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A Fluorescent based Enzyme Assay for Recombinant Human Lipoxygenase Enzyme Isoforms

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Abstract: Background: In humans, six functional Lipoxygenase (LOX) isoforms have been identified. Out of these isoforms, 5-LOX, 12-LOX and 15-LOX have been originally identified in leukocytes, platelets and reticulocytes, respectively. These isoforms generate lipid mediators of inflammation and have a role in the pathophysiology of asthma, rheumatoid arthritis, pain, atherosclerosis, cardiovascular diseases, renal diseases, psoriasis, carcinogenesis etc. Based on their pathophysiological role in a wide range of inflammatory diseases, there is a need to develop novel inhibitors for specific LOX isoforms. However, high throughput LOX screening assays to test novel agents against these enzyme isoforms are limited. **Results:** In this study, we have reported the development of a high throughput screening method for identification of novel LOX isoforms inhibitor. We have developed and standardized a 96-well microplate end point fluorescent assay using H2DCFDA dye for recombinant 5, 12 and 15-LOX isoforms tested against several existing LOX inhibitors and validate the assay by comparing known IC_{50} values. **Conclusion:** Taken together, we have been successful in generating stable human recombinant 5, 12 and 15-LOX enzyme isoforms along and using these stable clones, an end point fluorescence assay has been developed for high throughput screening of the LOX-inhibitors.

Key words: Lipoxygenase, 5-LOX, 12-LOX, 15-LOX, H2DCFDA, recombinant enzymes

INTRODUCTION

Leukotrienes (LTs) are lipid mediators of immune and inflammatory responses, with major roles in respiratory and cardiovascular diseases (Peters-Golden and Henderson, 2007; Haeggstrom and Funk, 2011). For LT biosynthesis, the precursor arachidonic acid (AA) is released from membrane phospholipids by Phospholipase A2 (PLA2) and is then metabolized by 5-lipoxygenase (5-LOX) aided by the 5-LOX-activating protein (FLAP). Thus, 5-LOX is one of the rate-limiting enzymes in the biosynthesis of LTs and targeting 5-LOX represents an attractive strategy for therapeutic intervention (Peters-Golden and Henderson, 2007; Haeggstrom and Funk, 2011; Drazen *et al*., 1999; Werz and Steinhilber, 2006).

Among six different known isoforms of lipoxygenase (LOX) enzymes, three classical isoforms, 5-LOX (Dixon *et al*., 1988; Matsumoto *et al*., 1988), 12-LOX (Funk *et al*., 1990; Izumi *et al*., 1990) and 15-LOX (Funk, 2005; Werz and Steinhilber, 2005; Kuhn *et al*., 2002) have been studied extensively and different assay methods have been used to study these isoforms (Auerbach *et al*., 1992; Breton *et al*., 1993; Waslidge and Hayes, 1995; Kratky *et al*., 1999; Halliwell and Whiteman, 2004; Oberthur *et al*., 2005; Rittmannsberger *et al*., 2005; Cho *et al*., 2006). 5-LOX has been found to play an important role in the pathophysiology of several inflammatory diseases such as asthma, rheumatoid arthritis, pain, atherosclerosis and cardiovascular disease (Drazen *et al*., 1999; Funk, 2005; Werz and Steinhilber, 2005, 2006). 12-LOX is implicated in inflammatory, cardiovascular, renal diseases and psoriasis and may also play a modulatory role in ADP-induced platelet aggregation (Funk *et al*., 1990; Izumi *et al*., 1990; Funk, 2005). The role of 15-LOX metabolites has been reported in carcinogenesis and atherogenesis (Izumi *et al*., 1990).

Within the past 25 years, several types of compounds that intervene with LT biosynthesis (redox, iron ligand and non-redox type 5-LOX inhibitors as well as FLAP inhibitors) have been synthesized (Werz and Steinhilber, 2006; Kratky *et al*., 1999; Halliwell and Whiteman, 2004) and several preclinical and clinical studies have been performed in order to evaluate their

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efficacy. However, with respect to 5-LOX inhibitors, there is a lack of selectivity over the related enzymes such as 12 and 15-LOX and/or COX enzymes. This is actually not surprising because all of these iron-containing redox-active enzymes share AA as common substrate that may bind to a common, more or less conserved, AA-binding pocket at the active site. Hence, during the process of identifying specific 5-LOX inhibitors, the selectivity for 12-LOX, 15-LOX and COX enzymes become important. In order to discard non-selective compounds at an early stage, the selectivity screen should be high throughput, robust and upstream in the screening path.

The common assay formats available for characterization of LOX inhibitors are: (1) determination of end product formation by high pressure liquid chromatography (HPLC), (2) spectrophotometeric measurement of end products, (3) colorimetric assays, (4) chemiluminescent detection and (5) determination of enzyme activity based on oxygen consumption (Auerbach *et al*., 1992; Breton *et al*., 1993; Waslidge and Hayes, 1995 ; Kratky *et al*., 1999 ; Halliwell and Whiteman, 2004; Oberthur *et al*., 2005; Rittmannsberger *et al*., 2005; Cho *et al*., 2006; Frohberg *et al*., 2006). However, each of these methods has their own limitations. None of these methods are easily adaptable for high throughput screening. Pufahl *et al*. (2007) have reported a novel fluorescence-based assay using 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA) for 5-LOX activity using several standard inhibitors *in vitro*. Recently, Kenyon *et al*. (2011) have reported a high throughput assay for screening of the inhibitors using platelet type human 12-LOX and Dahlstrom *et al*., (2010) have developed an assay for the detection of lipid hydroperoxides formed by human 15-LOX in enzyme or cellular assays based on the ability of lipid hydroperoxides to oxidize nonfluorescent diphenyl-1-pyrenylphosphine (DPPP) to a fluorescent phosphine oxide. However, in present study, we have extrapolated the fluorescence-based assay of 5-LOX isoforms using H2DCFDA for 12 and 15-LOX isoforms and characterized several known inhibitors e.g., zileuton, atreleuton and Nordihydroguaiaretic Acid (NDGA). Further, we have cloned and generated the recombinant human 5-LOX, 12-LOX and 15-LOX enzymes in-house and were successful in generating three-month stable batches for these enzymes.

MATERIALS AND METHODS

Materials: Atreleuton and NDGA were synthesized at the Medicinal Chemistry Department, Ranbaxy Research Laboratories and were dissolved in DMSO. Zileuton was obtained from Mediking Pharmaceuticals. The compounds were dissolved in DMSO and the final concentration of DMSO in all *in vitro* enzyme assays was 5%. AA was purchased from Sigma and stored at -80°C. H2DCFDA was obtained from Molecular Probes (Invitrogen, CA, USA). All other chemicals used were of AR grade and were purchased from Sigma. All the restriction enzymes and modifying enzymes were purchased from New England Biolabs, USA. Human RNA panel containing cervix RNA was procured from Ambion. The cell lines were purchased from Invitrogen, USA. The anti-12-LOX antibody was purchased from Santa Cruz, USA.

Pfu DNA polymerase was from Roche, *Germany*. Plasmid miniprep, PCR purification and DNA gel extraction kits were from Qiagen, Germany. Tris Cl, NaCl, $MgCl_2$, DTT and Protease Inhibitor Cocktail were from Sigma, USA. Cell culture plasticware were from Nunc, USA. Bac-to-Bac vector expression system, Tni cells, Express Five-SFM, Cellfectin, BluoGal, Fluorescein-isothiocyanate (FITC)-labeled goat anti-mouse IgG were from Invitrogen, USA. Anti-GST-HRP antibody was from Santa Cruz, USA. The anti-rabbit anti-GST antibody was from SantaCruz, USA.

Methods:

Cloning and expression of stable human recombinant LOX enzyme isoforms

Cloning and expression of 5-Lipoxygenase in baculovirus expression system: Human 5-LOX (GeneID: 240; protein ID: NP_000689.1, 2025bp ORF) was PCR amplified from human liver cDNA library (Clontech, USA) using 5-LOX forward 5'-GGAATTCCATATGCCCTCCTACACGGTCA CCGT -3' and 5-LOX reverse 5'-CCGGAATTCTCAGA TGGCCACACTGTTCGGA -3' primers following GC rich PCR amplification strategy (Sahdev *et al*., 2007). The resulting purified PCR product and in-house modified pFastBac-HTb-GST vector (Tiwari *et al*., 2010) were restriction digested with NdeI-EcoRI independently and ligated to obtain pFastBac-HTb-GST-5LOX plasmid clone (Fig. 1a). Sequence confirmed pFastBac-HTb-GST-5LOX plasmid was utilized to generate respective baculovirus as per manufacturer's (Invitrogen, USA) instructions. The resultant GST-5-LOX baculovirus was titered using BaculoELISA titer kit (Clontech, USA) and was utilized at increasing Multiplicity of Infection (MOI) for expression optimization. Un-infected and wild type *Autographa californica* multicapsid nuclear polyhedrosis virus (*Ac*MNPV) infected Tni cells served as negative controls. GST-5-LOX expression levels were analysed by SDS PAGE, Western blotting (Fig. 1b, c) and immunofluorescence microscopy (Fig. 1d, e). For catalytically active enzyme preparation, GST-5-LOX

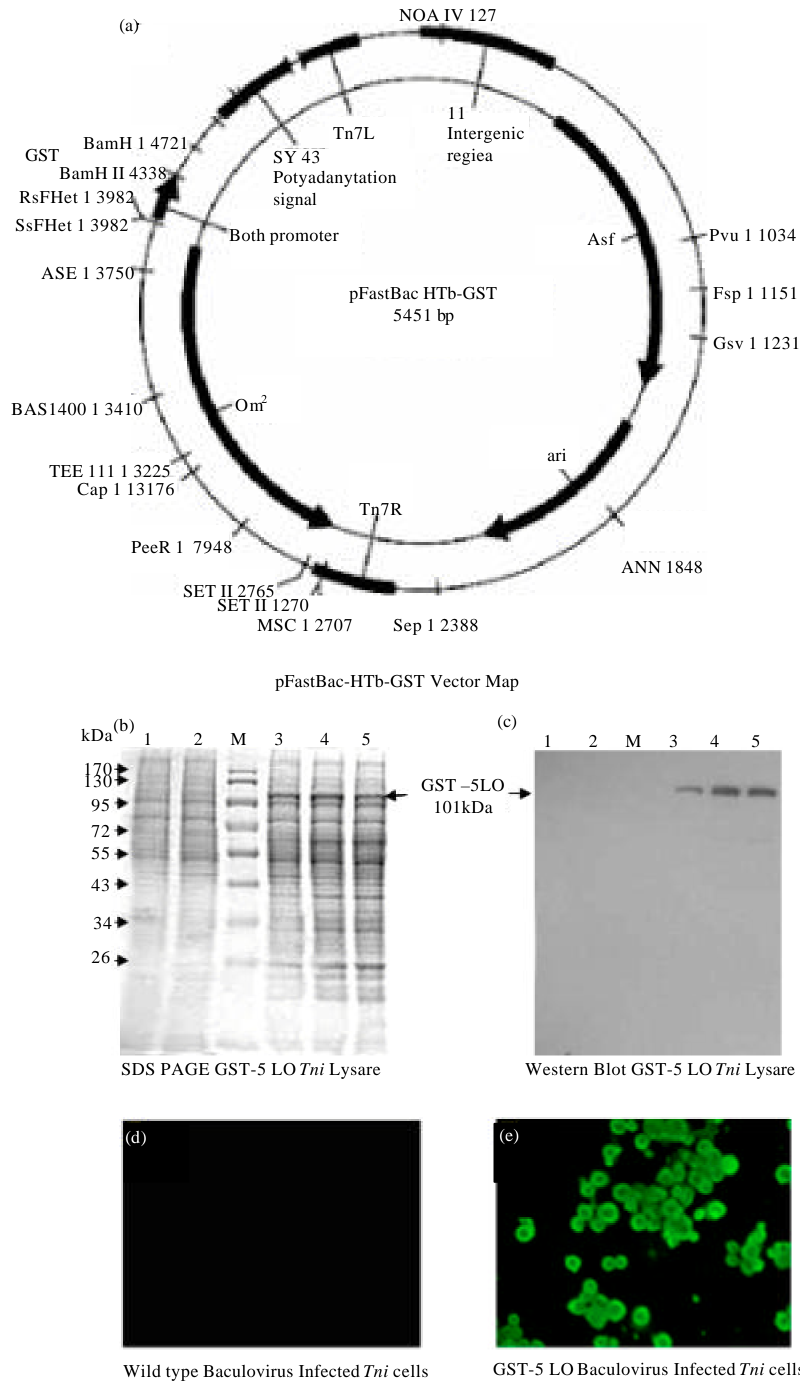

Fig. 1(a-e): (a) Vector map showing pFastBac-HTb-GST vector specifications. (b-c). SDS PAGE and Western Blot analysis of un-infected (lane-1), wild type (lane-2) and three different batches of GST-5LO baculovirus infected (lanes 3-5) Tni insect cell lysates. Pre-stained protein molecular weight marker is depicted as lane M, labelled with their respective band sizes. (d-e) Immunofluorescence images of wild type and GST-5LO baculovirus infected Tni cells, respectively

baculovirus infected Tni cell (at 1.0 M.O.I.) were lysed in buffer containing 10 mmol L^{-1} Tris Cl, 50 mmol L^{-1} NaCl, $MgCl_2$, 1 mmol L^{-1} DTT and 1X protease inhibitor cocktail by repeated freeze-thaw cycle (X3) and centrifuged at 13,000 rpm for 10 min at 4°C. Total protein concentration of the resultant supernatant estimation was done by Bradford assay. Finally, a fluorescence based GST-5-LOX *in vitro* enzyme activity assay to study Km, Vmax and IC_{50} of standard inhibitors were carried out.

Preparation of recombinant 12-LO bacmid DNA: Human 12-LOX cDNA was cloned into pFastBac HT B vector of baculovirus expression system (Invitrogen, USA) to generate recombinant baculovirus harboring h12LOX. Human cervix RNA was reverse transcribed using random hexamers, complete Open Reading Frame (ORF) of human 12-LOX gene was PCR amplified from the synthesized cDNA using a proof reading Phusion DNA polymerase (Finnzyme, USA). The primers used for the PCR amplification were:

- ALOX-Forward primer: 5' CTA AGC TGC TGG GGG GCG CCA TGG GCC 3'
- ALOX-Reverse primer: 5' GGG CTC AGA TGG TGA CAC TGT TCT CTA TGC AGC TGG GC 3'

The PCR amplification was performed for 30 cycles by following the PCR profile: denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec and polymerization at 72°C for 1 min with a 5 min initial denaturation at 95°C and a final polymerization at 72°C for 10 min. The resulting PCR product was gel purified and cloned in Zero blunt cloning vector (Invitrogen, USA). ALOX12 tentative positive clones were identified by antibiotic selection, restriction digestion analysis and selected clones were sequence confirmed.

Confirmed clone was digested with BamHI and XhoI and released insert was sub-cloned in pFastBac HTb vector. Selected positive clones were analyzed by restriction analysis and sequencing. DH10-Bac *E. coli* cells (Invitrogen, USA) were then transformed for pFastBac HT B-12-LOX plasmid for bacmid generation. The recombinant 12-LOX clones were checked for the presence of the gene specific insert by blue-white screening and the pure white clones were taken for colony PCR using M-13 forward and M-13 reverse primers. The PCR positive DH10-Bac-12-LOX colonies were cultured overnight to isolate the 12-LOX bacmid DNA (recombinant baculoviral DNA).

Spodoptera frugiperda and *Trichoplusia ni* insect cell strains viz. Sf21 and Tni (Invitrogen, USA) were respectively cultured in SF-900 serum free media (Invitrogen, USA) and iSEM, modified media developed in our laboratory (unpublished data) at 27°C in BOD incubator. Sf21 at a 2.5 X 10^6 density was transfected with 12-LOX DNA (7 μL) in a six well plate using Cellfectin poly cationic mix. Transfected cells were monitored for infection from 48 h.p.i and recombinant 12-LOX baculovirus were harvested 96 h.p.i. Subsequent virus amplification was carried out, as per supplier's instructions. Time course analysis of the expression was carried out at different time intervals in both Sf21 and Tni cells. To enhance the activity of 12-LOX protein expressed in Tni cells, hemin chloride was added to the cells at the time of infection, at a concentration of 1 μg mL^{-1}. The cell pellets were used for the analysis of protein expression by western blotting and the supernatant was collected and stored for further infections. The activity assay was done with the crude lysate of the cells with and without hemin treatment. The pellets of uninfected and infected Tni cells were lysed using cell lytic buffer (Sigma), as per supplier's instructions. The lysate was collected and stored in 20% glycerol. The protein estimation was done by Bicinchoninic Acid (BCA) method.

Cloning and expression of 15-lipoxygenase in baculovirus expression system: A 1500 bp fragment of 15-LOX PCR amplified from cDNA clone received from Origene was sub-cloned into pFast HT B vector (Invitrogen) at EcoRI and XhoI restriction sites. The recombinant construct was confirmed by restriction digestion and DNA sequencing. 15-LOX was expressed by infecting wild type Sf21 cells with the recombinant bacmid for 72 h. Infected cells were washed with Dulbecco's phosphate-buffered saline (2.7 mmol L^{-1} KCl, 1.5 mmol L^{-1} KH_2PO_4, 137 mmol L^{-1} NaCl, 8.0 mmol L^{-1} Na_2HPO_4). Washed cells were resuspended and lysed in lysis buffer (20 mmol L^{-1} Tris Cl, pH 8.0, 500 mmol L^{-1} NaCl, 10% Glycerol, 1% Triton X-100) by incubating on ice for 20 min. The homogenate was centrifuged at 13,000 rpm for 30 min. The resulting supernatant contained 15-LOX that was utilized for activity assays. Expression of the protein was confirmed with western blot utilizing polyclonal 15-LOX primary antibody and detected by secondary antibody conjugated with horseradish peroxidase using an Enhanced Chemiluminescence (ECL) method.

***In vitro* studies**

Kinetic studies of 5-LOX, 12-LOX and 15-LOX enzymes: The kinetic studies of 5, 12 and 15-LOX enzyme isoforms were done to determine Km of the substrate, AA and Vmax for the respective enzymes. This was done in

order to determine the concentration of the substrate, AA to be used in the study of the IC_{50} of the LOX inhibitors. Briefly, the enzymes were diluted properly in the assay buffer (Tris 50 mmol L^{-1}, EDTA 2 mmol L^{-1} and $CaCl_2$ 2 mmol L^{-1} and pH 7.5) and mixed with 10 μM of H_2DCFDA and the reaction mixture was incubated for 15 min. Subsequently, AA in the concentration range of 0.2-50 μM and ATP (5 μM $well^{-1}$) were added and fluorescence kinetics was studied in a multimode detector (Safire, Tecan) at 480 nm excitation/520 nm emission for 1 h at room temperature. The fluorescence units obtained were plotted against respective concentration of AA to get the Michaelis Menten plot and the values of Km and Vmax were determined from the plot.

***In vitro* enzyme inhibition assay for 5-LOX, 12-LOX and 15-LOX isoforms:** The enzyme assay is based on the oxidation of the substrate H_2DCFDA to the highly fluorescent 2', 7'-dichloro-fluorescein (DCF) product. Twenty microliter of assay buffer was added in the assay plate along with 5 μL of different concentrations of compound (in the range of 1 nmol L^{-1} to 10 μM) diluted in DMSO. The recombinant 5, 12 or 15-LOX enzyme isoforms (all were generated in-house) were diluted appropriately in assay buffer and added in the plate. The reaction mixture was incubated for 30 min. Subsequently, 10 μM of H_2DCFDA dye was added per well and incubated for 15 min. The reaction were started by addition of suitable concentration of arachidonic acid as determined from Km value and ATP and fluorescence was read in a multimode detector (Safire, Tecan) at 480 nm excitation/520 nm emission after an incubation of 1 h at room temperature.

Analysis of results: The Km and Vmax values were determined from the Michaelis Menten plot drawn by using Graph Pad Prism 4.2 software. The IC_{50} values were determined by using the non-linear curve-fitting program using Graph Pad Prism 4.2 software.

RESULTS

Stable expression of human recombinant 5, 12 and 15-LOX enzyme isoforms: Recombinant human 5, 12 15-LOX were expressed stably in the respective cells as described previously and characterized by Western blot, real-time PCR and HPLC analysis. The control cells failed to show any LOX activity or immunoreactivity. Quantitative RT-PCR confirmed that expression of one LOX isoform in respective cells did not induce spurious expressions of other LOX isoforms. These results suggest the specificity of the cells for screening inhibitors for a particular over-expressed LOX isoform.

***In vitro* Kinetic studies of 5-LOX, 12-LOX and 15-LOX enzymes:** The Km and Vmax of the human recombinant 5, 12 and 15-LOX isoforms were studied using different concentration of AA as shown in the Fig. 2a-c. The Km of substrate (AA) for human recombinant 5, 12 and 15-LOX isoforms were found to be 0.43, 0.57 and 1.2 μmol L^{-1}, respectively. Accordingly, these concentrations of the substrate were used to study the IC_{50} of the inhibitors (zileuton, atreleuton and NDGA) for the respective enzymes.

***In vitro* enzyme inhibition assay for human recombinant LOX isoforms**

Recombinant human 5-LOX enzyme inhibition assay using H2DCFDA: Assays were performed to check the effects of several LOX inhibitors on 5-LOX activity. Pufahl *et al*. (2007) have reported a fluorescent assay for purified 5-LOX using H2DCFDA based on the principle of oxidation of H2DCFDA to form a fluorescent product by the reactive oxygen species formed during the reaction. The fluorescent product formed was monitored at excitation wavelength of 480 nm and emission wavelength of 520 nm.

In present study, the inhibition profile of some common reference inhibitors such as zileuton, atreleuton and NDGA was studied by using recombinant human 5-LOX enzyme and has been shown in Fig. 3a. Out of these inhibitors, zileuton and atreleuton were specific 5-LOX inhibitors whereas NDGA was a non selective LOX inhibitor for 5, 12 and 15-LOX enzyme isoforms. All these compounds showed inhibition in a concentration dependent manner ranging from 1 to 10 μmol L^{-1}. The IC_{50} values of zileuton, atreleuton and NDGA for 5-LOX enzyme according to this assay has been specified in Table 1. In this assay, atreleuton and NDGA were found to be 4 and 15 fold more potent than zileuton, respectively and these values were in good agreement with the published data (Bell *et al*., 1992, 1997; Carter *et al*., 1999; Li *et al*., 2009; Malo *et al*., 1994; Nair and Funk, 2009; Zweifel *et al*., 2008). These IC_{50} values of zileuton was found to be consistent with recently published results using two additional 5-LOX enzyme assays, a spectrophotometric assay that measures 5-HPETE/5-HETE formation in a cuvette and a fluorescence-based assay using a fluorescein derivative. In these assays, zileuton inhibited 5-LOX with IC_{50} values of 0.92 and 0.12 μmol L^{-1}, respectively (Pufahl *et al*., 2007).

Development of an end point fluorescent assay to study the inhibition of recombinant human 12 and 15-LOX enzyme isoforms using H2DCFDA: This assay is based on the principle of oxidation of H2DCFDA to form a

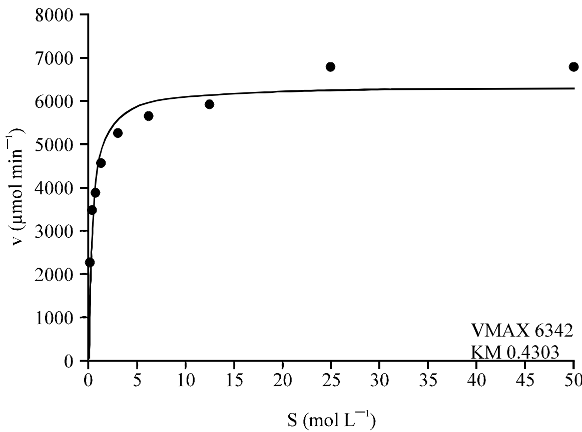

Fig. 2a: Michaelis Menten plot for recombinant human 5-LOX enzyme using varying concentration of arachidonic acid as substrate

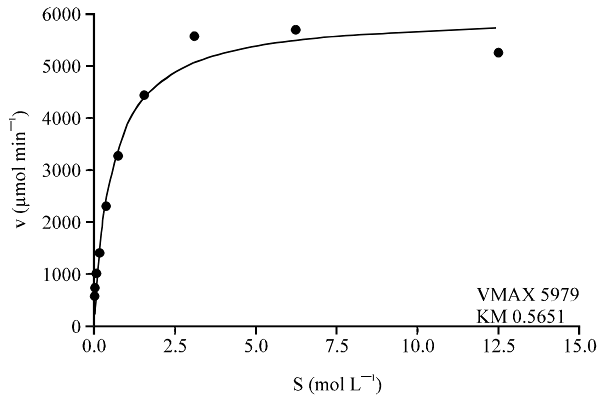

Fig. 2b: Michaelis Menten plot for recombinant human 12-LOX enzyme using varying concentration of arachidonic acid as substrate

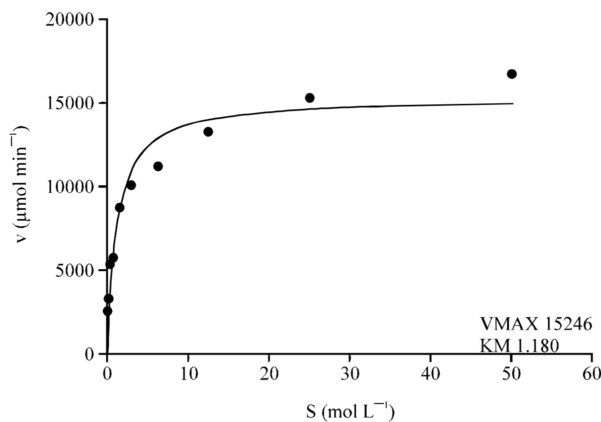

Fig. 2c: Michaelis Menten plot for recombinant human 15-LOX enzyme using varying concentration of arachidonic acid as substrate

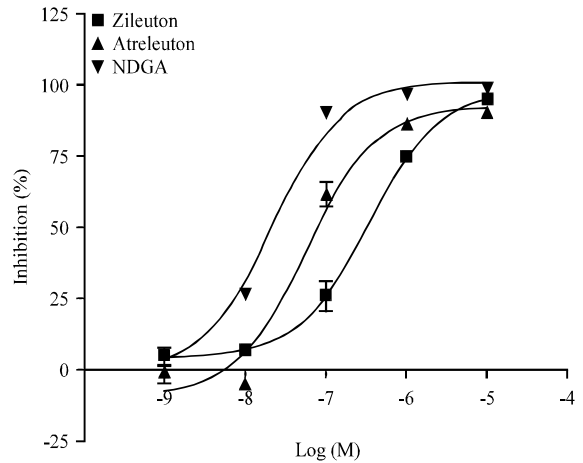

Fig. 3a: Inhibition of recombinant human 5-LOX enzyme by zileuton, atreleuton and NDGA. Each point represents means±SEM of three different experiments performed in duplicate

fluorescent product by the reactive oxygen species generated during a lipoxygenase reaction. The fluorescent product formed was monitored at excitation wavelength of 480 nm and emission wavelength of 520 nm. Pufahl *et al*. (2007) have reported a fluorescent assay for purified 5-LOX using H2DCFDA. Here, in this study, we have developed a fluorescent assay using H2DCFDA for 12 and 15-LOX enzyme isoforms based on the similar principle.

In this assay, NDGA, a general LOX isoforms inhibitor, was found to inhibit both 12 and 15-LOX enzymes in a concentration dependent manner ranging from 1 to 10 μmol L^{-1} as shown in Fig. 3b, c, respectively. The IC_{50} values of zileuton, atreleuton and NDGA for 12 and 15-LOX enzyme isoforms have been given in Table 1 and confirms the earlier published reports (Pufahl *et al*., 2007; Nair and Funk, 2009; Aleem *et al*., 2007). Recently, Nair and Funk (2009) have reported the IC_{50} values of NDGA for 12 and 15-LOX enzyme isoforms and these values were in between 0.3 to 1.0 μmol L^{-1} for 12-LOX and 0.1 to 0.3 μmol L^{-1} for 15-LOX enzyme. In our assay, zileuton and atreleuton have shown the IC_{50} values as shown in Table 1 for recombinant human 12 and 15-LOX enzymes, respectively. Thus, zileuton and atreleuton have shown significant selectivity towards 5-LOX enzyme isoforms, whereas NDGA was found to potently inhibit all the three isoforms. These data were found to be in line with the earlier published studies confirming the nature of these compounds.

Stability studies of recombinant human 5, 12 and 15-LOX enzyme isoforms: The stability studies of the 5, 12 and 15-LOX isoforms were done for different batches of

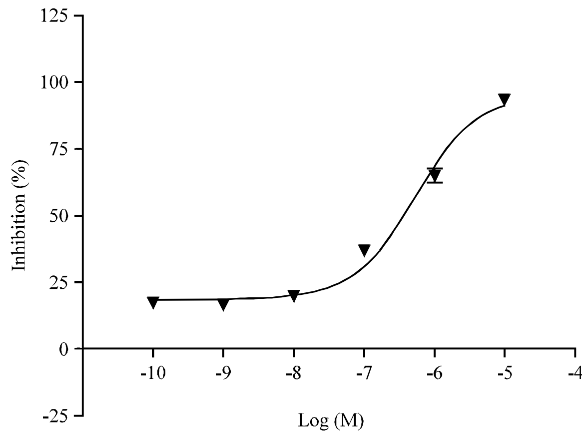

Fig. 3b: Inhibition of recombinant human 12-LOX enzyme by NDGA. Each point represents means±SEM of three different experiments performed in duplicate

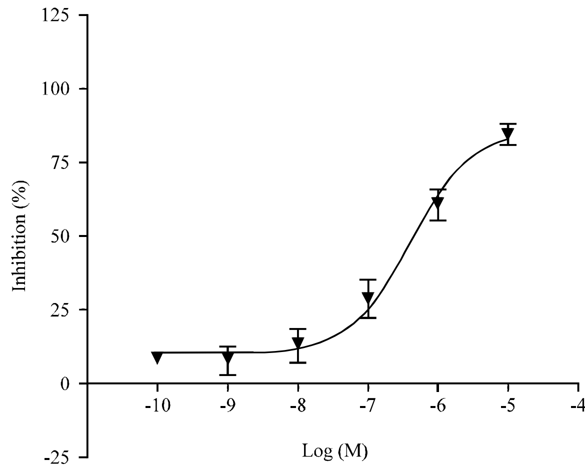

Fig. 3c: Inhibition of recombinant human 15-LOX enzyme by NDGA. Each point represents means±SEM of three different experiments performed in duplicate

Table 1: IC_{50} values of standard inhibitors for different isoforms of lipoxygenase enzymes:

Enzyme isoforms	IC_{50} values (µmol L^{-1}) Zileuton	Atreleuton	NDGA
5-LOX	0.32±0.06	0.08±0.01	0.02±0.0003
12-LOX	9.33±0.38	>10	0.38±0.06
15-LOX	17.80±0.49	1.72±0.20	0.30±0.07

enzyme to confirm the successful cloning of stable enzyme isoform. The intra-batch stability of these isoforms was also checked up to 90 days. The stability of recombinant human 5-LOX enzyme was checked by studying the IC_{50} values of zileuton whereas NDGA was used as a reference inhibitor to study the stability of 12

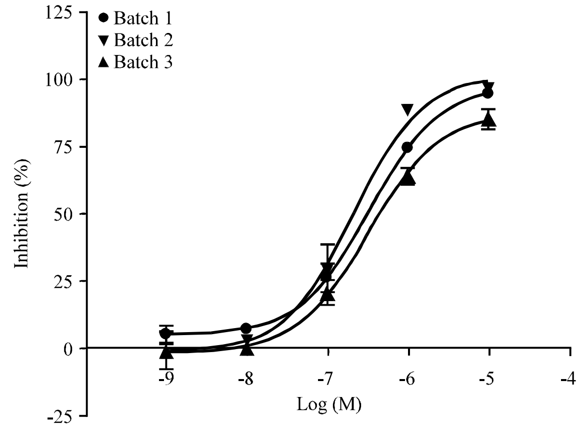

Fig. 4a: Inhibition of different batches of recombinant human 5-LOX enzyme by zileuton. Each point represents means±SEM of three different experiments performed in duplicate

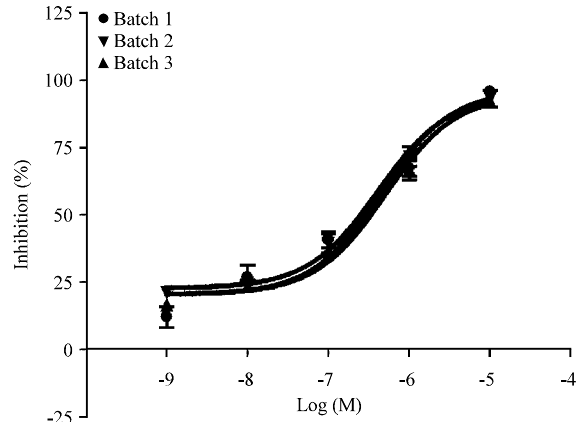

Fig. 4b: Inhibition of different batches of recombinant human 12-LOX enzyme by NDGA. Each point represents means±SEM of three different experiments performed in duplicate

and 15-LOX enzyme isoforms. The IC_{50} values of the respective inhibitors were also studied to confirm the intra-batch stability of 5, 12 and 15-LOX enzyme isoforms for up to 90 days. The enzymes were stored at -80°C during this period.

The stability of different batches of 5, 12 and 15-LOX enzyme isoform has also been checked using these standard inhibitors. Zileuton has shown IC_{50} values of 0.32±0.06, 0.22±0.06 and 0.44±0.05 µmol L^{-1} for three different batches of 5-LOX enzyme respectively (Fig. 4a). NDGA has shown IC_{50} values of 0.22±0.03, 0.36±0.06 and 0.35±0.06 µmol L^{-1}

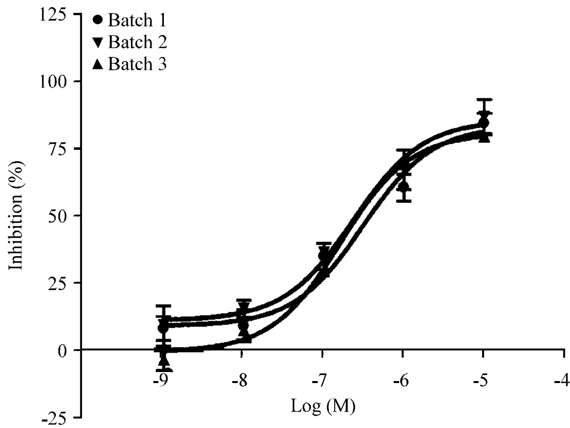

Fig. 4c: Inhibition of different batches of recombinant human 15-LOX enzyme by NDGA. Each point represents Mean±SEM of three different experiments performed in duplicate

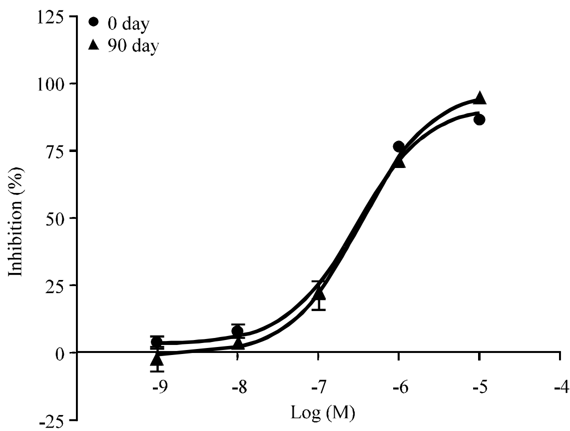

Fig. 5a: Inhibition of a single batch of recombinant human 5-LOX enzyme by zileuton stored at -80°C. Each point represents Mean±SEM of three different experiments performed in duplicate

for different batches of 12-LOX enzyme (Fig. 4b) and 0.39±0.10, 0.30±0.08 and 0.26±0.02 μmol L^{-1} for different batches 15-LOX enzyme (Fig. 4c), respectively. The IC_{50} values obtained for the reference inhibitor for the respective LOX enzyme isoforms were similar for different batches and hence confirms their stability.

Moreover, the intra-batch stability studies of 5, 12 and 15-LOX isoforms has also been done up to 90 days and the results have been shown (Fig. 5a-c). The IC_{50} values of zileuton for 5-LOX enzymes at 0 and 90 days were found to be 0.35±0.03 and 0.33±0.06 μmol L^{-1},

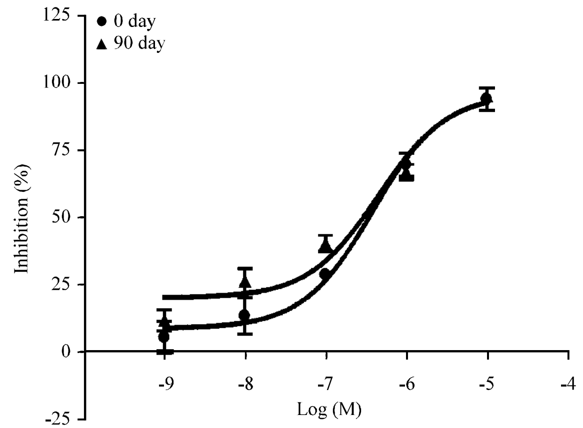

Fig. 5b: Inhibition of a single batch of recombinant human 12-LOX enzyme by NDGA stored at -80°C. Each point represents Mean±SEM of three different experiments performed in duplicate

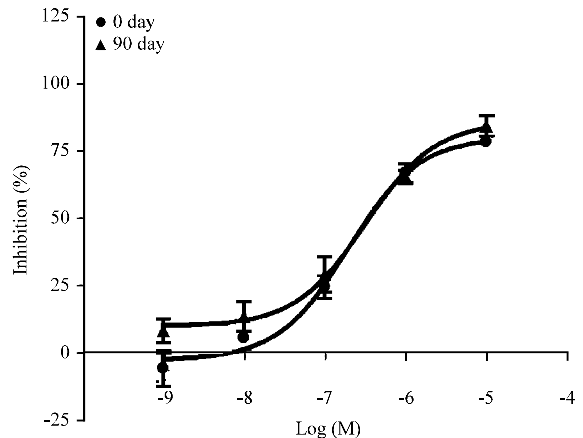

Fig. 5c: Inhibition of a single batch of recombinant human 15-LOX enzyme by NDGA stored at -80°C. Each point represents Mean±SEM of three different experiments performed in duplicate

respectively. NDGA has shown IC_{50} values of 0.30±0.11 and 0.36±0.04 μmol L^{-1} for 12-LOX enzyme and 0.43±0.14 and 0.49±0.04 μmol L^{-1} for 15-LOX enzyme at 0 and 90 days, respectively.

DISCUSSION

An *in vitro* end point fluorescent assay for 12 and 15-LOX enzymes was developed based on modification of previously published reports. The assay detects reactive oxygen species formed during lipoxygenase activity using H2DCFDA. Upon hydrolysis and subsequent oxidation, this non-fluorescent compound H2DCFDA is converted

into fluorescent Dichlorofluorescein (DCF) which can be measured. The end point fluorescence assay that we developed to study the inhibition of 12 and 15-LOX isoforms can either be used as a preliminary screening method for compounds or can be used to evaluate the selectivity of compounds towards 5-LOX enzyme isoform.

In present study, zileuton, atreleuton and NDGA inhibited the recombinant human 5-LOX in a concentration-dependent manner ranging from 1 nmol L^{-1} to 10 μmol L^{-1}. NDGA, being a non selective LOX inhibitor, inhibited all the three enzymes, 5, 12 and 15-LOX in a concentration-dependent manner ranging from 1 nmol L^{-1} to 10 μmol L^{-1}. The potency of these inhibitors used in our study was comparable to earlier published results for all the enzymes (Bell *et al*., 1992; Carter *et al*., 1999; Li *et al*., 2009; Malo *et al*., 1994; Nair and Funk, 2009; Zweifel *et al*., 2008; Aleem *et al*., 2007; Batt, 1992). As reported earlier by using different assay methods, the specific 5-LOX inhibitors e.g., zileuton and atreleuton, did not potently inhibit 12-LOX or 15-LOX in this fluorescence assays also. Zileuton, atreleuton and NDGA appeared to be potent inhibitors of 5-LOX isoform, whereas with respect to 12 and 15-LOX isoforms, only NDGA has shown significant inhibitory potency as reported earlier (Nair and Funk, 2009; Zweifel *et al*., 2008; Aleem *et al*., 2007).

There are other methods available for screening of compounds for inhibition of LOX isoforms. However, there are certain limitations associated with each of these methods. Several LOX inhibitors absorb light at 235 nm which is a limitation for spectrophotometric assays (Pufahl *et al*., 2007). Further, colorimetric detection techniques have low signal-to background ratio (Kratky *et al*., 1999) and chemiluminescent detection assays have short life time of measuring chemiluminescence (Waslidge and Hayes, 1995).

Moreover we have successfully generated in-house clones of recombinant human 5, 12 and 15-LOX isoforms using the baculovirus expression system and the stability of different batches of clones were confirmed by evaluating the IC_{50} values of standard compounds for the respective enzymes and the stability data of a single batch of enzymes for up to 90 days (stored at -80°C) has also been shown using the standard compounds for the respective LOX isoforms. The IC_{50} values obtained in these studies confirmed the inter batch and intra batch stability of the LOX enzyme isoforms cloned in-house.

Taken together, we have been successful in generating stable human recombinant enzymes namely 5, 12 and 15-LOX enzyme isoforms along with their end point fluorescence high throughput assay.

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