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Novel 3-substituted-1-aryl-5-phenyl-6-anilinopyrazolo[3,4-*d*]pyrimidin-4-ones: Docking, synthesis and pharmacological evaluation as a potential anti-inflammatory agents

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ABSTRACT

Novel 3-substituted-1-aryl-5-phenyl-6-anilino-pyrazolo[3,4-*d*]pyrimidin-4-ones of pharmacological significance were synthesized by the reaction of ethyl-(5-amino-3-methylthio-1-aryl-5-phenyl-2*H*-pyrazole)-4-carboxylates **3a–c** with S-methyl diphenyl thiourea independently to produce 1-aryl-3-thiomethyl-5-phenyl-pyrazolo[3,4-*d*]pyrimidines **4a–c** in DMF with catalytic amount of K_2CO_3, which on further treatment with different aromatic amines independently under same reaction conditions generated for compounds **5a–l**. The compounds were screened for the anti-inflammatory activity and evaluated for ulcerogenic potential. The compounds **5i** exhibited superior anti-inflammatory activity in comparison with diclofenac sodium and comparable activity with celecoxib at a dose of 25 mg/kg. The other compounds **4c**, **5c**, **5f** and **5l** were found as active with inhibition of edema in the range of 35–39 after 3 h of administration of test compounds. The ulcerogenic potential of active compounds was observed to be quite lesser as compared to standard. COX-2 docking score of the active compound **5i** was found to be better than standard celecoxib.

Pyrazole ring being predominant pharmacophore for the analgesic and anti-inflammatory activity as evoked in many drugs such as phenylbutazone, sulfinpyrazone, celecoxib.[1,2] Literature survey also revealed the importance of this ring in biology and proved its biological significance as anti-inflammatory,[3] COX inhibitory,[4] antimicrobial,[5] CDK inhibitory,[6] hypoglycemic[7] and CB_1 receptor antagonist[8] activities.

Condensed pyrimidines are reported in literature for array of biological activities. The annelation of the pyrazole ring on the pyrimidine nucleus lead to pyrazolopyrimidine which can be looked upon as the bio-isostere of purines (imidazole-pyrimidine). The biological significance of purine nucleus is well established; allopurinol and its congener oxypurinol and thiopurinol which contain pyrazolo[3,4-*d*]pyrimidine ring inhibit enzyme xanthine oxidase and interfere with the biosynthesis of uric acid, the causative agent for gout.[1] Pyrazolo[3,4-*d*]pyrimidine also exhibit analgesic and anti-inflammatory,[9,10] antifungal,[11] antimicrobial,[12] antiproliferative,[13] anticoagulant,[14] antiviral[15,16] and adenosine antagonistic[17] activities. The annual death rate amongst patients with rheumatoid and osteoarthritis due to serious adverse consequences of gastro duodenal ulceration (perforation and hemorrhage) has been estimated to be very high.[18] Hence, it put forth great need of NSAIDs to treat arthritis, osteoarthritis, acute pain, migraine, post operative pain and accidental pain with minimum adverse effects such as gastric ulcers. The present study describes the docking, synthesis and evaluation of novel 3-substituted-1-aryl-5-phenyl-6-anilinopyrazolo[3,4-*d*]pyrimidin-4-ones for anti-inflammatory activity and ulcerogenic potential which will further expand the scope of pyrazolopyrimidines (see Table 1 and Fig. 1).

In an attempt to design and develop novel potential anti-inflammatory agents, we synthesized target compounds by reacting ethyl bismethylthio-2-cyanoacrylate[19] **1** and aromatic hydrazines **2a–c** independently. The reaction was carried out in dimethyl formamide in the presence of catalytic amount of anhydrous K_2CO_3 for 1.5 to 2 h resulting in ethyl-(5-amino-3-methylthio-1-aryl-5-phenyl-2*H*-pyrazole)-4-carboxylates **3a–c**, which on independent treatment with *S*-methyl diphenyl thiourea[20] independently afforded the structurally diverse 6-anilino substituted pyrazolo[3,4-*d*]pyrimidines **4a–c**. Synthesis of intermediates and target compounds has been carried out as depicted in the Scheme 1. The compounds **4a–c** were reacted individually with aromatic amines like aniline, p-nitroaniline p-toludine and p-chloroaniline to produced the titled compounds 3-substituted-1-aryl-5-phenyl-6-anilino pyrazolo[3,4-*d*]pyrimidin-4-ones **5a–l** by nucleophilic substitution reaction.

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Table 1
Physical characterization of synthesized compounds **3a–c**, **4a–c** and **5a–l**

Compd	Mol. Formula	Mol. Weight	% Yield	M.P. (°C)	R_f value
3a	$C_{13}H_{15}N_3O_2S$	277	86	95–97	0.61
3b	$C_{13}H_{14}N_4O_4S$	322	80	161–165	0.70
3c	$C_{13}H_{16}N_4O_4S_2$	356	76	230–233	0.85
4a	$C_{24}H_{19}N_5OS$	425	86	200–203	0.54
4b	$C_{24}H_{18}N_6O_3S$	470	85	300–302	0.60
4c	$C_{24}H_{20}N_6O_3S_2$	504	89	240–242	0.85
5a	$C_{29}H_{22}N_6O$	470	69	250–253	0.66
5b	$C_{29}H_{21}N_7O_3$	515	96	325–327	0.32
5c	$C_{29}H_{23}N_7O_3S$	549	72	260–265	0.67
5d	$C_{29}H_{21}N_7O_3$	515	77	265–268	0.74
5e	$C_{29}H_{20}N_8O_5$	560	64	341–344	0.59
5f	$C_{29}H_{22}N_8O_5S$	594	70	270–275	0.67
5g	$C_{30}H_{24}N_6O$	484	88	253–256	0.65
5h	$C_{30}H_{23}N_7O_3$	529	65	333–337	0.58
5i	$C_{30}H_{25}N_7O_3S$	563	71	255–257	0.73
5j	$C_{29}H_{21}ClN_6O$	504	89	250–254	0.65
5k	$C_{29}H_{20}ClN_7O_3$	549	55	346–349	0.44
5l	$C_{29}H_{22}ClN_7O_3S$	584	68	272–274	0.63

% Yield: refers to the isolated pure compound.
R_f value-mobile phase used was either n-henxane: ethyl acetate 80:20 or chloroform: methanol 30:70.
Melting points were determined for isolated pure compounds and are uncorrected.

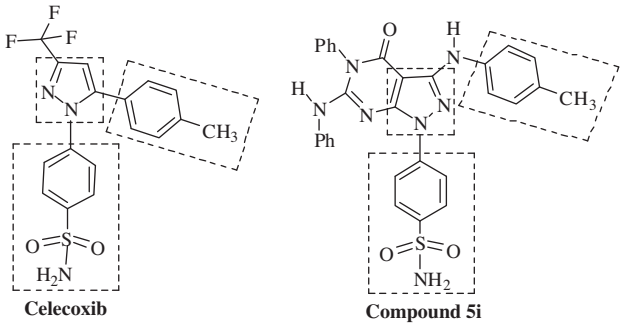


Figure 1. Pharmacophoric pattern of celecoxib and newly synthesized potent anti-inflammatory compound **5i**.

Having secured the intermediate carboxylates **3a–c**, pyrazolopyrimidines **4a–c** and series of 3-substituted-1-aryl-5-phenyl-6-anilino pyrazolo[3,4-*d*]pyrimidin-4-ones **5a–l**, in order to search for the potent compounds, newly synthesized molecules **4a–c** and **5a–l** were screened for anti-inflammatory activity and ulcerogenic potential of most active compounds was determined in order to investigate their potency and associated side effect.

All the experimental procedures and protocols for anti-inflammatory and ulcerogenic potential activities were reviewed and approved by the Institutional Animal Ethical Committee (IAEC) of Y.B. Chavan College of Pharmacy, Aurangabad, constituted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. The anti-inflammatory activity of the test compounds (**4a–c** and **5a–l**) was evaluated using *carrageenan-induced* rat paw edema method as described by Winter et al.[21] Few of the final compounds from the series **(4a–c** and **5g–i)** were selected for the determination ulcerogenic potential studies. The gastric lesions were counted and an ulcer index (UI) was calculated according to Szelenyl[22] and Thiemer.[23] Diclofenac sodium and celecoxib was used as a standard to compare anti-inflammatory activity and ulcerogenic potential of the test compounds. The percentage inhibition of edema in each test treated group was calculated using the formula as given below:

$$\%\text{Inhibition} = 100(1 - \Delta Vt/\Delta Vc)$$

where ΔVc and ΔVt is arithmetic mean of the increase in paw volume in the control and treated group respectively. The data was then analyzed statistically using ANOVA followed by Dunnett's test. All the results are expressed as mean ± S.E.M. The results are expressed as a percentage inhibition of edema formation (Table 2).

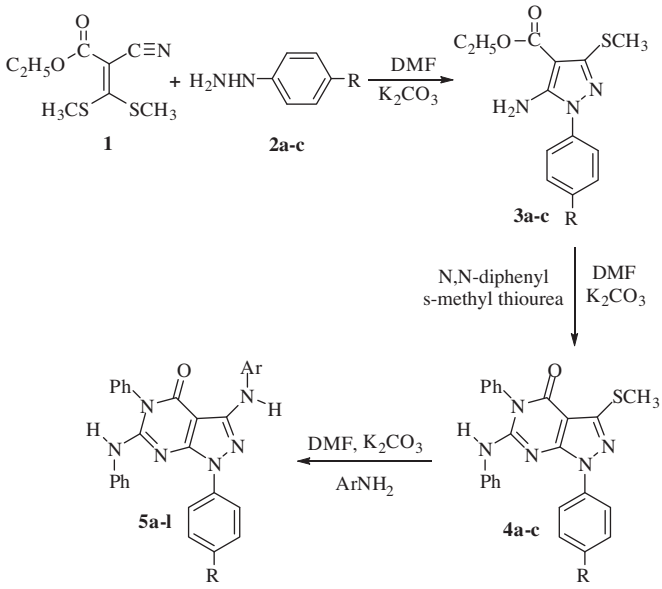
To predict the COX-2 selectivity, synthesized compounds **4a–c** and **5a–l**, standard compounds diclofenac and potent COX-2 inhibitor celecoxib were subjected to molecular docking studies on COX-2 receptor bonded with SC-588 in I 222 space group.[24] For the purpose of assessment of docking of ligands to protein active sites for estimation of binding affinities of docked compounds, an advanced molecular docking programme GLIDE (Schrodinger Inc., USA) version 4.5 was used. GLIDE; the Grid-based ligand docking with energetics algorithm approximates a systematic search of positions, orientations and conformations and eliminates unwanted conformations using scoring in the enzyme pocket via a series of hierarchical filters. Finally the conformations were refined via Monte Carlo sampling of pose conformation.[25,26] X-ray crystal structure of COX-2 was taken from PDB entry 6 COX having resolution of 2.80 Å (Angstroms). For docking studies all structures were prepared using protein preparation wizard in Maestro 9.0. Protein preparation was carried out in two steps viz. preparation and refinement. Chemical correctness was ensured and water molecules in crystal structure were deleted and hydrogen atoms were added at missing positions and bond order for crystal ligand and protein was adjusted and minimized up to 0.30 Å RMSD. Ligprep 2.2 module in maestro 9.0 build panel was used for ligand preparation which produced low energy conformations of ligand using OPLS 2005 force field. Glide provides three different levels of docking precisions viz. High Throughput Virtual Screening, HTVS; Standard precision, SP and Extra precision, XP. We carried out our calculations using XP docking mode as the tool is designed for better refinement in ligands.

Active compounds amongst **4a–c** and **5g–i** were selected for the ulcerogenic potential studies. The gastric lesions were counted and an ulcer index (UI) was calculated according to Szelenyl and Thiemer.[21,22]

$$\text{UI} = (n \text{ lesions I}) \times 1 + (n \text{ lesion II}) * 2 + (n \text{ lesions III}) \times 3$$

where I = presence of edema, hyperemia and single, submucosal, punctiform hemorrhages; II = presence of submucosal, hemorrhagic lesions with small erosions and III = presence of deep ulcer with erosions and invasive lesions.

In-vivo anti-inflammatory activity of 3-methylthio-1-aryl-5-phenyl-6-anilino-2*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **4a–c**, 1-aryl-5-phenyl-3-substituted-6-anilino-2H-pyrazolo[3,4-*d*]pyrimidin-4(5H)-one **5a–l** derivatives has been presented in Table 2 and some interesting trends were observed as to the effect of annelation and corresponding substitutions. It was found from the results that the nature of various substituent used affected the biological activity of the synthesized analogues. The activity data depicts the anti-inflammatory potential of synthesized compounds and majority of analogues were found to be active. Derivatives 3-methylthio-1-aryl-5-phenyl-6-anilino-2(*H*)-pyrazole[3,4-*d*]pyrimidin-4-(5*H*)-ones **4a–c** proved to be active variably, among them compound **4c** has percent inhibition of 68% as compared to diclofenac sodium having 75% after 1 h. This creates an emphasis for the presence of *p*-sulfamoyl substituted phenyl ring on first nitrogen atom of pyrazole nucleus in this series. This was further proved when we observed the similar pattern of the activity in the compounds **5c**, **5f**, **5i** and **5l** which also possess the *p*-sulfamoyl ($-SO_2NH_2$) phenyl substituent. All these derivatives exhibited comparable activity to diclofenac sodium and celecoxib at same dose level. The most active derivative **5i** possess all the substituents present in a well known molecule celecoxib. It exhibited the



Compd.	R	Compd.	R	Ar	Compd.	R	Ar
2a	H	**4b**	NO_2	---	**5f**	SO_2NH_2	p-Nitro phenyl
2b	NO_2	**4c**	SO_2NH_2	---	**5g**	H	p-Tolyl
2c	SO_2NH_2	**5a**	H	Phenyl	**5h**	NO_2	p-Tolyl
3a	H	**5b**	NO_2	Phenyl	**5i**	SO_2NH_2	p-Tolyl
3b	NO_2	**5c**	SO_2NH_2	Phenyl	**5j**	H	p-Chlorophenyl
3c	SO_2NH_2	**5d**	H	p-Nitro phenyl	**5k**	NO_2	p-Chlorophenyl
4a	H	**5e**	NO_2	p-Nitro phenyl	**5l**	SO_2NH_2	p-Chlorophenyl

Scheme 1. Synthesis of ethyl-5-amino-3-methylthio-1-aryl-2*H*-pyrazole)-4-carboxylate **(3a–c)**, 3-methylthio-1-aryl-5-phenyl-6-anilino-2*H*-pyrazolo[3,4-*d*]-pyrimidin-4-one **(4a–c)** and 3-substituted-1-aryl-5-phenyl-6-anilino pyrazolo[3,4-*d*]pyrimidin-4-ones**(5a-l)**.

Table 2
% Inhibition of rat paw edema and XP docking glide scores for test compounds and standards

Compd	% Inhibition ± SEM after 1 h	% Inhibition ± SEM after 2 h	% Inhibition ± SEM after 3 h	Glide score**
4a	46.09 ± 1.0*	36.63 ± 0.7*	25.44 ± 2.4	−5.6666
4b	42.14 ± 0.7	37.68 ± 0.8*	26.57 ± 1.6*	−4.7862
4c	68.85 ± 1.2*	54.11 ± 1.0*	35.2 ± 0.6*	−7.2379
5a	48.13 ± 2.5*	43.38 ± 0.8*	17.07 ± 2.0*	−3.6530
5b	43.94 ± 0.8	40.80 ± 0.6*	14.11 ± 1.6*	−6.3698
5c	60.24 ± 1.2*	54.84 ± 0.5*	39.65 ± 1.0*	−6.4872
5d	29.41 ± 0.7*	28.96 ± 0.6*	15.08 ± 1.0*	−3.3231
5e	23.87 ± 2.5*	19.38 ± 0.9*	16.76 ± 0.7*	−3.1175
5f	66.98 ± 1.0*	55.31 ± 1.3*	39.09 ± 1.5*	−3.5942
5g	55.70 ± 0.9*	53.40 ± 1.3*	32.92 ± 0.6*	−3.5431
5h	46.64 ± 0.7	39.78 ± 1.0*	30.24 ± 0.6*	−3.7546
5i	77.04 ± 0.5*	63.38 ± 0.7*	43.66 ± 0.6*	−6.5485
5j	47.55 ± 1.3*	44.00 ± 1.1*	28.65 ± 0.7*	−3.6397
5k	39.56 ± 1.4*	38.97 ± 0.4*	20.63 ± 0.7*	−3.6850
5l	69.79 ± 0.7*	56.33 ± 1.1*	38.30 ± 0.3*	−7.7415
Control	0.85 0.01	1.11 ± 0.02	1.08 ± 0.41	–
Diclofenac	75.77 ± 0.6*	57.19 ± 0. 7*	42.83 ± 2.3*	−4.8753
Celecoxib	78.09 ± 0.7*	58.59 ± 0.5*	39.62 ± 1.1*	−5.9177

n = 6, mean paw volume ± SEM anaylsed by ANOVA followed by Dunnett's test *p <0.05, **—denotes g-score or docking score obtained for XP docking with 6 COX (PDB ID).

percent inhibition of 77%, 63% and 43% after 1, 2 and 3 h of administration.

Prediction of COX-2 selectivity of compounds was evaluated with potent and selective COX-2 inhibitor celecoxib and to ascertain non-selective nature of synthesized derivatives; diclofenac was also subjected to COX-2 docking studies which revealed that the potent anti-inflammatory and less ulcerogenic molecule **5i** showed glide score −6.5485 which is more than that of celecoxib itself that is −5.9177. Among the other synthesized compounds, **4c**, **5b**, **5c**, **5i**, and **5l** predicted to have better COX-2 affinity than celecoxib with **4a** depicting COX-2 affinity comparable to celecoxib. Molecules **4c** and **5l** have exceptionally high docking scores in series which are −7.2379 and −7. 7415. Rest of the derivatives viz **4b**, **5a, 5d-h**, **5j and 5k** were shown docking scores in between −3.1175 to −4.7862 which is less than the score obtained for diclofenac which predicts their nonselective nature. Study of Binding

Table 3
Evaluation of ulcer index

Compd	Dose(mg/kg)	Time(days)	Ulcer Index
Control	–	4	0
Diclofenac	75	4	39.17 ± 1.08*
Celecoxib	75	4	9.58 ± 0.71*
4a	75	4	4.66 ± 1.14*
4b	75	4	7.50 ± 1.26
4c	75	4	7.83 ± 1.17*
5g	75	4	3.20 ± 1.27*
5h	75	4	16.72 ± 0.48*
5i	75	4	9.50 ± 1.84*

Each value represent the mean ± SEM, $n = 6$ analyzed by ANOVA followed by Dunnett's test $^{*}p < 0.05$.

modes of the synthesized test compounds **4a**–**c** and **5a**–**l** depicts pie-pie stacking between benzene rings and amino acids Proline (Pro A:538) of 6 COX, along with same type interaction for pyrazole nucleus of all synthesized derivatives with amino acid Phenyl Alanine (Phe B:142) as a common interaction. When docking interactions pertaining to discrete chemical substitutions attached were compared we observed that; the compounds substituted with p-nitro phenyl moiety on first nitrogen of pyrazole nucleus have ionic interactions with either Asparagine B:537 as in **5d**, **5e**, **5f** or Valine B:228 and with both as in case of compound **4a**, **4c**. Some of the typical binding modes noticed for molecules with high glide score were; for **4c**; amino function and oxygen belonging to sulphonamide group formed hydrogen bonding with Valine (Val A:228) and Histidine (His A:226).

Selected compounds **4a**–**c** and **5g**–**i** when tested for the possible ulcerogenic potential, the findings were that the compounds with phenyl substitution at first position and p-toludinyl substitution at third position showed very few sign of redness. Among the tested compounds, **5g** found to be least ulcerogenic with ulcer index of 3.20. The most active compound of the series **5i** showed the ulcer index of 9.5 whereas diclofenac sodium exhibited ulcer index of 39.17 and celecoxib exhibited of ulcer index with value 9.58. Ulcerogenic potential of substituents of compounds can be summarized as COOH > NO_2 > SO_2NH_2 > Phenyl > tolyl Table 3.

In summary, a series of novel 3-substituted-1-aryl-5-phenyl-6-anilino pyrazolo[3,4-*d*]pyrimidin-4-ones has been prepared and assigned structures by analytical and spectral data. The results of the anti-inflammatory activity of the series showed that the compounds exhibited moderate to good anti-inflammatory activity. The compound **5i** was found to be superior to reference drug diclofenac sodium and comparable to celecoxib where as compound **4c**, **5c**, **5f** and **5l** showed comparable activity diclofenac sodium. The ulcer index of the compound **5i** was found to be less than celecoxib and diclofenac sodium. The COX-2 docking score of compound **5i** was found to be better than celecoxib. Hence this series could generate precursors which can be further optimized and developed for a novel lead compounds for emerging drug design and drug discovery of anti-inflammatory compounds.

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Notes-Pharmacological Screening-Healthy Wistar Albino rats of either sex weighing 150–180 g were used for study. Diclofenac sodium and celecoxib at 25 mg/kg was administered orally as a standard drug for comparison of anti-inflammatory activity and ulcerogenic potential. The test compounds were administered orally at a dose of 25 mg/kg suspended in 1% Tween 80 with distilled water. The paw volumes were measured using the mercury displacement technique with the help of Ugo Basile Digital Plethysmometer at 0, 1, 2 and 3 h after carrageenan injection(0.1 ml, 1%w/v solution).
Docking-2D structures were converted to 3D stereoisomers were generated, charged structures were neutralized and most probable ionization state at user defined pH was determined. Conformations were generated using rapid torsion angle search approach followed by minimization in OPLS force field 2005. Evaluation is done with glide score (docking score) and single best pose is generated as output for particular ligand. G Score is expressed as Gscore = $a \times$ vdW + b*coul + Lipo + H bond + Metal + Bury P + Rot B + Site Where, vdW is Van der Waal's energy, Coul is coulomb energy, Lipo is lipophilic contact term, H-Bond is hydrogen-bonding term, Metal is metal binding term, BuryP is penalty for buried polar groups, RotB is penalty for freezing rotatable bonds, Site is polar interactions at the active site and the coefficients of vdW and Coul are $a = 0.0065$, $b = 0.130$. Chemscore and atom-atom pair function assigns score to lipophilic ligand atoms based on summation over a pair function; each term of which depends upon inter

atomic distance between a ligand atom and a neighboring lipophilic protein atom.

Evaluation of Ulcerogenic Potential-The male Wistar Albino rats weighing 170–200 g were divided into eight groups each comprising of six rats, including a control and standard group. The test compounds and standard diclofenac sodium (dose 75 mg/kg/day) were suspended separately in 4 ml of 1% w/v solution of tween 80 with water and administered orally on all 4 days. The rats of all groups were fasted for 12 h after the administration of last dose, thereafter they were sacrificed by decapitation and stomach was removed and opened along with greater curvature and washed with distilled water and Krebs solution.