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Comparative metabolism of DDAO benzoate in liver microsomes from various species

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ABSTRACT

DDAB (6,8-dichloro-9,9-dimethyl-7-oxo-7,9-dihydroacridin-2-yl benzoate) is a newly developed near-infrared fluorescent probe for human carboxylesterase 2 (hCE2), exhibiting high specificity and good reactivity for real-time monitoring the enzymatic activities of hCE2 in complex biological systems. In order to explore the applicability of DDAB in commonly used animal species, the interspecies difference in DDAB hydrolysis was carefully investigated by using liver microsomes from human and five experimental animals including mouse, rat, dog, minipig and monkey. Metabolite profiling demonstrated that DDAB hydrolysis could be catalyzed by all tested liver microsomes from different animals but displayed significant difference in the reaction rate. Chemical inhibition assays demonstrated that carboxylesterases (CEs) were the major enzymes involved in DDAB hydrolysis in all tested liver microsomes, indicating that DDAB was a selective substrate of CEs in a variety of mammals. However, the differential effects of loperamide (LPA, a specific inhibitor against hCE2) on DDAB hydrolysis among various species were observed. The apparent kinetic parameters and the maximum intrinsic clearances (CL_{max}) for DDAB hydrolysis in liver microsomes from different animals were determined, and the order of CL_{max} values for the formation of DDAO was CyLM > MLM ≈ PLM > RLM > HLM ≈ DLM. These findings were helpful for the rational use of DDAB as an imaging tool for CE2 in different mammals, as well as for translational researches on the function of mammalian CEs and CE2-associated drug-drug interactions.

1. Introduction

Carboxylesterases (CEs) belong to a superfamily of α/β-fold proteins, which could hydrolyze chemicals containing carboxylic acid ester, amide, or thioester (Satoh and Hosokawa, 1998). Numerous natural compounds including fatty acids, flavonoids, tanshinones and triterpenoids can inhibit enzymatic activities of hCEs (Wang et al., 2017). In human, two primary CEs including human carboxylesterase 1 (hCE1) and human carboxylesterase 2 (hCE2) have been identified and extensively studied in the past decade (Satoh and Hosokawa, 2006; Williams et al., 2010). hCE1 and hCE2 share 47% amino acid sequence identity, but exhibit differential tissue distribution and distinct substrate and inhibitor specificities (Imai et al., 2006; Hosokawa et al., 2007). Generally, hCE1 is primarily expressed in the liver, and prefers to hydrolyze the ester substrates with a small alcoholic group and a large, bulky acyl groups, such as enalapril and clopidogrel (Zhu et al., 2013; Thomsen et al., 2014). In contrast, hCE2 is expressed at relatively high levels in the small intestine, colon and liver, and prefers to hydrolyze the esters with a relatively large alcohol group and a small acyl group, such as anticancer drugs capecitabine and irinotecan (Quinney et al., 2005; Hatfield et al., 2011; Landowski et al., 2006).

As the major CEs isoform in the human intestine and tumor tissues,

Abbreviations: DDAB, 6,8-Dichloro-9,9-dimethyl-7-oxo-7,9-dihydroacridin-2-yl benzoate; DDAO, 1,3-Dichloro-9,9-dimethyl-9H-acridin-2(7)-one; BNPP, Bis(p–nitrophenyl) phosphate; EDTA, Ethylenedi-aminetetraacetic acid; LPA, Loperamide; EDTA, Ethylene diamine tetraacetic acid; HA, Huperzine A; CEs, Carboxylesterases; CE2, Carboxylesterase 2; hCE2, Human carboxylesterase 2; hCE1, Human carboxylesterase 1; CyLM, Cynomolgus monkey liver microsome; MLM, Mouse liver microsome; PLM, Pig liver microsome; HLM, Human liver microsome; DLM, Dog liver microsome; RLM, Rat liver microsome

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hCE2 exerts profound effects on oral bioavailability of ester drugs, the treatment outcomes as well as the adverse effects of ester anti-cancer agents (Hsieh et al., 2015). Of interest, the relative abundance variation of hCE2 between the tumor and normal tissues has been reported (Sanghani et al., 2003). Therefore, the investigation on the tissue-dependent and tumor-associated variations in both expression and function of CE2 is very helpful for the deeper understanding of the biological roles of this key enzyme in the detoxification and metabolic activation of xenobiotic compounds. As the primary catalyzing enzyme responsible for intestinal hydrolysis of irinotecan, CE2 is deemed as an important toxicity biomarker, correlating with the occurrence of severe or life-threatening toxicities of irinotecan, including diarrhea and neutropenia (Di-Paolo et al., 2011). CE2 may protect the central nervous system from toxic esters and maybe a component of blood-brain barrier (Zhang et al., 2002). CE2 has broad and overlapping xenobiotic substrate specificities, attracting the attention of the researchers in academia, the pharmaceutical industry, and regulatory agencies (Ross and Crow, 2007). However, limited by the shortcomings of the available CE2 probe such as *in vivo* imaging specificity or tissue penetration, it is still difficult to investigate the biological function of CE2 in living animals, including the functional changes in physiological and pathological states as well as the complex interaction between CE2 and xenobiotic.

DDAB is a newly developed colorimetric NIR fluorescent probe of hCE2 and has been successfully used for the rapid, selective and sensitive detection of hCE2 in living cells and living animals (Lei et al., 2017; Jin et al., 2016). Distinguished from the previous fluorescent sensors of CE2, DDAB is of high specificity, good tissue penetration, as well as minimal interference from background in complex biological systems (Jin et al., 2016). DDAB displays high efficiency in measuring the real activities of hCE2 as well as screening hCE2 modulators in complex biological samples. With low cytotoxicity and satisfactory cell membrane permeability, DDAB can be used not only for fluorescence imaging in living cells, but also to visualize endogenous CE2 in living mouse and various CE2-expressing tissues (Jin et al., 2016). Overall, DDAB offers a possibility for efficient monitoring of the biological functions of CE2 *in vivo* (Fig. 1).

Animal models are indispensable tools for further studies of pharmacological and physiological roles of CE2, and must be used in the pharmacological and toxicological tests of the drug candidate whose *in vivo* metabolism is prominently mediated by CE2 (Nishimuta et al., 2014; Mukai et al., 2015; Marques et al., 2014). The interspecies similarity and difference on the hydrolysis of DDAB, in spite of its superiority as the CE2 substrate, has not been systematically investigated, limiting the application of DDAB and interpretation of the relevant results in animals. In order to explore the applicability of DDAB in commonly used animal species, the interspecies difference in DDAB hydrolysis was investigated using liver microsomes of human and experimental animals including mouse, rat, dog, minipig, and cynomolgus monkey. The hydrolysis behaviors of DDAB in liver microsomes from different species were characterized with respect to the similarities and differences of metabolic profiles, involved enzymes, catalytic efficacy and inhibitory potency by known chemical inhibitors.

2. Materials and methods

2.1. Chemicals and reagents

DDAO (9H-(1,3-Dichloro-9,9-Dimethylacridin-2-One-7-yl) benzoate (also termed DDAB) was synthesized by the authors, and the purity is above 98% determined by HPLC-UV. DDAO was purchased from Tianjin Biolite Biotech (Tianjin, China). Bis(p–nitrophenyl)phosphate (BNPP), ethylene diamine tetraacetic acid (EDTA), huperzine A (HA), and loperamide (LPA) were purchased from TCI (Tokyo, Japan). Ethylene diamine tetraacetic acid (EDTA) and huperzine A (HA) were obtained from J & K Chemical Ltd. (Beijing, China). The stock solutions of these compounds were freshly prepared in DMSO and stored at 4 °C until use.

Liver microsomes of male ICR/CD-1 mouse (MLM, Lot. STOM), male Sprague-Dawley rat (RLM, Lot. BDVH), male beagle dog (DLM, Lot. UKHU), male Yucatan minipig (PLM, Lot. RUIB), male cynomolgus monkey (CyLM, Lot. ZDD) as well as male Mongolian human (HLM, Lot. DXOV) were purchased from Research Institute for Liver Diseases (RILD, Shanghai, China). All microsomal samples were stored at − 80 °C until use.

2.2. Incubation conditions

The incubation mixture, with a total volume of 200 μl, consisted of 100 mM potassium phosphate buffer (pH 7.4) and liver microsomes. In all experiments, DDAB was serially diluted to the required concentrations and the final concentration of DMSO did not exceed 1% (*v*/v) in the mixture. After pre-incubation at 37 °C for 3 min, the reaction was initiated by adding DDAB and further incubated at 37 °C in a shaking water bath. The reaction was terminated by the addition of ice-cold acetonitrile (200 μl). The mixture was kept on ice until it was centrifuged at 20,000 × *g* for 10 min at 4 °C. Aliquots of supernatants were stored at − 20 °C until analysis. Control incubations without microsomes were carried out to ensure that the metabolite formation was microsome-dependent. All incubations throughout the study were carried out in triplicate.

2.3. Kinetic analyses

The kinetic parameters of DDAB hydrolysis in liver microsomes from human and various animal species were determined under the incubation conditions in which the formation rates of DDAO were in the linear range in relation to the incubation time and protein concentration. Briefly, DDAB was incubated with the pooled HLM (5 μg protein/ml), MLM (0.5 μg protein/ml), RLM (2 μg protein/ml), and DLM

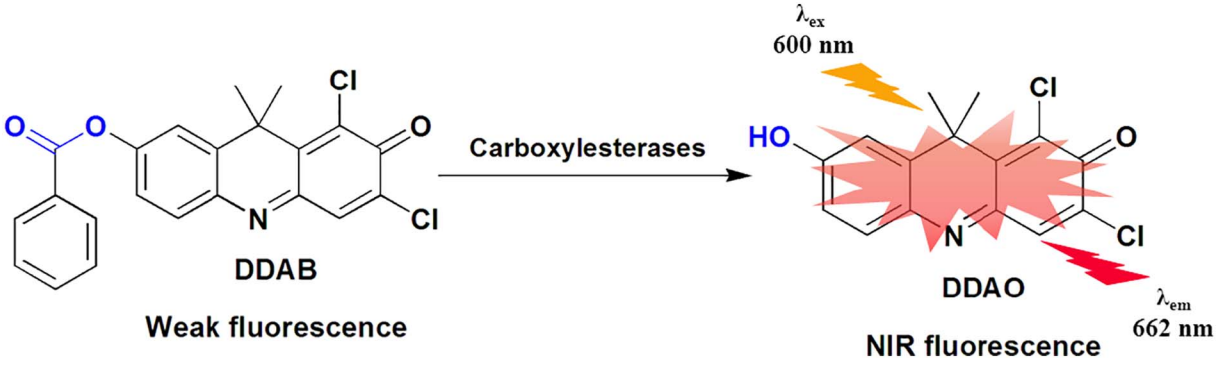

Fig. 1. The chemical structure of DDAB and its fluorescence response towards mammalian carboxylesterases in liver microsomes from different species.