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2-Amino-4-aryl thiazole: a promising scaffold identified as a potent 5-LOX inhibitor†

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Human 5-lipoxygenase (5-LOX) is an important enzyme in the biosynthesis of leukotrienes and is a target for asthma and allergy treatment. Zileuton is the only drug currently marketed that targets this enzyme ($IC_{50} \sim 1$ μM). So, the development of novel lead compounds is highly desirable. A series of 2-aryl indole, thiazolopyrazole acid, oxadiazolobenzothiophene, 1,4-disubstituted-1,2,3-triazole, 2-amino-4-aryl thiazole and 4,4′-(1,4-phenylene)bis(1,3-thiazole) derivatives when tested against this enzyme resulted in the identification of a potent compound (**1d**), *p*-fluoro substituted 2-amino-4-aryl thiazole, with an IC_{50} of ~10 μM. Another lead compound identified is (**4a**), a thiazolopyrazole acid derivative ($IC_{50} \sim 40$ μM). All the compounds exhibit poor DPPH radical scavenging activity which suggests that their action occurs not due to the disruption of the redox cycle of iron present in the enzyme (unlike zileuton) but through competitive inhibition, since the V_{max} remains constant but the K_m increases with an increase in inhibitor concentration. Molecular docking of **1d** and **4a** to the active site of 5-LOX also supports the experimental data, and suggests that their possible mechanism of action is through competitive inhibition. The current study identifies a promising lead molecule which could be improved further to match the activity of the commercial drug.

Introduction

Pain, fever, allergy, swelling, *etc.* are different symptoms of inflammatory diseases suffered by the majority of the human population.[1] Among these, asthma is an inflammatory disease of the respiratory tract affecting the majority of Indians.[2–4] The discovery of the arachidonic acid (AA) metabolic pathway,[5] and the identification of the enzymes involved in it[6] have provided a systematic approach for the development of anti-inflammatory drugs.[7] Leukotrienes are important mediators of inflammatory and allergic diseases.[8,9] 5-LOX is a key enzyme which catalyzes the two-step conversion of AA to leukotriene A4 (LTA4). It catalyzes the hydroperoxidation of polyunsaturated fatty acids.[10] Therefore, it is considered as a potential target in the treatment of asthma, atherosclerosis, prostate cancer, and allergies.[11–15] Different classes of 5-LOX inhibitors have been reported, which include redox, non redox and iron chelating agents (for a review see ref. 16). The only 5-LOX inhibitor which has entered the market is zileuton [*N*-(1-benzo[*b*]thien-2-ylethyl)-*N*-hydroxyurea],[17] an iron chelator which has weak reducing properties. It is used for the treatment of bronchial asthma (with an $IC_{50} = 0.5–1$ μM in stimulated leukocytes). However, weak potency, hepatic toxicity and a short half life are the therapeutic drawbacks of zileuton.[18] So, there is a strong need for the development of safer and efficacious drugs.

Generally, nitrogen and sulphur containing heterocycles have a significant role in the discovery of new drugs because of their physicochemical properties. Various substituted indoles, pyrazole scaffolds, compounds containing a thiazole ring moiety and substituted triazoles have been found to have numerous biological activities including anti-inflammatory activity.[19–25] Recently *N*-1-tosyl indoles with amino acids have been reported as effective 5-LOX inhibitors. Glycine and tryptophan residues at the end of the C-3 substituent have been identified as the most active amongst these compounds.[26] The benzothiophene pharmacophore is found to be a potent 5-LOX inhibitor and the aryl group has been identified to be responsible for inhibition.[27] 3-Amino-1-phenyl-4,5-dihydro-1*H*-pyrazol-5-one has been shown to be a potent human 5-LOX, COX 1 and COX-2 inhibitor.[28] A series of triazole based compounds have been reported as potent dual mPGES-1 and 5-LOX inhibitors. A bulky, halogenated phenylether together with a 2-amino group was found to be very potent for the activity of the compound.[29] In addition, 5-benzylidene-2-phenyl-thiazolinone derivatives have been observed to be effective 5-LOX inhibitors.[30] The 2-amino-5-thiazolyl moiety has been reported to have anti-inflammatory activity in acute carrageenan and chronic formalin induced rat paw edema models, which indicates that aliphatic oxime esters attached *via* a ketone bridge to the 5th position of thiazole is responsible for the activity.[31]

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Based on these observations, a variety of scaffolds including 2-aryl indoles, oxadiazolobenzothiophenes, thiazolopyrazole acid derivatives, 1,4-disubstituted-1,2,3-triazoles and 2-amino-4-aryl thiazoles bearing indole, benzothiophene, pyrazole, triazole and thiazole as a core structure, respectively, were synthesized and tested as possible 5-LOX inhibitors. Also the hitherto unreported oxadiazolobenzothiophenes are synthesised and their activities tested for the first time. Furthermore, in order to elucidate the mechanism of action of these compounds, their antioxidant properties were also determined. The experimental results were rationalized by performing mathematical modelling and docking of the best inhibitor with the target enzyme and were compared with those of zileuton.

Results and discussion

In vitro evaluation of 5-LOX inhibition

The inhibitory activity of 2-amino-4-aryl thiazoles (**1a–1f**), 2-aryl indoles (**2a–2e**), oxadiazolobenzothiophenes (**3a–3j**), thiazolopyrazole acid derivatives (**4a–4d**), 1,4-disubstituted-1,2,3-triazoles (**5a–5c**), and 4,4′-(1,4-phenylene)bis(1,3-thiazole)s (**6a–6f**) (Fig. 1) at a concentration of 50 μM in 2% DMSO against 5-LOX (isolated from recombinant plasmid) were evaluated *in vitro* by measuring the formation of 5-hydroxy eicosatetraenoic acid (5-HETE) from AA (Tables 1–5). Zileuton and MK-886 (a 5-LOX activating protein – FLAP inhibitor which was withdrawn from clinical trials due to high plasma absorption) (Fig. 2) were used as positive controls while DMSO (2%, v/v) was used as a vehicle control. 2%, v/v DMSO (vehicle control) did not show any inhibition of 5-LOX enzyme activity.

SAR studies. Amongst the 2-amino-4-aryl thiazole derivatives (**1a–1f**) tested, compound **1d** with a thiazole ring and an electron withdrawing group (fluoro substitution) at the *p*-position adjacent to the benzene ring is seen to be the most active with an IC_{50} of ~10 μM (Table 1). This activity is found to be closer to that of the standard drug, zileuton (IC_{50} ~ 1 μM) and FLAP inhibitor MK-886 (IC_{50} ~ 2 μM) compared to the other compounds in the series.[32] Interestingly, replacing fluorine with chlorine (**1b**), completely deactivates the compound, which is a less electronegative atom than fluorine. Replacing chlorine with a methyl group (**1c**) increases the activity threefold and with a nitro group (**1e**) fivefold compared to **1b**. When there is no substituent or R = H (**1a**), the compound is totally inactive while the addition of a hydroxy group at the *ortho* position to it (**1f**) increases activity 10–11 fold. Structure activity analysis of compounds (**1a–1e**) indicates that as log *P* of the compound increases, the 5-LOX inhibition decreases (correlation coefficient, $r = -0.6$), indicating that hydrophilic substituents at the *para* position enhance the activity. 2-Aryl indole (Table 2) derivatives (**2a–2e**), irrespective of substitution at the R position (halogen, nitro or hydroxyl group) exhibited poor activity. None of the oxadiazolobenzothiophenes (Table 3) tested showed any reasonable activity (<35%). **3g** with methyl substitution at R^2 and phenyl substitution at R^3 exhibiting the best activity (~32%). The activity was completely lost with hydrogen substitution at R^2 (**3a** > **3b** > **3c**). Potency decreased 5–6 fold in **3d** and **3f** compared to **3g** due to the steric hindrance of the *p*-methoxy phenyl group at the R^3 position. Compound **4a** of the thiazolopyrazole acid derivatives, having a *p*-fluoro phenyl ring in the R^2 position, exhibited good activity of ~70% (Table 4). Comparable activity was observed for compound **4c** in the same series when R^1 attached to the nitrogen of the adjacent benzene ring was substituted by an electron donating ethyl group. Replacement of F with Cl reduced the activity by 20 percent (~45%). Structure activity analysis of compounds (**4a–4d**) indicates that as log *P* of the compound increases, the 5-LOX

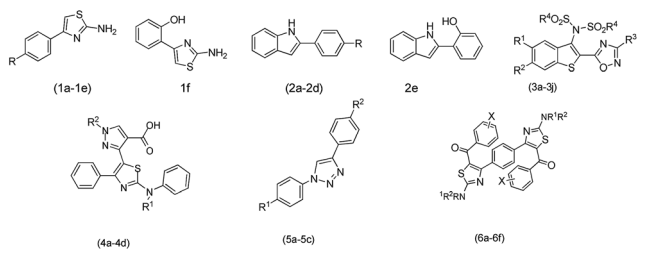


Fig. 1 Chemical structures of 2-amino-4-aryl thiazole derivatives (**1a–1f**), 2-aryl indole derivatives (**2a–2e**), oxadiazolobenzothiophene derivatives (**3a–3j**), thiazolopyrazole acid derivatives (**4a–4d**), 1,4-disubstituted-1,2,3-triazole derivatives (**5a–5c**), and 4,4′-(1,4-phenylene)bis(1,3-thiazole) derivatives (**6a–6f**). R, R^1, R^2, R^3 and R^4 groups are described in (Tables 1–6).

Table 1 The effects of 2-amino-4-aryl thiazole derivatives at 50 μM concentration on % 5-LOX inhibition and antioxidant activity (* at 40 μM conc.) Data are expressed as the means ± S.D of single determinations obtained in three to four independent experiments

S. No.	Compounds	R	% inhibition of 5-LOX ± SD	% scavenging of DPPH ± SD
	Zileuton		97.1 ± 1.3	45.6 ± 2.2
	MK-886		82.6*	35.5 ± 1.1
	Ascorbic acid		—	89.8 ± 0.3
1	**1a**	H	4.4 ± 3.1	11.0 ± 1.4
2	**1b**	Cl	7.3 ± 5.2	18.3 ± 0.1
3	**1c**	CH_3	23.4 ± 7.2	12.1 ± 3.1
4	**1d**	F	84.7 ± 1.0	20.1 ± 0.001
5	**1e**	NO_2	36.5 ± 3.1	9.6 ± 2.1
6	**1f**	H, *o*-OH	47.4 ± 2.1	20.6 ± 0.1

Table 2 Effects of 2-aryl indole derivatives at 50 μM concentration on % 5-LOX inhibition and antioxidant activity. Data are expressed as means ± S.D of single determinations obtained in three to four independent experiments

S. No.	Compounds	R	% inhibition of 5-LOX ± SD	% scavenging of DPPH ± SD
1	**2a**	H	10.2 ± 4.5	10.9 ± 2.4
2	**2b**	Cl	19.1 ± 6.3	11.4 ± 1.0
3	**2c**	F	19.7 ± 5.4	13.8 ± 3.3
4	**2d**	NO_2	8.28 ± 1.8	7.38 ± 4.5
5	**2e**	H, *o*-OH	14.0 ± 8.1	9.5 ± 5.3

Table 3 Effects of oxadiazolobenzothiophene derivatives at 50 μM concentration on % 5-LOX inhibition and antioxidant activity. Data are expressed as means ± S.D of single determinations obtained in three to four independent experiments

S. No.	Compounds	R^1	R^2	R^3	R^4	% inhibition of 5-LOX ± SD	% scavenging of DPPH ± SD
1	**3a**	H	H	C_6H_5	CH_3	25.2 ± 1.4	6.0 ± 2.5
2	**3b**	H	H	C_6H_5	C_2H_5	19.4 ± 1.4	3.6 ± 0.3
3	**3c**	Br	H	C_6H_5	CH_3	16.5 ± 0.9	4.7 ± 2.3
4	**3d**	Br	H	4-$OMeC_6H_5$	CH_3	5.8 ± 0.4	5.2 ± 0.3
5	**3e**	Br	H	4-ClC_6H_5	CH_3	15.2 ± 1.8	5.2 ± 1.2
6	**3f**	H	H	4-$OMeC_6H_5$	CH_3	4.5 ± 1.4	3.8 ± 1.0
7	**3g**	H	CH_3	C_6H_5	CH_3	32.0 ± 0.9	2.6 ± 0.1
8	**3h**	H	CH_3	4-$OMeC_6H_5$	CH_3	13.3 ± 0.9	3.7 ± 1.9
9	**3i**	H	CH_3	4-ClC_6H_5	CH_3	12.3 ± 1.4	3.2 ± 1.6
10	**3j**	H	OCH_3	4-$OMeC_6H_5$	CH_3	10.4 ± 0.4	3.6 ± 0.7

Table 4 Effects of thiazolopyrazole acid derivatives at 50 μM concentration on % 5-LOX inhibition and antioxidant activity. Data are expressed as means ± S.D of single determinations obtained in three to four independent experiments

S. No.	Compounds	R^1	R^2	% inhibition of 5-LOX ± SD	% scavenging of DPPH ± SD
1	**4a**	CH_3	4-F-C_6H_5	69.9 ± 3.8	8.2 ± 5.7
2	**4b**	CH_3	4-Cl-C_6H_5	45.2 ± 3.8	6.1 ± 0.3
3	**4c**	CH_2CH_3	H	65.7 ± 0.1	14.1 ± 8.5
4	**4d**	CH_2CH_3	C_6H_5	41.8 ± 4.8	12.1 ± 12.1

Table 5 Effects of 1,4-disubstituted-1,2,3-triazole derivatives at 50 μM concentration on % 5-LOX inhibition and antioxidant activity. Data are expressed as means ± S.D of single determinations obtained in three to four independent experiments

S. No.	Compounds	R^1	R^2	% inhibition of 5-LOX ± SD	% scavenging of DPPH ± SD
1	**5a**	Br	H	25.4 ± 0.9	12.0 ± 0.001
2	**5b**	Cl	H	24.1 ± 0.9	11.5 ± 4.8
3	**5c**	F	H	48.9 ± 1.3	13.3 ± 2.9

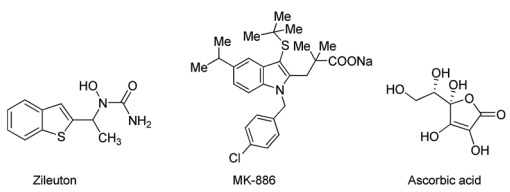


Fig. 2 Chemical structures of zileuton, MK-886 and ascorbic acid.

inhibition decreases (correlation coefficient, $r = -0.7$). A 1,4-disubstituted-1,2,3-triazole with a fluoro group, **5c**, exhibited 49% activity (Table 5). Replacement of F with Br or Cl reduced the activity by half (~25%). Of the compounds in the bisthiazole series (Table 6), the best compounds **6d** and **6f** share the same activity of around 59% with a nitro group at the X position irrespective of substitution at the NHR^1R^2 position. There is a reduction in activity when the nitro is replaced with H at the X position and *N*-methylaniline is replaced with *N*-ethylaniline at the NHR^1R^2 position (**6e**), compared to **6d** and **6f**. The activity is reduced by half when nitro is replaced with chloro at the X position and aniline is added at the NHR^1R^2 position (**6b**). So it appears that an electron withdrawing group (such as NO_2) is essential for good activity.

The V_{max} and K_m values for the control are 0.99 ± 0.01 nmol min^{-1} and 0.25 ± 0.04 μM, respectively. In the presence of 10 and 50 μM of compound **1d**, V_{max} almost remained the same at 0.90 ± 0.03 and 0.85 ± 0.11 nmol min^{-1} and the K_m of the enzyme increased to 0.38 ± 0.013 and 2.03 ± 0.12 μM, respectively. Similarly, the V_{max} and K_m values for the control are 1.21 ± 0.03 nmol min^{-1} and 0.10 ± 0.05 μM, respectively. In the presence of 10 and 50 μM of compound **4a**, V_{max} almost remained the same at 1.05 ± 0.03 and 1.33 ± 0.3 nmol min^{-1} and the K_m of the enzyme increased to 0.40 ± 0.09 and 3.44 ± 0.35 μM, respectively.

In vitro evaluation of antioxidant activity

Studies have suggested that most LOX inhibitors are also antioxidants or free radical scavengers.[33] Compounds which inhibit LOX and have the ability to reduce Fe^{3+} at the active site to the catalytically inactive Fe^{2+} in the enzyme are closely related.[34,35] LOXs contain a "non-heme" iron in the enzyme active site as Fe^{2+} in the native form and Fe^{3+} in the activated state. Redox inhibitors reduce Fe^{3+} to inactive Fe^{2+}. Iron chelating agents bind to the ferric iron and block the binding ability of AA without changing the iron state.[36]

The results show that all the compounds show poor antioxidant activity as measured by DPPH assay. Ascorbic acid exhibited

Table 6 Effects of 4,4′-(1,4-phenylene)bis(1,3-thiazole) derivatives at 50 μM concentration on % 5-LOX inhibition and antioxidant activity. Data are expressed as means ± S.D of single determinations obtained in three to four independent experiments

S. No.	Compounds	NHR^1R^2	X	% inhibition of 5-LOX ± SD	% scavenging of DPPH ± SD
1	**6a**	*N*,*N*-Dimethylamine	Cl	35.4 ± 2.6	2.8 ± 1.0
2	**6b**	Aniline	Cl	29.1 ± 1.7	2.2 ± 0.5
3	**6c**	*n*-Butylamine	Cl	42.6 ± 1.3	2.8 ± 0.5
4	**6d**	*N*-Methylaniline	NO_2	58.6 ± 1.7	3.6 ± 1.4
5	**6e**	*N*-Ethylaniline	H	52.0 ± 4.8	2.5 ± 1.8
6	**6f**	Pyrrolidine	NO_2	58.6 ± 5.3	2.8 ± 0.2

89.8% activity while zileuton and MK-886 showed 45.6% and 35.5% radical scavenging activity, respectively.

Molecular docking studies

To support the *in vitro* anti-inflammatory results exerted by **1d** and **4a**, they are docked to the active site of 5-LOX (Fig. 3 & 4) and compared with the results for zileuton (Fig. 5).

It is observed that in compound **1d**, the sulfur of the thiazole ring and the nitrogen of the amino group attached to the thiazole ring both form hydrogen bonds with the backbone oxygen of amino acid Ala606 in the active site of 5-LOX with a binding score of −4.78 kcal mol^{-1} (Fig. 3). However, the second best compound, **4a**, with a better binding score of −6.92 kcal mol^{-1} exhibits one H-bond between the nitrogen of its pyrazole ring

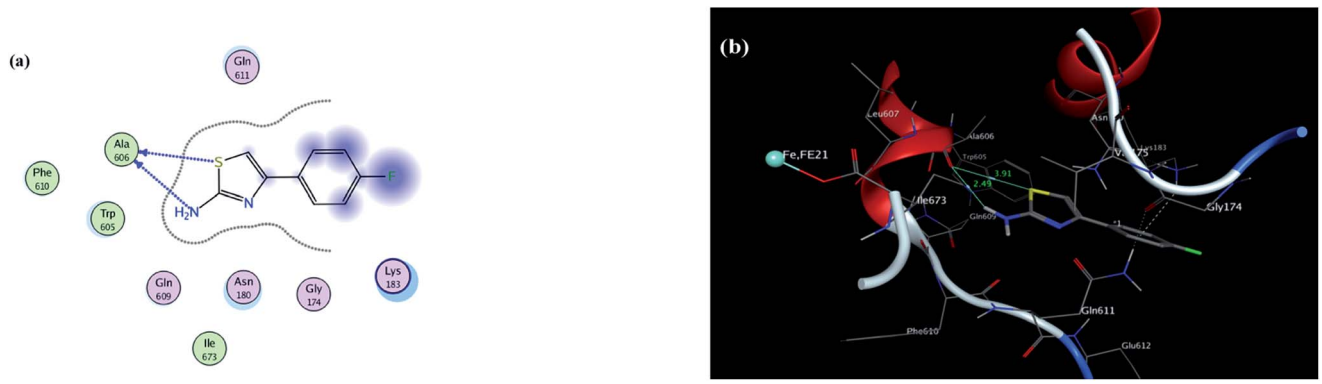


Fig. 3 Binding of compound **1d** with 5-LOX. (a) Ligplot showing residues involved in the interaction between the amino acids of the enzyme with compound **1d**. (b) Docked pose of compound **1d** in a stick model. Interactions are shown by dotted lines. Colours: carbon: grey, hydrogen: cyan, nitrogen: blue, sulphur: yellow, fluorine: green and oxygen: red.

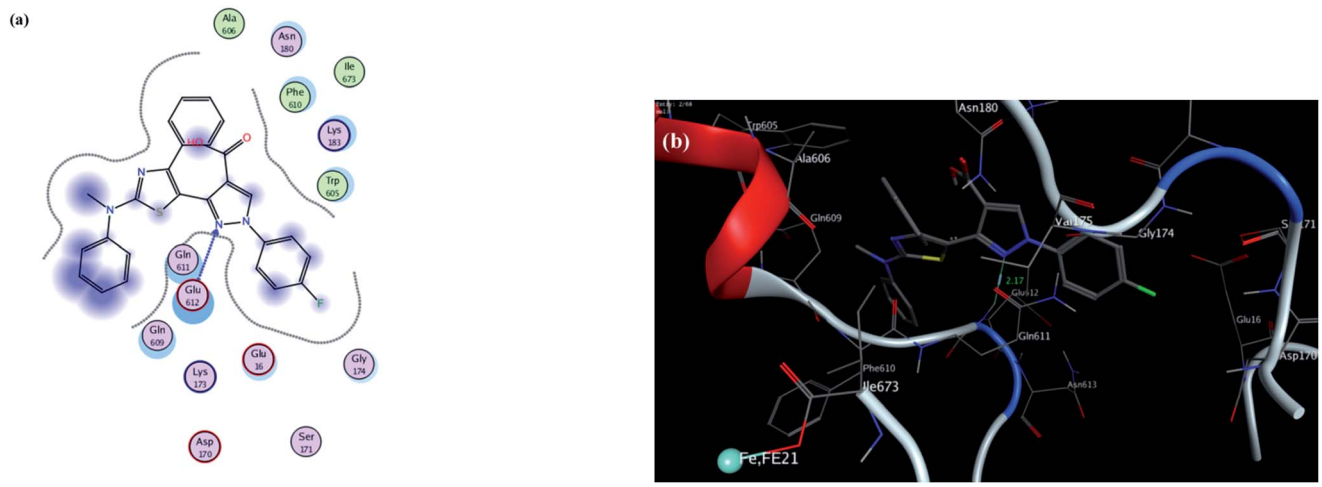


Fig. 4 Binding of compound **4a** with 5-LOX. (a) Ligplot showing residues involved in the interaction between the amino acids of the enzyme with **4a**. (b) Docked pose of **4a** in a stick model. Interactions are shown by dotted lines. Colours: carbon: grey, hydrogen: cyan, nitrogen: blue, sulphur: yellow, fluorine: green and oxygen: red.

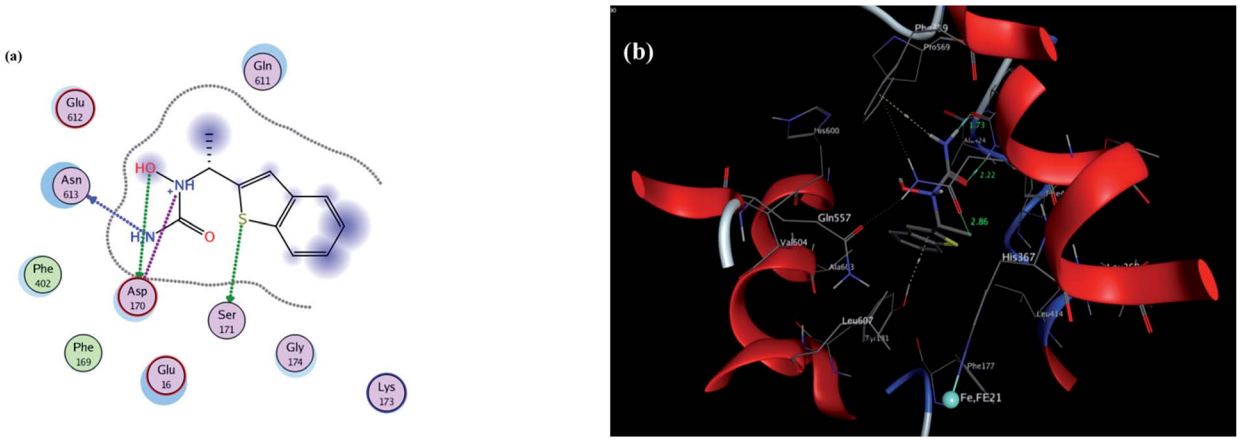


Fig. 5 Binding of zileuton with 5-LOX. (a) Ligplot showing residues involved in the interaction between the amino acids of the enzyme with zileuton. (b) Docked pose of zileuton in a stick model. Interactions are shown by dotted lines. Colours: carbon: grey, hydrogen: cyan, nitrogen: blue, sulphur: yellow, fluorine: green and oxygen: red.

and the amino acid Glu612, and is found to be oriented towards the residues in the binding pocket (Fig. 4). In comparison, the binding score of zileuton is -4.93 kcal mol^{-1} (Fig. 5). It is found that the nitrogen in the amino group of the ligand forms a H-bond with the backbone oxygen of residue Asn613. Another H-bond is seen between the oxygen atom of the ligand hydroxyl group and the sidechain of amino acid Asp170. One more H-bond is formed between the sulfur of the thiophene ring of zileuton and the side chain of amino acid Ser171. Also of importance is the metal contact between the nitrogen of the *N*-hydroxy urea group present in zileuton and iron in the enzyme (Fig. 5) which is responsible for electron transfer and thus the inhibition of the enzyme. The molecular docking results indicate that the mechanism of action of **1d** and **4a** is not similar to that of zileuton.

Insight into the potential mechanism of action

5-LOX inhibitors can be generally classified into three main categories: (1) redox (reductive or electron transfer type), (2) non redox (competitive or mixed inhibitors) and (3) iron chelators (iron ligands).[37,38] Several inhibitors have been reported in these classes such as zileuton, nordihydroguaiaretic acid (NDGA), and atreleuton as chelative and reductive; and setileuton (MK-0633) and PF-4191834 as competitive and non reductive inhibitors (Fig. 6).[39–41]

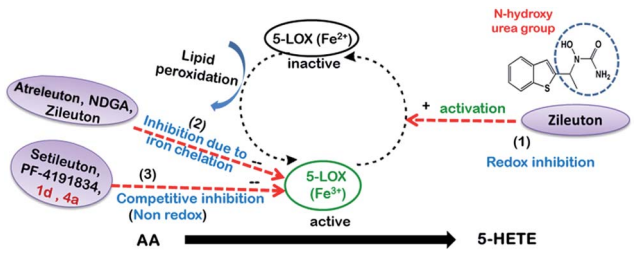


Fig. 6 Classification of 5-LOX inhibitors based on their mechanism. Commercial drugs and those in clinical trials are marked in black and our best inhibitors marked in red.

Nevertheless, zileuton is the only approved drug to emerge against the 5-LOX enzyme.[17] It is known to be a metal ion chelator and it also inhibits 5-LOX *via* the reduction of ferric iron (active state) to ferrous (inactive state) and is classed as a redox inhibitor.[29] However, the transfer of an electron from zileuton to the ferric iron of the enzyme does not occur in isolation, and electron transfer in its redox cycle is carried out through the *N*-hydroxyurea group present, which ultimately generates unstable intracellular lipid alkoxide and thiol radicals.[42]

Here from experimental data, it is visible that compounds **1d** (~85%) and **4a** (~70%) show very good 5-LOX inhibition while they exhibit poor DPPH radical scavenging activities (20% and 8% respectively). At the same time, docking studies indicate that they bind and interact with active site amino acids, thus blocking the substrate binding site. Poor antioxidant activity indicates that these compounds are not acting by reducing ferric iron. In contrast, zileuton has marvellous 5-LOX inhibition (~97%) and also possesses radical scavenging activity (~45%) which indicates that it acts by reducing the ferric iron and thus it is classified as a redox inhibitor. In addition, this study is also supported by docking studies which highlight that the *N*-hydroxy urea group is involved in the reduction of ferric iron.

Simultaneously, Lineweaver–Burke plots for both the compounds show that V_{max} remains almost the same and K_m value increases when the concentrations of the compounds are increased, suggesting them to be competitive inhibitors towards the 5-LOX activity of the protein.

From the experiments, binding energies in terms of Gibbs free energy for compounds **1d** and **4a** are calculated, where the value for the control is 3419.0 ± 0.03 J mol^{-1} which decreased to 2395.8 ± 0.02 J mol^{-1} in the presence of 10 μM and further to a negative value of -1754.12 ± 0.65 J mol^{-1} in the presence of 50 μM of inhibitor **1d**. For compound **4a** the value for the control is 5723.2 ± 0.04 J mol^{-1} which decreased to 2254.6 ± 0.06 J mol^{-1} in the presence of 10 μM and further to a negative value of -3047.4 ± 0.32 J mol^{-1} in the presence of 50 μM of inhibitor **4a**. The decreased and negative values of Gibbs free

energy indicate that the compound at higher concentrations has better binding affinity and stability with the protein 5-LOX.

Conclusions

The present study shows that among six different scaffolds tested, a 2-amino-4-aryl thiazole derivative with fluorine substitution (**1d**) possesses the highest anti-inflammatory activity (IC_{50} = 10 μM) followed by a thiazolopyrazole acid derivative (**4a** with IC_{50} = 40 μM). Both the compounds have electron withdrawing *p*-fluoro substitution on the phenyl ring. Moreover, they do not posses hydroxyl radical scavenging activity and hence exhibit poor antioxidant activity. So, their possible mechanism of action against this enzyme may be through competitive inhibition and non redox type (unlike zileuton). Indeed, the hepatotoxicity of zileuton is perhaps related to its mechanism of action, particularly to the *N*-hydroxyurea moiety which is a known toxicant, and it acts *via* the free radical-mediated lipid peroxidation of cell membranes.[43] It takes part in electron transfer in the redox cycle resulting in the elevation of serum liver enzyme.[44] In addition, the thiophene moiety which forms chemically reactive metabolites in liver is an alternative reason for its hepatotoxicity.[45,46] So, efforts are made to focus on the development of non redox type inhibitors for 5-LOX. Here, in the present work, we have identified a scaffold exhibiting a non redox type of inhibition which could be a significant contribution to the development of 5-LOX inhibitors.

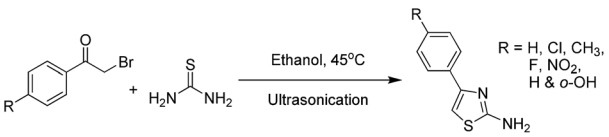
So, it can be concluded that the poor antioxidant activity but good anti-inflammatory activity of compounds **1d** and **4a** indicates that their possible mechanism of action is non redox type. Constant V_{max} and increasing K_m with an increase in inhibitor concentration indicates that they probably act by blocking the substrate binding site thereby possibly acting as a competitive inhibitor. In contrast, zileuton acts by non competitive (redox and iron chelation) inhibition. The docking studies also support these experimental observations. By further optimizing the structure and SAR of compound **1d**, it may be possible to achieve activity comparable or better than that of the commercial drug zileuton (which exhibits an IC_{50} of ~ 1 μM). Also, based on our studies on the other four scaffolds which exhibit poor 5-LOX inhibition, we wish to conclude that further studies and SAR in this direction may not bear useful leads.

Experimental section

Dimethyl sulfoxide (DMSO) was purchased from Merck, USA. Luria–Bertani (LB) medium was purchased from BD, Biosciences USA. AA, Zileuton and MK-886 were obtained from Cayman Chemical (Inalco, Milan, Italy). All other chemicals reported here were purchased from Sigma, St. Louis, USA. Reagents were of analytical grade and were purchased from HiMedia Biosciences, Mumbai, India. Fourier transform infrared (FT-IR) spectra were recorded using a Shimadzu IR Affinity-1 CE model with resolution 4 cm^{-1} and a Perkin-Elmer FT-IR (Spectrum 1000). ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded using a Bruker AMX (400 MHz) spectrophotometer. The solvents used were DMSO-*d*6, $CDCl_3$ and CD_3OD, and tetramethylsilane (TMS) was used as an internal standard. Mass spectra were recorded using an LC-MS instrument. All new compounds characterized gave satisfactory spectral data.

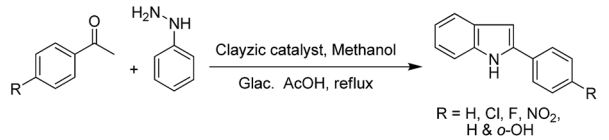
Chemistry

General procedure for the synthesis of 2-amino-4-aryl thiazoles. The 2-amino-4-aryl thiazole derivatives (**1a–1f**) were synthesized as described briefly here (Scheme 1).[47] Ethanolic solution of α-bromoarylethanones (1 mmol) and thiourea (1 mmol) were taken in boiling tubes and ultrasonicated at 45 °C in an ultrasonic bath. The reaction was monitored by TLC every 5 min. It was found that heterocyclisation was completed within 20–35 min. The reaction mixture was allowed to cool, and solvent was evaporated to obtain the products. Recrystallization of the crude products was carried out using ethanol and petroleum ether to obtain pure 2-amino-4-substituted phenyl-1,3-thiazoles.



Scheme 1 General synthesis of 2-amino-4-aryl thiazole derivatives (**1a–1f**).

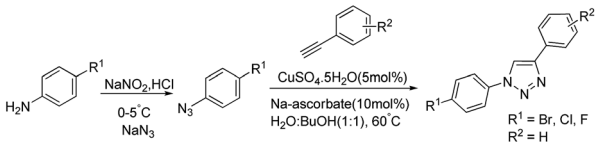
General procedure for the synthesis of 2-aryl indoles. The procedure for the synthesis of 2-aryl indoles (**2a–2e**) is briefly described here (Scheme 2).[48] Equimolar amounts of acetophenone (0.5 mmol) and phenylhydrazine (0.5 mmol) were dissolved in methanol. The mixture was then warmed in a water bath and glacial acetic acid was added until the solution became clear. Clayzic catalyst (200 mg) was added to the above reaction mixture, which was refluxed. The reaction was monitored by TLC using petroleum ether and ethyl acetate (4 : 1). Upon completion of the reaction (in 20 min), it was cooled to room temperature, filtered and poured over crushed ice. The product formed was then extracted with dichloromethane, rotary evaporated and further purified by column chromatography using petroleum ether and ethyl acetate (90 : 10).



Scheme 2 General synthesis of 2-aryl indole derivatives (**2a–2e**).

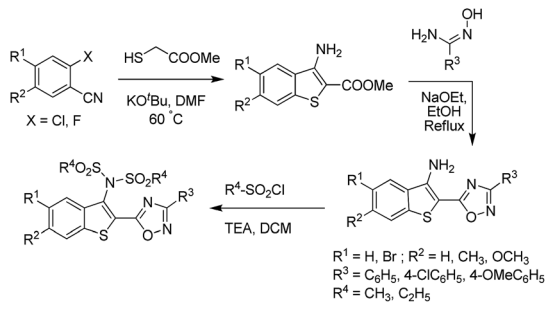
General procedure for the synthesis of 1,4-disubstituted-1,2,3-triazoles. Various 1,4-disubstituted-1,2,3-triazoles (**5a–5c**)

were synthesized by click chemistry reactions involving Huisgen [1,3] dipolar cycloaddition between azides and acetylenes to form triazoles with some modifications to the procedure (Scheme 3).[49]



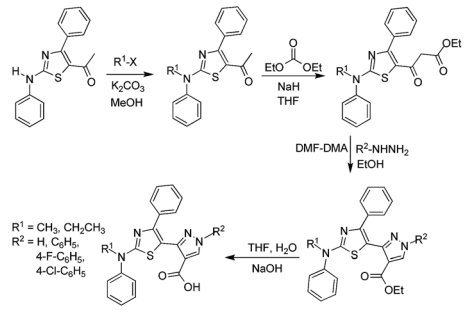
Scheme 3 General synthesis of 1,4-disubstituted-1,2,3-triazole derivatives (**5a–5c**).

General procedure for the synthesis of oxadiazolobenzo thiophenes. The procedure for the synthesis of 2-(5-substituted-[1,2,4]-oxadiazol-5-yl)-benzo[*b*]thiophen-3-yl bis sulfonamide derivatives (**3a–3j**) is briefly described here (Scheme 4). 3-Amino-substituted benzo[*b*]thiophene-2-carboxylic acid methyl esters were prepared from the reaction between *o*-halonitriles (1.0 eq.) and methyl 2-mercaptoacetate (1.0 eq.) in the presence of potassium *t*-butoxide (1.1 eq.) as a base in *N*,*N*-dimethylformamide at 80 °C for 3 h. The (*E*)-*N*′-hydroxy-benzimidamides were prepared from the reaction of the nitriles and hydroxylamine in ethanol at reflux for 2 h. The first reaction step product (1.0 eq.) was treated with different (*E*)-*N*′-hydroxy-benzimidamide (1.1 eq.) in ethanol with sodiumethoxide as base at 80 °C to get substituted-2-(3-substituted-1,2,4-oxadiazol-5-yl) benzo[*b*]thiophen-3-amines. These compounds were further treated with sulfonyl chloride (2.5 eq.) in dichloromethane and triethylamine as a base to get the 2-(5-substituted-[1,2,4]-oxadiazol-5-yl)-benzo[*b*]thiophen-3-yl bis sulfonamide title compounds.



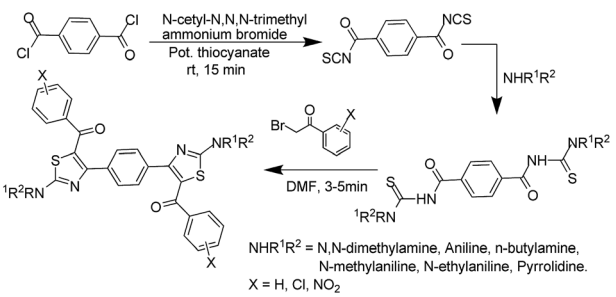
Scheme 4 General synthesis of oxadiazolobenzothiophene derivatives (**3a–3j**). These are novel compounds reported for the first time. Methodology and the spectral data are given in the ESI.†

General procedure for the synthesis of thiazolopyrazole acids. The procedure for the synthesis of pyrazol-3-yl thiazole 4-carboxylic acid derivatives (**4a–4d**) is described *as per* our previously reported methodology[50] with some modifications (Scheme 5). Pyrazole carboxylates (0.246 mmol), (synthesized *as per* the previously reported method) were dissolved in THF : water (2 ml : 1 ml), sodium hydroxide (0.370 mmol) was added and stirred at room temperature overnight. The reaction mixture after reaction completion was neutralized with citric acid and extracted with ethyl acetate (10 ml × 2). This ethyl acetate layer was washed with water (10 ml) and brine (10 ml). Then, the organic layer was separated, dried over sodium sulfate and concentrated to get the compounds.



Scheme 5 General synthesis of thiazolopyrazole acid derivatives (**4a–4d**).

General procedure for the synthesis of 4,4′-(1,4-phenylene) bis(1,3-thiazole)s. The 4,4′-(1,4-phenylene)bis(1,3-thiazole) derivatives (**6a–6f**) were synthesized as described briefly here (Scheme 6) *as per* our previously reported method.[51] The bis-thiazoles were obtained by treating substituted *N*,*N*′-bis(amino-carbonothioyl)terephthalamides, synthesized by the reaction of terephthaloyl chloride and potassium thiocyanate, with the appropriate *N*,*N*′-diaryl/alkyl amines and further heterocyclisation with the corresponding phenacyl bromides. Thus, the 4,4′-(1,4-phenylene)bis(1,3-thiazole)s[52] were synthesized by the condensation of *N*,*N*′-bis(amino-carbonothioyl)terephthalamides with the appropriate phenacylbromides in DMF.



Scheme 6 General synthesis of 4,4′-(1,4-phenylene)bis(1,3-thiazole) derivatives (**6a–6f**).

Biology

Expression and purification of human 5-LOX from *Escherichia coli* (*E. coli*). Recombinant human 5-LOX in a pT3 plasmid (a kind gift from Prof. Olof Rådmark, Karolinska Institute, Stockholm, Sweden) was expressed in *E. coli* BL21 bacteria.[53] A bacterial culture was grown at 37 °C overnight with shaking in LB medium (150 μg ml^{-1} ampicillin) to an OD_{600} above 0.5. The

expression of 5-LOX was induced with 0.5 mM of isopropyl-D-thiogalactopyranoside and the culture was cooled to 18 °C with shaking for overnight growth. Cells were then pelleted out by centrifugation at 5000 rpm and 4 °C for 15 minutes. Cell pellets were resuspended in a chilled buffer at pH 8.0, containing 50 mM triethanolamine HCl, 5 mM EDTA, 60 μg ml^{-1} trypsin inhibitor, 1 mM PMSF and 500 μg ml^{-1} lysozyme, with 30 minutes incubation in ice and then sonicated for 42 seconds. The cell lysate was centrifuged at 19 000 × *g* and 4 °C for 15 minutes. Ammonium sulfate was added to the supernatant (50% w/v) which was stirred in ice for 45 minutes, centrifuged at 16 000 × *g* and 4 °C for 30 minutes and aliquoted. Semi-purified 5-LOX was immediately used for activity assays.

5-LOX enzyme activity assay. Aliquots of semi-purified 5-LOX prepared as described were added to a mixture containing 25 mM of HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (pH 7.3), 0.4 M of ethylenediaminetetraacetic acid (EDTA), 10 mM of $CaCl_2$, and 4 mM of ATP at 4 °C. The total activity of the enzyme was measured by lipoxygenase reaction using UV absorbance at a λ_{max} of 236 nm with 100 μl of final reaction mixture containing 96.75 μl of assay mixture, 2 μl of DMSO (2% in final mixture), and 1.25 μl of the substrate (30 μM of AA). The test compounds dissolved in DMSO were added to the reaction mixture to a final concentration of 50 μM and the 5-LOX reaction was initiated by the addition of the substrate AA (30 μM). The activity was determined by measuring the conversion of AA to the product 5-HETE in terms of OD at λ_{236}. Percentage inhibition was calculated by comparing 5-LOX activity in the presence and absence of the inhibitor. Commercial drug zileuton and FLAP inhibitor MK-886 were used as positive controls. Each assay was repeated thrice. We compared the IC_{50} values of our two highly potent 5-LOX inhibitors to the IC_{50} of zileuton experimentally and with the literature. Zileuton displayed an IC_{50} value of ~1 μM, which is in good agreement with the literature value of 0.5 μM.[54,55]

Assay for mechanism of action. The 5-LOX activity of the protein in terms of 5-HETE produced was measured for between 1 and 30 μM of AA in the presence of 10 and 50 μM of compounds **1d** or **4a** in the same way as the activity assay with UV absorbance at a λ_{max} of 236 nm. The mechanism of inhibition was determined from a Lineweaver–Burke plot. Further, the binding energy of the substrate to the protein in terms of Gibbs free energy was estimated from the equilibrium constants for the control and in the presence of inhibitor using the formula:[56,57]

$$\Delta G^{\circ} = -RT \ln K_{eq}$$

where ΔG° = Gibbs free energy, R = gas constant, T = absolute temperature, and K_{eq} = equilibrium constant.

DPPH assay for antioxidant activity. The antioxidant activities of all the compounds were determined using the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). This is an indication of the radical scavenging ability of the compounds.[36] A solution of DPPH (0.10 mM) in methanol (HPLC grade) was prepared. Then, 4 μl of the test samples (2.5 mM) was mixed with 196 μl of the DPPH (0.10 mM) solution and the mixture was incubated for 30 min at room temperature in darkness. The absorbance was measured at 517 nm using a Multimode Plate reader (Enspire Perkin Elmer, version 4.10.3005.1440). Ascorbic acid was used as a reference compound. DPPH scavenging percentage was calculated using the formula:

$$\% \text{ inhibition} = \frac{(A_0 - A_s)}{A_0} \times 100$$

where A_0 = absorbance of the control (blank, without sample) and A_s = absorbance of the test samples. All tests were performed in triplicate.

Molecular modeling studies. Docking experiments were performed using Molecular Operating Environment (MOE; version 2011.10, Chemical Computing Group, Suite 910, Canada) to determine the binding free energies of ligands with different poses. The X-ray crystal structure of 5-LOX was obtained from the Brookhaven Protein Data Bank (http://www.rcsb.org/pdb). The protein target (PDB Code 3O8Y) was selected from the PDB database and all the heteroatoms (*i.e.*, nonreceptor atoms such as water, ions, *etc.*) were removed. Polar hydrogens were added and Kollmann charges were assigned using forcefield AMBER99; also the energy of the protein was minimized by using AMBER99 force field. The structures of the ligands were built in MOE and their energies were minimized using MMFF94x force field. The ligands were then docked into the protein for the best docking pose and its binding free energy. LondondG was used as the first and GBVI/WSAdG as the second rescoring methodology. The binding energies of the top 100 conformations were computed. The more negative the binding score, the more favorable the binding of the ligand to the protein was.

Acknowledgements

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