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Lead modification: Amino acid appended indoles as highly effective 5-LOX inhibitors

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ABSTRACT

N-1 tosyl indoles carrying amino acid as a part of C-3 substituent are identified with considerable 5-LOX inhibitory activities. On the basis of enzyme inhibitory activities and log*P*, it is found that these compounds are more suitable to use as ester prodrugs. In addition to the significant K_a and K_i for 5-LOX, advantageously the compounds under present investigation do not affect the viability of the cell. The experimental results were also supported by molecular docking of compounds in the active site of 5-LOX.

1. Introduction

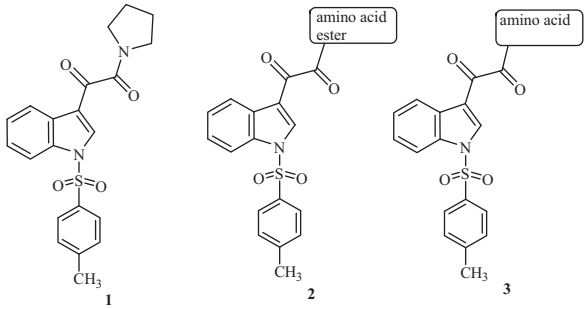
Symptomized with pain, increase in body temperature, redness, swelling, loss of function etc.; a large group of human population suffers from inflammatory diseases.[1] Discovery of arachidonic acid metabolic pathway,[2] identification of enzymes participating therein[3] and recognition of inflammatory metabolites led to systematic studies for development of anti-inflammatory drugs.[4] Starting with stimulatory role of phospholipases to release arachidonic acid from phospholipids, over-expression of cyclooxygenase-2 (COX-2) and lipoxygenase (LOX) enzymes cause more than normal production of inflammatory prostaglandins and leukotrienes.[5] Hence these enzymes are the targets of majority of anti-inflammatory drugs. Irrespective of the long journey of anti-inflammatory drugs, from aspirin to COXIBS[4,6]—targeting COX-2 and zileuton, MK-886 and MK-095—targeting LOX,[7] still the safety (devoid of side effects) of the drug/s is a primary issue.[8] This necessitates the search for such compounds which may prove as more efficacious and safer drugs. In continuation of our previous report,[9] another set of compounds is designed, synthesized and evaluated for inhibition of catalytic activity of various enzymes of arachidonic acid metabolic pathway.

Structural and functional diversity of naturally occurring indole has made it a privileged pharmacophore in drug development where indole based natural and synthetic compounds find myriad properties as anti-microbial,[10] anti-inflammatory,[11] anti-cancer agents,[12] 5-LOX inhibitors[13] as well as in the form of agrochemicals.[14] Keeping in mind the biological acceptability of indole and amino acids; herein, compounds **2**–**3** (Chart 1) were obtained by modifying the previous compound **1**.[9] While in compound **1**, tosyl group was optimized, here, presence of amino acid as part of C-3 substituent led to the development of highly potent 5-LOX inhibitors.

2. Result and discussion

2.1. Chemistry

The sequence of steps for the synthesis of target compounds is illustrated in Schemes 1 and 2. Oxalyl chloride was added to solu-



2-3: amino acid/ester; a) glycine, b) tryptophane, c) histidine, d) glutamic acid, e) proline

Chart 1. Based on previous compound **1**, design of new molecules **2** and **3**.

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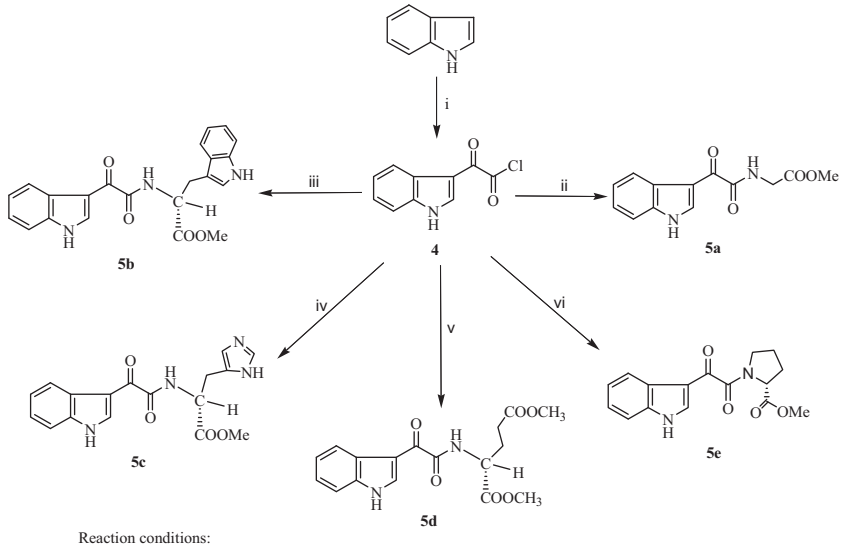


Reaction conditions:
i) $(COCl)_2$, dry ether, 0 °C
ii) Dry ACN, K_2CO_3, glycine methyl esterHCl, 0-5 °C
iii) Dry ACN, K_2CO_3, L-tryptophan methyl esterHCl, 0-5 °C
iv) Dry ACN, K_2CO_3, L-histidine methyl esterHCl, 0-5 °C
v) Dry ACN, K_2CO_3, L-glutamic methyl diesterHCl, 0-5 °C
vi) Dry ACN, K_2CO_3, L-proline methyl esterHCl, 0-5 °C

Scheme 1.

tion of indole in dry ether at 0 °C and resulting yellow colored solid (**4**) was filtered out from the reaction mixture. Compound **4**, taken in dry acetonitrile was treated with L-amino acid ester hydrochlorides in presence of K_2CO_3. After stirring the reaction mixture for 25–30 min, it was purified with column chromatography to get 3-substituted indole **5a**–**e** in 70–80% yield (Scheme 1). Taken in dry acetonitrile, compounds **5a**–**e** were treated with p-tolylsulfonyl chloride in presence of NaH keeping reaction temperature 0–5 °C. After usual work up, compounds **2a**–**e** were procured in 80–85% yield. Further, hydrolysis of compounds **2a**–**e** with NaOH provided compounds **3a**–**e** in quantitative yield (Scheme 2). All the compounds were characterized with NMR and high resolution mass spectra (Supporting information).

2.2. Enzyme immunoassays

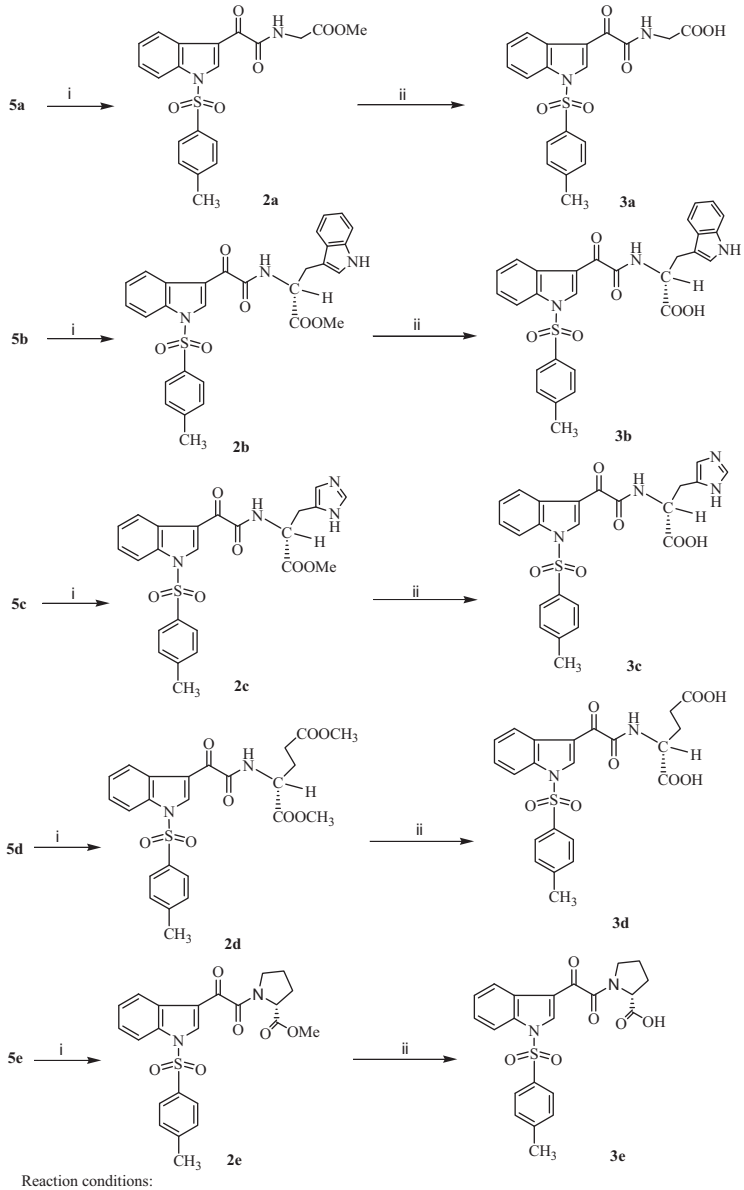
2.2.1. 5-LOX inhibitory activities

In vitro 5-LOX inhibitory activities of compounds **2**, **3** and **5** were checked at 0.01 μM, 0.1 μM, 1 μM, 10 μM and 100 μM concentrations using enzyme immunoassay kits.[15] The results of these experiments in terms of percentage inhibition of 5-LOX activity at different concn of the compounds and their respective IC_{50} are given in Table 1. Although compounds under present investigation may not have same mode of action as zileuton and MK 0591 but 5-LOX inhibitory data of these two drugs was taken for comparison. All the compounds except **5a**–**e** exhibited appreciable inhibition of 5-LOX activity. Comparison of enzyme immunoassay results of compounds **5** with **2** and **3** indicated that a substituent at N-1 position of indole is essential for inhibition of 5-LOX enzymatic activity. Amongst compounds **2**, it was observed that **2a** and **2b**, carrying respectively glycine and tryptophan functionality were most active with IC_{50} 9.7 nM and 52 nM. Compounds **3**, the corresponding acids of esters **2**, exhibited considerably higher 5-LOX inhibitory activity than their ester analogues except in case of compound **3e**. Here again, compounds **3a** and **3b** with glycine and tryptophan residue at the end of C-3 substituent were identified as the most active amongst the compounds under present investigation. IC_{50} of compounds **3a** and **3b** was found to be 8.6 nM and 10 nM, respectively. Moreover, compound **3d** also exhibited significant 5-LOX inhibitory activity with IC_{50} 97 nM. Compounds **3a** and **3b** were found to be showing inhibition of 5-LOX enzymatic activity comparable to that of zileuton[16] and MK 0591.[17]

Therefore, the trends of 5-LOX inhibitory activities of compounds **2**, **3** and **5** indicate that compounds **3** are more suitable for their use as 5-LOX inhibitors. Since a proper balance between lipophilicity and hydrophilicity is one of the primary criteria to adjudge the suitability of compound for medicinal purpose, partition coefficient of compounds **2**, **3** and **5** was calculated ($C\log P$).[18] $\log P$ for compounds **5** was found to be 0.3–1.5 (Table 2) which is quite low. In presence of tosyl group at N-1 position of compounds **5**, the resulting compounds **2** have desirable $\log P$, ~2. Experimental $\log P$ of compounds **2** (determined by octanol–water partition) was quite close to the theoretical values. However, compounds **3** exhibited quite low partition coefficient. Hence, it may be recommended to use compound **2** (instead of compound **3**) as 5-LOX inhibitors which may undergo hydrolysis under the action of esterase (present in sufficient amount in the cell) to form compounds **3** when administered to the biological system. In vitro hydrolysis of compounds **2** to **3** using HEPES buffer as medium was successfully performed. Interestingly, 5-LOX inhibitory activities of compounds **2**, checked through enzyme immunoassay performed in presence of pig liver esterase, were found to be quite close to those of compounds **3** (Table 1) indicating the in situ enzymatic (esterase) hydrolysis of compounds **2** to **3**. It seems that substituent at N-1 position of indole might be responsible for maintaining appropriate hydrophilic-lipophilic balance in the compound. However, more information about the role of different substituents present on indole moiety of compounds **2**–**3** was obtained from the results of docking studies of these compounds in the active site of 5-LOX enzyme.

2.2.2. Enzyme immunoassays with COX-2 and PLA_2

In order to check if these compounds also inhibit the catalytic activity of other enzymes of arachidonic acid pathway, their enzyme immunoassays with COX-2, COX-1 and PLA_2 were per-



Scheme 2.

formed.[19] However, we did not observe change in the catalytic activity of these three enzymes in presence of compounds **2**, **3** and **5**. Hence, similar to the 5-LOX selectivity of zileuton,[20] amongst other enzymes of arachidonic acid pathway, compounds **2** and **3** also target 5-LOX only and do not seem to be the frequent hitters. The selective 5-LOX inhibitory activity of zileuton for the treatment of disorders like asthma is well documented.[20]

2.2.3. K_a (Association constant) and K_i (inhibitor constant) of compounds 2 and 3 for 5-LOX

Based on the results of 5-LOX enzyme immunoassay, interactions of compounds **2** and **3** with 5-LOX were investigated with the help of UV–visible spectral studies[21] and the association constant of compounds **2** and **3** with 5-LOX were calculated using Eq. 1.[22]

$$1/A_f - A_{obs} = 1/A_f - A_{fc} + 1/K_a[A_f - A_{fc}][L] \quad (1)$$

where A_f = absorbance of free host, A_{obs} = absorbance observed, A_{fc} = absorbance at saturation

As it is apparent from the data given in Table 3, compounds **2** and **3** exhibited appreciable K_a for 5-LOX. Similar to the results of enzyme immunoassay, compounds **2a** and **3a** with their respective K_a 2.32×10^6 and 2.07×10^6 showed significant association with 5-LOX. Moreover, the K_i (inhibitor concentration required to produce half maximum inhibition) of compounds **2** and **3** for 5-LOX (Table 3) were also calculated with Eq. (2).[23]

$$K_i = IC_{50}/1 + [L]/K_d \quad (2)$$

2.2.4. Cell viability assay

To check the toxicity of the compounds towards mammalian cells, the cell viability assay was performed with compounds **2a** and **3a**. Mammalian HeLa cells were treated with compounds **2a** and **3a** and analyzed the culture using MTT assay at different time points. As it is evident from Figure 1, there was no change in the growth of HeLa cells even with 100 μg/ml (~0.25 M) of compounds **2a** and **3a**.

Table 1
5-LOX inhibitory activities of compounds **2**, **3** and **5**

Compd	% Inhibition of 5-LOX at compound concn					IC_{50}* (μM)	IC_{50}# (μM)
	100 μM	10 μM	1 μM	0.1 μM	0.01 μM		
5a	70	55.5	47.8	46.2	40.0	3.5	–
5b	65	49.8	43.4	41.1	35.0	–	–
5c	65	45.5	40.8	37.7	30.0	–	–
5d	55	51.2	44.8	40	36	8.33	–
5e	60.6	59.8	40.2	25.5	22.5	5.46	–
2a	98	85	67.67	51.5	50	0.0097	0.0080
2b	80.4	75.5	62.70	55.3	45.5	0.052	0.014
2c	63.8	58.18	45.3	40.5	30	4.33	2.98
2d	65	60.33	51.8	35.19	18.46	0.90	0.13
2e	72	69.33	54.5	48.7	38.50	0.33	0.75
3a	97.5	91.2	78.4	63.5	57.4	0.0086	–
3b	85	81.5	67.3	56.5	51	0.010	–
3c	64.7	55.5	48.7	28.5	20	2.80	–
3d	90.6	87.59	59.5	50.5	42.0	0.097	–
3e	74.3	68.8	53.5	45	27.3	0.60	–
Zileuton						0.3	–
MK 0591						1.6#	–

* ±0.5 of two experiments; #value in nM.
# IC_{50} of compounds **2** checked in presence of pig liver esterase.

Table 2
C log*P* of compounds **2**, **3** and **5**

Compd	*C*log*P*	Compd	*C*log*P*	Experimental log*P*	Compd	*C*log*P*
5a	0.579	**2a**	2.003	2.21	**3a**	−0.474
5b	1.52	**2b**	2.220	2.34	**3b**	1.872
5c	0.323	**2c**	2.020	1.98	**3c**	−0.05
5d	0.661	**2d**	2.010	2.12	**3d**	−0.295
5e	1.213	**2e**	2.061	2.19	**3e**	0.565

Table 3
Association constant (K_a) of compound **2** and **3** with 5-LOX

Compd	K_a (M^{-1})	K_i (μM)
2a	2.32×10^6	0.0095
2b	6.67×10^5	0.037
2c	1.01×10^4	4.16
2d	1.06×10^5	0.82
2e	1.2×10^5	0.32
3a	2.07×10^6	0.0084
3b	3.6×10^5	0.0099
3c	3.7×10^4	2.53
3d	1.1×10^5	0.095
3e	2.6×10^5	0.51

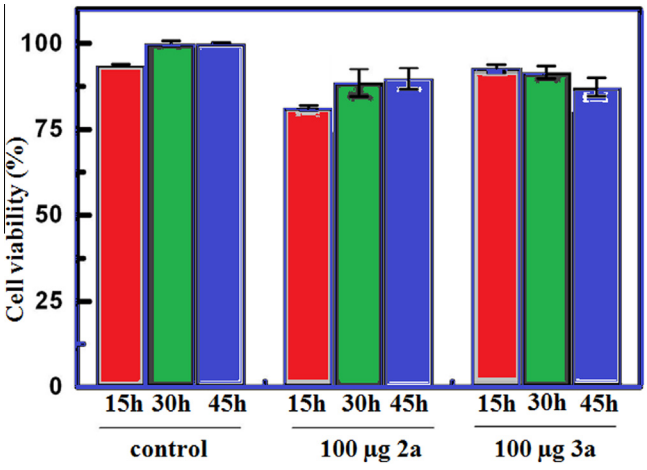


Figure 1. Growth of HeLa cells was not affected by compounds **2a** and **3a**.

2.3. Docking studies

Since the compounds under present investigation showed appreciable inhibition of the catalytic activity of 5-LOX, it was planned to scrutinize the mode of interactions of compounds **2** and **3** with amino acids in the active site of 5-LOX. For this purpose, molecular docking of these compounds in the active site of the enzyme was performed. Using ArgusLab 4.0.1,[24] compounds **2** and **3** were docked in the active site of 5-LOX (pdb ID 3v99).[25] It was observed that most of these compounds exhibit H-bond interactions with active site amino acids of 5-LOX but with significant difference in their respective docking score (Table 4). Compound **2a** showed H-bond interaction with Q554 and Q557 through its COOMe group and indole N (Fig. 2), respectively while compound **2b** through its ketonic O and amidic O interacted with F177, A410 and N413 (Fig. S33). However, docking of compound **3a** in the active site of 5-LOX showed that this compound interact more effectively with the active site amino acids in comparison to the interactions of compound **2a** with the amino acids of same enzyme. Compound **3a** exhibited H-bond interactions with Q554, Q557, V604 and N554 through sulfonyl oxygen, indole ring N, amidic O and carboxyl O, respectively (Figs. 3–5). Compound **3b** also interacted with active site amino acids through its sulfonyl O and carboxyl O (Fig. 6). It seems that similar to the mode of action of indomethacin, the most potent compounds in the present studies also interact with 5-LOX through their carboxyl functionality. For comparing the molecular interactions of compounds **2** and **3** with

Table 4
Docking score of compounds **2** and **3**

Compd	Docking score (K cal/mol)
2a	−20.0
2b	−9.0
2c	−11.5
2d	−13.0
2e	−14.2
3a	−25.4
3b	−24.2
3c	−13.7
3d	−17.0
3e	−13.8
Zileuton	−9.18
MK886	−9.96

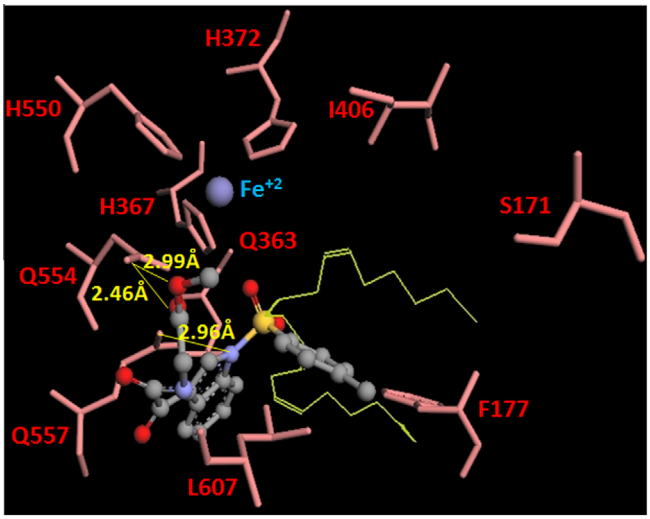


Figure 2. Compound **2a** docked in the active site of 5-LOX. Hs' are omitted for clarity.

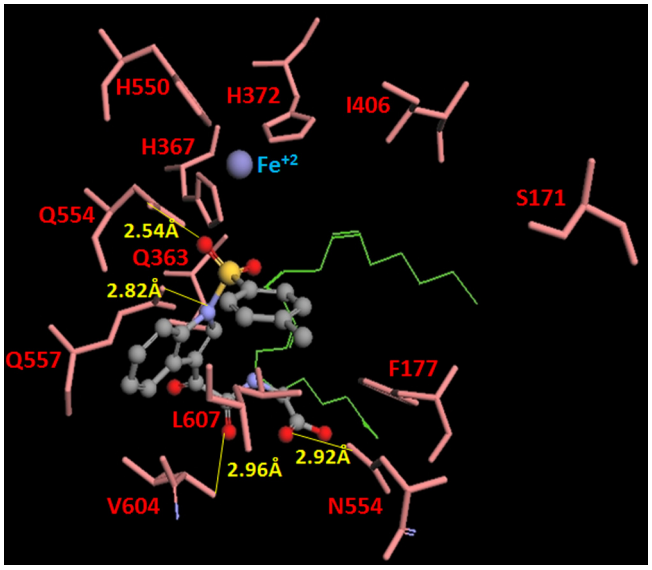


Figure 3. Compound **3a** docked in the active site of 5-LOX. (pdb ID 3V99).

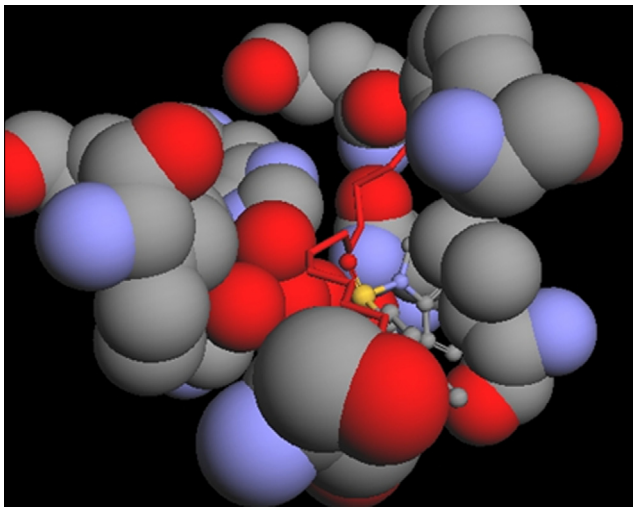


Figure 4. Compound **3a** docked in the active site of 5-LOX. Compound **3a** (ball and stick model) is placed in the same pocket where AA (red wire) is present.

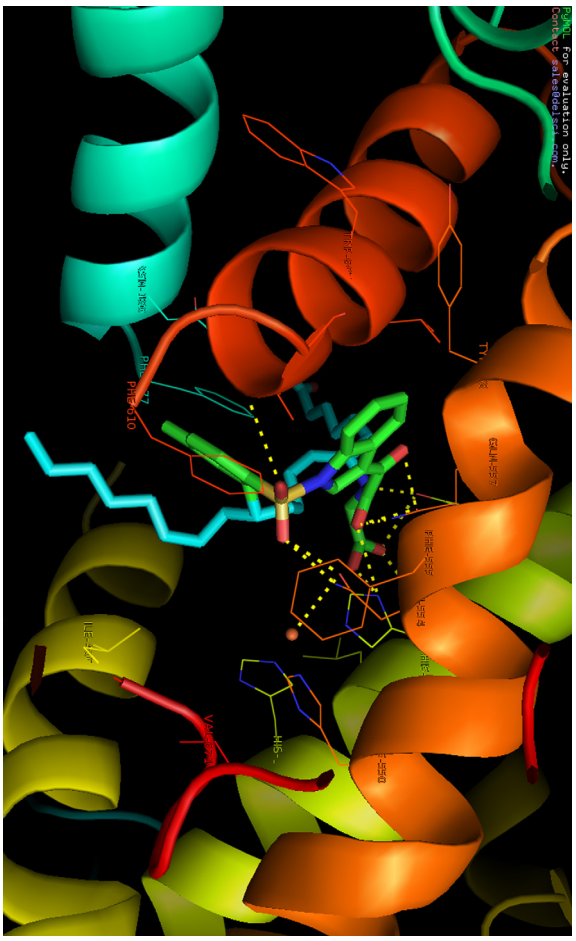


Figure 5. Crystal coordinates of 5-LOX in association with compound **3a** (generated in pymol). Dotted lines indicate H-bond interactions of compound **3a** with amino acid residues.

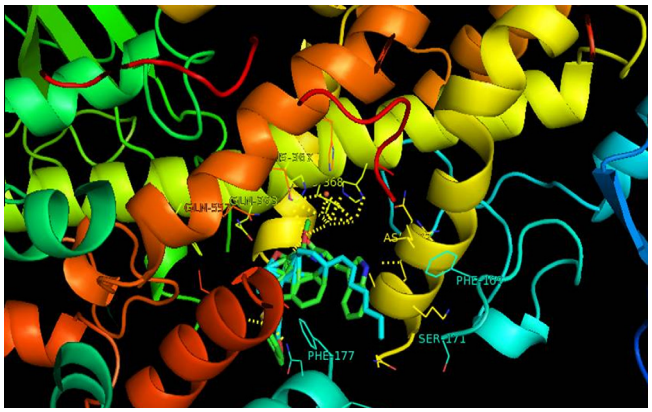


Figure 6. Crystal coordinates of 5-LOX in association with compound **3b** (green) (generated in Pymol). Dotted lines indicate H-bond interactions between the compound and amino acids.

that of zileuton and MK886 in the active site of 5-LOX, these two standard drugs were also docked in the active site of 5-LOX. However, H-bond interactions of zileuton (Fig. S41) and MK886 (Fig. S42) as well as their respective docking scores were less in comparison to compounds **2a**, **3a** and **3b**. Therefore, supporting the experimental results, the molecular docking of the compounds

in the active site of 5-LOX provided an insight into the mode of interactions between the compounds and amino acids.

3. Conclusions

In conclusion, improving over the previous compound, amino acid appended indoles were identified with considerable increase in their biological activity. Two compounds exhibited IC_{50} for 5-LOX in nm range. Physico-chemical parameters and in vitro investigations indicate the use of compounds **2** as prodrugs of their more active form **3**. Appreciable K_a and K_i of compounds **2a** and **3a** for 5-LOX, supported by molecular docking results and their non-toxicity to mammalian cells indicate the suitability of these compounds for further investigations. More compounds of this category by introducing two/three amino acids at C-3 position of indole will also be explored for their 5-LOX inhibitory potential.

4. Experimental Procedure

4.1. 5-LOX inhibtory activities

For 5-LOX inhibition studies, solutions of compounds at 0.01 μM, 0.1 μM, 1 μM, 10 μM and 100 μM concentrations were prepared in DMSO. 10 μL of each compound from the above concentrations was added to 90 μL solution (in assay buffer) of 5-LOX enzyme taken in the wells of a 96-well plate. Each compound was tested in duplicate and the average of two values with deviation <5% was taken for calculation. Two wells were taken as blanks (assay buffer + AA) and four wells were taken as positive controls (enzyme in assay buffer + AA). The reaction was initiated by the addition of 10 μL of the substrate (AA). After shaking the 96-well plate on a shaker for 5 min, 100 μL of the chromogen (developing reagent) was added to each well. The plate was again shaken for 5 min and read at 490 nm in a microplate scanning spectrophotometer. Percent 5-LOX inhibitory activity was determined using the mean of the two values for each sample from the calculations given below:Lipoxygenase activity (μmol/min/ml) = [A500/min/9.47 mM^{-1}] × [0.21 ml/0.09 ml] × sample dilutionwhere A500/min = A500 (sample)/min − A500 (blank)/5 min, 9.47 mM^{-1} = -Extinction coefficient of chromogen, 0.21 ml = Total volume of the solution in each well and 0.09 ml = Volume of the enzyme solution used.Percent 5-LOX inhibition = [{L.A. (P.C.) − L.A. (I.)}/L.A. (P.C.)] × 100

L.A. (P.C.) − Lipoxygenase Activity of positive control, L.A. (I.) − Lipoxygenase Activity of inhibitor.

IC_{50} values were determined from the graph between Percent Inhibition versus concn of inhibitor using KaleidaGraph 3.5.

4.2. 5-LOX inhibtory activities of compounds 2 in presence of pig liver esterase

This assay was performed through the same procedure as above except that compound **2** was treated with pig liver esterase (extracted by following reported procedure) before it is added to the 5-LOX enzyme.

4.3. COX-1/2 and PLA_2 inhibitory activities

COX-1/2 and PLA_2 enzyme inhibitory activities of compounds **2** and **3** were checked as per the protocol given with each enzyme kit.[19]

4.4. UV–visible spectral studies for calculating K_a

Same protocol was used as did previously for studying compound–DHFR interactions.[20]

4.5. Cell viability assay

Effect of the compounds **2a** and **3a** on the growth of cells was monitored using MTT assay. Performing the experiment in triplicate, HeLa cells (4000 cells/well) were incubated in a 96-well plate in the presence of 100 μg/ml of compound **2a** and **3a** with final volume of 200 μL at 37 °C in a humidified chamber. Cells with solvent only (DMSO) were taken as a control. After certain intervals, 20 μL of MTT solution (5 mg/ml in PBS) was added to each well and incubated for another 4 h. After removing the supernatant at the end of 4 h, the resultant formazan crystals were dissolved in 200 μL DMSO and absorbance (A) was measured at 570 nm by a microplate reader. The percentage viability of the cells was calculated by using the following equation.

Percentage of cell viability = (absorbance of test samples/absorbance of the control sample) × 100.

4.6. Docking Procedure

Compounds were built using the builder toolkit of the software package ArgusLab 4.0.1 and energy minimized using semi-empirical quantum mechanical method PM3. The crystal coordinates of 5-lipoxygenase (pdb ID 3V99) were downloaded from protein data bank, carrying arachidonic acid as ligand in its binding site. 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 docking programme implements an efficient grid based docking algorithm which approximates an exhaustive search within the free volume of the binding site cavity. The conformational space was explored by the geometry optimization of the flexible ligand (rings were 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 (docking score in Kcal/mol). H-bond and hydrophobic interactions between the compound and enzyme were explored.

Conflict of interest

The authors declare they have no conflict of interests.

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Supplementary data

Supplementary data (experimental data, NMR spectra, mass spectra of compounds and docking structures) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.01.027.

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