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Aristolochic acid and its derivatives as inhibitors of snake venom L-amino acid oxidase

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ABSTRACT

Snake venom L-amino acid oxidase (LAAO) exerts toxicity by inducing hemorrhage, pneumorrhagia, pulmonary edema, cardiac edema, liver cell necrosis *etc*. Being well conserved, inhibitors of the enzyme may be synthesized using the template of the substrate, substrate binding site and features of the catalytic site of the enzyme. Previous findings showed that aristolochic acid (AA), a major constituent of *Aristolochia indica*, inhibits Russell's viper venom LAAO enzyme activity since, AA interacts with DNA and causes genotoxicity, derivatives of this compound were synthesized by replacing the nitro group to reduce toxicity while retaining the inhibitory potency. The interactions of AA and its derivatives with LAAO were followed by inhibition kinetics and surface plasmon resonance. Similar interactions with DNA were followed by absorption spectroscopy and atomic force microscopy. LAAO-induced cytotoxicity was evaluated by generation of reactive oxygen species (ROS), cell viability assays, confocal and epifluorescence microscopy. The hydroxyl (AA-OH) and chloro (AA-Cl) derivatives acted as inhibitors of LAAO but did not interact with DNA. The derivatives significantly reduced LAAO-induced ROS generation and cytotoxicity in human embryonic kidney (HEK 293) and hepatoma (HepG2) cell lines. Confocal images indicated that AA, AA-OH and AA-Cl interfered with the binding of LAAO to the cell membrane. AA-OH and AA-Cl significantly inhibited LAAO activity and reduced LAAO-induced cytotoxicity.

1. Introduction

L-amino acid oxidase (LAAO, E.C. 1.4.3.2.) is a flavoenzyme that catalyzes stereospecific oxidative deamination of L-amino acids to α-keto acids resulting in production of ammonia and hydrogen peroxide (H_2O_2) (Bhattacharjee et al., 2015). Venoms of snakes, insects and marine animals contain LAAO that acts as an effective deterrent against enemies and/or predators. The cytotoxic action of LAAO is mostly due to high concentration of H_2O_2 locally formed near the binding site of the enzyme on the cell surface leading to apoptosis (Geyer et al., 2001). The crystallographic structures of four snake venom (SV)-LAAOs have been solved. The structure/amino acid sequence of the enzymes are conserved to the extent of 90%. Further, a highly conserved β-α-β motif in the N-terminal region is observed in FAD binding (Du and Clemetson, 2002). These features provide additional advantages towards successful designing of inhibitors of this class of enzymes. Despite all reports on inhibitors of LAAO are limited (Yu and Qiao, 2012).

Among snakes, Viperidae, Crotalidae and Elapidae venoms are rich sources of LAAO. The amount of LAAO in snake venoms varies

Abbreviations: AA, 8-methoxy-6-nitro-phenanthro-(3,4-d)-1,3-dioxolo-5-carboxylic acid or aristolochic acid; AA-OH, hydroxyl derivative of aristolochic acid; AA-Cl, chloro derivative of aristolochic acid; AFM, atomic force microscope; BSA, bovine serum albumin; CT-DNA, calf thymus deoxyribonucleic acid; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified eagle medium; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; H_2DCF-DA, 2′,7′-dichlorodihydro-fluorescein diacetate; HRP, horse radish peroxidase; LAAO, L-amino acid oxidase; L-Phe, L-phenylalanine; L-Lys, L-lysine; MD, molecular dynamics; MTT, 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide; NATA, N-acetyl tryptophan amide; NAT, N-acetyl tryptophan; OAB, *o*-amino benzoic acid; RITC, rhodamine-B-isothiocyanate; ROS, reactive oxygen species; RP-HPLC, reverse phase-HPLC; RU, response units; RVV, Russell's viper venom; SPR, surface plasmon resonance.

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between 0.1 and 30% (Izidoro et al., 2014). LAAO constitutes about 1.2% and 20% of crude venoms of *Crotalus adamanteus* and *Daboia russelii russelii* respectively (Raibekas and Massey, 1996; Mandal and Bhattacharyya, 2008). SV-LAAOs are involved in the pathogenesis of snakebite-induced inflammation, pneumorrhagia, pulmonary interstitial edema, fusion of pulmonary alveoli, cardiac interstitial edema and necrosis of liver cells. They can also stimulate lymphocytes, monocytes and neutrophils to release proinflammatory interleukins (IL-2, IL-6, IL-8, IL-12), tumor necrosis factor (TNF-α) and lipid mediators prostaglandin E2 (PGE2) and leukotriene B4 (LTB4) (Tan and Choy, 1994; Wei et al., 2007, 2009; Pontes et al., 2014, 2016; Costa et al., 2017). LAAO isolated from *Daboia russelii russelii* venom (Russell's viper venom, RVV) dose-dependently inhibited ADP- and collagen-induced platelet aggregation with IC_{50} values of 0.27 and 0.82 μM respectively (Chen et al., 2012). It appears that LAAO may synergize with other venom components to enhance bleeding after RVV envenomation.

In general, substrate analogs are molecule of choice for inhibition of enzymes as they are well recognized by the catalytic site of an enzyme. Vinylglycine as a suicide substrate/inhibitor of LAAO was reported (Marcotte and Walsh, 1976). The crystallographic structures of LAAO predict that different electrostatic and hydrophobic patches within the catalytic funnel play a significant role in the interaction of substrate analogs. Based on this knowledge, inhibition of two LAAO isoforms of RVV was explained. The inhibitors were 'substrate analogs' like N-acetyl tryptophan amide (NATA), N-acetyl tryptophan (NAT), *o*-amino benzoic acid (OAB) (Mandal and Bhattacharyya, 2008). Subsequently, interaction of LAAO from *C. adamanteus* with the suicide substrate L-propargylglycine through covalent modification of the active site His 223 was established (Mitra and Bhattacharyya, 2013). Despite this, there are very few reports on inhibitors of snake venom LAAOs (Ushanandini et al., 2006; Leanpolchareanchai et al., 2009; Pithayanukul et al., 2010).

Aristolochic acids (AA) are a class of compounds commonly found in the plants of Aristolochiaceae family. They are believed to be the causative agent of 'Balkan Disease'. In the Balkan region, exposure to AA from wheat contaminated with seeds of *Aristolochia clematitis* apparently caused Balkan endemic nephropathy (Grollman et al., 2007; Stiborova et al., 2008; De Broe, 2012; Gokmen et al., 2013; Report on Carcinogens, 2016). The major nephrotoxic constituent of AA is aristolochic acid I (AAI). Metabolic activation of AAI by cytosolic and microsomal oxidoreductase enzymes like NAD(P)H:quinoneoxidoreductase, cytochrome P450, cyclooxygenases *etc* leads to the formation of a cyclic N-hydroxylactam in human hepatic and renal microsomes. In contrast to other nitroaromatic arenes that preferentially react with the C-8 position of guanine, activated AAI forms adducts with the exocyclic amino groups of purine bases, *viz*, 7-(deoxyadenosine-N^6-yl) aristolactam and 7-(deoxyguanosine-N^2-yl) aristolactam This reactive species can react with the peripheral amino group of purine bases generating bulky DNA adducts and it produces mutagenic and carcinogenic effects (Scheme 1) (Stiborova et al., 2008). AAI may directly cause acute renal toxicity and apoptosis through endoplasmic reticulum (ER) stress pathway or mitochondrial cell death pathway or p53 mediated pathway in renal tubular cells (Grollman et al., 2007; Gokmen et al., 2013). The US Food and Drug Administration have banned consumption of products that contain AA or its derivatives (Report on Carcinogens, 2016).

The subspecies *Aristolochia indica* grows abundantly in the Indian subcontinent and it contains only AAI. In rural India, an aqueous decoction of its root is used as a drink against Russell's viper envenomation. A paste of the root or leaves is also applied topically at the site of bite (Bhattacharjee and Bhattacharyya, 2014). The aqueous extract of the root inhibits LAAO (Bhattacharjee and Bhattacharyya, 2013). Here we report that AA is an inhibitor of LAAO. The structure of AA was modified to reduce toxicity while retaining its inhibitory potency against LAAO.

2. Materials and methods

2.1. Purification of LAAO from snake venom

Daboia russelii russelii (Russell's viper venom, RVV) and *Naja naja* venoms were collected from Mr Dipak Mitra, Calcutta Snake Park as desiccated crystals. RVV (100 mg) was dissolved in 10 mM Na-phosphate, pH 7.5 and after centrifugation, the supernatant was applied to Sephadex G-100 (Sigma-Aldrich) gel filtration column (85 × 0.5 cm) pre-equilibrated with the buffer containing 100 mM NaCl (Chakrabarty et al., 2000). The active fraction was dialyzed against the original buffer and was fractionated by a CM Sephadex C-50 (Sigma-Aldrich) column (50 × 20 mm) pre-equilibrated in 10 mM Na-phosphate, pH 7.5. Unabsorbed fractions were eluted after washing with 3-column volumes of equilibrating buffer. Bound fractions were eluted after application of a linear gradient of 0–0.5 M NaCl (Sigma-Aldrich) in the same buffer in 3 column volumes. Flow rate was 20 ml/h, fraction size was 1 ml and elution of fractions was followed at 280 nm. All chromatographic separations were performed at +4 °C. The LAAO activity was found in two bound fractions, pooled and denoted as $LAAO_1$ and $LAAO_2$ (Mandal and Bhattacharyya, 2008). The purified isoforms migrated as single band in SDS-PAGE that corresponds to 60 kDa (result not shown). The venoms (100 mg) of *C. adamanteus* (Sigma-Aldrich) and *N. naja* were dissolved in 10 mM Na-phosphate, pH 7.5 and after centrifugation, the supernatant was applied to a Sephadex G-200 (Sigma-Aldrich) gel filtration column (85 × 0.5 cm) pre-equilibrated with the same buffer. LAAO (~120 kDa in dimeric form) in each set was eluted in the first fraction where most of the protein components were separated. Henceforth, the crude RVV and the purified LAAO enzyme from RVV is denoted by RVV and RVV-LAAO respectively.

2.2. Synthesis of derivatives

The lactam derivative of AA was synthesized by heating AA (50 mg) at +65 °C for 3.5 h with HCl (12 N) in presence of Sn to reduce the $-NO_2$ group to $-NH_2$ group. It was neutralized with NaOH to yield aristolactam which was extracted by ether-water phase separation (yield 75%). The AA and its lactam derivative were spotted on preparative thin layer chromatography (TLC) plates (Sigma-Aldrich) and separated using mixture of toluene and ethyl acetate (1:1, v/v). Chromatograms were developed at +25 °C and spots were viewed under short wavelength UV irradiation (254 nm, Mineralight lamp, Model No. UVGL-25, UVP, Upland, USA). The AA and its lactam derivative migrated as single spots of R_f 0.50 and 0.46 respectively. In MS analysis, a peak of 316 Da indicated the presence of the lactam. A reduced mass of 301 Da was probably generated from the dissociation of the $-OCH_3$ moiety from the lactam. From this data, lactam ring formation was indicated. As the $-NO_2$ group was converted to $-NH_2$ group, under neutralized conditions, it reacted with neighboring −COOH group to form 5-membered lactam ring.

To synthesize amide derivative of AA, 20 μl of $SOCl_2$ was added to AA (50 mg) and stirred for 1 h. Then excess liquor ammonia was added to the reaction mixture, the product was extracted by ether and analyzed by TLC using mixture of toluene and ethyl acetate (1:1, v/v). Distinct bands of R_f 0.06, 0.11, 0.21, 0.275, 0.58, 0.73 and 0.89 were observed, which were extracted from silica gel by ether, dried and analyzed by MS that indicated presence of a component of 363 Da corresponding to the Mw of -$CONH_2$ derivative. As this derivative was highly unstable, further analysis was not done.