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High-throughput screening assays for cyclooxygenase-2 and 5-lipoxygenase, the targets for inflammatory disorders

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High-throughput screening (HTS) involves testing of compound libraries against validated drug targets using quantitative bioassays to identify 'hit' molecules that modulate the activity of target, which forms the starting point of a drug discovery effort. Eicosanoids formed via cyclooxygenase (COX) and lipoxygenase (LOX) pathways are major players in various inflammatory disorders. As the conventional non-steroidal anti-inflammatory drugs (NSAIDs) that inhibit both the constitutive (COX-1) and the inducible (COX-2) isoforms have gastric and renal side effects and the recently developed COX-2 selective anti-inflammatory drugs (COXIBs) have cardiac side effects, efforts are being made to develop more potent and safer anti-inflammatory drugs. Current assay methods for these enzymes, such as oxygraphic, radioisotopic, spectrophotometric etc. are not compatible for screening of large number of compounds as in drug discovery programs. In the present study, HTS-compatible assays for COX-1, COX-2 and 5-LOX were developed for screening of compound libraries with the view to identify potential anti-inflammatory drug candidates. A spectrophotometric assay involving co-oxidation of tetramethyl-p-phenylene diamine (TMPD) during the reduction of prostaglandin G_2 (PGG_2) to PGH_2 was adopted and standardized for screening of compounds against COX-1 and COX-2. Similarly, the HTS-compatible FOX (ferrous oxidation-xylenol orange) based spectrophotometric assay involving the formation of Fe^{3+}/xylenol orange complex showing absorption in the visible range was developed for screening of compounds against 5-LOX.

Keywords*:* High-throughput screening, Cyclooxygenase, Lipoxygenase, NSAIDs, COXIBs, CLOXIBs

The inflammatory response helps localize and eliminate the injurious agents and remove damaged tissue components, so that the body can begin to heal. Chronic inflammation has, however, certain pathological consequences. It has a role in psoriasis, dermatitis, arthritis (rheumatoid arthritis), inflammatory CNS diseases, multiple sclerosis, chronic obstructive pulmonary disease, chronic lung inflammatory conditions, inflammatory bowel disease, ulcerative colitis, Crohn's disease, cardiovascular disease and cancer. Inflammatory diseases increase the risk for development of many cancer types including bladder, cervical, gastric, intestinal, oesophageal, ovarian, prostate and thyroid[1]. Elevated levels of arachidonic acid (AA)-derived eicosanoids have been observed in blood and tissues of patients with acute or chronic inflammatory conditions. Eicosanoids formed via cyclooxygenase (COX) and lipoxygenase (LOX) pathways are the major players in the inflammation[2].

COX-1 and COX-2 are prostaglandin synthases which catalyze sequential synthesis of prostaglandin G_2 (PGG_2) and PGH_2 from AA by virtue of intrinsic COX and peroxidase activities[3]. Despite the similar enzymatic activities, COX-1 and COX-2 genes have distinct properties and differing expression patterns. While COX-1 is constitutively expressed, COX-2 is upregulated in response to growth factors, tumor promoters and cytokines[3]. Thus, COX-2, in addition to being the target for anti-inflammatory drugs is emerging as an increasingly promising pharmacological target for the prevention and treatment of many human cancers[4].

LOXs are a group of dioxygenases involved in the insertion of one molecule of O_2 at different sites in

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Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; COXIBs, COX-2 selective inhibitors; CLOXIBs, COX-2/5-LOX dual inhibitors; FOX, ferrous oxidation-xylenol orange; 15-(S)-HETE, 15-(S)-hydroxyeicosatet-raenoic acid; 15-(S)-HPETE, 15-(S)-hydroperoxyeicosatetraenoic acid; HTS, high-throughput screening; LOX, lipoxygenase; NSAIDs, non-steroidal anti-inflammatory drugs; PGG_2, prostaglandin G_2; TMPD, tetramethyl-p-phenylene diamine.

AA. The particular site of incorporation is tissue and enzyme-specific. LOXs catalyze the oxygenation of poly-unsaturated fatty acids (PUFA) containing a 1, 4 *cis*, *cis*-pentadiene system producing a 1-hydroperoxy-2, 4-trans, *cis*-pentadiene product. Four main types of LOXs with positional specificities occur in animal tissues, i.e. 5-LOX, 8-LOX, 12-LOX and 15-LOX. 5-LOX found in mast cells, macrophages and neutrophils, is involved in the synthesis of 5-hydroperoxy eicosatetraenoic acid (5-HPETE) and leukotriene B_4 (LTB_4), C_4 (LTC_4) and D_4 (LTD_4)[5]. 5-Hydroxy eicosatetraenoic acid (5-HETE), a reduced product of 5-HPETE has strong chemotactic activity for neutrophils, eosinophils, monocytes and macrophages. LTC_4 and LTD_4, which are enzymatically-derived from LTA_4 have been shown to be the active bronchoconstrictor components of slow-reacting substance of anaphylaxis (SRS-A)[6]. In comparison to histamine, LTC_4 and LTD_4 are 1000-times more potent bronchoconstrictors and probably extremely important mediators of asthma and allergic reactions[7].

5-LOX, 8-LOX and 12-LOX have shown a pro-carcinogenic role, whereas 15-LOX is found to be anti-carcinogenic. Products of 12R-LOX are associated with various skin diseases[3,8] and 15-LOX products are associated with atherosclerosis[9]. The role of various LOX metabolites in regulating carcinogenesis has been studied[8-11]. 5-LOX has been found to be a critical regulator for leukemia cancer stem cells in chronic myeloid leukemia (CML). Treatment of CML mice with a 5-LOX inhibitor also impairs the function of leukemia cancer stem cells[12]. Inhibition of 5-LOX by vitamin E[13] and benzyl propargyl ethers[14] has been reported.

COX-1, COX-2 and 5-LOX have been employed as the targets in developing anti-inflammatory drugs like aspirin, indomethacin, ibuprofen, celecoxib, zileuton etc[15]. As most of these drugs are associated with side effects such as gastric ulceration, disturbance in kidney ionic balance, cardiac side effects, etc., efforts are being made to develop safer anti-inflammatory drugs[15].

High-throughput screening (HTS) is the process for screening large number of molecules against selected biological targets, so as to identify potential modulators of their activity[16]. This study has been aimed at developing HTS compatible assays for COX-1, COX-2 and 5-LOX in order to enable screening of large compound libraries to identify potential anti-inflammatory drug candidates.

Materials and Methods

Chemicals

Arachidonic acid (AA), N,N,N',N''-tetraramethyl-phenylenediamine (TMPD), Tris, EDTA, diethyldithio carbamate (DDC), Tween-20, hematin, glycerol, phenol, ammonium sulphate, butylated hydroxytoluene (BHT), xylenol orange and nordihydroguaretic acid (NDGA) were purchased from Sigma Chemical Co. (St. Louis, MO). Indomethacin was supplied by Cayman Chemical Co. (Ann Arbor, MI). Celecoxib and rofecoxib were a generous gift from Unichem Laboratories (Mumbai, India). COX-1 was extracted and purified from Ram seminal vesicles. Recombinant human COX-2 was expressed in insect cells as per the methods described earlier[17].

Purification of COX-1

Ram seminal vesicles were collected from local slaughterhouse and stored at –80°C until used. Few hours before homogenization, the Ram seminal vesicles were removed from the freezer and stored at 4°C for thawing. The tissue was weighed, minced into small pieces and homogenized in the buffer containing 100 mM Tris-HCl (pH 8.0), 5 mM EDTA and 5 mM DDC. The homogenization was done in a blender initially and later in a Potter-Elvehjem homogenizer. The homogenate was filtered through two layers of cheese cloth to remove fat and waste material. The filtrate was centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant obtained above was again centrifuged at 1,00,000 *g* for 1 h 10 min at 4°C to obtain microsomal pellet. The microsomal pellet obtained above was suspended in minimum volume of solubilization buffer containing 100 mM Tris-HCl (pH 8.0), 5 mM EDTA, 5 mM DDC and 1% Triton X-100 with slow stirring at 4°C for 30 min and the resulting supernatant was used as the enzyme source for assays.

Expression and extraction of recombinant human COX-2

Spodoptera frugiperda (Sf9) cells were maintained at 28°C in Grace's insect culture medium supplemented with 10% fetal bovine serum, 3.3 g/l yeastolate, 3.3 g/l lactalbumin hydrolysate and 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells at 60% confluence were infected with baculovirus containing human COX-2 (hCOX-2 cDNA sequence cloned into BamH1 site of the baculovirus expression vector pVL941). After 72 h of infection, the cells were collected by centrifugation at

2,000 rpm for 5 min at 4°C. The pellet was suspended in minimum volume of Tris-HCl buffer (50 mM, pH 7.2) containing 5 mM EDTA, 300 mM sucrose, 5 mM diethyldithiocarbamate, 1 µg/ml pepstatin and 1 mM phenol and sonicated for 3 min. The cell lysate was subjected to centrifugation (1,00,000 *g* for 1 h) at 4°C and the microsomal pellet obtained was suspended in Tris-HCl buffer (25 mM, pH 7.2) containing 0.5% glycerol, 0.1% Tween 20 and 1 mM phenol. This microsomal fraction was used for further studies.

Extraction and partial purification of 5-LOX

5-LOX from potato tubers was purified by the method described by Reddanna *et al*[18]. 250 g of peeled potatoes cut into small pieces were taken and 500 ml of 100 mM potassium phosphate buffer (pH 6.3) containing 2 mM sodium metabisulphite, 1 mM EDTA and 2 mM ascorbic acid was added to it and homogenized at 4°C. The homogenate was passed through several layers of cheese cloth and the filtrate collected was centrifuged at 10,000 rpm for 20 min at 4°C. Supernatant collected was measured and proceeded with 15% ammonium sulphate precipitation (with continuous stirring for 1 h) at 4°C. This was again centrifuged at 15,000 rpm for 30 min at 4°C to remove the precipitate. The supernatant obtained was subjected to 15-45% ammonium sulfate fractionation with continuous stirring for 1 h at 4°C and then subjected to centrifugation at 15,000 rpm for 30 min at 4°C. The pellet was collected and suspended in 40 mM phosphate buffer (pH 6.3). The clear supernatant obtained after centrifugation was taken for further purification on DE-52 anion-exchange column chromatography.

5-LOX was further purified by dialysis for 24 h against 4 l phosphate buffer (pH 6.3) with continuous stirring (buffer was changed 4-times). This dialyzed sample was passed through DE-52 column (anion-exchange) and elution was done with 50 mM potassium phosphate buffer containing 0-0.4 M KCl. The 1 ml fractions with good activity were pooled and employed as the source of 5-LOX.

Spectrophotometric assay for COX

Enzymatic activities of COX-1 and COX-2 were measured according to the method described elsewhere[19] with slight modifications using a chromogenic assay based on the oxidation of TMPD during the reduction of PGG_2 to PGH_2. The assay mixture contained Tris-HCl buffer (100 mM, pH 8.0), hematin (15 µM), EDTA (3 µM), enzyme (100 µg, COX-1 or COX-2) and test compound. The mixture was preincubated at 25°C for 15 min and then the reaction was initiated by the addition of AA and TMPD in total volume of 1 ml. The enzyme activity was measured by estimating the initial velocity of TMPD oxidation for the first 25 s of the reaction, following the increase in absorbance at 603 nm. A low rate of non-enzymatic oxidation observed in the absence of COX-1 and COX-2 was subtracted from the experimental value while calculating the percent inhibition. The effect of different concentrations of indomethacin and celecoxib on COX-1 and COX-2 was examined under the same experimental conditions.

Standardization of assays compatible to HTS for COX

Initially COX assays were standardized on spectrophotometer in 1 ml cuvette. The enzyme activity was checked and dose-dependent inhibition was observed with the known standard inhibitors i.e., indomethacin for COX-1 and celecoxib for COX-2. Later, the assay protocols were standardized on ELISA and later on HTS, which was compatible to 96-well plates. To each well, 160 µl of buffer, 10 µl of test drug and 10 µl of enzyme was added and kept for incubation for 5 min. To this mixture, 10 µl of TMPD and 10 µl of AA (final conc. 133 µM) were added and readings were taken at 620 nm in BMG Polarstar Galaxy plate reader.

Spectrophotometric assay for 5-LOX

This is colorimetric assay, where the hydroperoxides formed by enzymatic reaction oxidize ferrous sulfate to ferric sulfate. The formed ferric sulfate can form complex with xylenol orange to form ferric-xylenol orange complex which is blue in color. The color developed can be measured at 590 nm. Typical reaction mixture contains 490 µl of PBS, 10 µl of enzyme and 10 µl of AA (final conc. 133 µM). The reaction mixture was incubated for 2 min. Then 490 µl of FOX reagent was added and this mixture was incubated for 1 min and the color developed was measured at 590 nm.

Standardization of assay compatible to HTS for 5-LOX

HTS compatible assay for LOXs was done based on the FOX assay on 96-well plates. To each well, 85 µl of PBS, 10 µl of test drug, and 10 µl of enzyme was added and incubated for 5 min and then 10 µl of AA (final conc. 133 µM) was added. The reaction mixture was incubated for 2 min. Then to each well,

85 µl of FOX reagent was added and kept for 1 min incubation and readings were taken at 590 nm in BMG Polarstar Galaxy plate reader.

Results and Discussion

Inhibitory studies of COX-1 and COX-2

The enzymatic oxygenation of AA via the COX and LOX pathways play a key role in the mediation of inflammation. As a result, the key enzymes of these pathways COX-1, COX-2 and 5-LOX have become the targets for the development of anti-inflammatory drugs. Current methods of assays of these enzymes, such as oxygraphic, radioisotopic, spectrophotometric etc. are not compatible for screening of large number of compounds as in drug discovery program[20]. This study, therefore, was aimed at developing assay methods compatible for HTS.

Initially COX assays were standardized on spectrophotometer in 1 ml cuvette. The enzyme activity was checked and dose-dependent inhibition was observed with the known standard inhibitors i.e., indomethacin for COX-1 (Fig. 1) and celecoxib for COX-2 (Table 1). The inhibition of COX-2 is also shown on wave length and time scans (Figs 2 & 3). COX-1 showed an IC_{50} value of 28 nM and COX-2 of 50 nM with the standard inhibitors indomethacin and celecoxib, respectively. These studies validated the assay methods employed in the present study.

Table 1—Spectrophotometric assay of COX-2 activity in the presence of increasing concentrations of celecoxib (a selective COX-2 inhibitor)

Celecoxib (nM)	Absorbance difference	Activity (Units/mg)	Inhibition (%)
0	0.547	1655.56	0
10	0.466	1410.41	14.80
50	0.251	759.68	54.11
100	0.145	438.86	73.49
200	0.079	239.10	85.55

One unit was defined as the amount of enzyme that caused the oxidation of 1.0 nmole of TMPD/min at 25 °C

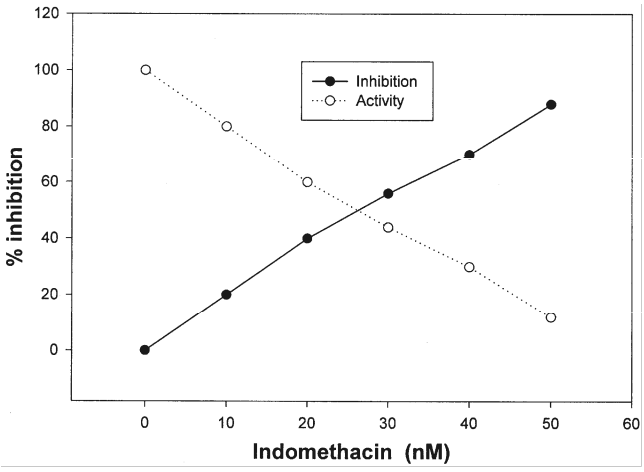

Fig. 1—Inhibition of COX-1 activity by its specific inhibitor indomethacin [The IC_{50} value obtained from the graph was 28 nM]

High-throughput screening of compounds

After validation of the assays on spectrophotometer, COX assays were standardized on 96-well plates. The enzyme activity was checked and dose-dependent inhibition was observed with the

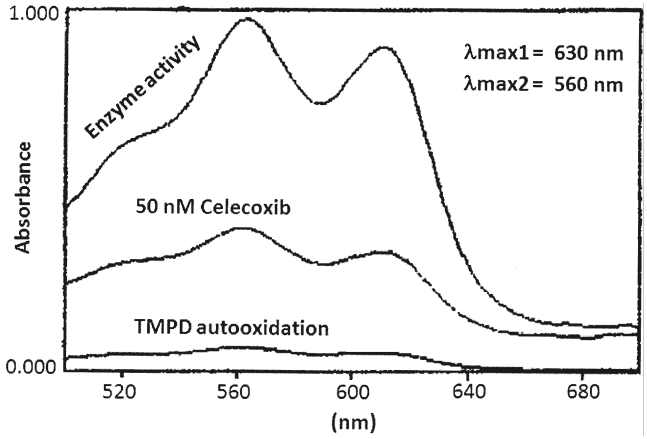

Fig. 2—Wave length scan showing the TMPD oxidation products, as a measure of COX-2 activity in the presence and absence of celecoxib [Non-enzymatic autooxidation of TMPD is also shown. Wave length scan of the TMPD oxidation product was done with active COX-2 enzyme and in the presence or absence of 50 nM celecoxib, a standard inhibitor. The graph shows two peaks at 630 nm and 560 nm respectively]

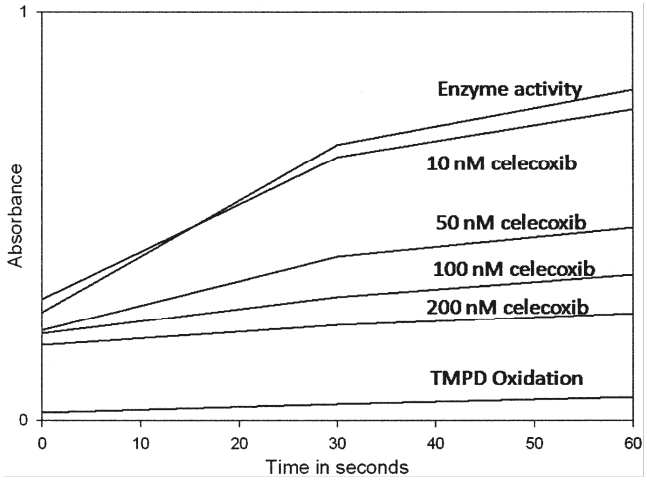

Fig. 3—Time scan showing the increase in the absorbance at 630 nm with reference to time in the presence of increasing concentrations of celecoxib [Celecoxib showed dose-dependent inhibition in COX-2 activity with an IC_{50} value of 50 nM]

Table 2—COX-2 inhibition data obtained from HTS assays

Celecoxib (nM)	Absorbance difference	Activity (Units/mg)	Inhibition (%)
0	0.081	392.24	0
100 nM	0.040	193.70	50.61
250 nM	0.026	125.88	67.90

One unit was defined as the amount of enzyme that caused the oxidation of 1.0 nmole of TMPD/min at 25 °C

Table 3—5-LOX inhibition data obtained from HTS assays

NDGA (μM)	Absorbance difference	Activity (Units/mg)	Inhibition (%)
0	1.533	100	0
0.25	1.253	81.73	18.26
2.5	1.136	74.10	23.90
5	0.763	49.77	50.22

One unit of LOX activity was defined as 1.0 μmole O_2 consumed/min at 25 °C

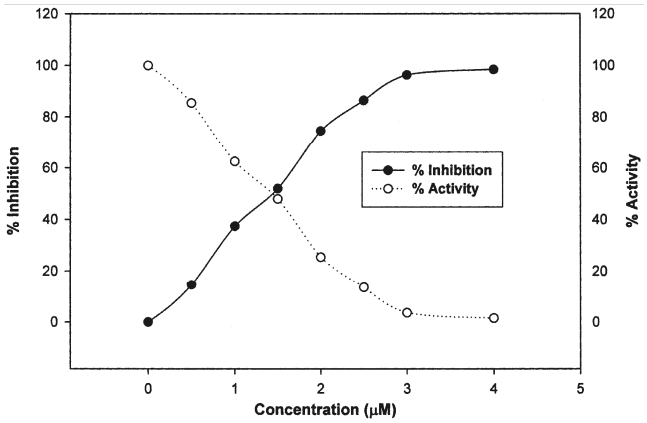

Fig. 4—Inhibition of 5-LOX activity by its specific inhibitor NDGA [The IC_{50} value obtained from the graph was 1.5 μM]

known standard inhibitor i.e., celecoxib for COX-2 (Table 2) showing an IC50 value of 100 nM on 96-well plates.

Inhibitory studies of 5-LOX employing spectrophotometric and HTS assays

Initial standardization and validation of 5-LOX assay was carried on spectrophotometer in 1 ml cuvette by employing NDGA as the standard inhibitor (Fig. 4). From these studies NDGA showed an IC_{50} of 1.5 μM. After this initial validation on spectrophotometer, LOX assay was further standardized on 96-well plates. Dose-dependent inhibition of 5-LOX was observed with NDGA and IC_{50} value was found to be 5 μM (Table 3).

Overexpression of COX-2 has been detected in a number of tumors including colorectal, breast as well as pancreatic and lung cancers[21-24], where it correlates with a poor prognosis. Moreover, overexpression of COX-2 has been reported in CML and colon cancer cells[15,25]. COX-2 is known to play a role in different steps of cancer progression by increasing proliferation of mutated cells[7], down regulation of apoptosis and thus affect the efficacy of anticancer therapies[26-30]. It is also implicated in metastasis by affecting apoptosis induced by loss of cell anchorage (anoikis)[31].

The NSAIDs such as aspirin and sulindac, which are commonly administered for the relief of pain and inflammation, are inhibitors of COX. However, as most conventional NSAIDs are associated with side effects such as gastric ulceration, disturbance in kidney ionic balance etc., efforts are being made to develop new drugs without these problems[3,15]. The discovery of COX-2 as the inducible form in all inflammatory conditions has paved the way for the development of COX-2 specific inhibitors-COXIBs which do not show gastric and renal side effects. However, due to the cardiac side effects that surface on long-term use, rofecoxib (vioxx) has been withdrawn from the market. Thus, the need for safer COXIBs still exists[32]. As the activation of 5-LOX during the inhibition of COX is found responsible for some of the side effects of NSAIDs and COXIBs, there is also focus on the development of COX-2/5-LOX dual inhibitors (CLOXIBs)[15]. The HTS assays reported in this study would be very useful for screening of compound libraries for the identification of COXIBs/CLOXIBs and thus in the development of safer anti-inflammatory drug discovery programs.

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