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In vitro inhibition of lysine decarboxylase activity by organophosphate esters

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ABSTRACT

Organophosphate esters (OPEs), a major group of organophosphorus flame retardants, are regarded as emerging environmental contaminants of health concern. Amino acid decarboxylases catalyze the conversion of amino acids into polyamines that are essential for cell proliferation, hypertrophy and tissue growth. In this paper, inhibitory effect of twelve OPEs with aromatic, alkyl or chlorinated alkyl substituents on the activity of lysine decarboxylase (LDC) was assessed quantitatively with an economic and label-free fluorescence sensor and cell assay. The sensor comprises a macrocyclic host (cucurbit[7]uril) and a fluorescent dye (acridine orange) reporter. The twelve OPEs were found to vary in their capacity to inhibit LDC activity. Alkyl group substituted OPEs had no inhibitory effect. By contrast, six OPEs substituted with aromatic or chlorinated alkyl groups inhibited LDC activity significantly with IC_{50} ranging from 1.32 μM to 9.07 μM. Among them, the inhibitory effect of tri-*m*-cresyl phosphate (TCrP) was even more effective as an inhibitor than guanosine 5′-diphosphate-3′-diphosphate (ppGpp) (1.60 μM), an LDC natural inhibitor *in vivo*. Moreover, at non-cytotoxic concentrations, these six OPEs showed perceptible inhibitory effects on LDC activity in PC12 living cells, and led to a marked loss in the cadaverine content. Molecular docking analysis of the LDC/OPE complexes revealed that different binding modes contribute to the difference in their inhibitory effect. Our finding suggested that LDC, as a new potential biological target of OPEs, might be implicated in toxicological and pathogenic mechanism of OPEs.

1. Introduction

Due to the ongoing worldwide phase-out and restriction of the brominated flame retardants (BFRs) since the early 2000s and the recent banning of polybrominated diphenyl ethers by the Stockholm Convention on persistent organic pollutants, the production and application of organophosphate esters (OPEs) as substitutes for BFRs has increased dramatically [1–3]. In 2004, OPEs accounted for 14% of the global production volume of flame retardants, compared to 21% for BFRs. OPEs are man-made industrial chemicals used as flame retardants, plasticizers, antifoaming agents and additives in various household and industrial products such as building and insulation materials, textiles, furniture, floor polishes, paints, lubricants, hydraulic fluids, cables and electronics [4–6]. OPEs are the derivatives of phosphoric acid with different substitutes including alkyl chains (*e.g.*, tri-*n*-butyl phosphate (T*n*BP)), partly halogenated alkyl chains (*e.g.*, tri(2-chloroethyl) phosphate (TCEP)) as well as aromatic contents (*e.g.*, triphenyl phosphate (TPhP)) [7–9]. Similar to BFRs, OPEs are not chemically bound in the fire-proofed material and can easily leach into the environment *via* volatilization, abrasion and dissolution. As a result, OPEs have been frequently detected in the environment, mainly in the aquatic environment (*e.g.*, river, groundwater, surface water, drinking water, wastewater) (0.015 ng L^{-1}–24 μg L^{-1}) [10,11], indoor air (0.05 ng m^{-3}–730 mg m^{-3}) [12,13] and dust (0.04–1800 μg g^{-1}) [6,14], sediment (0.05–24,000 μg kg^{-1}) [12,15] and marine coastal biota (0.025–810 μg kg^{-1}) [15,16]. Especially, the concentration of OPEs detected in house dust is comparable, or in some cases exceeds concentrations of BFRs.

OPEs have been reported to induce various toxic effects including skin irritation, neurotoxicity, reproductive toxicity, carcinogenicity and developmental toxicity [4]. In some previous studies, esterases, such as the neuropathy target receptors, have been identified as important biological targets of OPEs. Animal data showed that some OPEs including tributoxyethyl phosphate (TBEP), TCEP, TPhP and tri-*m*-cresyl phosphate (TCrP) caused the red blood cell cholinesterase activity in rats to decrease

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significantly after long-term exposure [17]. TCrP and TPhP remarkably inhibited acetyl cholinesterase (AChE) activity in cholinergic nerve synapses by phosphorylating a serine hydroxyl group at the active site of the enzyme, which resulted in the accumulation of neurotransmitter acetylcholine in nicotinic and muscarinic receptor and subsequently caused the impairment of neurological and neurobehavioral functions [18]. *In vitro* experiments revealed that five arylphosphates including diphenyl phosphate, tribenzyl phosphate, tricyclohexyl phosphate, diphenyl methyl phosphate and TPhP were relatively effective inhibitors of human monocyte carboxylesterase (CBE) activity, but the alkylphosphates (tributyl phosphate and triethyl phosphate) had no inhibitory activity [19]. In addition, TPhP has been shown to bind with androgen receptor (AR) by using a radiolabeled competitive binding assay [20].

Amino acid decarboxylases, which catalyze the conversion of amino acids (*e.g.* lysine, arginine, histidine, ornithine) into polyamines (*e.g.* cadaverine, agmatine, histamine, putrescine), play dual roles in acid resistance and the synthesis of polyamines [21]. Polyamines, as naturally occurring organic cations, are found in plants, animals and microbes. Early studies demonstrated that polyamines (putrescine, spermidine and cadaverine) are essential for optimal growth and viability [22,23]. Some polyamines such as spermidine and hypusine are requirement for cell proliferation in eukaryotic cells [24,25]. Recently, this paradigm has extended to mammals, with the observation that polyamine is essential for tissue development and growth. For example, polyamines are necessary for blood-vessel development occurring in response to damage to normal tissues or tumor growth [26]. In addition, Polyamine levels have also been associated with the normal growth and hypertrophy of several tissues-including skin, breast, kidney and heart-in rodents [27]. The role of polyamines in tissue repair might be to facilitate tissue remodelling, as has been reported for certain types of lung damage [28]. Polyamines have also been implicated in the development and function of both male and female reproductive organs [29]. Other studies indicated that polyamine-synthesis inhibitors disrupted intestinal development in mice. Inhibition of polyamine synthesis also suppressed wound healing and decreased hormone responsivity in rodents [30,31]. In addition, some diseases including cancers are closely associated with the abnormal polyamine level [32]. Given the important biological functions of polyamines, inhibition of the enzymes responsible for their *in vivo* synthesis, *i.e.*, amino acid decarboxylases by exogenous chemicals is worth investigating.

In this work, we investigated the inhibitory activity of twelve structurally diverse OPEs on lysine decarboxylase (LDC), an amino acid decarboxylase, using a fluorescence-based enzyme activity assay developed in our laboratory. These OPEs carry different substituents including alkyl, chlorinated alkyl and aromatic groups. By combining the fluorescence sensing assay, *in vitro* cell experiments and molecular docking, the structural requirements for the inhibition of LDC by OPEs, as well as LDC as a possible cellular target of OPEs, were investigated and assessed in detail.

2. Materials and methods

2.1. Chemicals and materials

Lysine, cadaverine, acridine orange (AO), cucurbit[7]uril (CB7), lysine decarboxylase (LDC, from *Bacterium cadaveris*), 2,4,6-trinitrebenzenesulfonic acid (TNBS), and 1,7-diaminoheptane (DAH) were purchased from Sigma–Aldrich (St. Louis MO, USA). Guanosine 5′-diphosphate, 3′-diphosphate (ppGp) was obtained from Trilink BioTechnologies (San Diego, CA). Trimethyl phosphate (TMP), triethyl phosphate (TEP), TCEP, tri-*n*-propyl phosphate (TPrP), tris(2-chloroisopropyl)phosphate (TCPP), tri(2-chloro-1-(chloromethyl)ethyl) phosphate (TDCP), TPhP, T*n*BP, TBEP, TCrP, 2-ethylhexyl diphenyl phosphate (EHDPP) and tris(2-ethylhexyl) phosphate (TEHP) were purchased from Dr. Ehrenstorfer Gmbh (Germany) (Fig. 1). Benzoyl chloride was from TCI (Tokyo, Japan), and BCA protein assay kit was from ComWin Biotech (Beijing, China). HPLC grade acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ, USA). Sodium dodecyl sulfate (SDS), glycerol and 2-mercaptoethanol were acquired from Amresco (Ohio, USA). NH_4OAc, Tris–HCl, HCl and K_2CO_3 were all obtained from Sinopharm Chemical Reagent Beijing Co. Ltd (Beijing, China). De-ionized water (18.2 MΩ cm) obtained from a Millipore ultrapure water system (Millipore, Bedford, USA) was used throughout the experiment.

2.2. Fluorescence enzyme activity assay

In our work, a self-assembled host-guest inclusion complex was employed as fluorescence sensor for the enzyme activity assay of LDC. The complex was composed of macrocyclic host CB7 and fluorescent dye AO. Competitive binding of enzyme product cadaverine with CB7 displaces AO from CB7, leading to reduced fluorescence signal. If LDC activity is inhibited, the fluorescence intensity would remain unchanged (Fig. 2). Steady-state fluorescence was measured on a Horiba Fluoromax-4 spectrofluorimeter (Edison, NJ, USA). The excitation and emission wavelength was 485 nm and 510 nm, respectively. Excitation and emission slits were all set at 3 nm.

First of all, the binding interaction of AO with CB7 was investigated by successive addition of a known amount of CB7 into 0.5 μM AO solution. With the optimized CB7/AO concentration, fluorescence displacement measurements were carried out to examine the binding affinity of lysine (LDC substrate) and cadaverine (LDC product) with CB7. It was performed by successive addition of a known amount of lysine or cadaverine into a mixed solution of 0.5 μM AO and 5 μM CB7. In the LDC activity assay, an LDC stock solution was prepared by dissolving 500 μg solid in 1 mL HCl–NH_4OAc buffer (10 mM, pH 6.0). Enzyme reaction was performed in an Eppendorf tube containing a 100 μL solution of 8.0 μg mL^{-1} LDC, 50 μM lysine, 0.5 μM AO, and 5 μM CB7, and the temperature was thermostatically controlled at 37.0 ± 0.1 °C. The reaction was monitored by recording AO fluorescence emission spectrum with time. For the LDC inhibition assay, 8.0 μg mL^{-1} LDC and different concentrations of OPEs were mixed in HCl-NH_4OAc buffer (pH 6.0) and incubated for 5 h at 37.0 ± 0.1 °C. Then, 0.5 μM AO, 5 μM CB7 and 50 μM lysine were added. Fluorescence spectra were recorded after reacting for 1.5 h. The change of fluorescence intensity *versus* time was taken as a relative reaction rate, and was plotted as a function of inhibitor concentration. 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 [33]. The IC_{50} can be readily converted into the inhibition constants K_i by considering the enzyme concentration [34]:

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

2.3. Cell culture, treatment and viability test

Pheochromocytoma PC12 cells, a cell line derived from rat adrenal medulla, were obtained from ATCC (Manassas, VA, USA), and maintained in Dulbecco's modified Eagle's medium supplemented with 6% fetal calf serum, 6% horse serum, 100 units/mL penicillin and 100 μg/mL streptomycin in a humidified CO_2 incubator at 37 °C. All culture reagents were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Approximated 0.5–1×10^5 cells mL^{-1} in 96-well plates was seeded for cell viability assay. The exposure was performed in DMEM with or without (DMSO control) OPEs (up to 250 μM), respectively. The change of