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N-1, C-3 substituted indoles as 5-LOX inhibitors—In vitro enzyme immunoaasay, mass spectral and molecular docking investigations

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ABSTRACT

Based upon the structures of some known 5-LOX inhibitors, a set of five compounds carrying appropriate substituents at N-1 and C-3 of indole were synthesized and investigated for 5-LOX inhibitory activities. Fifty percent inhibitory concn (IC_{50}) of these compounds ranges from 0.6 to 5 μM and found to be comparable to that of clinically used 5-LOX inhibitor, zileuton. The compounds under present investigations exhibited appreciable interactions with 5-LOX as apparent from their association constants calculated from the mass spectral data. Compound **5a** with a tosyl group at N-1 and pyrolidinyl-1,2-dione substituent at C-3 of indole, exhibiting IC_{50} 0.6 μM and stoichiometry of 1:7 in the enzyme–compound complex was identified as highly potent 5-LOX inhibitor and seems to be suitable for further investigations.

Due to the production of pro-inflammatory prostaglandins and leukotrienes, arachidonic acid (AA) metabolism is being the target of the drugs used for treatment of allergic and inflammatory diseases.[1] While prostaglandins are generated through COX-1 and COX-2 mediated channels[2] of AA metabolism, leukotrienes are produced by 5-lipoxygenase (5-LOX) through the participation of 5-LOX activating protein (FLAP).[3] Besides allergy and inflammation, leukotrienes also play pivotal role in instigation of asthma, hypersensitivity, atherosclerosis, stroke, osteoporosis as well as certain types of cancer.[4] Hence, decreasing leukotriene biosynthesis through the inhibition of 5-LOX has been extensively studied as a potential target for the development of novel therapies of 5-LOX associated diseases.[4a,5] Four different classes of 5-lipoxygenase inhibitors have been identified as redox inhibitors, iron chelating agents, active site-directed inhibitors or competitive reversible inhibitors and inhibitors of FLAP.[3c,6] To the best of our knowledge, zileuton and a few cysteinyl-LT receptor antagonists are the limited drugs being clinically used for the treatment of LOX-mediated diseases.[7] MK-886 and MK-0591 are the other two most important 5-LOX inhibitors tested for the treatment of LOX mediated diseases (Chart 1).

The uniqueness of indole in the biological systems, playing crucial roles in the form of amino acid, growth hormone and alkaloids and its presence at or near the catalytic/molecular recognition sites of enzymes are the advantages for making indole as part of the drug molecule. Similar to the benzothiophene fragment present in zileuton and central core of MK-886 and MK-0591, indole moiety was derivatized to procure new compounds for studying their 5-LOX inhibitory activities. In vitro enzyme immunoassay, stoichiometry, association constants and molecular interactions of these compounds for 5-LOX were investigated.

The reaction sequence adopted for the preparation of target compounds is illustrated in Scheme 1. 2-(1*H*-Indol/5-methoxy-1*H*-indole-3-yl)-2-oxoacetyl chloride (**2**) (93%) was obtained from the reaction of 1*H*-indole/5-methoxy-1*H*-indole (**1**) with oxalyl chloride at 0–5 °C using dry ether as the reaction medium. Treatment of **2** with pyrrolidine and morpholine in presence of K_2CO_3 in acetonitrile gave respective compounds **3**(**a**,**b**) and **4**(**a**,**b**) in 71–83% yield. N-1 substitution of compounds **3** and **4** was achieved through their reactions with various halides in presence of NaH in dry acetonitrile to get target compounds **5**(**a–c**) and **6**(**a**,**b**) in 75–94% yields. All the compounds were characterised by NMR spectra, mass spectra and CHN analysis.

In vitro 5-LOX inhibitory activities of compounds **5** and **6** were determined at 0.01, 0.1, 1 and 10 μM concentrations by using enzyme immuno assay kit according to the instructions provided by the kit manufacturer.[8] Compounds **5**(**a–c**) and **6**(**a**,**b**) exhibited more than 50% inhibition of 5-LOX activity at 10 μM concentration. The presence of morpholinyl moiety at the end of C-3 substituent and cinnamyl and tosyl groups at N-1 in compounds **5c** and **6b** resulted in appreciable inhibition of 5-LOX activity with IC_{50} 5 and 1 μM, respectively (Table 1). Compounds with pyrolidinyl as a part of C-3 substituent and tosyl, benzoyl and cinnamyl groups at N-1 (**5a**, **5b** and **6a**) showed significant inhibition of 5-LOX activity with respective IC_{50} 0.6, 1 and 1 μM. These compounds seem to be

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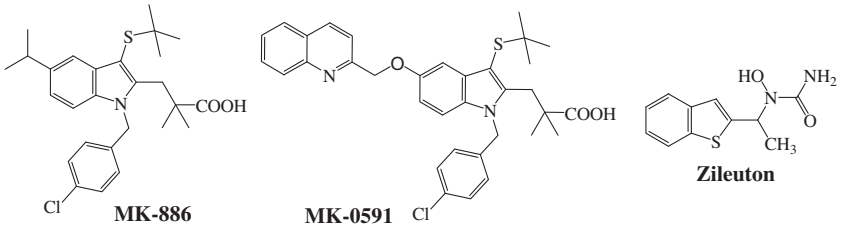
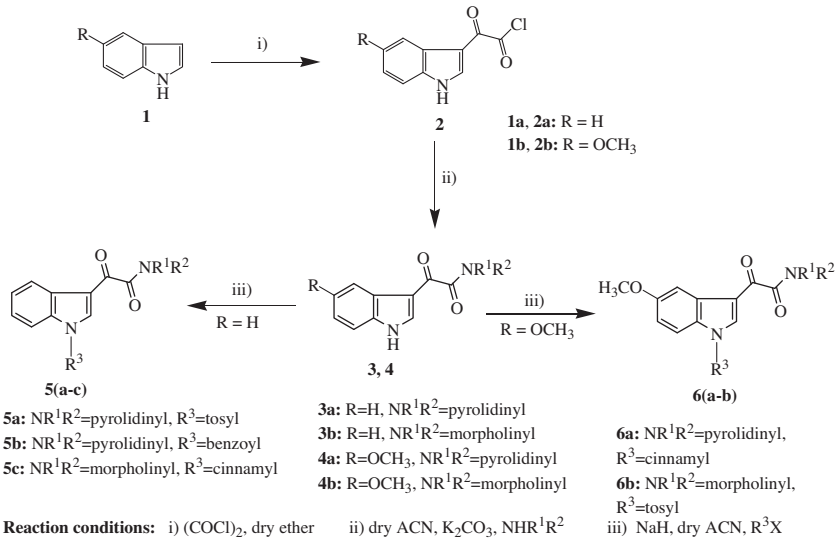


Chart 1. 5-LOX inhibitors.



Scheme 1.

Table 1
5-LOX inhibitory activities of compounds **5** and **6**

Compound	% Inhibition of 5-LOX				IC_{50} (μM)
	10 μM	1 μM	0.1 μM	0.01 μM	
5a	95.4	63.8	27.8	0.9	0.6
5b	59.6	54.2	17.4	6.8	1
5c	59.2	40.7	21.1	2.5	5
6a	68.6	54.5	25.6	5.9	1
6b	59.2	53.8	22.0	4.7	1
Zileuton					3.7
MK-0591					5

Table 2
Stoichiometry of the enzyme–compound complex and association constants

Compound	*m*/*z* Free enzyme (a)	*m*/*z* Enzyme–compd complex (b)	(b) − (a)	Stoichiometry (enz:compd)	Association constant (K_a)
5a	86,957	89,641	2684	1:7	1.2×10^5
5b	86,957	88,335	1378	1:4	0.99×10^5
5c	86,957	88,203	1246	1:3	0.53×10^5
6a	86,957	89,274	2317	1:6	1.06×10^5
6b	86,957	88,812	1855	1:4	1.96×10^5
Zileuton	86,957	89,656	2699	1:9	0.26×10^4

better 5-LOX inhibitors than the clinically used zileuton (IC_{50} 3.7 μM) and MK-0591 (IC_{50} 5 μM).[9] Therefore, the compounds with appropriate combination of substituents at N-1 and C-3 of indole, investigated here, seems to have sufficient potential for 5-LOX inhibition.

The enzyme inhibition data of the test compounds led us to further investigate the affinity and strength of association of these compounds with 5-LOX. Since electrospray ionisation (ESI) mode of mass spectrometer proved to be a versatile tool for studying the enzyme–ligand interactions, the data corresponding to 5-LOX inhibitory concentration of the compounds was supported with mass spectra of 5-LOX in combination with compounds **5** and **6**. Mass spectrum of solution of compound **5a** and 5-LOX in ACN–H_2O (7:3) was recorded in the +ve mode at source temperature 180 °C and collision energy 5–8 eV. Similar mass spectra were obtained using ACN–H_2O (1:9) as medium. Comparison of mass spectra of enzyme recorded in ACN–H_2O (7:3) and in pure water indicated no change in the enzyme spectrum in ACN medium. The compound–enzyme additive mass peak was observed at *m*/*z* 89641. Difference in the mass of compound–enzyme complex and pure enzyme divided by the mass of the compound gave **5a**–5-LOX stoichiometry 7:1 (Table 2, Fig. 1). Similarly, the compound–enzyme additive mass peaks were observed for other compounds and stoichiometries were calculated. In comparison to the stoichiometry of zileuton with 5-LOX as calculated from mass spectra shown in Figure 2, it was observed that the compounds under present investigation have better affinity for 5-LOX. The association constants of enzyme–compound complexes were calculated using the intensity of various mass peaks as equivalent to the concentration of that species.[10] In parallel with their enzyme inhibitory concn (obtained from enzyme immunoassay), the mass spectral technique also supported that all the five compounds exhibited interactions with 5-LOX better than that of zileuton as

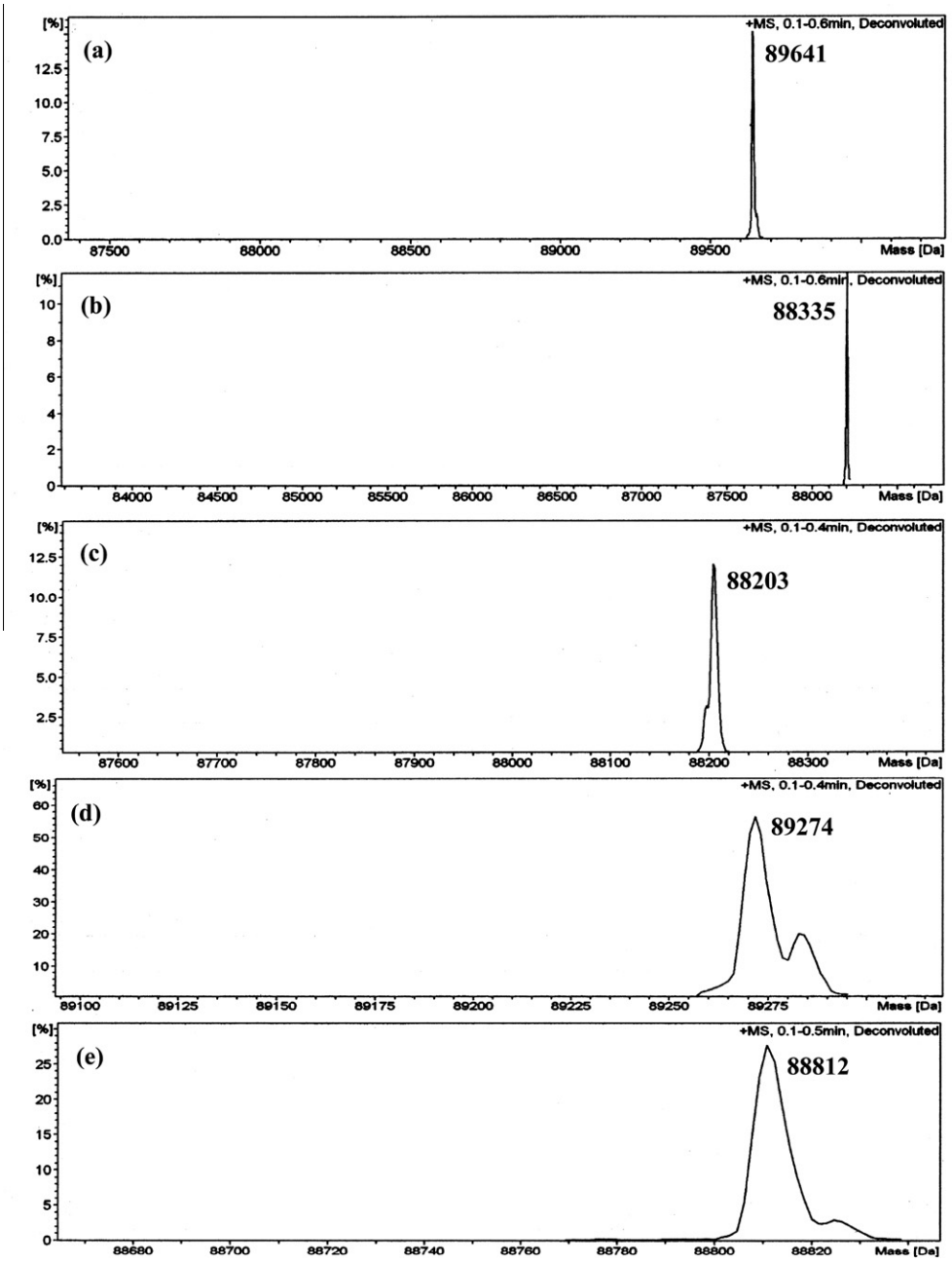


Figure 1. Deconvoluted mass spectra of (a) **5a**–5-LOX complex; (b) **5b**–5-LOX complex; (c) **5c**–5-LOX complex; (d) **6a**–5-LOX complex; (e) **6b**–5-LOX complex.

evident from their association constants with the enzyme (Table 2 and Table S1).

The results of above two experiments favouring the suitability of the compounds for inhibition of 5-LOX incited to take molecular modelling into consideration for looking into the nature of interactions between the compound and active site amino acids of the enzyme. Compounds were built using the builder toolkit of the software package ArgusLab 4.0.1[11] and energy minimized using semi-empirical quantum mechanical method PM3. The crystal coordinates of 5-LOX (pdb ID 3V99)[12] were downloaded from protein data bank. Structure of this protein carries arachidonic acid as a ligand in its binding site. In the molecule tree view, the active site of the enzyme is defined as 15 Å around the ligand. The molecule to be docked in the active site of the protein was pasted in the work space carrying the structure of the enzyme. The conformational space was explored by the geometry optimization of the flexible ligand (rings are treated as rigid) in combination with the incremental construction of the ligand torsions. Thus, docking occurs between the flexible ligand parts of the compound and enzyme. The ligand orientation was determined by a shape scoring function based on Ascore and the final positions were ranked by lowest interaction energy values. H-bond and hydrophobic interactions between the compound and enzyme were explored.

Dockings of the clinically used 5-lipoxygenase inhibitor, zileuton in the active site of 5-LOX showed H-bond interactions with amino acids A672, H372 and N554 through its hydroxy group, carbonyl group and amino group, respectively (Fig. 3). Docking of compounds **5a** and **5c** in the active site of 5-LOX indicated H-bond interactions with F177 and Q413 amino acid residues through the oxygen of carbonyl group and morpholine ring (Fig. 4 and Fig. S3),

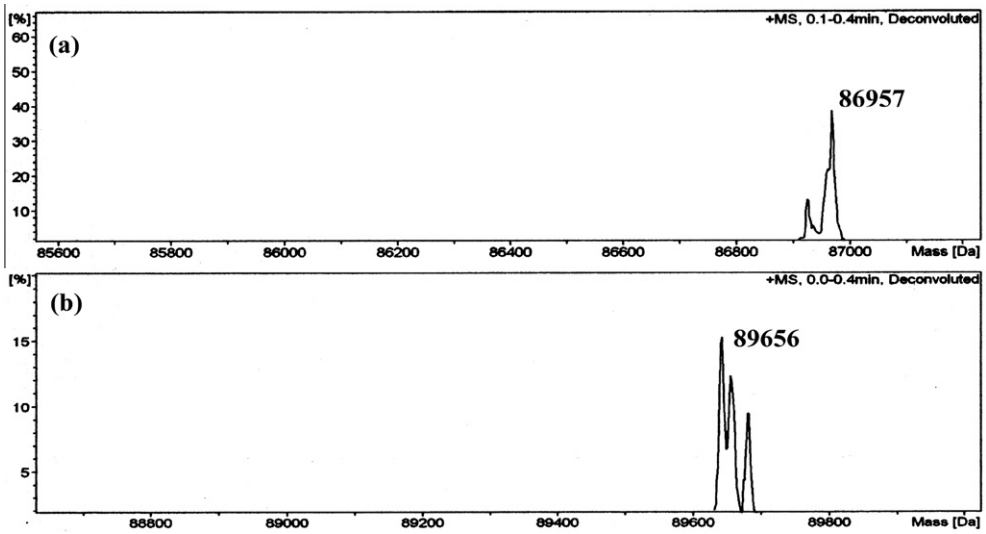


Figure 2. Deconvoluted mass spectra of (a) 5-LOX and (b) 5-LOX + zileuton.

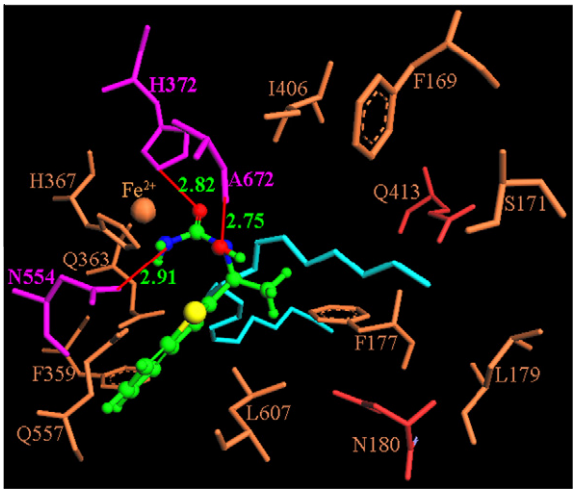


Figure 3. Zileuton (green) docked in the active site of 5-LOX showing H-bonding interactions with amino acids Ala672, His372 and Asn554 (shown in pink colour). Arachidonic acid in the active site is shown in light blue colour.

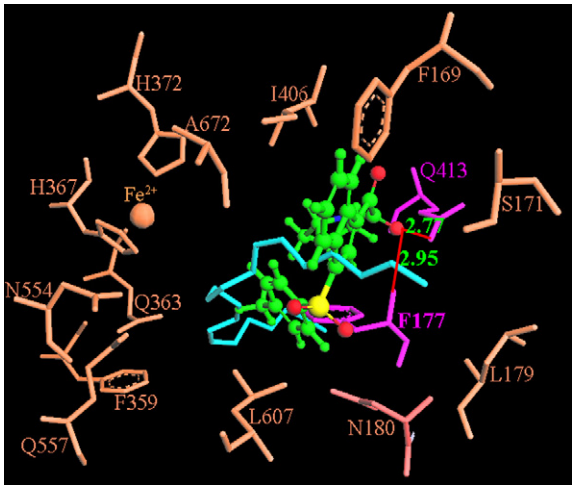


Figure 4. Compound **5a** (green) docked in the active site of 5-LOX. H-bonds between carbonyl oxygen and amino acids Phe177 and Gln413 (pink) of the active site are clearly visible. Arachidonic acid in the active site is shown in light blue colour.

while compound **6b** interacted through its carbonyl oxygen and morpholine oxygen with F177, Q413 and K409 amino acid residues (Fig. S2). Compound **5b** used its benzoyl carbonyl oxygen and nitrogen of the indole ring to form H-bonds with Q557 and N554 amino acid residues (Fig. S1). Compound **6a** showed H-bond interactions of oxygen of carbonyl and methoxy group with amino acids H367, H372 and N180 in the active site of enzyme 5-LOX (Fig. 5). Besides hydrogen bond interactions, there are numerous non-bonding interactions observed between the compound and the amino acids in the active site. The indole ring in compounds **5a**, **5b** and **6b** filled the cavity formed by amino acids I406, F169, S171; H367, L607, Q363, Q557 and L607, F177, Q413, respectively. N-1 benzoyl group and cinnamyl group in compounds **5b** and **5c** showed a close approach to the amino acids A672, H372, H367, N554 in the active site of 5-LOX, while the morpholine ring and pyrrolidine ring in compounds **6b** and **6a** showed non-bonding interactions with amino acids K409, S171, F169, I406 and A672, H372, H367, Q363, respectively. Similarly the methoxy groups in compounds **6b** and **6a** showed a close approach to amino acids F177, Q413 and N180, L607, F177, respectively. It was observed that compounds **5b** and **6a** interacted with the same amino acids

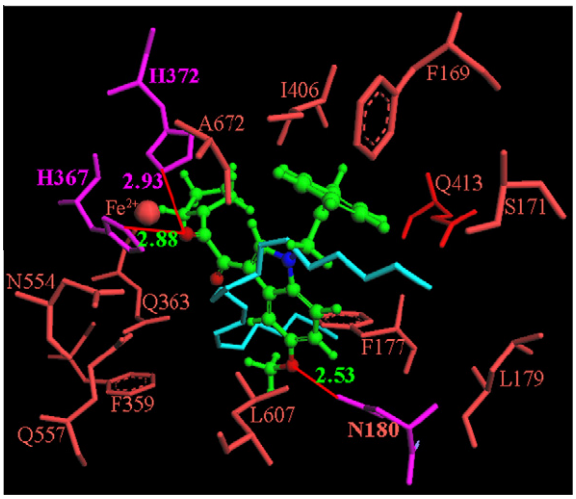


Figure 5. Compound **6a** (green) docked in the active site of 5-LOX showing H-bonding interactions through its carbonyl oxygen and methoxy oxygen with amino acids Asn180, His372 and His367 (pink). Arachidonic acid in the active site is shown in light blue colour.

of 5-LOX (N554 and H372) where zileuton was bound. All these compounds follow Lipinski's rule of 5 (Table S2).

In conclusion, indole based compounds synthesized through an easy synthetic methodology exhibited significant 5-LOX inhibitory activities. The affinity, strength and nature of interactions of the compounds with the enzyme were explored through mass spectroscopy and molecular modelling techniques. Compound **5a** with a tosyl group at N-1 and pyrolidinyl-1,2-dione substituent at C-3 of indole, exhibiting IC_{50} 0.6 μM and stoichiometry of 1:7 in the enzyme–compound complex was identified as highly potent 5-LOX inhibitor. This series of compounds seems to have significant potential for inhibition of 5-LOX and is under further refinement for improving the biological profile of the molecules.

Acknowledgements

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Supplementary data

Supplementary data (experimental procedures, characterisation of compounds, ^{1}H NMR spectra of comopunds, calculation of association constants, Lipinski's values and Figures S1–S3) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.12.068.

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