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Cellular target recognition of perfluoroalkyl acids: *In vitro* evaluation of inhibitory effects on lysine decarboxylase

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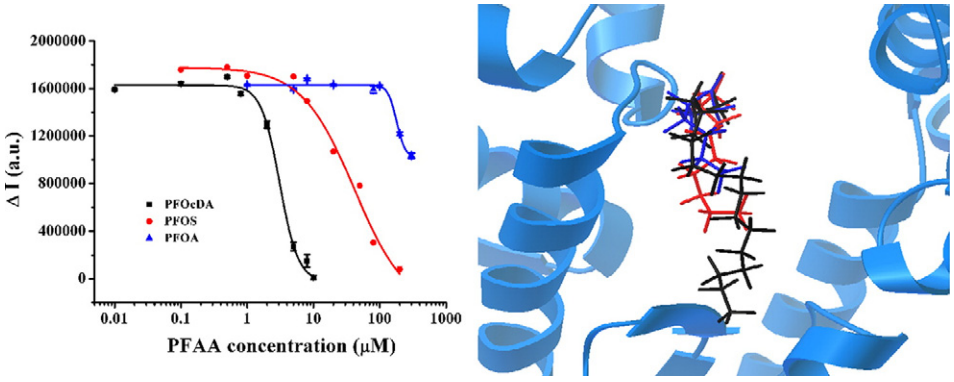
HIGHLIGHTS

- Inhibitory effects of PFAAs on lysine decarboxylase activity were evaluated.
- Four different methods were employed to investigate the mechanisms.
- The long chain PFAAs showed inhibitory effect compare with 4–6 carbon chain.
- The long chain PFAAs bound with LDC differently from the short ones.
- The results in cells correlate with those obtained from fluorescence assay.

GRAPHICAL ABSTRACT



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ABSTRACT

Perfluoroalkyl acids (PFAAs) have been shown to bind with hepatic peroxisome proliferator receptor α, estrogen receptors and human serum albumin and subsequently cause some toxic effects. Lysine decarboxylase (LDC) plays an important role in cell growth and developmental processes. In this study, the inhibitory effect of 16 PFAAs, including 13 perfluorinated carboxylic acids (PFCAs) and 3 perfluorinated sulfonic acids (PFSAs), on lysine decarboxylase (LDC) activity was investigated. The inhibition constants obtained in fluorescence enzyme assays fall in the range of 2.960 μM to 290.8 μM for targeted PFCAs, and 41.22 μM to 67.44 μM for targeted PFSAs. The inhibitory effect of PFCAs increased significantly with carbon chain (7–18 carbons), whereas the short chain PFCAs (less than 7 carbons) did not show any effect. Circular dichroism results showed that PFAA binding induced significant protein secondary structural changes. Molecular docking revealed that the inhibitory effect could be rationalized well by the cleft binding mode as well as the size, substituent group and hydrophobic characteristics of the PFAAs. At non-cytotoxic concentrations, three selected PFAAs inhibited LDC activity in HepG2 cells, and subsequently resulted in the decreased cadaverine level in the exposed cells, suggesting that LDC may be a possible target of PFAAs for their *in vivo* toxic effects.

1. Introduction

Perfluoroalkyl acids (PFAAs) are a family of fluorine-containing chemicals with unique physical and chemical properties that have been widely used in industrial and commercial products. Two most widely known PFAAs are perfluorooctane sulfonate (PFOS) and

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perfluorooctanoic acid (PFOA). Due to the extraordinary stability of the carbon–fluorine bond, these compounds are resistant to chemical, biological and thermal degradation. Therefore, PFAAs have been found in environments and accumulate in the bodies of humans and wildlife (Giesy and Kannan, 2002; Sundström et al., 2011; Raymer et al., 2012; Houde et al., 2006). A number of studies have demonstrated the adverse effects of PFAAs on experimental animals, mostly of PFOA and PFOS (Lau et al., 2007). In 2009, PFOS was added to the list of persistent organic pollutants of the Stockholm Convention to reduce and eventually eliminate its production and use. In spite of all these policies and bans, due to the nature of persistence, bioaccumulation and biomagnifications through the food chain (Conder et al., 2008) of PFAAs, their risks to human health are still worthy of long-term concern.

Numerous studies have been published regarding the toxicity and human health effects of PFAAs, including loss of body weight, hepatotoxicity, interference of lipid transportation and metabolism, endocrine effects, tumorigenicity, reproductive toxicity and developmental toxicity (Thorsten et al., 2011). Early toxicological studies of PFAAs (fatty acids like structures) focused on the mechanisms involving ligand-dependent activation of the hepatic peroxisome proliferator receptor α (PPARα), which induces enzymes responsible for β-oxidation, fatty acid ω-oxidation and cholesterol homeostasis (Andersen et al., 2008; DeWitt et al., 2009). However, due to the observation of increased liver cancer incidence by PFAAs in PPARα knockout mice, the role of estrogenic activity of PFAAs in their hepatocarcinogenesis has attracted increasing attentions recently (Ito et al., 2007; Guyton et al., 2009). Some PFAAs were found to bind to estrogen receptors (ERs) and recruit coactivator peptides *in vitro*, and induce ER-mediated transcriptions in cells (Benninghoff et al., 2011; Gao et al., 2013). Several studies also found depression of thyroid hormone levels in PFOS-exposed rats (Lau et al., 2003; Wang et al., 2011). The interactions between PFAAs and thyroid hormone transport proteins were also observed (Chang et al., 2007). *In vitro* binding between PFAAs and human serum albumin was also evaluated as a possible route for disrupting fatty acid transport in blood (Chen and Guo, 2009; Hebert and MacManus-Spencer, 2010). Although some biological targets have been gradually revealed for exploring molecular mechanisms underlying PFAA toxicity, there are still other proteins *in vivo* to potentially interact with PFAAs (Weiss et al., 2009; White et al., 2011; Zhang et al., 2013).

Lysine decarboxylase (LDC) is a key enzyme involved in the production of cadaverine by the decarboxylation of lysine. Cadaverine is known to be involved in a number of growth and developmental processes (Bagni and Tassoni, 2001) and the biosynthesis of a broad range of alkaloids (Leistner and Spenser, 1973) with beneficial pharmacological properties, such as cytotoxic, antiarrhythmic, oxytocic, hypoglycemic, and antipyretic activities (Ohmiya et al., 1995; Michael, 2008). Inhibition of cadaverine synthesis suppressed wound healing in rodents (Calandra et al., 1996). Some diseases including cancers are closely associated with the abnormal cadaverine level (Gerner and Meyskens, 2004). Although LDC plays an important role in many biological processes, information on the molecular regulatory mechanisms is limited.

In the present study, we employed a fluorescence sensing method to measure the inhibitory effect of 16 PFAAs on LDC activity. The selected PFAAs are structurally diverse, with different carbon chain lengths (4–18 carbons) and functional groups (carboxylic or sulfonic acid). By combining the fluorescence sensing assay, circular dichroism (CD) spectroscopy, *in vitro* cell experiments and molecular docking, the relationship between PFAA structure and their LDC inhibitory effect, as well as LDC as a potential cellular target of PFAAs, was investigated and analyzed.

2. Material and methods

2.1. Chemicals

Lysine, cadaverine, cucurbit[7]uril (CB7), lysine decarboxylase (LDC, from *Bacterium cadaveris*), 2,4,6-trinitrobenzenesulfonic acid (TNBS), 1,7-diaminoheptane (DAH), 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT), perfluorobutyric acid (PFBA, 98%), perfluoropentanoic acid (PFPA, 97%), perfluorohexanoic acid (PFHxA, ≥97%), perfluoroheptanoic acid (PFHpA, 99%), perfluorooctanoic acid (PFOA, 96%), perfluorononanoic acid (PFNA, 97%), perfluorodecanoic acid (PFDA, 98%), perfluoroundecanoic acid (PFUnA, 95%), perfluorododecanoic acid (PFDoA, 96%), perfluorotridecanoic acid (PFTrDA, 97%), perfluorotetradecanoic acid (PFTeDA, 97%), perfluorobutane sulfonate (PFBS, 97%), perfluorohexane sulfonate (PFHxS, ≥98.0%) and perfluorooctane sulfonate (PFOS, ≥98.0%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Perfluorohexadecanoic acid (PFHxDA, 95%) and perfluorooctadecanoic acid (PFOcDA, 97%) were obtained from Alfa Aesar (Ward Hill, MA, USA). Dapoxyl was from Molecular Probes (Eugene, OR, USA). Guanosine 5′-diphosphate, 3′-diphosphate (ppGpp) was used as received from Trilink BioTechnologies (San Diego, CA). (Fig. S1 in the supplementary data) Benzoyl chloride was from TCI (Shanghai, China). All chemicals are of analytical grade. BCA protein assay kit was obtained from ComWin Biotech (Beijing, China).

2.2. Fluorescence enzyme activity and inhibition assay

To investigate the inhibitory effect of PFAAs on LDC, a proper enzyme activity assay is needed. In 2007, Henning et al. described a novel concept for the determination of LDC enzymatic activity, using macrocyclic receptors and fluorescent dyes such as CB7/Dapoxyl and CX4/DBO (Henning et al., 2007). This product-competitive displacement method is simple, convenient and label-free. Briefly, a self-assembled host–guest inclusion complex CB7/Dapoxyl was employed as a fluorescence reporter pair for the enzymatic activity assay of LDC. Competitive binding of the enzyme product cadaverine with CB7 displaces Dapoxyl from CB7, leading to reduced fluorescence intensity. If the enzymatic activity is inhibited, the fluorescence intensity would remain unchanged (Scheme 1). LDC activity assay was performed according to the following protocol. The reaction mixture containing 20 μg mL^{-1} LDC, 50 μM lysine, 2.5 μM Dapoxyl and 30 μM CB7 in a total volume of 100 μL was incubated at 37 °C for 1.5 h. For the inhibition assay, inhibitors at varying concentrations were included in the reaction mixture. The change in fluorescence intensity ($\Delta I = I_0 - I$) was taken as relative LDC activity, and was plotted as a function of inhibitor concentration. I and I_0 are the fluorescence intensity of CB7/Dapoxyl in the presence and absence of LDC. The dose–response curve was fitted with a sigmoidal model (Origin Lab 8.0, Northampton, MA, USA) and analyzed with the Hill equation to obtain IC_{50} value (Copeland, 2003), which can be readily converted into the inhibition constants K_i by considering the enzyme concentration (Nau et al., 2009):

$$IC_{50} = K_i + \frac{1}{2}[E].$$

Steady-state fluorescence was measured on a Horiba FluoroMax-4 spectrofluorimeter (Edison, NJ, USA). The excitation and emission wavelength were 336 nm and 380 nm, respectively. Excitation and emission slits were both set at 4 nm.

2.3. Circular dichroism spectroscopic measurement

Circular dichroism (CD) spectra measurement of LDC (150 μg mL^{-1} in HCl–NH_4OAc buffer) was carried out in the absence and presence of PFAAs on a JASCO J-815 spectropolarimeter (Tokyo, Japan) with a 10 mm path length quartz cuvette. The CD spectra were recorded from 190 to 300 nm at a scan rate of 50 nm/min and 1 s response time. Three scans were averaged for protein secondary structure analysis, which was performed with the JWSSE-513 program installed on the CD instrument. In the experiments, different concentrations of PFAAs in acetonitrile were incubated with LDC in NH_4OAc buffer. The final