a wide range of pH values from 7.4 to 8.3. The glutarate ester–prodrugs (**3h**, **3i**) were confirmed to dissolve between pH 8.3 and 10.1. If the vicinity of the ester has a relatively bulky structure, it is thought to have high stability, but the water solubility decreases as the carbon length increases. Compared to the corresponding linear alkyl esters **3a** and **3h**, the solubility of **3b** and **3i** to which the methyl group was substituted was not greatly changed, but the stability was high. These results suggest that 3,3-dimethylglutarate ester **3i** has both excellent stability and excellent solubility.

3.5. Hydrolysis reaction in hCES solution

There was no evidence that the ester compounds were activated by hCES1 because these metabolic reactions were performed in HLM that contained various hydrolytic enzymes. We compared the hydrolyzing activities of hCES1, hCES2, and AADAC (Fig. 6). The best synthesized prodrug was considered to be the prodrug **3i** because it showed good solubility in a buffer solution of pH 8.3 and high stability. Using this prodrug **3i**, hydrolysis was carried out in each enzyme solution to confirm the metabolic activation enzyme. In the hydrolysis reaction of prodrug **3i**, no metabolite was detected in the hydrolysis reaction with CES2 or AADAC, but the presence of the metabolite was confirmed only in the hydrolysis reaction with CES1. It was found that indomethacin ester is likely to be specifically hydrolyzed by hCES1.

The hydrolysis mechanisms by CES of the synthesized phenytoin prodrugs **3i** are shown (Fig 7). First, nucleophilic attack to carbonyl carbon is caused by the hydroxyl group of serine which the nucleophilicity of which is enhanced by the hydrogen bond with histidine. The basicity of histidine is also enhanced by the hydrogen bond with glutamic acid. Second, an acyl–enzyme complex is formed by elimination of the hydroxymethyl phenytoin. The hydroxymethyl phenytoin is spontaneously decomposed to form formaldehyde and phenytoin (**1**). Finally, the acyl-enzyme complex undergoes a nucleophilic addition-elimination by water, the corresponding dicarboxylic acid is eliminated from the acyl-enzyme complex to form CES. The initial nucleophilic addition rate decreases with the bulkiness of the ester. X-ray structural analysis shows that the active center of the hCES1 consists of a rigid site and a flexible site, and is known to have a wide space (Bencharit et al., 2012). On the other hand, in the amino acid sequence of hCES2, one of the loops constituting the flexible site is deleted, and the flexibility of the active center of hCES2 is considered to be inferior to that of hCES1. The differences in substrate specificity between hCES1 and hCES2 are thought to be due to differences in site size and flexibility. It is considered that hCES2 does not catalyze the hydrolysis of compounds with bulky acyl groups, because the steric hindrance of the active center prevents the bond between acyl groups. The alkoxy group of prodrug **3i** has a planar hydantoin group adjacent to the ester and is less bulky, but the acyl group of prodrug **3i** has a relatively bulky tertiary carbon. Esters with large acyl groups and flat, small alkoxy groups are more easily recognized by hCES1 (Mizoi et al., 2020). Therefore, it is considered that the prodrug synthesized this time was specifically metabolically activated by hCES1.

4. Conclusions

We designed, synthesized, and evaluated water-soluble prodrugs with consideration of the substrate recognition ability of hCES1. It was confirmed that all synthesized phenytoin prodrugs (**3a**–**3j**) were efficiently metabolized in HLM solution and converted to phenytoin (**1**). Since these prodrugs (**3a**–**3j**) had much lower hydrolysis rates in HIM solution than in HLM solution, it was estimated that hCES1 is the main metabolic activation enzyme of these prodrugs (**3a**–**3j**). Of these prodrugs, glutarate ester–type prodrugs showed good solubility (>10 mg/mL) in buffers with pH values from 8.3 to 10.1. The more stable structure is the 3,3-dimethylglutarate ester prodrug **3i**. The introduction of 3,3-dimethylglutaric acid is thought to be applicable to improving the water solubility of other alcohol- or amine-containing pharmaceuticals. It is expected that prodrug research will be further developed using the findings obtained in this study.

Acknowledgements

We thank the Human and Animal Bridging (HAB) Research Organization (Japan) for providing human livers. This work was supported by JSPS KAKENHI Grant Number 19K16421.

References

Agarwal, S.P., Blake, M.I., 1968. Determination of the pKa' value for 5,5-diphenylhydantoin. J. Pharm. Sci. 57, 1434–1435.

Bencharit, S., Morton, C.L., Hyatt, J.L., Kuhn, P., Danks, M.K., Potter, P.M., Redinbo, M.R., 2003. Crystal structure of human carboxylesterase 1 complexed with the Alzheimer's drug tacrine: from binding promiscuity to selective inhibition. Chem. Biol. 10, 341–349.

Bosch, J., Roca, T., Domènech, J., Suriol, M., 1999. Synthesis of water-soluble phenytoin prodrugs. Bioorganic Med. Chem. Lett. 9, 1859–1862.

Casey Laizure, S., Herring, V., Hu, Z., Witbrodt, K., Parker, R.B., 2013. The role of human carboxylesterases in drug metabolism: Have we overlooked their importance? Pharmacotherapy 33, 210–222.

Fukami, T., Yokoi, T., 2012. The Emerging Role of Human Esterases. Drug Metab. Pharmacokinet. 27, 466–477.

Furihata, T., Hosokawa, M., Fujii, A., Derbel, M., Satoh, T., Chiba, K., 2005. Dexamethasone-induced methylprednisolone hemisuccinate hydrolase: Its identification as a member of the rat carboxylesterase 2 family and its unique existence in plasma. Biochem. Pharmacol. 69, 1287–1297.

Gupta, D., Bhatia, D., Dave, V., Sutariya, V., Varghese Gupta, S., 2018. Salts of Therapeutic Agents: Chemical, Physicochemical, and Biological Considerations. Molecules 23, 1719.

Hamada, Y., 2016. Novel prodrugs with a spontaneous cleavable guanidine moiety. Bioorg. Med. Chem. Lett. 26, 1685–1689.

Haque, E.; Kikuchi, T.; Kanemitsu, K.; Tsuda, Y., 1986. NII-Electronic library service. Chem. Pharm. Bull. 34, 430–433.

Hosokawa, M., 2008. Structure and catalytic properties of carboxylesterase isozymes involved in metabolic activation of prodrugs. Molecules 13, 412–431.

Hosokawa, M., Endo, T., Fujisawa, M., Hara, S., Iwata, N., Sato, Y., Satoh, T., 1995. Interindividual variation in carboxylesterase levels in human liver microsomes. Drug Metab. Dispos. 23, 1022–1027.

Hosokawa, M., Furihata, T., Yaginuma, Y., Yamamoto, N., Koyano, N., Fujii, A., Nagahara, Y., Satoh, T., Chiba, K., 2007. Genomic structure and transcriptional regulation of the rat, mouse, and human carboxylesterase genes. Drug Metab. Rev. 39, 1–15.

Jamerson, B.D., Dukes, G.E., Brouwer, K.L., Donn, K.H., Messenheimer, J.A., Powell, J.R., 1994. Venous irritation related to intravenous administration of phenytoin versus fosphenytoin. Pharmacotherapy 14, 47–52.

Jansod, S., Afshar, M.G., Crespo, G.A., Bakker, E., 2016. Biosensors and Bioelectronics Phenytoin speciation with potentiometric and chronopotentiometric ion-selective membrane electrodes 79, 114–120.

Jornada, D.H., dos Santos Fernandes, G.F., Chiba, D.E., de Melo, T.R.F., dos Santos, J.L., Chung, M.C., 2015. The prodrug approach: a successful tool for improving drug solubility. Molecules 21, 42.

Kamendulis, L.M., Brzezinski, M.R., Pindel, E. V, Bosron, W.F., Dean, R.A., 1996. Metabolism of cocaine and heroin is catalyzed by the same human liver carboxylesterases. J. Pharmacol. Exp. Ther. 279, 713–717.

Miwa, M., Ura, M., Nishida, M., Sawada, N., Ishikawa, T., Mori, K., Shimma, N., Umeda, I., Ishitsuka, H., 1998. Design of a novel oral fluoropyrimidine carbamate, capecitabine, which generates 5 fluorouracil selectively in tumours by enzymes concentrated in human liver and cancer tissue. Eur. J. Cancer 34, 1274–1281.

Mizoi, K., Takahashi, M., Haba, M., Hosokawa, M., 2016. Synthesis and evaluation of atorvastatin esters as prodrugs metabolically activated by human carboxylesterases. Bioorg. Med. Chem. Lett. 26, 921–923.

Mizoi, K., Takahashi, M., Sakai, S., Ogihara, T., Haba, M., Hosokawa, M., 2020. Structure-activity relationship of atorvastatin derivatives for metabolic activation by hydrolases. Xenobiotica 50, 261–269.

Müller, D.G., Stella, V.J., Lötter, A.P., 1994. Determination of the position of the ester bond in a micellar prodrug of phenytoin. Int. J. Pharm. 102, 109–116.

Patel, S.I., Birnbaum, A.K., Cloyd, J.C., Leppik, I.E., 2015. Intravenous and Intramuscular Formulations of Antiseizure Drugs in the Treatment of Epilepsy. CNS Drugs 29, 1009–1022. https://doi.org/10.1007/s40263-015-0289-0

Sanches, B.M.A., Ferreira, E.I., 2019. Is prodrug design an approach to increase water solubility? Int. J. Pharm. 568, 118498.

Satoh, T., Hosokawa, M., Atsumi, R., Suzuki, W., Hakusui, H., Nagai, E., 1994. Metabolic Activation of CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin, a novel antitumor agent, by carboxylesterase. Biol. Pharm. Bull. 17, 662–664.

Shi, D., Yang, J., Yang, D., LeCluyse, E.L., Black, C., You, L., Akhlaghi, F., Yan, B., 2006. Anti-influenza prodrug oseltamivir is activated by carboxylesterase human carboxylesterase 1, and the activation is inhibited by antiplatelet agent clopidogrel. J. Pharmacol. Exp. Ther. 319, 1477–1484.

Stella, V.J., 1996. A case for prodrugs: Fosphenytoin. Adv. Drug Deliv. Rev. 19, 311–330.

Stella, V.J., Martodihardjo, S., Terada, K., Rao, V.M., 1998. Some relationships between the physical properties of various 3- acyloxymethyl prodrugs of phenytoin to structure: Potential in vivo performance implications. J. Pharm. Sci. 87, 1235–1241.

Stella, V.J., Nti-Addae, K.W., 2007. Prodrug strategies to overcome poor water solubility. Adv. Drug Deliv. Rev. 59, 677–694.

Takahashi, M., Ogawa, T., Kashiwagi, H., Fukushima, F., Yoshitsugu, M., Haba, M., Hosokawa, M., 2018. Chemical synthesis of an indomethacin ester prodrug and its metabolic activation by human carboxylesterase 1. Bioorg. Med. Chem. Lett. 28, 997–1000.

Takahashi, M., Takani, D., Haba, M., Hosokawa, M., 2020. Investigation of the chiral recognition ability of human carboxylesterase 1 using indomethacin esters. Chirality 32, 73–80.

Takahashi, M., Uehara, T., Nonaka, M., Minagawa, Y., Yamazaki, R., Haba, M., Hosokawa, M., 2019. Synthesis and evaluation of haloperidol ester prodrugs metabolically activated by human carboxylesterase. Eur. J. Pharm. Sci. 132, 125–131.

Takai, S., Matsuda, A., Usami, Y., Adachi, T., Sugiyama, T., Katagiri, Y., Tatemitsu, M., Hirano, K., 1997. Hydrolytic profile for ester- or amide-linkage by carboxylesterases pI 5.3 and 4.5 from human liver. Biol. Pharm. Bull. 20, 869–873.

Taketani, M., Shii, M., Ohura, K., Ninomiya, S., Imai, T., 2007. Carboxylesterase in the liver and small intestine of experimental animals and human 81, 924–932.

Tanino, T., Ogiso, T., Iwaki, M., Tanabe, G., Muraoka, O., 1998. Enhancement of oral bioavailability of phenytoin by esterification, and in vitro hydrolytic characteristics of prodrugs. Int. J. Pharm. 163, 91–102.

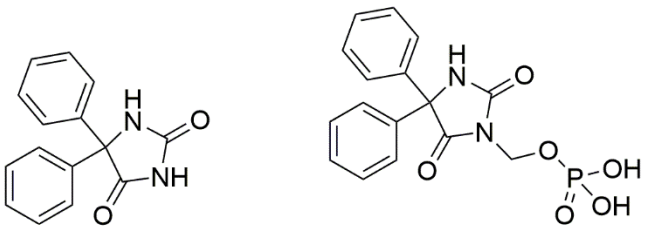
Varia, S.A., Schuller, S., Sloan, K.B., Stella, V.J., 1984. Phenytoin prodrugs III: water-soluble prodrugs for oral and/or parenteral use. J. Pharm. Sci. 73, 1068–1073.

Wang, D., Zou, L., Jin, Q., Hou, J., Ge, G., Yang, L., 2018. Human carboxylesterases: a comprehensive review. Acta Pharm. Sin. B 8, 699–712.

Yoshida, T., Fukami, T., Kurokawa, T., Gotoh, S., Oda, A., Nakajima, M., 2017. Difference in substrate specificity of carboxylesterase and arylacetamide deacetylase between dogs and humans. Eur. J. Pharm. Sci. 111, 167–176.

Zhang, J., Burnell, J.O.E.C., Dumaual, N., Bosron, W.F., 1999. Binding and Hydrolysis of Meperidine by Human Liver Carboxylesterase hCE-1 1 290, 314–318.

Zhang, Weiyi, Yang, J., Fan, J., Wang, B., Kang, Y., Liu, J., Zhang, Wensheng, Zhu, T., 2019. An improved water-soluble prodrug of propofol with high molecular utilization and rapid onset of action. Eur. J. Pharm. Sci. 127, 9–13. https://doi.org/10.1016/j.ejps.2018.09.024



phenytoin (**1**) fosphenytoin (**2**)

Fig. 1 Chemical structures of phenytoin (**1**), fosphenytoin (**2**).

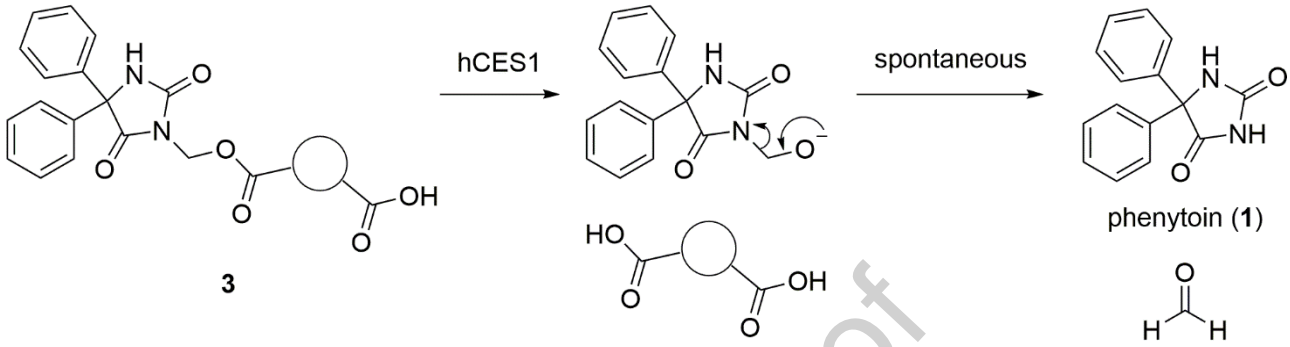


Fig. 2 Proposed mechanism for metabolic activation of phenytoin prodrugs **3**.

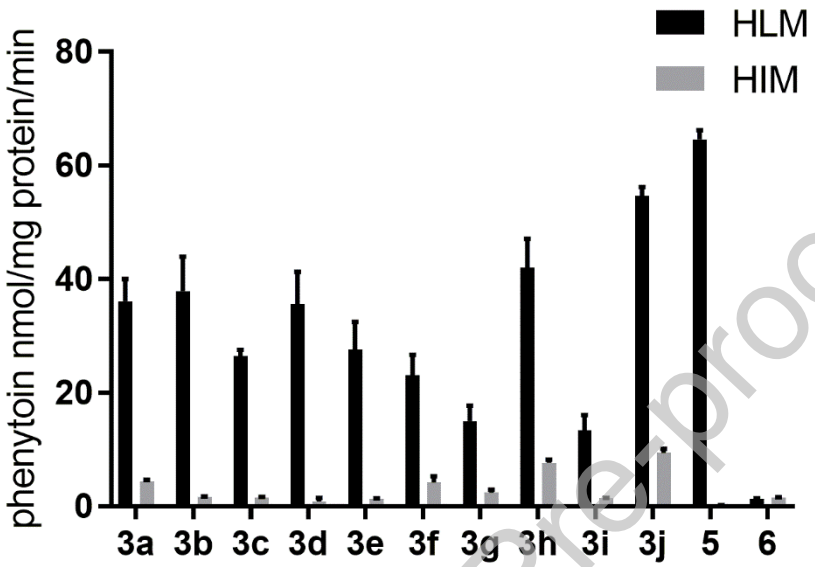


Fig. 3 Hydrolytic rates of phenytoin prodrugs (**3a**–**3j**), temocapril (**5**) and methylprednisolone hemisuccinate (**6**) in HLM solution and HIM solution. The rate of formation of hydrolysis products was measured using the methods described in the materials and methods. The prodrugs (**3a**–**3j**, **5** and **6**) were incubated for 0.5 h (HLM) or 3 h (HIM) at 37 °C. Values are means ± SD (n = 3).

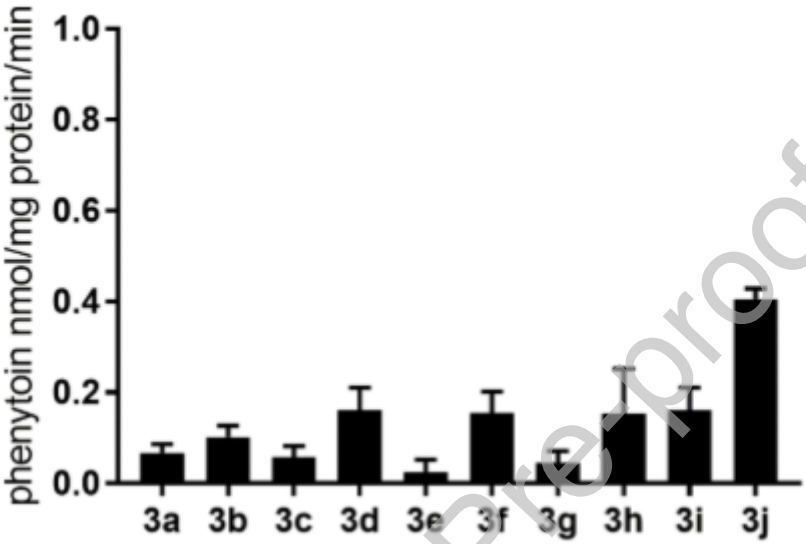


Fig. 4 Hydrolytic rates of phenytoin prodrugs (**3a**–**3j**) in human plasma solution. Phenytoin (**1**) production ratio was measured using the methods described in the materials and methods. The prodrugs (**3a**–**3j**) were incubated for 3 h at 37 °C. Values are means ± SD (n = 3).

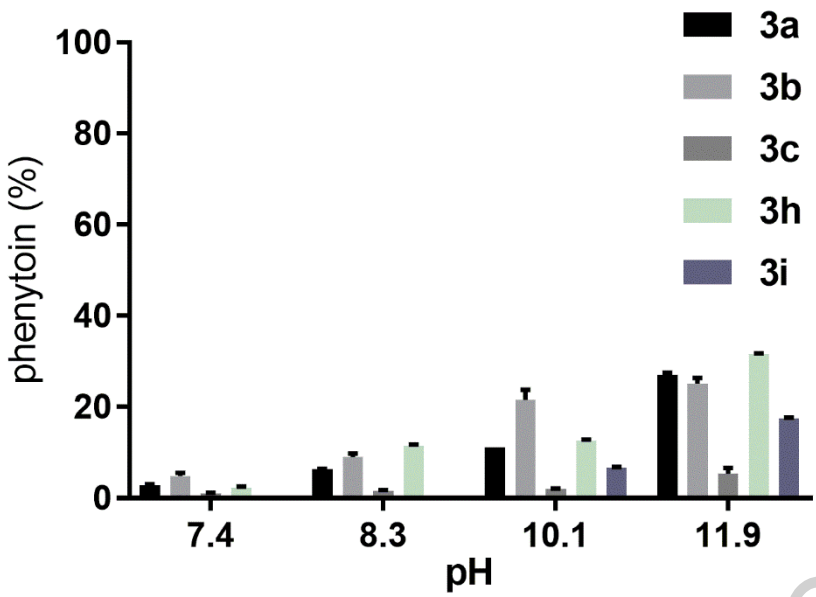


Fig. 5 *Production ratios of phenytoin (**1**).* The ratios were calculated as the amount of phenytoin (**1**) produced relative to the amount of phenytoin prodrug dissolved in buffers (pH 7.4, 8.3, 10.1, 11.9). Decomposed phenytoin (**1**) ratio was measured using the methods described in the materials and methods. The prodrugs were exposed to the buffers for 1.5 h at 25–30 °C. Values are means ± SD (n = 3).

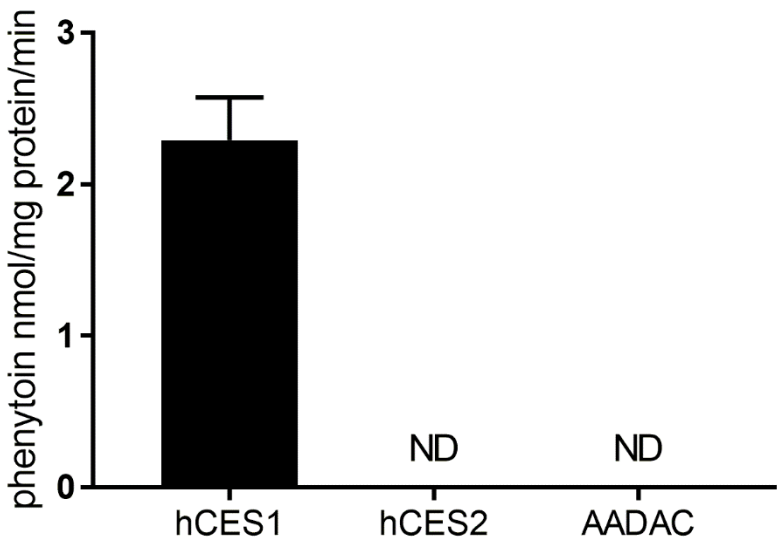


Fig. 6 Hydrolytic rates of phenytoin prodrug **3i** in solutions of hCES1, hCES2, and AADAC. Phenytoin (**1**) production ratio was measured using the methods described in the materials and methods. The prodrugs (**3a**–**3j**) were incubated for 0.5 h at 37 °C. Values are means ± SD (n = 3). ND = not detectable under these assay conditions.

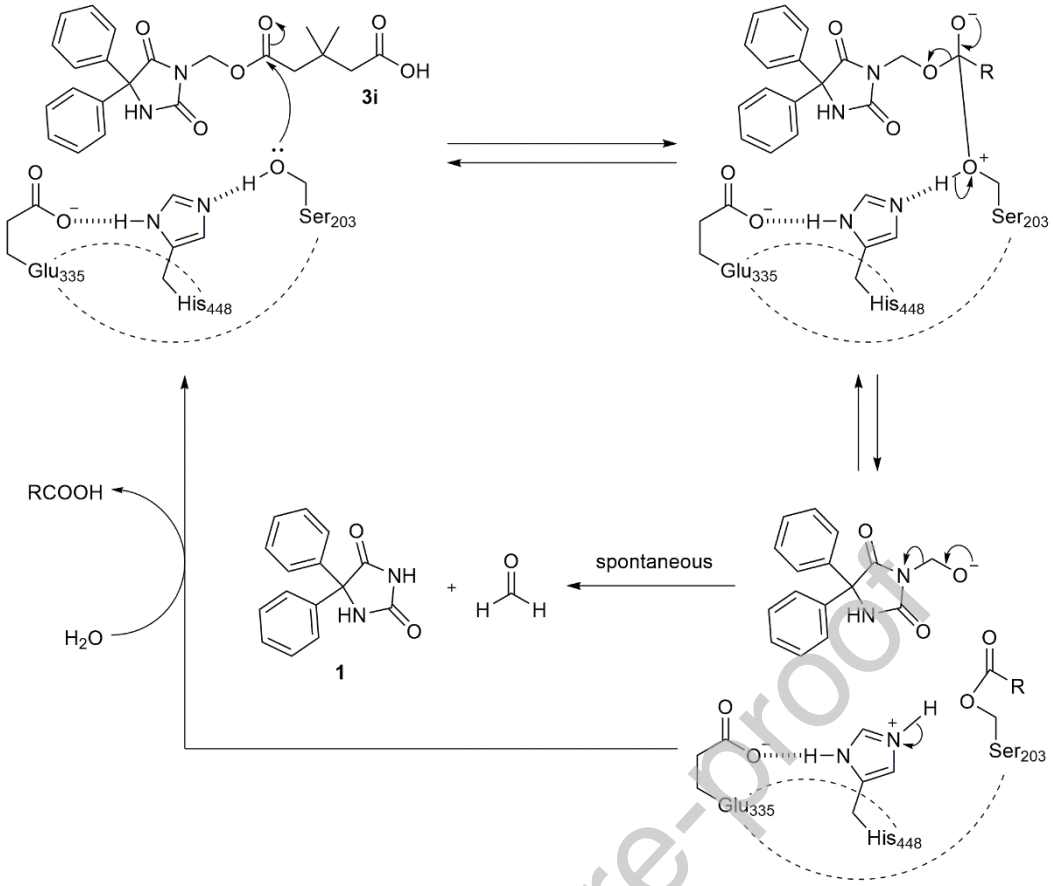
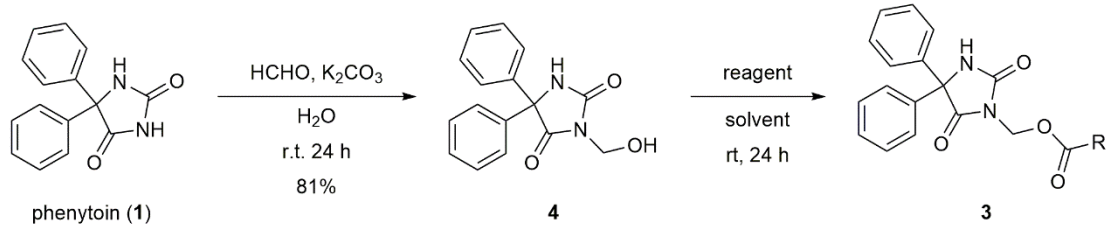
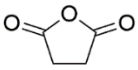
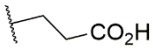
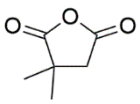
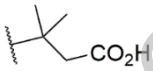
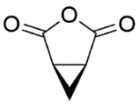
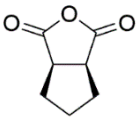
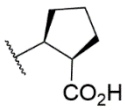
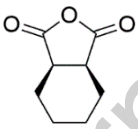
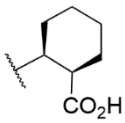
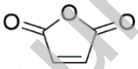
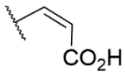
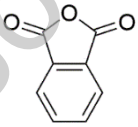
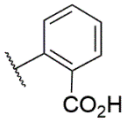
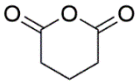
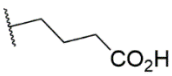
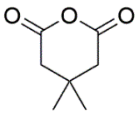
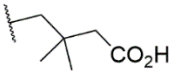
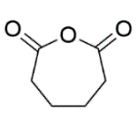
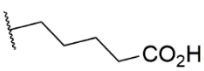


Fig. 7 Proposed mechanism for the hydrolysis of phenytoin prodrug **3i** by CES.

Table 1

Synthesis of ester prodrugs **3** from phenytoin (**1**).



compound	reagent	solvent	R	yield (%)[a]	pKa[c]
3a		pyridine		89.8	4.21
3b		pyridine		61.5 (2 : 1)[b]	4.38
3c		pyridine		56.2	3.95
3d		pyridine		56.4	4.28
3e		pyridine		67.9	4.27
3f		acetone		18.8	3.85
3g		MeCN		11.5	3.25
3h		pyridine		41.2	4.46
3i		pyridine		49.2	4.48
3j		pyridine		32.0	4.65

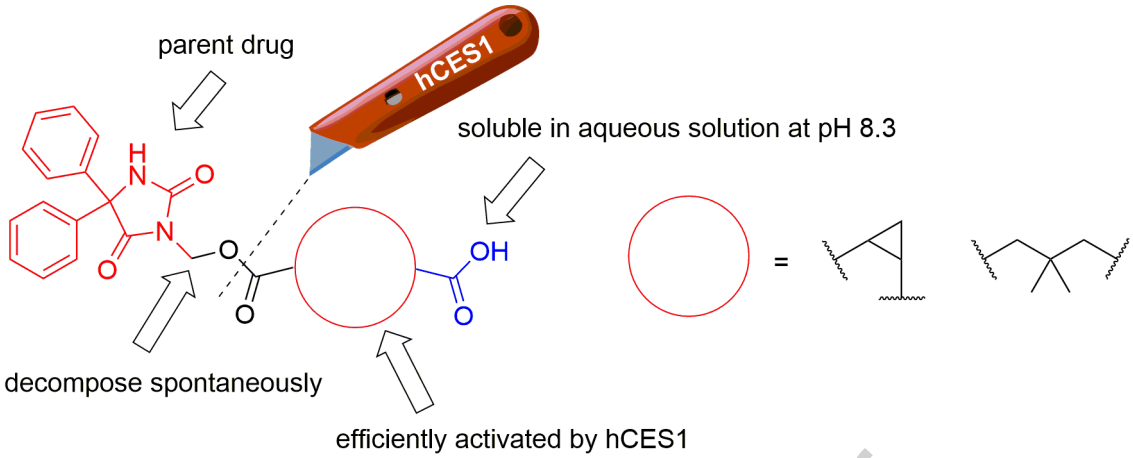
[a] Isolated yield [b] A mixture of 3,3-dimethyl– and 2,2-dimethyl–isomer [c] Calculated using ChemDraw Professional 15.0.

Table 2

Solubility of phenytoin prodrugs (**3a**–**3j**) and phenytoin (**1**) in buffers (pH 7.4, 8.3, 9.2, 10.1, 11.0, 11.9).

	Solubility (mg/mL)					
	pH 7.4	pH 8.3	pH 9.2	pH 10.1	pH 11.0	pH 11.9
3a	7.55±0.29	6.82±0.12	7.41±0.23	7.00±0.12	6.09±0.83	3.09±0.25
3b	6.67±1.40	6.88±0.78	4.88±0.64	3.64±0.26	3.43±0.50	2.21±0.17
3c	4.13±0.81	4.52±0.23	5.46±0.11	4.38±0.20	3.17±0.95	3.21±0.92
3d	0.15±0.03	0.16±0.03	0.12±0.02	0.13±0.02	0.15±0.004	0.14±0.02
3e	0.31±0.13	5.54±0.48	2.12±0.24	2.61±0.18	2.81±1.20	1.53±0.09
3f	3.32±0.66	1.77±0.25	2.67±0.04	2.67±0.18	1.88±0.39	1.66±0.33
3g	1.53±2.04	2.04±0.15	3.54±0.41	3.43±0.43	1.77±0.49	1.92±0.39
3h	7.54±0.97	>10.0	>10.0	>10.0	>10.0	6.94±0.45
3i	2.71±0.12	>10.0	>10.0	>10.0	9.48±0.89	8.71±0.59
3j	0.40±0.11	4.94±0.36	2.64±0.41	4.06±0.41	1.64±0.50	1.24±0.15
1	< 0.1	< 0.1	0.22±0.002	1.26±0.04	1.73±0.04	8.63±0.35

Graphical abstract



Credit Author Statement

Category 1 Conception and design of study: M. Takahashi, M. Haba, M. Hosokawa;

acquisition of data: M. takahashi, Y.J. Lee, T. Kanayama, Y. Kondo, K. Nishio, K. Mukai;

analysis and/or interpretation of data: M. takahashi, Y.J. Lee, T. Kanayama, Y. Kondo, K. Nishio, K. Mukai.

Category 2 Drafting the manuscript: M. takahashi, M. Hosokawa;

revising the manuscript critically for important intellectual content: M. Haba, M. Hosokawa.

Category 3 Approval of the version of the manuscript to be published: M. takahashi, Y.J. Lee, T. Kanayama, Y. Kondo, K. Nishio, K. Mukai, M. Haba, M. Hosokawa.