Chem Biol Drug Des 2016; **87:** 508–516

Research Article

Development of 2-(Substituted Benzylamino)-4-Methyl-1, 3-Thiazole-5-Carboxylic Acid Derivatives as Xanthine Oxidase Inhibitors and Free Radical Scavengers

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A series of 2-(substituted benzylamino)-4-methylthiazole-5-carboxylic acid was designed and synthesized as structural analogue of febuxostat. A methylene amine spacer was incorporated between the phenyl ring and thiazole ring in contrast to febuxostat in which the phenyl ring was directly linked with the thiazole moiety. The purpose of incorporating methylene amine was to provide a heteroatom which is expected to favour hydrogen bonding within the active site residues of the enzyme xanthine oxidase. The structure of all the compounds was established by the combined use of FT-IR, NMR and MS spectral data. All the compounds were screened *in vitro* for their ability to inhibit the enzyme xanthine oxidase as per the reported procedure along with DPPH free radical scavenging assay. Compounds 5j, 5k and 5l demonstrated satisfactory potent xanthine oxidase inhibitory activities with IC_{50} values, 3.6, 8.1 and 9.9 μM, respectively, whereas compounds 5k, 5n and 5p demonstrated moderate antioxidant activities having IC_{50} 15.3, 17.6 and 19.6 μM, respectively, along with xanthine oxidase inhibitory activity. Compound 5k showed moderate xanthine oxidase inhibitory activity as compared with febuxostat along with antioxidant activity. All the compounds were also studied for their binding affinity in active site of enzyme (PDB ID-1N5X).

Key words: febuxostat, free radical, methylene amine, molecular docking, xanthine oxidase

Received 17 June 2015, revised 19 October 2015 and accepted for publication 25 October 2015

Xanthine oxidase (XO, E.C. 1.1.3.22) is a complex metalloflavoprotein in the purine scavenging pathway which catalyses the hydroxylation of hypoxanthine to xanthine and xanthine to uric acid (UA) with simultaneous production of hydrogen peroxide and super oxide anions (1–3). In last two steps of biosynthesis process, excessive production of uric acid ultimately leads to the deposition of monosodium hydrogen urate crystals in the joints of humans which may lead to the hyperuricemic condition called gout (4,5). The reduction of plasma levels of UA in blood can prevent gout, which is possible with XO inhibitors that block the production of UA from purine metabolism (6). There is an irresistible acceptance that XO serum levels are increased in several pathological conditions like hepatitis, inflammation, ischaemia-reperfusion, cancer and ageing. Thus, the selective inhibition of XO may outcome for the broad spectrum therapeutics like gout, cancer, inflammation, oxidative damage, cardiovascular and ischaemic-reperfusion therapy (7,8). Allopurinol (**1**) a known XO inhibitor and an analogue of hypoxanthine is widely used in treatment of gout for last many years. However, in some cases, severe life-threatening side-effects have been reported which include a toxicity syndrome dramatized by eosinophilia, vasculitis, rash hepatitis and progressive renal failure due to purine backbone of allopurinol and its analogs (9,10). Therefore, there is an immense need of developing non-purine alternatives to allopurinol with potent XO inhibitory activity and possessing fewer adverse effects. In the year 2009, USFDA approved Febuxostat (**2**), a thiazole derivative, since then multitudinous non-purine inhibitors have attracted worldwide attention. Outstanding potency and associated toxicology profile was observed during clinical trials, and diverse febuxostat analogues have been designed (11,12). Recently, in June 2013 in Japan one of the drugs topiroxostat (**3**) (FYX-051) was approved for the treatment of gout and hyperuricemia (13,14).

In addition, a somewhat higher incidence of toxicological profile was observed in clinical study of above described drugs along with large amount of superoxide anion generation by XO involved in the various pathological conditions. Development of XO inhibitors with free radical scavengers potential may prove to be promising agents to treat Gout. This encouraged the researchers to develop structurally diverse molecules without purine backbone with promising XO inhibitory activity such as N-(1,3-diaryl-3-oxopropyl)

 doi: 10.1111/cbdd.12686

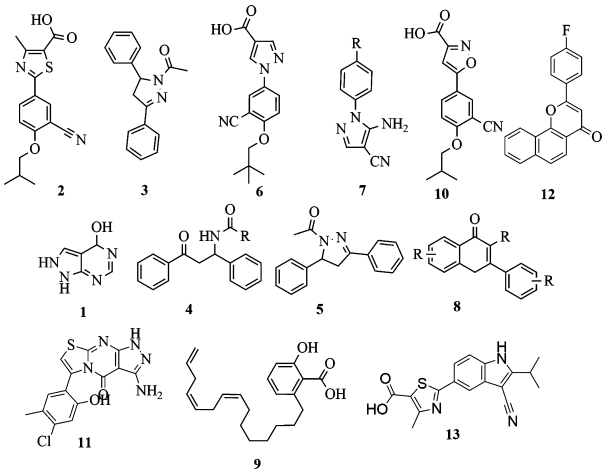

Figure 1: Chemical structures of reported xanthine oxidase inhibitors.

amides (**4**) (15),1-acetyl-3,5-diaryl-4,5-di-hydrol(1H) pyrazoles (**5**) (16)**,** Y-700 (**6**) (12), N-aryl-5-amino-4-cyanopyrazole **(7)** (17), flavonoids (**8**) (18), anacardic acid (**9**) (19), thiazolo-pyrazolyl 5-phenylisoxazole-3-carboxylic acid (**10**) (20), naphthoflavones (**11**) (21), and pyrazolo[3,4-d] thiazolo[3,2-a]pyrimidin-4-one (**12**) (22), 2-(3-cyano-2-isopropyl-1H-indol-5-yl)-4-methylthiazole-5-carboxylic acid (**13**) (23), derivatives (Figure 1). It was assumed worthwhile to rationally design with pharmacophoric approaches to develop structural analogous of febuxostat as non-purine XO inhibitors with antioxidant activity for diminishing the associated toxicities. In this article, we describe the synthesis and evaluation of 2-(benzylamino)-4-methyl-1,3-thiazole-5-carboxylic acid derivatives (Figure 2) as potent XO inhibitors and free radical scavengers.

Experimental

Materials and Method

All the chemicals and reagent used were of LR grade and purchased from S.D. fine Chemicals, Merk India, CDH Spectrochem, etc. All reactions were monitored, and the purity of the compounds was checked by thin layer chromatography (TLC) using precoated silica gel G plates at 254 nm under UV lamp/iodine vapours using toluene/ethyl acetate/formic acid or benzene/acetone as eluent. Melting points were determined by the open capillary method with electric melting point apparatus and are uncorrected. IR spectra were recorded on Bruker FT-IR spectrophotometer, and ^{1}H and ^{13}C-NMR spectra were recorded on Bruker DPX 400 MHz spectrophotometer (Bruker Bio Spin Corporation, Billerica, MA, USA) using DMSO-d_6 or $CDCl_3$ as solvent. Mass spectra (ESI-MS) were obtained by JEOL-AccuTOF JMS-T100LS mass spectrometer (JEOL USA, Inc., Peabody, MA, USA), and elemental analysis was performed on a Vario-EL III CHNOS. Elemental analysis was ±0.4%, that is within the theoretical values.

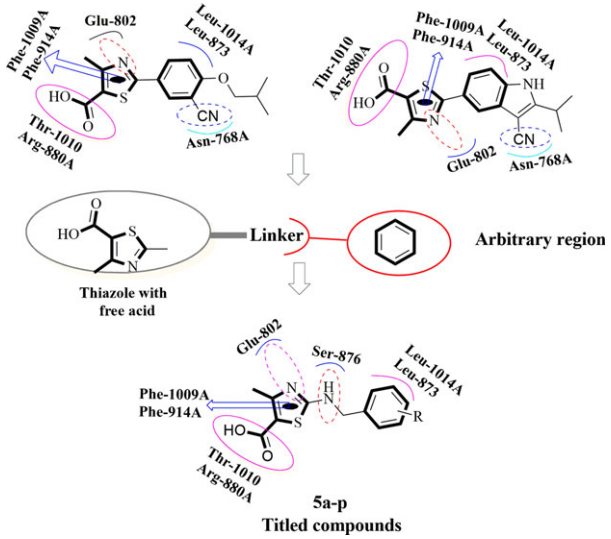

Figure 2: Chemical modification of febuxostat and 2–(3–cyano–2–isopropylindol–5–yl)–4–methylthiazole–5–carboxylic acid for development of 2–(substituted benzylamino)–4–methyl–1,3–thiazole–5– carboxylic acid derivatives (**5a**–**p**) showing similarity in binding interaction within the active site of xanthine oxidase.

Synthesis of ethyl 2-amino-4-methyl-1,3-thiazole-5-carboxylate (3) (24)

To a mixture of anhydrous ethanol (100 mL) and thiourea (0.098 mole), ethyl-2-chloro acetoacetate (0.097 mole) was added drop-wise under constant stirring at room temperature. Once addition of ethyl-2-chloro acetoacetate is completed, reaction mixture was heated at 70–80 °C for 15 min. Then reaction mass was cooled to room temperature, and the solid precipitate was isolated, washed with 100 mL of cold ethanol and further washed with saturated sodium bicarbonate solution to obtain white solid which was finally dried under vacuum at 50 °C for 8 h.

Yield: 91%; m.p.: 170–174 °C; IR (KBr *v* max): 3222, 3051,1598, 1415, 1299, 698 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6); δ 1.36 (s, 3H, J = 7.2 Hz, CH_3), 2.62 (s, 3H, CH_3), 4.32 (s, 2H, J = 7.2 Hz, CH_2CH_3), 5.09 (bs, 2H, D_2O exchangeable NH_2); ^{13}C-NMR (100 MHz, $CDCl_3$); 13.17 ($CH_2\underline{CH}_3$), 17.43 (thiazole CH_3), 62.41($\underline{CH}_2CH_3$), 117.18, 156.06, 161.43(C=N), 165.55(C=O); ESI-MS: *m/z* 187.07 (M+H); Anal. Calcd. for $C_7H_{10}N_2O_2S$: C,45.15; H, 5.41; N, 15.04%. Found: C, 45.13; H, 5.42; N, 15.09%.

Synthesis of substituted ethyl 2-(benzylamino)-4-methyl-1,3-thiazole-5-carboxylate (4a-p)

To a solution of substituted benzaldehyde (0.001 mole) in 10 mL of methanol, ethyl 2-amino-4-methyl-1,3-thiazole-5-carboxylate **(3)** (0.0012 mole) was added and stirred at room temperature. In the saturated reaction mixture, 50 mg iodine (0.0004 mole) was added with stirring at room temperature till all iodine was dissolved. To the stir-

red solution, 55 mg (0.0014 mole) of sodium borohydride was slowly added, further stirred at room temperature till the completion of reaction. A solid precipitate obtained was filtered, washed with water, dried and further recrystallized with ethanol to give solid products **(4a-p)** (25,26). The progress of the reaction and purity of the compound were checked by TLC, using toluene:ethylacetate:formic acid (5:4:1) as mobile phase.

Ethyl 2-(benzylamino)-4-methyl-1,3-thiazole-5-carboxylate (4a)

Yield: 83%; m.p.: 165–167 °C; IR (KBr *v* max): 3097, 2955, 1701, 1485, 1336, 1029, 789 $cm^{-1;}$ ^{1}H-NMR (400 MHz, DMSO-d_6); δ 1.36 (d, 3H, J = 7.4 Hz, CH_3), 2.69 (s, 3H, CH_3), 4.30 (s, 2H, CH_2), 4.36 (q, 2H, J = 7.4 Hz, CH_2CH_3), 7.26- 7.45 (m, 5H, Ar-H), 9.55 (bs, 1H, D_2O exchangeable NH); ^{13}C-NMR (100 MHz, $CDCl_3$); 13.91 ($CH_2\underline{CH_3}$), 17.26 (thiazole CH_3), 49.97(CH_2NH), 61.01($\underline{CH_2}CH_3$), 115.18, 121.32, 127.17, 129.83, 140.10, 151.52, 162.43(C=N), 165.91(C=O); ESI-MS: *m/z* 277.13 (M+H); Anal. Calcd. for $C_{14}H_{16}N_2O_2S$: C, 60.85; H, 5.84; N, 10.14%. Found: C, 60.89; H, 5.83; N, 10.16%.

Synthesis of substituted 2-(benzylamino)-4-methyl-1,3-thiazole-5-carboxylic acid acetamide derivatives (5a-p) (27)

Substituted ethyl 2-(benzylamino)-4-methyl-1,3-thiazole-5-carboxylate **(4a-p)** (0.075 mole) and potassium carbonate (0.3 mole) were added to a mixture of methanol:water (9:1). The solution was refluxed with stirring until the reaction was completed. The clear solution was cooled and neutralized with glacial acetic acid and stirred for 1 h. Precipitate obtained was filtered, washed with water, dried and recrystallized with ethanol. The progress of the reaction and purity of the compounds were checked by TLC, using benzene:acetone (8:2) as mobile phase.

2-(Benzylamino)-4-methyl-1,3-thiazole-5-carboxylic acid (5a)

Yield: 67%; m.p.:102–104 °C; IR (KBr *v* max): 3220, 3031, 1697, 1599, 1426, 1283, 1029, 809 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$); δ 2.28 (s, 3H, CH_3), 4.36 (bs, 2H, CH_2), 6.17 (bs, 1H, D_2O exchangeable NH), 7.06–7.33 (m, 5H, Ar-H), 10.06 (bs, 1H, COOH); ^{13}C-NMR (100 MHz, $CDCl_3$); 17.36 (thiazole CH_3), 48.38 (CH_2NH), 101.67, 118.55, 123.03, 129.47, 140.55, 148.38, 165.29 (C=O); ESI-MS: *m/z* 248.11 (M+H); Anal. Calcd. for $C_{12}H_{12}N_2O_2S$: C, 58.05; H, 4.87; N, 11.28%. Found: C, 58.11; H, 4.86; N, 11.30%.

In silico *bioactivity study*

All the synthesized compounds were evaluated for their oral bioavailability, physicochemical properties, toxicity and online pharmacological activity by utilizing these online software Osiris Property Explorer, Molinspiration and PASS computational study. Lipophilicity (c-log P), water solubility (c-log S), molecular weight (MW), number of rotatable bonds (NROTB) of Lipinski's rule of five, drug likeness and toxicity were calculated using online Molinspiration property calculation toolkit and online OSIRIS Property explorer. With the help of Osiris Property Explorer software, toxicities were predicted which indicated that the synthesized compounds would be free of mutagenicity, tumorigenicity, reproductive side-effects and irritation (28–33). These all synthesized compounds were also predicted for their pharmacological activity by online PASS computer program (prediction of activity spectra for substances) (34,35).

Molecular docking studies

Molecular docking studies were carried out using Glide XP Docking protocol in SCHRODINGER 9.4 (36,37). Co-crystal structure of xanthine dehydrogenase (XDH) with febuxostat (PDB entry-1N5X) was selected based on superior crystal structure parameters and compared with other XO or XDH structures. There was no difference seen in the binding sites and co-crystal structures of XO and XDH. Using LIGPREP module within Maestro BUILD, ligand was prepared by default setting. The tautomeric forms of ligands were generated at physiological pH which includes *keto* and *enol* forms of ligands. Thirty conformations for each ligand were generated, and lowest energy conformers were used for the docking analysis. Protein was prepared, optimized and minimized by Protein Preparation Wizard using OPLS-2005 force field. Active site for docking was defined as a grid box of dimensions 25 × 25 × 25 $Å^3$ around the centroid of the ligand assuming that the ligands to be docked are of similar size as the co-crystallized ligand. Using Glide XP module, the docking of molecules was carried out with Epik state penalties for different ionizations and tautomeric states. Different docking poses of ligands were generated and analysed for interpretation of final results.

Xanthine oxidase inhibitory activity

Xanthine oxidase (XO) assay of 2-(benzylamino)-4-methyl-1,3-thiazole-5-carboxylic acid derivatives **5a-p** was evaluated using Bovine milk XO (grade 1, ammonium sulphate suspension, purchased by Sigma Aldrich). UV–visible spectrophotometer (EI 2371**)** was used for measuring uric acid formation at 293 nm at 25 °C under aerobic condition. The reaction mixture containing 1 mL xanthine (0.15 mM), 2.5 mL potassium phosphate buffer (50 mM, pH 7.4) and 0.5 mL of XO solution (0.405 U/mL) was incubated for 5 min at 25 °C. Inhibition of XO activity by various inhibitors was measured by following the decrease in the uric acid formation at 293 nM at 25 °C. The blank was prepared without enzyme solution. Febuxostat was used as positive control. The enzyme was preincubated for 5 min, with test compounds ranging from 6.25 to 100 μM (six concentrations in triplicate) dissolved in DMSO (1% v/v) (38,39), and the reaction started by the addition of xanthine. DMSO (1% v/v) did not interfere with the enzyme

activity. All the experiments were performed in triplicate, and values were expressed as mean of three experiments (40).

Xanthine oxidase activity was expressed as percentage inhibition of XO, as:

$$\text{XO Inhibition rate}\,(\%) = \frac{\text{Abs.C} - \text{Abs.S}}{\text{Abs.C}} \times 100,$$

where S and C represent the absorbance's (Abs.) of test compound and control, respectively. The IC_{50} values, that is the μg concentration of inhibitor necessary for 50% inhibition, were determined using GraphPad (Prism) software.

DPPH free radical scavenging assay

The stable 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for the determination of free radical scavenging activity of synthesized 2-(benzylamino)-4-methyl-1,3-thiazole-5-carboxylic acid derivatives **5a-p** by Koleva *et al.* method (41). Different concentrations of test compounds (6.25–100 μM) in methanol were added separately to an equal volume of 100 μM methanolic solution of DPPH, and the reaction mixture was kept at room temperature for 15 min. The absorbance of the reaction mixture was recorded at 515 nm using a UV–visible spectrophotometer. The control sample contained DPPH and methanol excluding test compounds. Ascorbic acid was used as standard. Free radical scavenging (%) activity was calculated using the formula.

$$\text{Free radical scavenging}\,(\%) = \frac{\text{Abs.C} - \text{Abs.S}}{\text{Abs.C}} \times 100,$$

where C represents control and S represents sample reaction mixture as described above in the method. The concentration of test compounds having 50% radical scavenging activity (IC_{50}) was calculated using Graph Pad Software, Inc. (La Jolla, CA, USA).

Results and Discussion

Chemistry

The target compounds, 2-(benzylamino)-4-methyl-1,3-thiazole-5-carboxylic acid derivatives (**5a-p**), were synthesized as outlined in Figure 3. Commercially available 2-chloroethylacetoacetate was cyclized with thiourea to form ethyl 2-amino-4-methyl-1,3-thiazole-5-carboxylate ester (**3**) (24). Subsequently, reaction was carried out by simply stirring 1.0 equivalent of an substituted aldehyde and 1.2 equivalents of ethyl 2-amino-4-methyl-1,3-thiazole-5-carboxylate in methanol in the presence of the $NaBH_4$/I_2. This one-pot procedure involved iodine catalysed *in situ* formation of an imine at room temperature which was ultimately reduced with sodium borohydride. The substituted benzyl amino esters **(4a-p)** were hydrolysed (27) with potassium carbonate (after acidification) to obtain substituted 2-(benzylamino)-4-methyl-1,3-thiazole-5-carboxylic acid derivatives (**5a-p**) as target compounds.

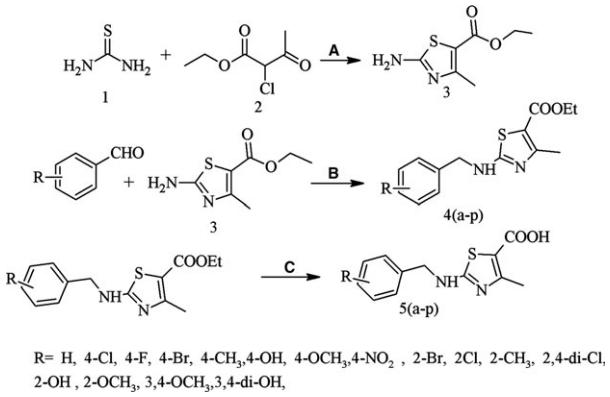

Figure 3: Synthetic scheme for 2–(substituted benzylamino)–4–methyl–1,3–thiazole –5–carboxylic acid derivatives (**5a**–**p**). Reagent and conditions: (A) ethanol, reflux 70 °C (B) $NaBH_4$, iodine and methanol, stirring rt. (C) Potassium carbonate, methanol: water (9:1) stirring with reflux.

All synthesized compounds were characterized by combined use of IR, ^{1}H and ^{13}C-NMR and mass spectroscopy. All spectral values were in accordance with the assumed structures. IR spectral data displayed the presence of carbonyl function of carboxylic acid which appeared as stretching band at 1598 cm^{-1}, N-H stretching band appeared at 3222 cm^{-1}, C-N stretching at 1105 cm^{-1} for compound **5a**. In ^{1}H NMR spectral analysis of compound **4**, the ester proton resonated as a doublet and quartet at δ 1.36 and 4.36 ppm, which disappeared in spectrum of compound **5a,** and a new peak of carboxylic acid was observed at high down-field values at δ 10.06 ppm (bs, 1H) integrating for one proton. The signal due to NH proton was observed at δ 6.17 ppm. The methylene group of -CH_2NH- appeared as broad singlet at δ4.36 ppm. Furthermore, in ^{13}C NMR spectrum of the compound **5a**, signal due to the carbonyl carbon was observed at 165.29 ppm, respectively. No ester signal was found in the spectrum of compounds **5a,** and a new signal for -CH_2NH- appeared at 48.38 ppm. The elucidation of spectral data showed successful synthesis of desired compounds.

In-silico computational studies

Physicochemical properties

For development of XO inhibitors with enhanced pharmacological profile, the prediction of physicochemical properties is the most emphasizing parameter. Increased lipophilicity with poor water solubility is one of the important parameters. Using online Osiris Property explorer

and Molinspiration property calculation toolkit, the drug-likeness characters such as lipophilicity (clogP), water solubility (clogS), molecular weight (MW), number of rotatable bonds (NROTB) and drug-likeness score of Lipinski's rule of five (28–30) were calculated for the targeted synthesized compounds (**5a-p**) (Table 1)**.** The solubility (clogS) of synthesized compounds was found in an acceptable range (<−4). Moreover, the lipophilicity-related clogP quantifying target-oriented drug-likeness properties, drug potency, pharmacokinetics and toxicity analysis has been used for many years. Compounds with clogP value <5 have more favourable drug-likeness profile (31,32). Among the series, all the compounds were found with <5 clogP value which signify their applicability for oral route of administration. Other physicochemical parameters of topological polar surface area (TPSA) were also calculated for observing poorly membrane permeable with less CNS bioavailability which are useful for selecting oral drug candidates. Compounds with TPSA values >60 Å are commonly picked for oral drug molecules (33). In case of present series of synthesized compounds **5a-p,** the TPSA values were found in the range of 62.21–108.04 Å, which impressed further structural optimization for the development of new derivatives. The drug-likeness properties were calculated by Molinspiration property calculation toolkit, and it suggested that compounds having zero or negative values should not be considered as drug-like applicant. In distinction of all synthesized compounds influencing drug-likeness scores >0, all compounds except **5h** and **5i** showed maximum-likeness score in between 1.52 and 5.00 as depicted in Table 1. This online prediction software helped in quickly identifying a set of probable compounds and also useful for screening of compounds for pharmacological activities.

PASS prediction

Potential biological activities of a compound based on a comparison of its chemical structure with a database of available quantitative structure activity relationships (2D QSAR) of over 250 000 compounds exhibiting more than 4000 kinds of biological activities including pharmacological effects, mechanism of action, toxic and adverse effects, interaction with metabolic enzymes and transporters, influence on gene expressions can be predicted by PASS online software (34,35). Keeping in view the features of XOIs, PASS prediction of synthesized compounds was performed to eliminate the synthesized compounds with probability of activity (Pa) <0.400. These compounds were selected for gout treatment which have net probability of activity over inactivity (Pa-Pi), greater than 0.400, which added the evidence in their favour of being active (Table 1). Further it was proved by *in vitro* screening of the synthesized compounds which gave potential drug candidates. The PASS prediction software helped in fastly recognizing a set of probable compounds having much more reliable results of docking. Poorly docked compounds, with either low dock scores or poorly docked poses, were found to be inactive.

Molecular docking studies

Enzyme–inhibitor interaction was exposed by molecular docking studies on XO (PDB entry-1N5X) using Glide XP Docking protocol in SCHRODINGER 9.4 (36,37,42). Molecular docking variations include Vander Waals and electrostatic interactions between active site and ligand which is the basis of force field scoring. Molecular docking studies of synthesized compounds with active site of XO were carried out, and results were compared with febuxostat. The most tightly bound part of febuxostat was its carboxylate

Table 1: *In silico* physicochemical properties for oral bioavailability and bioactivity of the test compounds as evaluated utilizing computational predictive software

Comp. ID	Gout treatment Pa	Pa-Pi	Dock score Kcal/mol	XP score	c LogP	c LogS	H-bond donors	H-bond Acceptors	Mol.Wt.	Rotatable bonds	Drug likeliness	Drug score	TPSA	Toxicity
5a	0.57	0.56	−10.54	−10.66	2.32	−3.22	2	4	248.30	4	2.8	0.85	62.21	NM, NC
5b	0.51	0.51	−11.71	−11.82	2.92	−3.96	2	4	282.75	4	4.54	0.79	62.21	NM, NC
5c	0.50	0.49	−11.71	−11.82	2.42	−3.54	2	4	266.29	4	2.81	0.83	62.21	NM, NC
5d	0.49	0.49	−10.49	−10.60	3.04	−4.06	2	4	327.20	4	1.52	0.69	62.21	NM, NC
5e	0.56	0.56	−10.24	−10.36	2.66	−3.57	2	4	262.33	4	2.07	0.79	62.21	NM, NC
5f	0.54	0.532	−11.71	−11.71	1.97	−2.93	3	4.75	264.30	4	3.64	0.89	82.44	NM, NC
5g	0.49	0.48	−10.52	−10.64	2.25	−3.24	2	4.75	278.33	5	3.89	0.86	71.45	NM, NC
5h	0.48	0.47	−11.59	−11.82	1.39	−3.68	2	5	293.30	5	−6.77	0.42	108.04	SM
5i	0.47	0.47	−10.70	−10.81	3.04	−4.06	2	4	327.20	4	−0.81	0.50	62.21	NM, NC
5j	0.48	0.48	−12.20	−12.31	2.92	−3.96	2	4	282.75	4	3.29	0.78	62.21	NM, NC
5k	0.54	0.54	−12.32	−12.44	2.66	−3.57	2	4	262.33	4	2.68	0.81	62.21	NM, NC
5l	0.47	0.47	−12.24	−12.36	3.53	−4.70	2	4	317.19	4	3.91	0.67	62.21	NM, NC
5m	0.53	0.53	−11.53	−11.64	1.97	−2.93	3	4.75	264.30	4	2.78	0.87	82.44	NM, NC
5n	0.48	0.47	−11.89	−12.00	2.25	−3.24	2	4.75	278.33	5	3.09	0.85	71.45	NM, NC
5o	0.49	0.48	−9.53	−10.05	2.18	−3.26	2	5.50	308.35	6	5.0	0.86	80.68	NM, NC
5p	0.56	0.55	−10.27	−10.27	1.62	−2.63	3	4.75	280.30	4	3.83	0.90	102.67	NM, NC

Pa, Probability of being active; Pi, probability of being inactive; cLogP, lipophilicity; cLogS, water solubility; TPSA, topological polar surface area; NM, non-mutagenic; NC, non-carcinogenic; SM, slightly mutagenic; NP, not predicted.

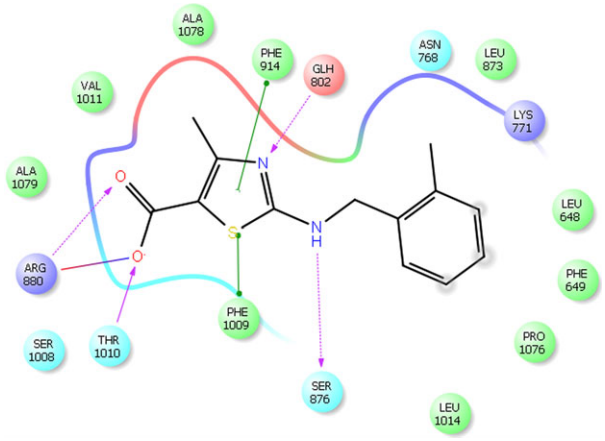

Figure 4: Two-dimensional docking pose of compound **5k** in the active site of xanthine oxidase.

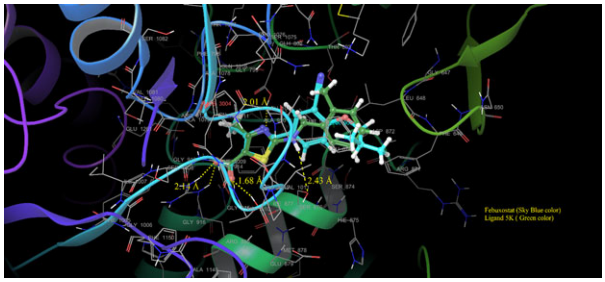

Figure 5: Three-dimensional docking pose of compound **5k** in the active site of xanthine oxidase.

group (43) which was also present in our inhibitors and in docking studies that binds with same amino acids at same places. It showed the binding pattern as the oxygen atoms interacted with side chain of guanidinium group of Arg 880, hydrogen bonds (2.14 Å) to the side chain and hydroxyl with backbone amide of Thr1010 (1.68 Å). The carboxylate group of GlH802 (2.01 Å) was located at N-3 of the thiazole ring, which led to formation of hydrogen bond between the thiazole nitrogen and carboxylate side chain. Whole thiazole ring was inserted between two phenylalanine residues Phe914 (4.00 Å) and Phe1009 (4.85 Å) with PI-PI interaction; these interactions may support the stabilization of the binding positions and also help in substrate recognition. The linker -CH_2NH- introduced in between benzyl and thiazole rings has hydrogen binding pattern with -NH- and amino acid Ser876, which favours the confirmation of expansion of lead molecules. The benzylamino part of synthesized derivatives was introduced in between Leu873 and Leu1014 with Asn768-nitrile bond, and this introduction provided guidance for the alignment of benzylamino part. Hydrophobic amino acids Leu 648, Lys771, Thr803, Leu1014, Asn768, Pro1076, Leu873, Phe1009, Phe 914, Val1011, Ser876, Arg880, Ser1008, Thr1010, Ala1078 and Ala1079 appeared as birdcage (Figure 4) for derivatives **5j, 5k** and **5l** mainly comprising of hydrophobic interactions. The electron donating groups 2-CH_3, 4-OH, 2, 6-di-Cl and 2-OCH_3 were surrounded by amino acids Leu648, Lys 771. Significant docking score of most active compounds was found to be −12.32, −12.24 and −12.20 for derivative **5j, 5k** and **5l,** respectively**,** when compared with febuxostat −12.71 Kcal/mol. According to molecular docking analysis of **5j, 5k** and **5l,** we may suggest that presence of large aromatic thiazole rings in

Table 2: In vitro IC_{50} value of XO inhibitory and free radical scavenging activities of derivatives

Comp. ID	Compound	XO inhibition (%)	*In-vitro* IC_{50} (μM)	Free radical inhibition (%)	Free radical scavenging activity IC_{50} (μM)
5a	H	39	98.1 ± 0.1	69	23.8 ± 0.5
5b	4-Cl	30	>100	29	>100
5c	4-F	71	30.3 ± 0.3	23	>100
5d	4-Br	60	43.2 ± 0.51	18	>100
5e	4-CH_3	34	>100	63	29.3 ± 0.47
5f	4-OH	76	21.5 ± 0.21	58	47.3 ± 0.35
5g	4-OCH_3	36	>100	68	25.0 ± 0.88
5h	4-NO_2	–	ND	–	ND
5i	2-Br	31	>100	32	>100
5j	2-Cl	79	9.9 ± 0.30	39	59.0 ± 0.90
5k	2- CH_3	89	3.6 ± 0.7	84	15.3 ± 0.52
5l	2,4-di-Cl	84	8.1 ± 0.4	33	61.7 ± 0.45
5m	2-OH	35	>100	59	34.6 ± 0.68
5n	2-OCH_3	81	14.7 ± 0.32	73	19.6 ± 0.12
5o	3,4-di-OCH_3	39	ND	49	49.1 ± 0.24
5p	3,4-OH	68	60.2 ± 0.54	79	17.6 ± 0.87
–	Febuxostat	98	0.03 ± 0.01	ND	ND
–	Ascorbic acid	ND	ND	96	13.4 ± 0.50

ND, Not Determined, % inhibitor, calculated at 100 μM concentration; **IC_{50}** value ± SEM.

the structure of 2-(benzylamino)-4-methyl-1,3-thiazole-5-carboxylic acid derivatives which fit a position in the active site of XO, favoured its binding and inhibition towards XO (Figure 5).

Biological activity

In vitro XO inhibitory activity of compounds **5a-p** was carried out spectrophotometrically by measuring uric acid (38–40) levels at 295 nm (Table 2). Febuxostat was used as a reference drug**.** Overall, most of the synthesized 2-(benzylamino)-4-methyl-1,3-thiazole-5-carboxylic acid derivatives exhibited XO inhibitory activity, but less than that of febuxostat. Among the limited number of synthesized 2-(substituted benzylamino)-4-methyl-1,3-thiazole-5-carboxylic acid derivatives, XO inhibition potency can be inferred due to the presence of thiazole rings with free carboxylic acid in the synthesized derivatives which may be responsible for comparatively potent XO inhibition. Comparison of inhibitory effects among all synthesized 2-(benzylamino)-4-methyl-1,3-thiazole-5-carboxylic acid derivatives, leading to an observation that the presence of electron donating groups like methoxy, methyl and hydroxyl at *para* followed by *ortho* position in **5c, 5f, 5j, 5k, 5l,** and **5n,** enhances the Xanthine oxidase inhibitory activity. Within the series of synthesized compounds, most of them exhibited potency levels in the micromolar range; however, compounds **5a, 5b, 5g, 5i, 5m** and **5o** were found to be less active in comparison with electron donating group derivatives. The insertion of chlorine atom at *ortho* and *para* position in **5l** as electron withdrawing group resulted into a general increase in the potency of inhibitor's in contrast to the insertion of electron-releasing groups in **5h, 5g** and **5o.** The presence of methyl group at *ortho* position improved the inhibitory property as compared to *para* position in **5k**. Compounds **5j, 5k** and **5l** emerged as most potent XO inhibitor molecule among the series of synthesized derivatives. IC_{50} values of all these compounds were depicted in Table 2, and percentage inhibition has been shown in Figures 6 and 7.

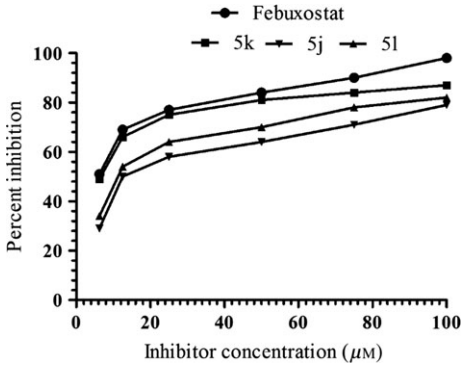

Figure 6: Percent inhibition pattern of xanthine oxidase inhibitors (febuxostat, **5j, 5k** and **5l**) at different concentrations.

Antioxidant potential of 2-(substituted benzylamino)-4-methyl-1,3-thiazole-5-carboxylic acid derivatives was also evaluated using DPPH assay (41). DPPH is a stable free radical and accepts electron or hydrogen radical to

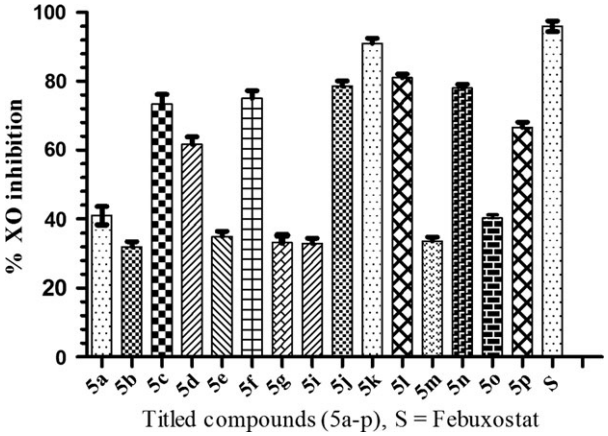

Figure 7: Percent inhibition of xanthine oxidase by 2-(substituted benzylamino)−4−methyl−1,3 −thiazole−5−carboxylic acid derivatives (**5a-p**).

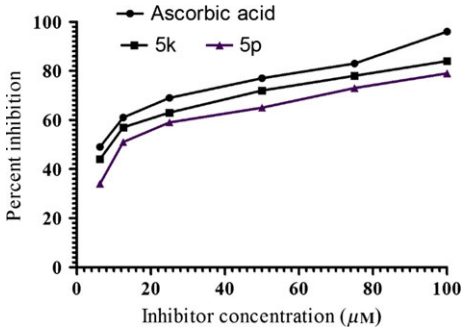

Figure 8: Percent inhibition pattern of DPPH free radical scavengers (ascorbic acid, **5k** and **5p**) at different concentrations.

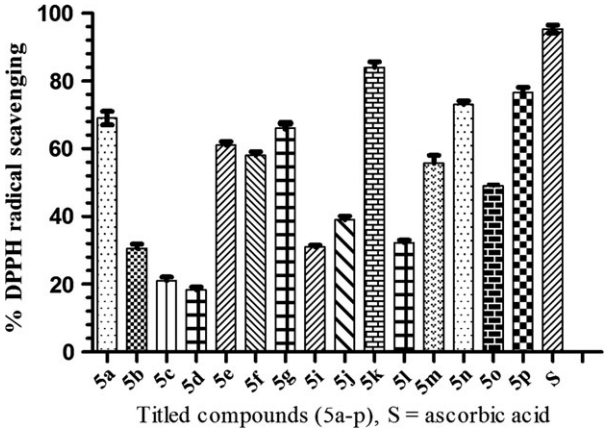

Figure 9: Percent inhibition of DPPH free radical scavenging potential of 2−(substituted benzylamino)−4−methyl−1,3−thiazole−5−carboxylic acid derivatives (**5a-p**).

become a stable diamagnetic molecule. Ascorbic acid was used as a reference compound**.** Derivatives **5a, 5k, 5n and 5p** showed moderate free radical scavenging activity on the basis of their IC_{50} values, and the presence of electron or hydrogen radical releasing groups OCH_3, OH, CH_3 and H in the structures of 5**a, 5e, 5g, 5j, 5k, 5m, 5o** and **5p** may be responsible for reduction of DPPH free radical that imparts scavenging activity to them. Although all synthesized compounds possess moderate to most potent free radical scavenging activity but, derivatives **5j, 5k,** and **5l** have potent XO inhibitory along with free radical scavenging, IC_{50} values of the compounds of interest (Table 2) and percentage inhibition pattern have been shown in Figures 8 and 9.

Conclusion

In conclusion, a series of thiazole derivatives (**5a-p)** as structural analogue of Febuxostat was synthesized and evaluated for *in vitro* xanthine oxidase inhibitory and free radical scavenging activity. These synthesized compounds were characterized by different spectral analysis. Compounds **5k, 5j** and **5l** demonstrated satisfactory potent XO inhibitory activities with IC_{50} values, 3.6, 8.1 and 9.9 μM, respectively, whereas compounds **5k**, **5n** and **5p** demonstrated potent antioxidant activities having IC_{50} values of 15.3, 17.6 and 19.6 μM, respectively, along with XO inhibitory activity. Molecular docking was further performed to study the XO inhibitory activity. It was found that several interactions with the various amino acid residues in the binding site of XO might play a crucial role in its XO inhibitory activity. All these data suggested that these compounds might serve as entrants for the treatment of XO based disorders and as lead compounds for further designing of new potential inhibitors of XO.

Acknowledgments

Authors are thankful to Department of Pharmaceutical Chemistry, Jamia Hamdard, New Delhi, for providing necessary facilities. Thanks are due to Jamia Hamdard, IIT Delhi, and CDRI, Lucknow, for providing spectral data. One of the authors (Md Rahmat Ali) is grateful to the UGC (Moulana Azad National Fellowship), New Delhi, India, for providing financial assistance.

The authors declare nil conflict of interests.

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