Bioorganic & Medicinal Chemistry Letters 28 (2018) 997–1000

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Chemical synthesis of an indomethacin ester prodrug and its metabolic activation by human carboxylesterase 1

Masato Takahashi *, Tomohiro Ogawa, Hiroshi Kashiwagi, Fumiya Fukushima, Misaki Yoshitsugu, Masami Haba, Masakiyo Hosokawa

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

ARTICLE INFO

Article history:
Received 28 December 2017
Revised 16 February 2018
Accepted 16 February 2018
Available online 21 February 2018

Keywords:
Indomethacin
Prodrug
Carboxylesterase
Thioester
Chiral recognition

ABSTRACT

It is necessary to consider the affinity of prodrugs for metabolic enzymes for efficient activation of the prodrugs in the body. Although many prodrugs have been synthesized with consideration of these chemical properties, there has been little study on the design of a structure with consideration of biological properties such as substrate recognition ability of metabolic enzymes. In this report, chemical synthesis and evaluation of indomethacin prodrugs metabolically activated by human carboxylesterase 1 (hCES1) are described. The synthesized prodrugs were subjected to hydrolysis reactions in solutions of human liver microsomes (HLM), human intestine microsomes (HIM) and hCES1, and the hydrolytic parameters were investigated to evaluate the hydrolytic rates of these prodrugs and to elucidate the substrate recognition ability of hCES1. It was found that the hydrolytic rates greatly change depending on the steric hindrance and stereochemistry of the ester in HLM, HIM and hCES1 solutions. Furthermore, in a hydrolysis reaction catalyzed by hCES1, the V_{max} value of *n*-butyl thioester with chemically high reactivity was significantly lower than that of *n*-butyl ester.

In recent years, many types of prodrug have been developed for improvement of bioavailability and reduction of side effects. Since various enzymes including carboxylesterases (CESs) participate in the metabolic activation of prodrugs, there has been much discussion about the properties of these enzymes. By catalyzing the hydrolysis of various ester, amide and thioester derivatives, CESs are involved in the metabolic inactivation or activation of many drugs, biological materials, natural products, foods and environmental materials.[1] CESs are categorized into five groups (CES1–CES5) based on the amino acid sequence homology, and the CES1 or CES2 group is the major group of CESs in mammals.[2–4] Substrates with a small alcoxy group, such as methyl ester derivatives (cocaine[5] and methylphenidate)[6] and ethyl ester derivatives (meperidine,[7] temocapril,[8] and oseltamivir),[9] are mainly catalyzed by the CES1 isozyme. Conversely, substrates with a small acyl group, such as benzoate derivatives (cocaine),[5] (1,4′-bipiperidin)-1′-ylate derivatives (CPT-11 with a huge alkoxy group)[10] and acetate derivatives (heroin),[11] are mainly catalyzed by the CES2 isozyme. The organ distribution of CESs has also been investigated, and it has been shown that CESs have ubiquitous tissue distribution profiles.[12,13] The CES1 family is mainly distributed in the liver and lung, whereas the CES2 family is mainly distributed in the small intestine and kidney. Therefore, it has been suggested that the substrates catalyzed by CES1 or CES2 are specifically metabolized in each tissue. Previous experiments by our group showed that atorvastatin prodrugs are not hydrolyzed in human intestine microsomes (HIM) or human CES2 (hCES2) solution but are hydrolyzed in human liver microsomes (HLM) and human CES1 (hCES1) solution[14]

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, diclofenac, ibuprofen and indomethacin are one of the most widely prescribed group of drugs. However, long-term use of NSAIDs might cause gastroduodenal mucosal injury.[15] For decreasing the side effects of indomethacin, prodrugs such as acemethacin[16,17] and indomethacin farnecil[18] have been developed. Recently, results of studies on an oral prodrug of indomethacin in which a cilexetil, pivoxil[19] or glycosyl[20] group was substituted have also been reported. Some studies have suggested that an indomethacin-conjugated anticancer prodrug is effective for targeting cancer cells.[21,22] Thus, many researchers have attempted to synthesize indomethacin prodrugs. However, there has been little study on the design and synthesis of an indomethacin prodrug activated by hCES1.

The purpose of the present study was to synthesize a new indomethacin ester prodrug activated by hCES1 and to investigate the

* Corresponding author.
E-mail address: matakahashi@cis.ac.jp (M. Takahashi).

https://doi.org/10.1016/j.bmcl.2018.02.035
0960-894X/

detailed substrate specificity of hCES1 for theoretical design of prodrugs. First, the chemical synthesis of indomethacin ester and thioester prodrugs was carried out. Then the synthesized prodrugs were subjected to hydrolysis reactions in HLM, HIM and hCES1 solutions, and the hydrolysis rates of these prodrugs were evaluated.

Indomethacin derivatives (**2a**–**2g**) were prepared according to the reported synthetic method (Scheme 1).[23] Treatment of indomethacin and alcohol compounds with dicyclohexylcarbodiimide (DCC) in the presence of dimethylaminopyridine (DMAP) provided the *n*-butyl ester **2a**,[23,24] *sec*-butyl ester **2b**, isobutyl ester **2c** and neopentyl ester **2e** in moderate yields. Although the yields were low, other esters such as *tert*-butyl ester **2d**,[25] 1-phenylpropyl ester **2f** and *n*-butyl thioester **2g** were synthesized by the same method. Substrates with a chiral center, such as *sec*-butyl ester **2b** and 1-phenylpropyl ester **2f**, were synthesized using their respective chiral alcohol compounds. The structures of the synthesized compounds were confirmed by ^{1}H NMR, ^{13}C NMR, and IR analyses.

First, the synthesized prodrugs (**2a**–**2g**) were subjected to hydrolysis reaction in HLM solution, and the hydrolysis rates were investigated (Fig. 1). The results showed that *n*-butyl ester **2a** had the highest hydrolysis rate among all of the prodrugs (**2a**–**2g**). A comparison of the C4-alkyl ester derivatives (**2a**–**2d**) showed that the hydrolysis rate greatly differs depending on the structure and configuration of carbon atoms of the alkoxy group. Although neopentyl ester **2e** has a bulky quaternary carbon, the hydrolysis rate of **2e** was not significantly reduced in comparison with that of *tert*–butyl ester **2d**. Therefore, it was thought that the hydrolysis rate in HLM solution is greatly influenced by steric hindrance of the neighboring carbon of the ester. In the *sec*-butyl ester derivative **2b** with a chiral center, the hydrolysis rate of the (*S*)-isomer was 2.1-times higher than that of the (*R*)-isomer. Surprisingly, 1-phenylpropyl ester **2f**, in which the hydrolysis rate of the (*R*)-isomer was 8.2-times higher than that of the (*S*)-isomer, has a reversed enantioselectivity from *sec*-butyl ester **2b**. Even for substrates with the same *n*-butyl group, it was found that the hydrolysis rate of thioester **2g** was 2.7-times lower than that of ester **2a**. The results suggested that indomethacin ester derivatives are more effectively hydrolyzed than are indomethacin thioester derivatives in HLM solution.

Next, prodrugs (**2a**–**2g**) were subjected to hydrolysis reaction in HIM solution, and the hydrolysis rates were investigated (Fig. 2). The data showed that the hydrolysis rates of C4-alkyl ester prodrugs (**2a**–**2d**) in HIM solution are similar to those in HLM solution. In the *sec*-butyl ester derivative **2b**, the hydrolysis rate of the (*S*)-isomer was 1.9-times higher than that of the (*R*)-isomer. Despite the high enantioselectivity in HLM solution, the hydrolysis rates of 1-phenylpropyl ester isomers **2f** were almost the same as those in HIM solution. The hydrolysis rate of *n*-butyl thioester **2g** was slightly higher than that of *n*-butyl ester **2a**. In terms of enantioselectivity or functional group selectivity, different results were obtained for the hydrolytic rates of indomethacin prodrugs in HLM and HIM solutions. On the whole, the hydrolytic rates of all of the prodrugs (**2a**–**2g**) in HIM solution were significantly lower than those in HLM solution. The results therefore suggested that all of the prodrugs (**2a**–**2g**) are efficiently hydrolyzed not in the human intestine but in the human liver.

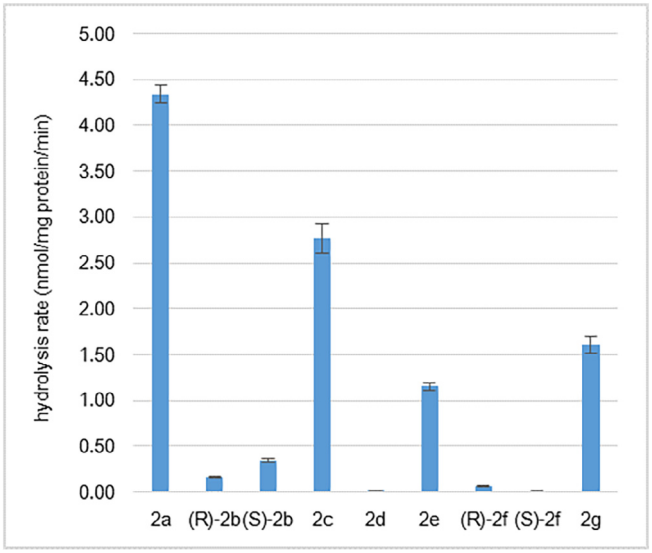
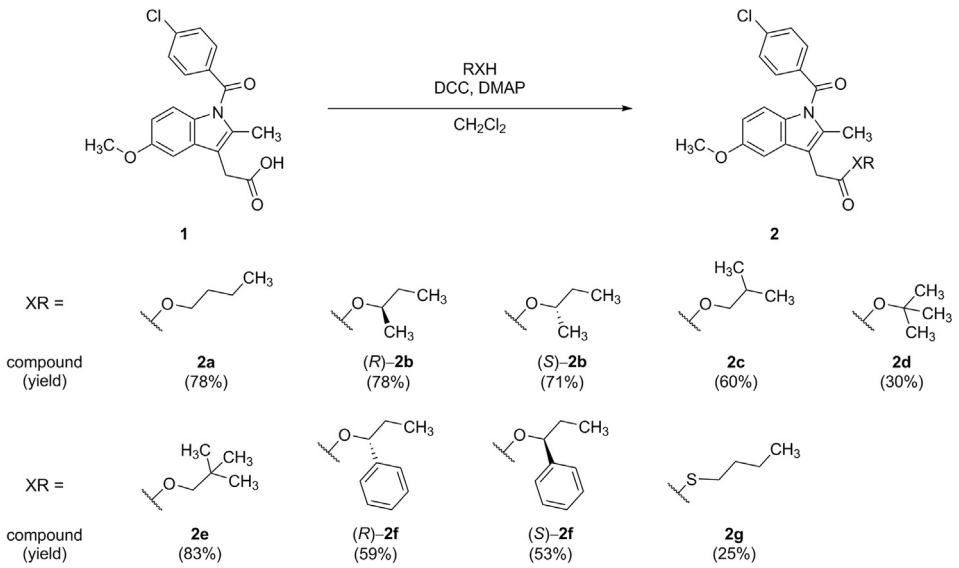


Fig. 1. Hydrolysis rates of indomethacin ester derivatives (**2a**–**2f**) and a thioester derivative (**2g**) in HLM solution. Values are means ± S.D. (n = 3).

Finally, the prodrugs (**2a**–**2g**) were subjected to hydrolysis reaction in hCES1b solution, and the kinetic parameters were determined (Table 1). hCES1b is also referred to as CES1A1, which is mainly expressed in the human liver. The K_m and V_{max} values were



Scheme 1. Synthesis of indomethacin ester derivatives (**2a–2f**) and a thioester derivative **2g**.

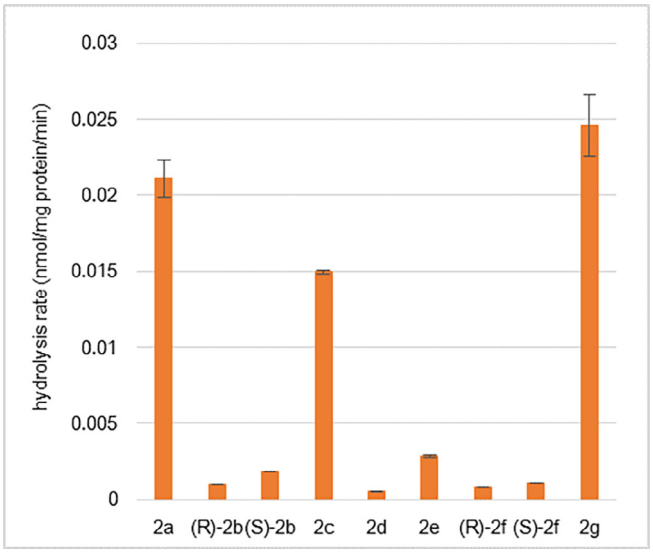


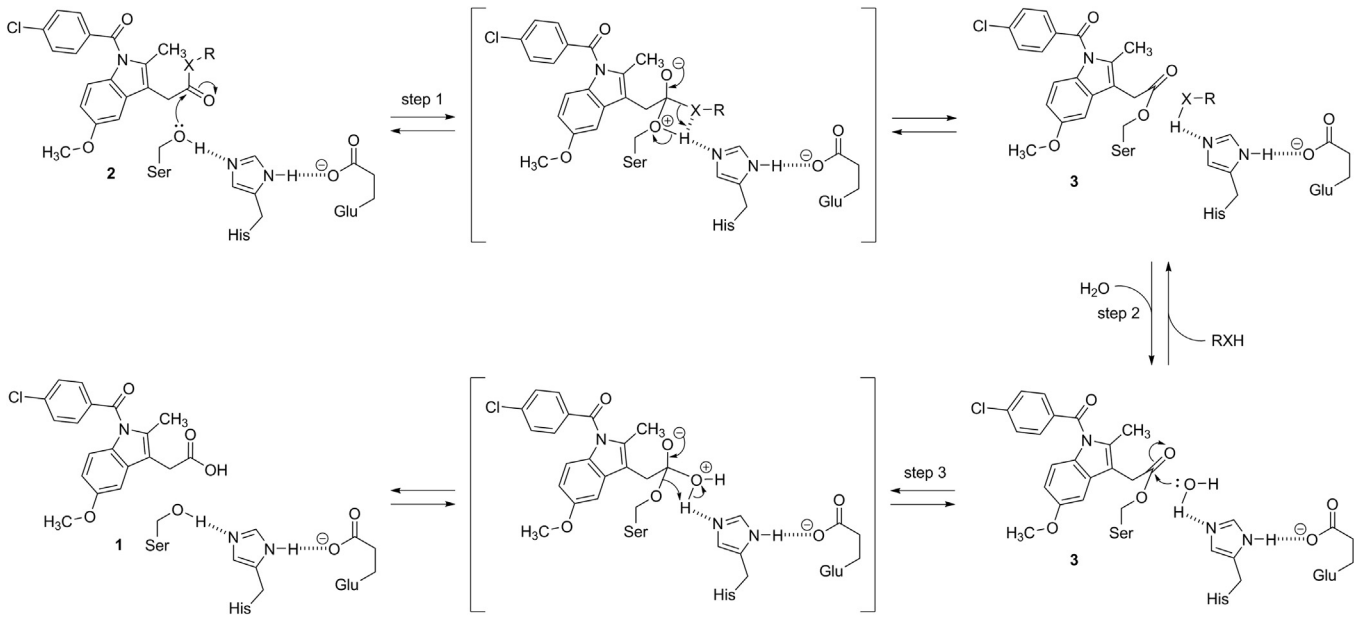
Fig. 2. Hydrolysis rates of indomethacin ester derivatives (**2a**–**2f**) and a thioester derivative (**2g**) in HIM solution. Values are means ± S.D. (n = 3).

Table 1
Kinetic parameters of indomethacin ester derivatives (**2a**–**2f**) and a thioester derivative (**2g**). Values are means ± S.E. (n = 3) nd: not detected.

Compound	V_{max} (nmol/mg protein/min)	K_m (mM)	CL_{int} (mL/mg protein/min)
2a	1.512 ± 0.041	4.4 ± 0.4	0.34
(***R***)-**2b**	0.049 ± 0.003	10.1 ± 2.1	0.0048
(*S*)-**2b**	0.103 ± 0.009	48.5 ± 10.3	0.0021
2c	1.221 ± 0.082	13.7 ± 2.9	0.089
2d	nd	nd	nd
2e	0.322 ± 0.014	4.6 ± 0.7	0.069
(*R*)-**2f**	0.021 ± 0.001	2.9 ± 0.4	0.007
(*S*)-**2f**	0.002 ± 0.0001	6.5 ± 1.1	0.0003
2g	0.800 ± 0.016	2.1 ± 0.3	0.38

calculated by the Michaelis-Menten equation using nonlinear regression analysis with software (GraphPad Prism 7), and CL_{int} was calculated by K_m and V_{max}. The V_{max} values for all of the prodrugs (**2a**–**2g**) show patterns similar to those for the hydrolytic activities in HLM solution. The *n*-butyl ester **2a** has the highest V_{max} value among all of the prodrugs (**2a**–**2g**). The V_{max} value of neopentyl ester **2e** with a bulky quaternary carbon is slightly lower than that of *tert*-butyl ester **2d**. Therefore, it is thought that hCES1b strongly recognizes the neighboring carbon of the ester. In the *sec*-butyl ester **2b**, the V_{max} value of the (*S*)-isomer is 2.1-times higher than that of the (*R*)-isomer. Furthermore, in the 1-phenylpropyl ester **2f**, the V_{max} value of the (*R*)-isomer is 10.2-times higher than that of the (*S*)-isomer. Previous studies showed that CESs have a chiral recognition ability for some drugs, such as propranolol esters,[26,27] flurbiprofen esters[26] and methyl phenidate.[6] These drugs have a functional group, such as a phenyl, amino or fluoro group, that is substituted next to the chiral center. In this study, it was found that hCES1 recognizes a simple alkyl ester such as *sec*-butyl ester. The V_{max} and K_m values of *n*-butyl thioester **2g** are lower than those of *n*-butyl ester **2a**. The *n*-butyl thioester **2** has the highest CL_{int} value among all of the prodrugs (**2a**–**2g**).

Thus, it was shown that the values of V_{max} and K_m differ depending on the structure of the ester. This difference can be explained by the reaction mechanism of hydrolysis by CES. The hydrolysis reaction by CES is a typical ping–pong–bi–bi reaction,[1] which is considered to proceed similarly for indomethacin hydrolysis reaction (Scheme 2). First, an acyl-enzyme complex **3** is formed by nucleophilic addition–elimination in the ester **2** by the hydroxyl group of Ser_{203} activated by Glu_{336} and His_{450} in CES (step 1). Subsequently, an exchange reaction between alcohol (or thiol) and water occurs (step 2), and finally indomethacin (**1**) is produced, causing a nucleophilic addition–elimination reaction in the acyl–enzyme complex **3** by water (step 3). In this reaction, steps 1 and 2 affect V_{max} and K_m depending on the type of ester. In the case of esters **2b** and **2f** in which the bulky alkoxy group is substituted, it can be assumed that the value of V_{max} was small since it is thought that it is difficult for nucleophilic attack of the hydroxyl group of Ser_{203} in step 1. Next, although the chemical reactivity of thioester was high in aqueous solution[28], the V_{max}



Scheme 2. Proposed mechanism for the hydrolysis reaction by CES.

value of thioester **2g** was smaller than that of ester **2a**. This is because exchange reaction between thiol and water in step 2 is difficult to proceed in solution with a high concentration. Although the hydrolysis reaction proceeds easily in a dilute solution, the amount of thiol produced by the reaction also increases a solution with a high concentration, presumably because the reverse reaction of step 1 proceeds. Since thiol has higher nucleophilic ability than that of alcohol, it is thought that the reverse reaction of step 1 proceeds more easily in thiol than in alcohol. Also, in the usual hydrolysis reaction, since carboxylic acid is formed in one step, the reverse reaction to the thioester does not occur. This is thought to be the reason for the V_{max} and K_m values of *n*-butyl thioester **2g** are lower than those of *n*-butyl ester **2a**. Thioester type prodrugs with a low K_m value may become prodrugs with a high metabolic activation rate at a low concentration.

In conclusion, this study showed that indomethacin ester prodrugs have the possibility of specific metabolic activation in the liver since these prodrugs were highly hydrolyzed in HLM solution but not in HIM solution. The results suggested that the hydrolysis rates of indomethacin prodrugs in hCES1 solution greatly change depending on the steric hindrance on the neighboring carbon of the ester. In addition, there were new findings for the chiral recognition ability of hCES1: Even in simple alkyl esters such as **2b**, there is a 2.1-times difference in hydrolysis rate between (*R*)-**2b** and (*S*)-**2b**, and for a substrate with a phenyl group, such as 1-phenylpropyl ester **2f**, there is 10.2-times difference in hydrolysis rate between (*R*)-**2f** and (*S*)-**2f**. Although the V_{max} value of a thioester is lower than that of an ester, a thioester has a higher CL_{int} value than that of an ester. The results provide important information regarding the relationship between the structure of an ester prodrug and the rate of metabolic activation. Further elucidation of the substrate specificity for CESs is desired for theoretical prodrug design.

Acknowledgement

We thank the Human and Animal Bridging (HAB) Research Organization (Japan) for providing human livers. This work was supported by JSPS KAKENHI Grant Number JP17K15519.

A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2018.02.035.

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