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Synthesis and biological evaluation of new pyrazolone–pyridazine conjugates as anti-inflammatory and analgesic agents

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ABSTRACT

A new series of pyrazolone–pyridazine conjugates **3** and **4a–l** were synthesized and characterized by spectroscopic means and elemental analyses. All compounds were tested in vivo for their anti-inflammatory and analgesic properties against diclofenac, as reference compound. The synthesized compounds were also evaluated for their ability to inhibit the production of certain inflammatory cytokines such as TNF-α and IL-6 in serum samples. The ulcerogenic potential of the synthesized compounds was also determined. IC_{50} values for inhibition of COX-1 and COX-2 enzymes were investigated in vitro for the most active candidates. Molecular docking was performed on the active site of COX-2 to predict their mode of binding to the amino acids. Among the synthesized derivatives, compounds **4c** and **4e** showed good analgesic and anti-inflammatory activities with lower ulcer index than the reference drug.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are a class of compounds used for the treatment of pain, fever and inflammation, particularly arthritis.[1,2] The pharmacological activity of NSAIDs is related to the suppression of prostaglandin biosynthesis from arachidonic acid by inhibiting cyclooxygenases (COXs), which exist in three distinct isoforms: COX-1, COX-2 and COX-3.[3–5] The COX-1 is a constitutive enzyme and is responsible for the production of cytoprotective prostaglandins in the gastrointestinal (GI) tract, thrombogenesis and homeostasis, while COX-2 is an inducible enzyme, which is induced in response to the release of several pro-inflammatory mediators such as endotoxins, mitogens or cytokines including tumor necrosis factor α (TNFα) and interleukins (ILs) such as IL-6 and IL-1.[6,7] Further, COX-3 which is considered as a variant of COX-1, is present in central nervous system and has been proposed to be another target for anti-inflammatory agents.[8] However, the beneficial effects of NSAIDs are usually balanced by their side effects especially on the GI system due to inhibition of COX-1 isoform. Many NSAIDs interact with both COX-1 and COX-2 isoforms and non-selectively inhibit their enzymatic activity, leading to reduction of prostaglandins PGE2 and PGI2 production, which possess an adverse ulcerogenic effect.[9,10] Chronic use of NSAIDs may induce GI haemorrhage, ulceration, perforation as well as nephrotoxicity, which greatly limit their therapeutic usefulness.[11,12] Therefore, drugs that selectively inhibit COX-2 isoform (coxibs) have been marketed as a new generation NSAIDs.[13] However, prospective examination of coxibs has revealed unexpected cardiovascular adverse effects such as myocardial infarction.[14] COX-2 selective inhibition also will lead to depletion of PGI2 and further increase in thromboxane formation which may cause atherosclerosis.[15,16] Several clinical studies showed an increased cardiovascular risks in treated patients with coxibs compared to others treated with traditional NSAIDs by five folds.[17,18] On the other hand, coxibs showed similar renal side effects to that exhibited by traditional NSAIDs as they reduce acutely medullary blood flow, sodium excretion and urine volume.[19] Therefore, there exists an unmet medical need to develop novel gastrointestinal-sparing NSAIDs with an improved safety profile.

Since the development of antipyrine, the first pyrazoline derivative used in the management of pain, inflammation and fever, great attention has been focused on pyrazoline derivatives as a potent class of anti-inflammatory, analgesic and antipyretic agents,[20–23], (Fig. 1). However, some potent analgesic and anti-inflammatory pyrazoline derivatives including dipyrone and phenylbutazone possess GI side effects that limit the clinical use of these drugs.[24]

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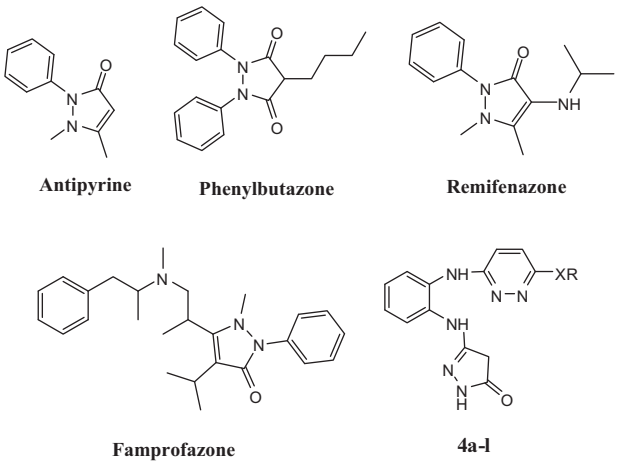


Figure 1. Some reported pyrazolone-containing anti-inflammatory agents and the newly synthesized compounds.

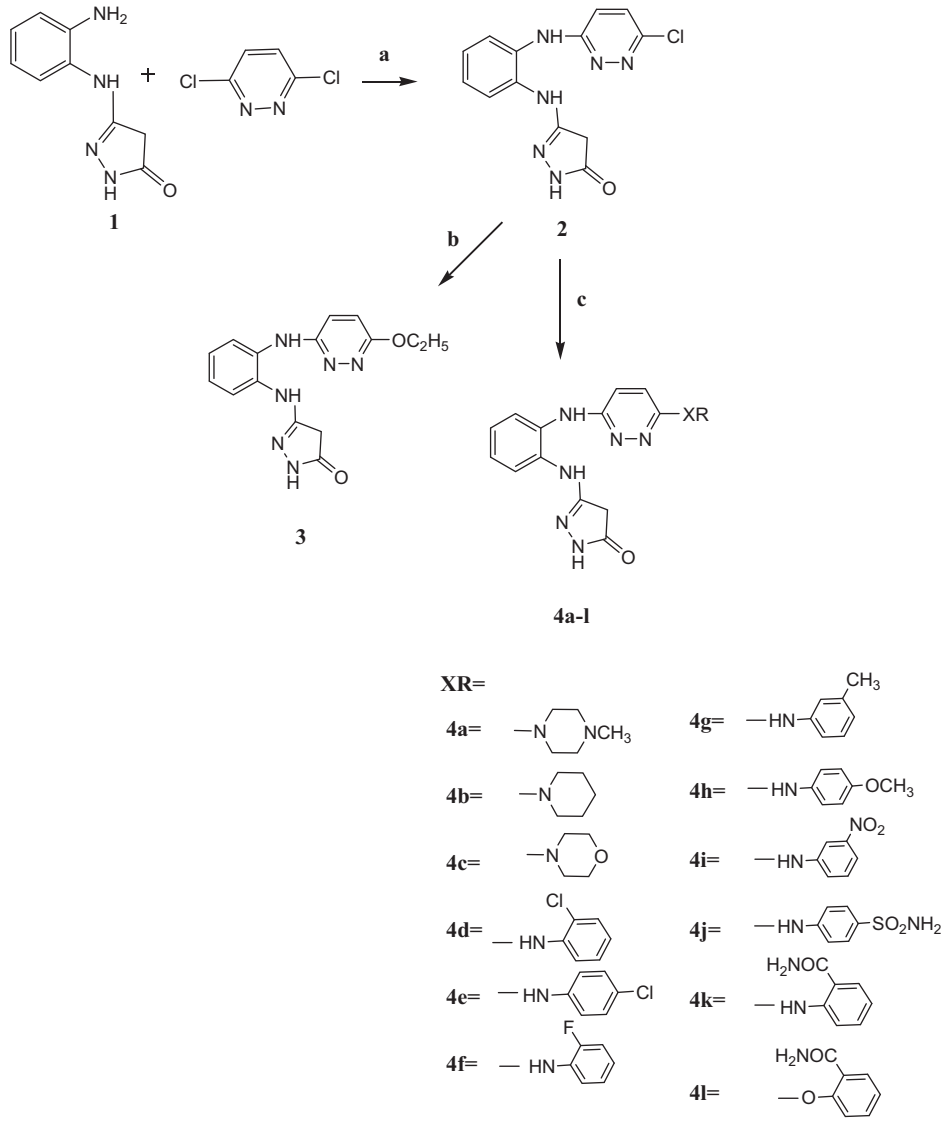
On the other hand, a large number of pyridazine and pyridazinone derivatives have been reported as analgesic and anti-inflammatory agents without gastrointestinal side effect.[25–27] Literature survey showed that pyridazine and pyrazole cores were often used as versatile scaffolds to develop new compounds endowed with interesting pharmacological properties against cyclooxygenase and lipoxygenase pathways.[28–30]

Encouraged with the well-documented anti-inflammatory properties associated with these heterocyclic cores, herein we report the synthesis of new pyrazolone derivatives in combination with pyridazine scaffold. The analgesic and anti-inflammatory activities were investigated for the title compounds utilizing the acetic acid-induced writhing test and the carrageenan-induced hind paw edema test, respectively. All the compounds were also evaluated for the irritative and ulcerogenic action on gastric mucosa.

2. Results and discussion

2.1. Chemistry

The synthetic route to the target compounds **3** and **4a–l** is outlined in Scheme 1. Briefly, 3-(2-aminophenylamino)-1*H*-pyrazol-5(4*H*)-one **1**, was prepared according to a procedure previously described.[31] Reaction of **1** with an equimolar amount of the commercially available 3,6-dichloropyridazine was achieved via



Scheme 1. Reagents and conditions: (a) isopropanol/K_2CO_3/reflux 4 h; (b) sodium ethoxide/ethanol, reflux 30 min; (c) amine/*n*-butanol/reflux 6 h.

nucleophilic displacement of chlorine to give **2**, which is the key intermediate for the production of the title compounds. The structure of compound **2** was confirmed by elemental and spectral analyses. IR spectrum for compound **2** revealed a band at 2954 cm^{-1} corresponding to aliphatic CH of the pyrazoline ring and a band at 1643 cm^{-1} corresponding to (C=O) group. ^1H NMR spectrum for compound **2** showed a singlet signal at δ 2.58 ppm corresponding to CH_2 of pyrazoline ring and an increase in aromatic proton multiplet signals at δ 7.11–7.50 ppm. The latter was further reacted with sodium ethoxide to give the corresponding *O*-ethyl derivative **3**, which showed the appearance of a triplet and a quartet signals in the ^1H NMR spectrum at δ 1.23 ppm and 3.87 ppm, assignable to CH_3 and CH_2 protons, respectively of $-OCH_2CH_3$ function.

On the other hand, reaction of **2** with a number of primary and secondary amines afforded the title compounds **4a**–**l**. Analytical and spectral data (IR, ^1H NMR, ^{13}C NMR and mass spectra) of the synthesized compounds were in full agreement with the proposed structures.

2.2. Molecular docking

In 2003, it was reported that a series of anti-inflammatory drugs inhibits PGE_2 synthesis by interacting with Tyr 385 and Ser 530 in the active site of COX-2 enzyme and that compounds which interact with such amino acids exhibit anti-inflammatory activity.[32] Moreover, interactions with Arg 120 may also play a role in the inhibitory effect.[32] Tyr-385 was found to be essential for the cyclooxygenase activity of PGH synthase and that nitration of this residue could be prevented by indomethacin. We conclude that Tyr 385 is at or near the cyclooxygenase active site of PGH synthase and the tyrosine residue could be involved in the first step of the cyclooxygenase reaction, which is the abstraction of the 13-proS hydrogen from arachidonate.[33] Acetylation of Ser 530 of sheep prostaglandin endoperoxide (PGG/H) synthase by aspirin causes irreversible inactivation of cyclooxygenase enzyme. Thus, Ser 530 does lie in a highly conserved region, probably involved in cyclooxygenase catalysis.[34] Arg 120 is located near the mouth of the hydrophobic channel that forms the cyclooxygenase active site of prostaglandin endoperoxide H synthases COX-1 and COX-2. Arg 120 forms an ionic bond with the carboxylate group of arachidonate and this interaction is an important contributor to the overall strength of arachidonate binding to COX-1.[35]

Molecular docking of all synthesized compounds was performed on the active site of COX-2 enzyme. To perform accurate validation of the docking protocol, docking of the co-crystallized ligand (Diclofenac) should be carried out to study the scoring energy (S), root mean standard deviation (rmsd) and amino acid interactions. Docking was performed using London dG force and refinement of the results was done using Force field energy. Diclofenac is fitted in the active site pocket with $S = -11.9408$ kcal/mol and rsmd = 0.3682. Diclofenac interacts with the two amino acids Tyr 385 and Ser 530 by three hydrogen bonds, the two hydrogen bonds lengths with Ser 530 were 2.7 Å and 2.9 Å while that with Tyr 385 was 2.7 Å as displayed in (Figs. 2 and 3). The dichlorophenyl group forms van der Waals interactions with Val-349, Ala-527, Leu-531, and Val-523. Following the aforementioned docking protocol, all the synthesized compounds were docked on the active site of COX-2 enzyme. Docking scores, amino acids interactions were summarized in Table 1.

All synthesized compounds were fit on the active site of COX-2 enzyme with energy scores range −11.3566 to −12.9831 kcal/mol. Compound **4c** showed the best docking score of −12.9831 kcal/mol. Compounds **2**, **3**, **4a** and **4f** showed interaction with one amino acid of the active site of COX-2, while compounds **4b** and **4e** interacted with two amino acids. Further, compounds **4c** and **4d**

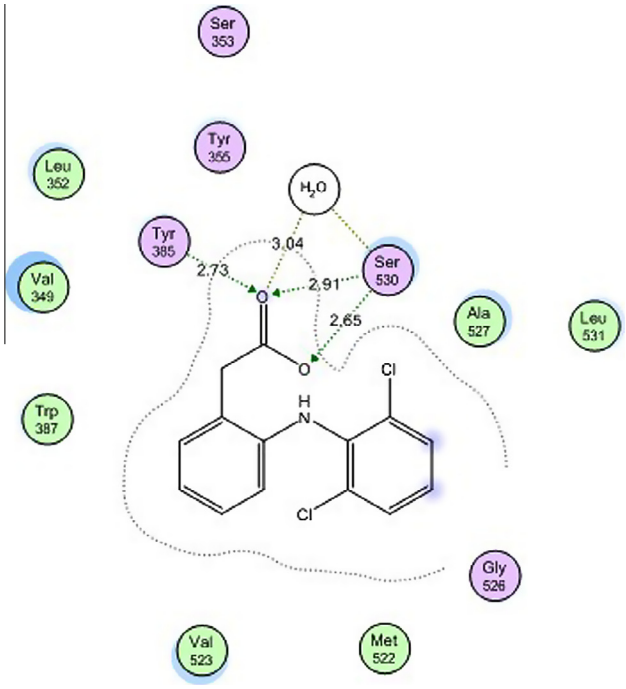


Figure 2. Diclofenac 2D interactions with active site of COX-2.

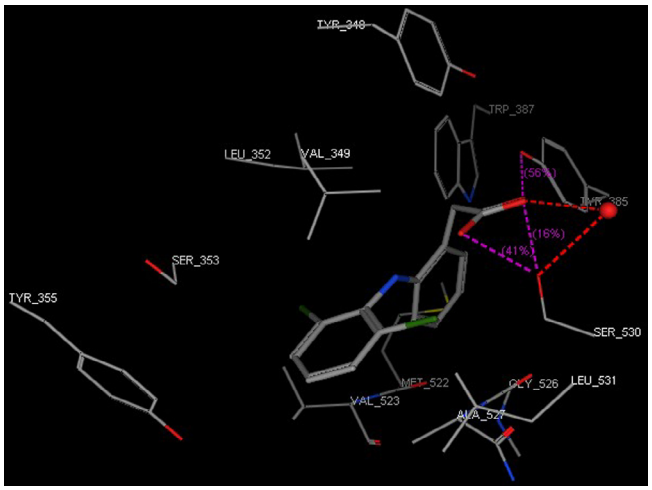


Figure 3. Diclofenac 2D interactions with active site of COX-2.

exhibited interaction with three amino acids of the active site of COX-2. The 2D interactions of the compounds **4c** and **4d** were displayed in Figures 4 and 5, respectively.

In light of good docking scores as well as good amino acid interactions displayed by all synthesized compounds, especially **4b**–**e**, it could be concluded that these compounds might possess promising anti-inflammatory activity.

2.3. Biological screening

2.3.1. Analgesic activity

In the present study, the test compounds showed variable degrees of percentage inhibition against acetic acid induced writhing. Diclofenac sodium induced higher degree of protection against writhing (83.3 ± 3.5%) compared to the tested compounds. Compounds **4b**, **4c**, **4d** and **4e** produced significant inhibition of 55.6 ± 5.1, 63.7 ± 5.4, 57.2 ± 4.3 and 68.3 ± 5.3%, respectively

Table 1
Binding scores and amino acid interactions of the docked compounds on the active site of COX-2 enzyme

Compound No.	S kcal/mol	Amino acid interactions	Interacting groups
2	−11.3973	Ser 530[a]	NH
3	−11.3566	Ser 530[a]	C=O (H_2O)
4a	−11.4265	Ser 530[a]	C=O
4b	−12.2006	Arg 120[a]	N pyrazoline
		Ser 530[a]	C=O (H_2O)
4c	−12.9831	Arg 120[b]	Pyridazine
		Ser 119[a]	NH
		Tyr 355[a]	N pyridazine
4d	−12.7081	Arg 120[a]	N pyridazine
		Ser 530[a]	C=O (H_2O)
		Tyr 385[a]	NH
4e	−12.7081	Tyr 385[a]	C=O
		Ser 530[a]	C=O(H_2O)
4f	−11.3094	Ser 530[a]	C=O(H_2O)
4g	−11.4281	Arg 120[a]	N pyrazoline
4h	−11.4730	Arg 120[a]	N pyrazoline
4i	−12.0538	Tyr 355[a]	N pyrazoline
4j	−12.1480	Arg 120[a]	N pyrazoline
4k	−11.6912	Arg 120[a]	N pyrazoline
4l	−11.4787	Arg 120[a]	N pyrazoline

[a] H-bond.
[b] Arene-cation.

compared to control group at $P < 0.05$, (Table 2). Dose response curves representing the degree of protection at different doses, against acetic acid induced writhing exhibited by the most active test compounds and diclofenac sodium, are shown in Figure 6.

2.3.2. Anti-inflammatory activity

Screening the anti-inflammatory activity for the selected compounds that demonstrated the most promising results in the writhing test **4b**, **4c**, **4d** and **4e**, revealed that these compounds exert good to moderate anti-inflammatory effect ranging from 62.3 ± 5.8% to 35.4 ± 2.8%, (Table 2). Furthermore, determination of serum TNF-α level highlighted that the compounds **4a**, **4b**, **4c**, **4d** and **4e** significantly reduced its serum level compared to the control group. Similarly, compounds **4b**, **4c**, **4d** and **4e** showed significant reduction in serum IL-6 level compared to vehicle treated mice, (Table 2).

2.3.3. In vitro COX-1 and COX-2 inhibition assay

In an attempt to explore the mechanism of anti-inflammatory effect produced by the newly synthesized compounds, in vitro assay for COX-1 and COX-2 enzymes inhibition was conducted. The most active compounds **4b**, **4c**, **4d**, **4e** were screened adopting enzyme-immuno assay (EIA) kit (Cayman Chemical Company, USA). The COX-1 and COX-2 inhibitory activity of the compounds was evaluated following a method described by Gautam et al.[36] IC_{50} values for the most active compounds were determined using celecoxib as the reference drug. Furthermore, selectivity index for COX-1/ COX-2 inhibition was calculated for each compound. IC_{50} values for COX-1 and COX-2 inhibition as well as the selectivity index were listed in Table 3. From the screening results, it was obvious that compounds **4b**, **4c**, **4d** and **4e** showed good inhibitory activity on both COX-1 and COX-2 enzymes with IC_{50} values range 2.86–4.45 μM for COX-1 and 0.39–0.84 μM for COX-2. Among the tested compounds, **4c** demonstrated the highest activity with IC_{50} value of 2.86 μM for COX-1 and 0.39 μM for COX-2, and exerting the highest selectivity index (SI) of 7.36.

2.3.4. Acute ulcerogenesis

Ulcerogenicity studies revealed that the most active compounds **4b**, **4c**, **4d** and **4e**, showed high ulcerogenic indices compared to the vehicle group. However, the indices registered with compounds **4b** and **4e** were significantly lower than diclofenac sodium. Further, the scores assigned for the histologic sections of mice gastric mucosa treated with compounds **4b**, **4c**, **4d** and **4e** revealed that all the four compounds produced significantly higher histologic scores compared to the vehicle group. Compound **4d** exhibited histologic score almost similar to that showed with mice treated with diclofenac sodium (Fig. 7).

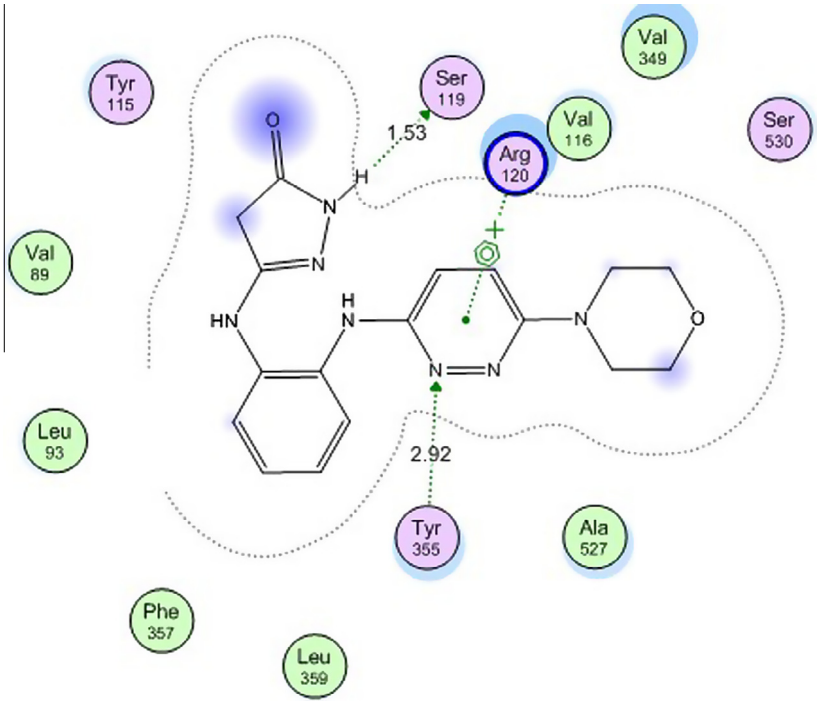


Figure 4. Compound **4c** on the active site of COX-2 enzyme.

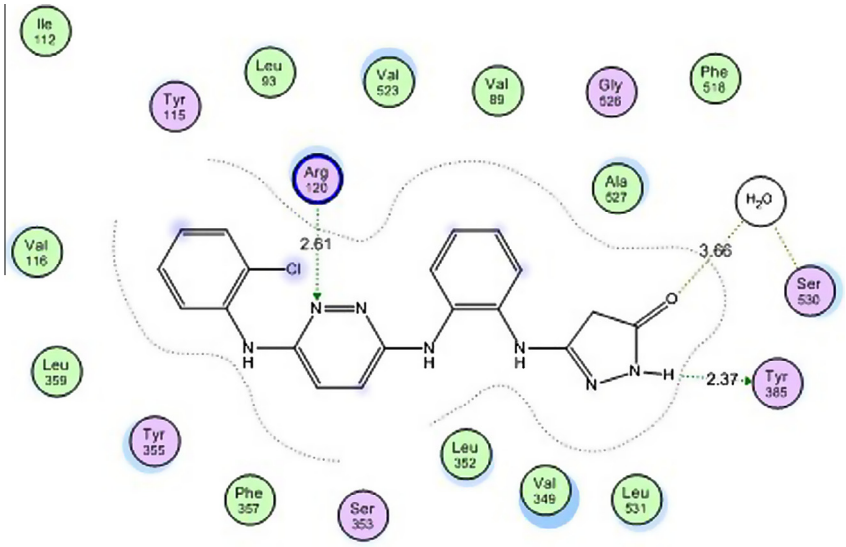


Figure 5. Compound **4d** on the active site of COX-2 enzyme.

Table 2
Percentage protection against writhing and percentage inhibition of carrageenan-induced paw edema method Effect of diclofenac sodium and the test compounds on serum TNF-α and IL-6 levels in mice tested by carrageenan-induced paw edema method

Compound	Analgesic activity (% protection)[a]	Anti-inflammatory activity (% inhibition)[a]	TNF-α (ng/ml)[b]	IL-6 (ng/ml)[b]
Vehicle	0	0	15.48 ± 0.31	23.8 ± 2.5
Diclofenac sodium	**83.3 ± 3.5***	**61.5 ± 5.14***	**5.01 ± 0.73***	**7.6 ± 0.35***
2	33.4 ± 2.8*	11.2 ± 1.1	13.2 ± 1.2	21.4 ± 1.8
3	18.2 ± 1.6	10.2 ± 0.92	12.4 ± 1.1	22.4 ± 2.5
4a	41.2 ± 3.8*	35.3 ± 3.2*	9.34 ± 0.83*	19.5 ± 1.6
4b	**55.6 ± 5.1****	**59.4 ± 5.3***	**7.5 ± 0.54***	16.5 ± 1.3*
4c	**63.7 ± 5.4***	**62.3 ± 5.8***	**7.2 ± 0.43***	**11.8 ± 0.87***
4d	**57.2 ± 4.3***	**54.3 ± 4.7***	**6.4 ± 0.48***	**10.65 ± 0.74***
4e	**68.3 ± 5.3***	**35.4 ± 2.8**	**7.12 ± 0.57***	**9.76 ± 0.58***
4f	36.1 ± 2.9*	11.2 ± 2.3	11.2 ± 1.3	17.3 ± 1.2
4g	35.32 ± 3.2*	12.4 ± 1.6	12.4 ± 1.2	18.4 ± 1.4
4h	19.43 ± 1.2	7.3 ± 0.5	14.4 ± 1.3	25.7 ± 1.2
4i	13.2 ± 1.1	5.4 ± 0.87	13.5 ± 1.2	18.3 ± 1.2
4j	11.3 ± 1.3	15.4 ± 1.5	16.7 ± 1.5	21.43 ± 1.5
4k	13.2 ± 1.2	18.2 ± 2.1	12.5 ± 0.94	22.3 ± 1.9
4l	14.2 ± 1.2	9.3 ± 2.4	11.98 ± 1.3	22.6 ± 2.1

Bold values indicate the most active compound in each assay.

[a] The vehicle group was pretreated with 1% administered orally carboxymethylcellulose solution. The test compounds were (25 mg/kg) thirty minutes prior to testing. Data are presented as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's post hoc test. *$P < 0.05$ compared to the vehicle group.

[b] All compounds were administered to mice (25 mg/kg, p.o.) thirty minutes prior to testing. TNF-α: tumor necrosis factor-α, IL-6: interlukin-6. Data are presented as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons.*$P < 0.05$ compared to the vehicle group.

Based on these biological results, It was clear that compound **2** showed a weak anti-inflammatory effect of 11.2% rat paw edema inhibition and a moderate analgesic effect of 33.4% protection in writhing test. Both activities were decreased with the ethoxy derivative **3** to 10.2% and 18.2% for rat paw edema inhibition assay and writhing test, respectively. On the other hand, when the ethoxy substituent in compound **3** was substituted by the *N*-methylpiperazine scaffold, compound **4a**, the anti-inflammatory and analgesic activities were markedly raised to more than three folds (35.3% and 41.2% respectively). Moreover, the results showed that other aliphatic amines such as piperidine and morphline, compounds **4b** and **4c** respectively, also 2- and 4-chloro substituted aromatic amines, compounds **4d** and **4e** respectively exhibited a remarkable activity. However, replacement of the ethoxy group in compound **3** by certain aromatic amines such as 2-flouroaniline **4f**, 3-methylaniline **4g**, 4-methoxyaniline **4h**, 3-nitoaniline **4i**, or amides including sulfanilamide **4j**, 2-aminobenazmide **4k** and 2-hydroxybenzamide **4l**, did not improve the activity. Among all tested compounds, **4c** was found to be the most active compound with 62.3% inhibition in rat paw edema test and 55.6% protection in writhing test. Compounds **4b**, **4d** and **4e** showed 59.4%, 54.3% and 35.4%, respectively in rat paw edema test and 55.6%, 57.2% and 68.3%, respectively in writhing test. Regarding ulcergenicity, compounds **4b** and **4e** showed lower ulcerogenic effect than the reference drug diclofenac. Moreover, **4e** demonstrated the least ulcerogenic potential among all tested compounds. Compounds **4c** and **4d** were the most active compounds regarding their effect on the inhibition of TNF-α and IL-6 production in serum. Additionally, compound **4c** was the most potent inhibitor of COX-1 and COX-2 with selectivity index (SI) of 7.3. Also compound **4c** showed the best docking score of -12.9831 kcal/mol. and three amino acids interaction with the active site of COX-2 enzyme in the molecular docking study.

These results can lead us to some conclusions about structure activity relationship of the newly synthesized compounds that can be summarizes as: neither substitution on pyridazine ring with

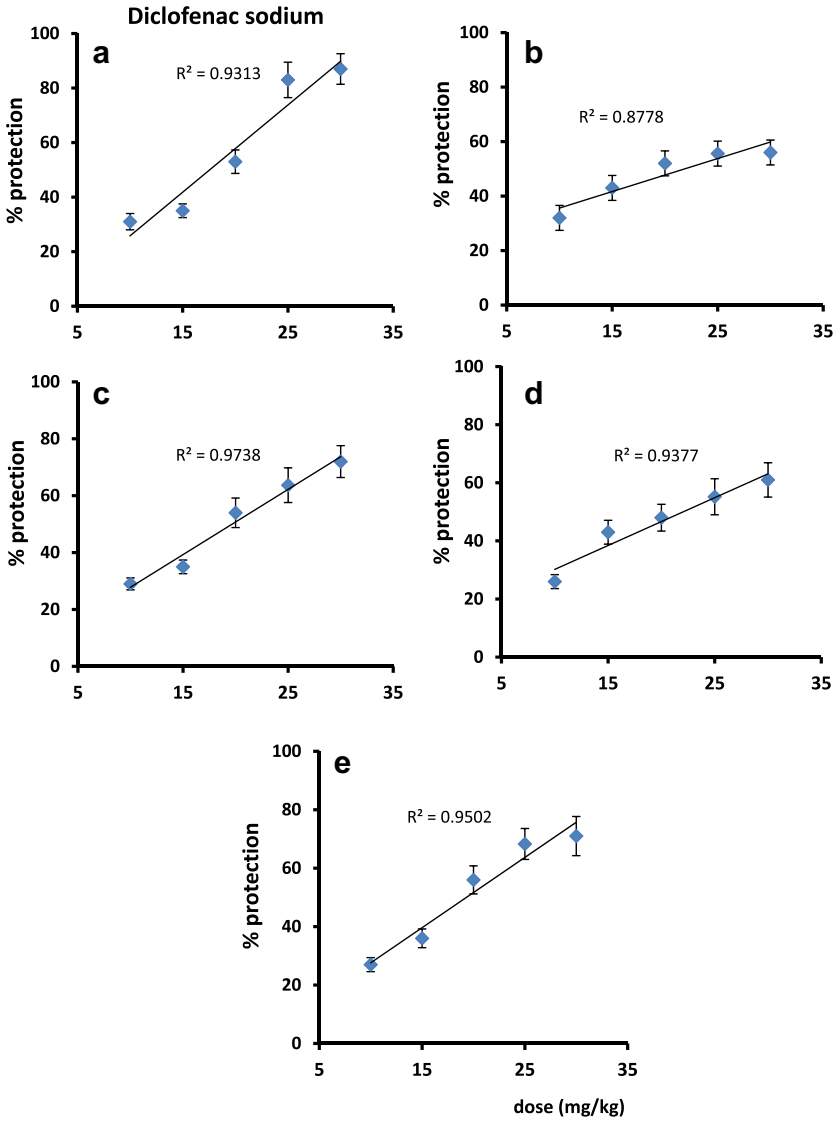


Figure 6. Dose response curves for the percentage protection calculated after administration diclofenac sodium and the most active test compounds (**4b**, **4c**, **4d** and **4e**) in the writhing test. 0.1% acetic acid solution was injected intraperitoneally to induce writhing in mice. The different compounds were tested at 10, 15, 20, 25 and 30 mg/kg (p.o.) in male albino mice. Data are presented as mean ± SEM.

Table 3
COX-1 and COX-2 inhibition IC_{50} and selectivity index (SI)

Compound	IC_{50} (μM)		Selectivity index (SI)
	COX-1	COX-2	
4b	3.15	0.50	6.31
4c	2.86	0.39	7.36
4d	4.02	0.72	5.51
4e	4.46	0.84	5.30
Celecoxib	>30	0.22	>200

electron withdrawing chloro **2** nor the more bulky ethoxy substituent **3** showed good anti-inflammatory or analgesic activity. On the other hand, substitution with bulky aliphatic secondary amines **4a–c** showed very good anti-inflammatory and analgesic activity with the highest activity attributed to the morpholine derivative **4c** decreased with the more lipophilic and more electron withdrawing piperidine derivative **4b** and much falling with the lipophilic and bulkier methyl piperazine derivative **4a**. The last series of substitutions comprising aromatic amine substituents **4d–k** showed that the most active compound was the ortho chloro derivative **4d** and when chloro substitution to para position activity has remarkably decreased. Also substititutions with more electron withdrawing groups like flouro, methoxy, nitro, sulfamoyl or carboxamide substituents and electron donating group like methyl on ortho, meta or para positions led to major decrease in activity **4e–k**. The biological evaluation of ortho carboxamido ether derivative **4l** showed also decreased both anti-inflammatory and analgesic activities.

Molecular docking study revealed that all the newly synthesized compounds have interactions with the amino acids of the active site of COX-2 enzyme. However these interactions fall into three categories according to the number of interacted amino acids which can be summarized as: The first category of compounds interacted with three amino acids on the active site. These compounds are **4c** and **4d** which showed very good anti-inflammatory activity. The second category interacts with two amino acids of the active site as presented by compound **4b** and **4e** with also good biological activity less than or approximate to the activity of the first category. The third category interacts with only one amino acid of the active site with much less activity as presented by compounds **2**, **3** and **4f–l**.

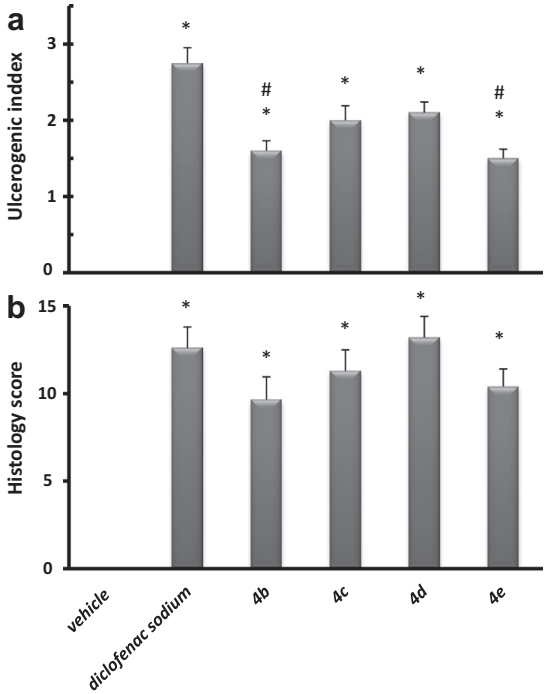


Figure 7. Ulcerogenic index after macroscopic examination of the gastric mucosa and histology score for histologic sections stained with hematoxylin and eosin. The test compounds were administered orally (50 mg/kg) to fasting mice. Data are presented as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's post hoc test. $^{*}P < 0.05$ compared to the vehicle, $\#P < 0.05$ compared to diclofenac sodium.

3. Conclusion

In light of biological results, we can conclude that compounds **4b** and **4c** can be considered as good candidates for the search of novel analgesic and anti-inflammatory agents urged by their in vivo and in vitro activities and lower ulcerogenic effect compared to diclofenac as the reference drug. Further, compound **4c** showed good activity as COX-1 and COX-2 inhibitor in vitro with a selectivity index of 7.3. It also exhibited good inhibition effect on the serum production of TNF-α and IL-6. Compound **4c** showed good docking score and three amino acids interaction with the active site of COX-2 enzyme.

4. Experimental

4.1. Chemistry

All chemicals and reagents were obtained from Aldrich (Sigma–Aldrich, St. Louis, MO, USA), and were used without further purification. Reactions were monitored by TLC, performed on silica gel glass plates containing 60 GF-254, and visualization on TLC was achieved by UV light or iodine indicator. IR spectra were determined on Shimadzu IR 435 spectrophotometer (KBr, cm^{-1}). ^{1}H NMR spectra were carried out using a Mercury 300-BB 300 MHz using TMS as internal standard. ^{13}C NMR spectra were carried out using a Mercury 300-BB 75 MHz using TMS as internal standard. Chemical shifts (δ) are recorded in ppm on δ scale, Micro analytical Center, Cairo University, Egypt. Mass spectra were recorded on Shimadzu Qp-2010 plus Spectrometer, Micro analytical Center, Cairo University, Egypt. Elemental analyses were carried out at the Micro analytical Center, Cairo University, Egypt. Melting points were determined with Stuart apparatus and are uncorrected. Progress of the reactions was monitored using TLC sheets precoated with UV fluorescent silica gel Merck 60F 254 using acetone/benzene (1:9) and were visualized using UV lamp.

All chemicals were obtained from Aldrich, Fluka, or Merck chemicals. The starting material 3-(2-aminophenylamino)-1*H*-pyrazol-5(4*H*)-one (**1**) was prepared as reported.[31]

4.1.1. 3-[2-(6-Chloropyridazin-3-ylamino)phenylamino]-1*H*-pyrazol-5(4*H*)-one (2)

A mixture of **1** (3.02 g, 0.01 mol), 3,6-dichloropyridazine (1.48 g, 0.01 mol) and anhydrous K_2CO_3 (1.96 g, 0.02 mol) in isopropanol (30 mL) was heated under reflux for 4 h. The reaction mixture was concentrated under reduced pressure to half its volume. The precipitated crystalline solid was filtered, washed with water, and recrystallized from ethanol to give **2**.

Yield: 80%; mp 151–152 °C; IR (KBr) (cm^{-1}): 3350–3260 (NH), 3182–3055 (aromatic CH), 2954 (aliphatic CH), 1643 (C=O); ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 2.58 (s, 2H, CH_2 pyrazole), 7.11–7.50 (m, 6H, aromatic CH), 8.60 (s, 1H, NH, D_2O exchangeable), 9.60 (s, 1H, NH, D_2O exchangeable), 10.41 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (100.63 MHz, DMSO-d_6) δ ppm: 35.06(1), 115.07(3), 121.86(4), 139.06(1), 149.72(3), 167.24(1); EIMS (% rel abundance): 304 [M+2] (27.54), 302 [M+] (25.42). Anal. Calcd for $C_{13}H_{11}ClN_6O$: C, 51.58; H, 3.66; N, 27.76; Found C, 51.50; H, 3.62; N, 27.70.

4.1.2. 3-[2-(6-Ethoxypyridazin-3-ylamino)phenylamino]-1*H*-pyrazol-5(4*H*)-one (3)

An appropriate **2** (0.312 g, 0.001 mol) in absolute ethanol (25 mL) containing metallic sodium (0.46 g, 0.02 mol) was heated under reflux for 0.5 h. The reaction mixture was concentrated under reduced pressure, poured into ice cold water then acidified by dilute hydrochloric acid. The precipitated crystalline solid was filtered, washed with water, and recrystallized from ethanol.

Yield: 66%; mp 259–260 °C; IR (KBr) (cm^{-1}): 3380–3255 (NH), 3187–3032 (aromatic CH), 2924–2854 (aliphatic CH), 1697 (C=O); ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 1.23 (t, 3H,CH_3), 2.51 (s, 2H, CH_2 pyrazole), 3.87 (q, 2H, CH_2), 7.10–7.50 (m, 6H, aromatic CH), 9.40 (s, 1H, NH, D_2O exchangeable), 10.20 (s, 1H, NH, D_2O exchangeable), 12.21 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (100.63 MHz, DMSO-d_6) δ ppm: 21.53(1), 34.63(1), 49(1), 115.09(2), 122.21(4), 138.54(1), 149.00(2), 168.46(2), 172.49(1); EIMS (% rel abundance): 314 [M+2] (4.99), 313 [M+1] (6.82), 312 [M](4.72). Anal. Calcd for $C_{15}H_{16}N_6O_2$: C, 57.68; H, 5.16; N, 26.91; Found C, 57.62; H, 5.10; N, 26.88.

4.1.3. General procedure for 4a–l

A mixture of **2** (0.312 g, 0.01 mol), anhydrous K_2CO_3 (1.96 g, 0.02 mol) and the appropriate substituted amine (0.01 mol) in *n*-butanol (30 mL) was heated under reflux for 4 h. The reaction mixture was concentrated under reduced pressure to half its volume. After cooling the precipitated crystalline solid was filtered, washed with water and recrystallized from ethanol.

4.1.3.1. 3-{2-[6-(4-Methylpiperazin-1-ylamino)pyridazin-3-ylamino]phenylamino}-1*H*-pyrazol-5(4*H*)-one (4a). Yield: 70%; mp 195–196 °C; IR (KBr) (cm^{-1}): 3390–3270 (NH), 3186–3062 (aromatic CH), 2978–2870 (aliphatic CH), 1651 (C=O); ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 2.49 (s, 2H, CH_2 pyrazole), 2.50 (s, 3H, CH_3), 3.29–3.31 (m, 4H, piperazine), 3.64–3.67 (m, 4H, piperazine), 7.10–7.48 (m, 6H, aromatic CH), 8.60 (s, 1H, NH, D_2O exchangeable), 9.15 (s, 1H, NH, D_2O exchangeable), 12.21 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (100.63 MHz, DMSO-d_6) δ ppm: 35.10(3), 55.00(2), 117.00(1), 118.00(1), 119.00(1), 121.76(4), 127.00(1), 140.00(1), 149.74(2), 167.23(2); EIMS (% rel abundance): 368 [M+2] (24.34), 367 [M+1] (28.76), 366 [M+] (32.74). Anal. Calcd for $C_{18}H_{22}N_8O$: C, 59.00; H, 6.05; N, 30.58; Found C, 58.95; H, 6.01; N, 30.52.

4.1.3.2. 3-{2-[6-(Piperidin-1-yaminol)pyridazin-3-ylamino]phenylamino}-1*H*-pyrazol-5(4*H*)-one (4b). Yield: 65%; mp 210–211 °C; IR (KBr) (cm^{-1}): 3350–3200 (NH), 3186–3055, (aromatic CH), 2958–2866 (aliphatic CH), 1651 (C=O); ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 2.48 (s, 2H, CH_2 pyrazole), 2.50–2.51 (m, 6H, $3CH_2$ piperidine, J=1.8 Hz), 3.29–3.30 (m, 4H, $2CH_2$ piperidine, J=1.8 Hz), 7.10–7.47 (m, 6H, aromatic CH), 8.60 (s, 1H, NH, D_2O exchangeable), 9.40 (s, 1H, NH, D_2O exchangeable), 12.2 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (100.63 MHz, DMSO-d_6) δ ppm: 35.11(3), 40.61(3), 121.77(3), 149.74(4), 167.24(4), 172.52(1); EIMS (% rel abundance): 351 [M+] (21.31). Anal. Calcd for $C_{18}H_{21}N_7O$: C, 61.52; H, 6.02; N, 27.90; Found C, 61.40; H, 6.08; N, 27.83.

4.1.3.3. 3-{2-[6-(Morpholinoamino)pyridazin-3-ylaminol]phenylamino}-1*H*-pyrazol-5(4*H*)-one (4c). Yield: 75%; mp 201–202 °C; IR (KBr) (cm^{-1}): 3300–3250 (NH), 3182–3055 (aromatic CH), 2958–2920 (aliphatic CH), 1647 (C=O); ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 2.48 (s, 2H, CH_2 pyrazole), 2.50–2.51 (m, 4H, $2CH_2$ morpholine), 3.20–3.29 (m, 4H, $2CH_2$ morpholine), 7.10–7.49 (m, 6H, aromatic CH), 8.61 (s, 1H, NH, D_2O exchangeable), 9.35 (s, 1H, NH, D_2O exchangeable), 10.20 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (100.63 MHz, DMSO-d_6) δ ppm: 34.84(1),115(3),121.83(6),139.11(4),149.74(4),167.21(1);EIMS (% rel abundance): 354 [M+1] (28.57), 353 [M+] (38.7). Anal. Calcd for $C_{17}H_{19}N_7O_2$: C, 57.78; H, 5.42; N, 27.75; Found C, 57.76; H, 5.38; N, 27.71.

4.1.3.4. 3-{2-[6-(2-Chloroanilino)pyridazin-3-ylamino]phenylamino}-1*H*-pyrazol-5(4*H*)-one (4d). Yield: 65%; mp 173–174 °C; IR(KBr) (cm^{-1}): 3370–3280 (NH), 3186–3035 (aromatic CH), 2978–2866 (aliphatic CH), 1651 (C=O); ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 2.49 (s, 2H, CH_2 pyrazole), 7.15–7.50 (m, 10H, aromatic CH), 8.60 (s, 1H, NH, D_2O exchangeable), 10.20 (s, 2H, 2NH, D_2O exchangeable), 10.40 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (100.63 MHz, DMSO-d_6) δ ppm: 34.95(1), 112.89(4), 115.07(2), 121.83(2), 127.89(2), 128.79(4), 130.50(1), 149.21(1),152.38(1),167.38(1); EIMS (% rel abundance): 395 [M+2] (10.87), 393 [M+] (6.51). Anal. Calcd for $C_{19}H_{16}ClN_7O$: C, 57.94; H, 4.09; N, 24.90; Found: C, 57.98; H, 4.12; N, 24.85.

4.1.3.5. 3-{2-[6-(4-Chloroanilino)pyridazin-3-ylamino]phenylamino}-1*H*-pyrazol-5(4*H*)-one (4e). Yield: 60%; mp 169–170 °C; IR (KBr) (cm^{-1}): 3375–3282 (NH), 3186–3040 (aromatic CH), 2980–2868 (aliphatic CH), 1655 (C=O); ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 2.49 (s, 2H, CH_2 pyrazole), 6.57–7.49 (m, 10H, aromatic CH), 8.60 (s, 1H, NH, D_2O exchangeable), 10.10 (s, 2H, 2NH, D_2O exchangeable), 10.30 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (100.63 MHz, DMSO-d_6) δ ppm: 34.95(1), 112.91(3), 115.07(1), 121.72(2), 127.89(4), 128.79(1), 130.50(2), 149.74(1), 157.12(2), 167.24(1), 172.13(1); EIMS (% rel abundance): 395 [M+2] (15.20), 393 [M+] (15.79). Anal. Calcd for $C_{19}H_{16}ClN_7O$: C, 57.94; H, 4.09; N, 24.90; Found: C, 57.89; H, 4.06; N, 24.95.

4.1.3.6. 3-{2-[6-(2-Fluoroanilino)pyridazin-3-ylamino]phenylamino}-1*H*-pyrazol-5(4*H*)-one (4f). Yield: 68%; mp 192–193 °C; IR (KBr) (cm^{-1}): 3350–3240 (NH), 3186–3059 (aromatic CH), 2962–2850 (aliphatic CH), 1651 (C=O); ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 2.48 (s, 2H, CH_2 pyrazole), 7.09–7.51 (m, 10H, aromatic CH), 8.61 (s, 1H, NH, D_2O exchangeable), 9.40 (s, 2H, 2NH, D_2O exchangeable), 10.21 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (100.63 MHz, DMSO-d_6) δ ppm: 34.09(1), 108.91(1), 113.17(1), 118.34(1), 121.79(6), 125.89(1), 127.88(1), 130.50(1), 146.11(1), 149.73(2), 167.22(2), 172.33(1); EIMS (% rel abundance): 377 [M+] (12.98); Anal. Calcd for $C_{19}H_{16}FN_7O$: C, 60.47; H, 4.27; N, 25.98; Found: C, 60.44; H, 4.23; N, 25.93.

4.1.3.7. 3-{2-[6-(4-Methoxyanilino)pyridazin-3-ylamino]phenylamino}-1*H*-pyrazol-5(4*H*)-one (4g). Yield: 55%; mp 206–207 °C; IR (KBr) (cm^{-1}): 3354–3247 (NH), 3182–3059 (aromatic CH), 2962–2858 (aliphatic CH), 1651 (C=O); ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 2.43 (s, 2H, CH_2 pyrazole), 4.20 (s, 3H, CH_3O), 7.01–7.49 (m, 10H, aromatic CH), 8.61 (s, 1H, NH, D_2O exchangeable), 9.36 (s, 2H, 2NH, D_2O exchangeable), 12.20 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (100.63 MHz, DMSO-d_6) δ ppm: 24.00(1), 35.08(1), 114.01(1), 115.02(2), 118.00(1), 121.80(6), 128.79(1), 134.50(1), 139.23(1), 149.73(2), 167.22(2), 172.43(1); EIMS (% rel abundance): 390 [M+1] (19.78),389 [M+] (24.18). Anal. Calcd for $C_{20}H_{19}N_7O_2$: C, 61.69; H, 4.92; N, 25.18; Found: C, 62.74; H, 4.93; N, 25.23.

4.1.3.8. 3-{2-[6-(3-Methylanilino)pyridazin-3-ylamino]phenylamino}-1*H*-pyrazol-5(4*H*)-one (4h). Yield: 70%; mp 199–200 °C; IR (KBr) (cm^{-1}): 3356–3247 (NH), 3188–3057 (aromatic CH), 2962–2855 (aliphatic CH), 1656 (C=O); ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 2.48 (s, 2H, CH_2 pyrazole), 2.51 (s, 3H, CH_3), 7.09–7.49 (m, 10H, aromatic CH), 8.60 (s, 1H, NH, D_2O exchangeable), 10.10 (s, 2H, 2NH, D_2O exchangeable), 10.21 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (100.63 MHz, DMSO-d_6) δ ppm: 35.10(1), 55.11(1), 115.00(1), 118.24(1), 121.77(6), 123.00(1), 127.79(1), 131.50(1), 142.00(1), 149.74(3), 167.24(2), 172.00(1); EIMS (% rel abundance): 374 [M+1] (17.13), 373 [M+] (25.55). Anal. Calcd for $C_{20}H_{19}N_7O$: C, 64.33; H, 5.13; N, 26.26; Found C, 64.28; H, 5.09; N, 26.21.

4.1.3.9. 3-{2-[6-(3-Nitroanilino)pyridazin-3-ylamino]phenylamino}-1*H*-pyrazol-5(4*H*)-one (4i). Yield: 68%; mp 195–196 °C; IR (KBr) (cm^{-1}): 3310–3250 (NH), 3186–3059 (aromatic CH), 2958–2870 (aliphatic CH), 1651 (C=O); ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 2.49 (s, 2H, CH_2 pyrazole), 7.10–7.49 (m, 10H, CH aromatic), 8.61 (s, 1H, NH, D_2O exchangeable), 9.40 (s, 2H, 2NH, D_2O exchangeable), 10.30 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (100.63 MHz, DMSO-d_6) δ ppm: 35.09(1), 115.91(1), 119.07(1), 121.79(6), 124.80(1), 134.50(1), 136.00(1), 147.22(1), 149.74(3), 167.23(2), 172.00(1); EIMS (% rel abundance): 405 [M+1] (18.15),404 [M+] (13.13); Anal. Calcd for $C_{19}H_{16}N_8O_3$: C, 56.43; H, 3.99; N, 27.71; Found: C, 56.40; H, 4.02; N, 27.67.

4.1.3.10. 4-{6-[2-(5-Oxo-4,5-dihydro-1*H*-pyrazol-3-ylamino)phenylamino)pyridazin-3-ylamino}benzenesulfonamide (4j). Yield: 75%; mp 196–198 °C; IR(KBr) (cm^{-1}): 3475–3275 (NH), 3113–3055 (aromatic CH), 2962–2866 (aliphatic CH), 1674 (C=O); ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 2.49 (s, 2H, CH_2 pyrazole), 7.10–7.49 (m, 10H, CH aromatic), 7.92 (2H, NH_2, D_2O exchangeable), 8.60 (s, 1H, NH, D_2O exchangeable), 9.60 (s, 2H, 2NH, D_2O exchangeable), 10.20 (s, 1H,NH, D_2O exchangeable); ^{13}C NMR (100.63 MHz, DMSO-d_6) δ ppm: 35.09(1), 112.91(1), 116.00(1), 118.17(1), 121.79(6), 128.80(1), 129.21(1), 134.50(1), 142.00(1), 149.73(2), 167.22(2), 172.00(1); EIMS (% rel abundance): 440 [M+2] (0.15), 439 [M+1] (0.17), 438[M] (0.21). Anal. Calcd for $C_{19}H_{18}N_8O_3S$: C, 52.05; H, 4.14; N, 25.56; Found: C, 52.10; H, 4.18; N, 25.55.

4.1.3.11. 2-{6-[2-(5-Oxo-4,5-dihydro-1*H*-pyrazol-3-ylamino) phenylamino]pyridazin-3-ylamino}benzamide (4k). Yield: 75%; mp 187–188 °C; IR (KBr) (cm^{-1}): 3478–3272 (NH), 3119–3055 (aromatic CH), 2962–2868 (aliphatic CH), 1670 (C=O); ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 2.50 (s, 2H, CH_2 pyrazole), 7.10–7.52 (m, 10H, CH aromatic), 7.90 (s, 2H, NH_2, NH_2, D_2O exchangeable), 8.61 (s, 1H, NH, D_2O exchangeable), 9.45 (s, 2H, 2NH, D_2O exchangeable), 10.20 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (100.63 MHz, DMSO-d_6) δ ppm: 32.46(1), 114.77(1), 119.61(1), 122.21(1), 124.57(1), 125.43(1), 127.47(1), 127.70(3),

129.26(1), 134.46(1), 135.68(1), 138.03(1), 144.71(1), 145.94(1), 147.34(1), 157.60(1), 160.72(1), 171.23(1); EIMS (% rel abundance): 404 [M+2] (9.84), 403 [M+1] (9.84), 402[M] (11.64). Anal. Calcd for $C_{20}H_{18}N_8O_2$: C, 59.69; H, 4.51; N, 27.85; Found: C, 59.73; H, 4.57; N, 27.82.

4.1.3.12. 2-{6-[2-(5-Oxo-4,5-dihydro-1*H*-pyrazol-3-ylamino) phenylamino]pyridazin-3-yloxy}benzamide (4l). Yield: 70%; mp 171–172 °C; IR (KBr) (cm^{-1}): 3470–3278 (NH), 3115–3058 (aromatic CH), 2962–2869 (aliphatic CH), 1674 (C=O); ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 2.49 (s, 2H, CH_2 pyrazole), 7.10–7.85 (m, 10H, CH aromatic), 8.21 (2H, NH_2, D_2O exchangeable), 8.52 (s,1H, NH, D_2O exchange), 9.80 (s,1H,NH, D_2O exchangeable), 10.80 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (100.63 MHz, DMSO-d_6) δ ppm: 32.57(1), 119.63(1), 127.08(3), 127.24(2), 129.27(2), 134.54(2), 138.04(2), 144.73(2), 147.10(2), 157.53(2), 170.11(1); EIMS (% rel abundance): 403[M] (16.90). Anal. Calcd for $C_{20}H_{17}N_7O_3$: C, 59.55; H, 4.25; N, 24.3; Found: C, 59.51; H, 4.20; N, 24.28.

4.2. Molecular docking

All the molecular modeling studies were carried out on an Intel Pentium 1.6 GHz processor, 512 MB memory with Windows XP operating system using Molecular Operating Environment (MOE, 10.2008) software. All the minimizations were performed with MOE until a RMSD gradient of 0.05 kcal mol^{-1} $Å^{-1}$ with MMFF94X forcefield and the partial charges were automatically calculated. The X-ray crystallographic structure of COX-2 enzyme with its co-crystallized ligand (Diclofenac) in the file (PDB ID: 1PXX) was obtained from the protein data bank. The enzyme was prepared for docking studies where: (i) Ligand molecule was removed from the enzyme active site. (ii) Hydrogen atoms were added to the structure with their standard geometry. (iii) MOE Alpha Site Finder was used for the active sites search in the enzyme structure and dummy atoms were created from the obtained alpha spheres. (iv) The obtained model was then used in predicting the ligand enzyme interactions at the active site.

4.3. Pharmacological activity

4.3.1. Animals

Male healthy Swiss albino mice with a weight of 25–32 g were used in the current experiment. Mice were purchased from the Modern Veterinary Office for Laboratory Animals (Cairo, Egypt). Animals were allowed to habituate for one week before starting the experiment then divided into different experimental groups, 6 mice each with food and water *ad libitum*. All experiments were conducted between 16:00 and 18:00 h to ensure animal activity and to eliminate circadian influence on animal behavior. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the Faculty of Pharmacy, Suez Canal University, Ismailia, Egypt.

4.3.2. Preparation of the tested compounds and the standard drug

The test compounds and diclofenac sodium were suspended in a 1% sodium carboxymethylcellulose (CMC) aqueous solution. Therapies were given to mice by oral gavage in a volume equals 5 mL/kg. Control mice received appropriate volumes of the dosing vehicle (5 mL/kg). Diclofenac sodium (25 mg/kg) was used as a reference analgesic anti-inflammatory drug. Glacial acetic acid was purchased from ADWIC Company (Cairo, Egypt) and used to prepare 1% acetic acid solution. Carrageenan (Sigma–Aldrich®, St. Louis, Missouri, USA) was freshly prepared as suspension (1% w/v in 0.9% saline, injected as 0.05 mL/mouse).

4.3.3. Analgesic activity

Analgesic activity was tested using acetic acid-induced writhing method.[37] Mice were habituated for 30 min individually in the testing cage prior to acetic acid injection. Screening of the analgesic activity was performed after administration of the test compounds (25 mg/kg, p.o.) and compared to an equal dose of diclofenac sodium as the reference control. Control mice received oral doses of 1% CMC suspension. Thirty minutes after drug administration, acetic acid solution (0.1 mL, 1%, ip) was given to each mouse to induce writhing.

The total number of abdominal contractions (writhing movements) was counted for the next 15 min, starting with the 5th minute after acetic acid injection. The analgesic activity was expressed as follows:

% Analgesic activity = $\{(n-n')/n\} \times 100$, where n = mean number of writhes of control group and n' = mean number of writhes of test group. The test compounds with percentage protection greater than 50%, a dose response curve was plotted using the data taken from different five doses (10, 15, 20, 25 and 30 mg/kg).

4.3.4. Anti-inflammatory activity

The anti-inflammatory activity was tested using carrageenan-induced hind paw edema method.[38] Animals were divided in groups of six each. One group of mice was kept as control and the mice of other groups were pre-treated with the test compounds suspended in 1% CMC aqueous solution, given orally thirty minutes before carrageenan injection. Carrageenan solution was injected into the subplantar tissue of the right hind paw of each mouse, whereas control mice were injected with saline. The paw volume was measured before and four hours after injection of carrageenan. Mean values of the treated groups were compared with the mean value of the control group. The percentage inhibition of inflammation was calculated by applying the following formula:

Anti-inflammatory activity (% inhibition) = $(V_c - V_t)/V_c \times 100$, where V_c = edema volume in control group, V_t = edema volume in groups treated with the test compounds.

Thereafter, mice were sacrificed by decapitation under anesthesia using a mixture of ketamine (50 mg/kg, ip) and xylazine (10 mg/kg, ip). A laparotomy was performed, and blood samples were withdrawn from the heart. Thirty minutes after collection, blood samples were centrifuged at 2000*g* for 15 min. Serum samples were separated, collected and stored at −80 °C until used for enzyme linked immunosorbent assay (ELISA). ELISA kits for TNF-α and IL-2 (Glory Science Co., Ltd, Del Rio, TX, USA) were used for determination of tissue levels of these markers. The assays were carried out following the instructions of the manufacturer using an automated ELISA reader (Europe S.A., Belgium). The most biologically active compounds were further screened for ulcerogenic potential.

4.3.5. Acute ulcerogenesis

Compounds that showed analgesic and anti-inflammatory activity were tested for acute ulcerogeneic potential.[39] Food pellets were removed 24 h before administration of the test compounds. Ulcerogenicity was evaluated after oral administration of the test compound or diclofenac at a dose of 50 mg/kg. Control mice were treated orally with the vehicle (1% CMC suspension). Mice were treated with the test compounds, then fed with a normal palatable diet for 18 h. Mice were then anesthetized with a mixture of ketamine (50 mg/kg, ip)/xylazine (10 mg/kg, ip) and sacrificed by decapitation. The stomach was removed, opened along the greater curvature then washed with distilled water and cleaned gently in saline. The gastric mucosa of the mice were examined by means of a magnifying glass. For each stomach, the severity of mucosal damage was assessed by measuring ulcerogenic index that is, severity of drug to cause mucosal damage, which is the difference

between the mean score of each treated group and the mean score of the control group.

For histopathological examination, the glandular area of gastric tissue samples were fixed in neutral buffered formalin for 24 h. Stomach sections were dehydrated with ethanol, passed through xylene then embedded in paraffin. Thereafter, the 5-μm thick paraffin sections were stained with hematoxylin and eosin (H&E). The specimens were assessed according to the reported method.[40] In a brief, a 1 cm length of each histological section was assessed for epithelial cell loss (a score of 0–3), edema in the upper mucosa (a score of 0–4), hemorrhagic damage (a score of 0–4) and presence of inflammatory cells (a score of 0–3). The sections were assessed blindly by an experienced pathologist.

4.3.6. In vitro COX-1 and COX-2 inhibition assay

In vitro inhibition of COX-1 and COX-2 enzymes was determined using COX (ovine) inhibitor screening assay EIA kit according to manufacturer's instructions.[41] COX catalyzes the first step in the biosynthesis of arachidonic acid to PGH_2. The $PGF_2\alpha$ produced from PGH_2 by reduction with stannous chloride is measured by EIA. The tested compounds were dissolved in DMSO. The enzyme COX-1 and COX-2 (10 μL), heme (10 μL) and samples (20 μL) were added to the supplied reaction buffer solution (950 μL, 0.1 M Tris–HCl, pH 8 containing 5 mM ethylenediamine tetraacetate (EDTA) and 2 mM phenol). The mixture of these solutions were incubated for a period of 10 min at 37 °C, after that COX reactions were initiated by adding arachidonic acid (10 μL, making final concentration 100 μM) solution. The COX reactions were stopped by addition of HCl (1 M, 50 μL) after 2 min and then saturated stannous chloride (100 μL) was added and again incubated for 5 min at room temperature. The $PGF_2\alpha$ formed in the samples by COX reactions was quantified by EIA. The pre-coated 96-well plate was incubated with samples for 18 h at room temperature. After incubation, the plate was washed to remove any unbound reagent and then Ellman's reagent (200 μL), which contains substrate to acetyl cholinesterase, was added and incubated for 60–90 min (until the absorbance of B_o well is in the range 0.3–0.8 A.U.) at room temperature. The plate was then read by an ELISA plate reader at 410 nm. The IC_{50} of inhibition of COX-1 and COX-2 was calculated by the comparison of the sample treated incubations to control incubations. Celecoxib was used as reference standard in the study.

4.3.7. Statistical analysis

Data were collected, tabulated and expressed as mean ± SEM. One-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test were used to test statistical differences between the groups for parameters with Gaussian distribution. However, parameters with non-Gaussian distribution were analyzed using Kruskal–Wallis test (non-parametric ANOVA) followed by Dunnett's test for multiple comparisons. Linear regression lines were plotted using Microsoft Excel 2007 whereas other statistical analyses were performed employing the Statistical Package for Social Sciences, version 17 (SPSS Inc., Chicago, IL, USA). All P values reported are two-tailed and $P < 0.05$ was considered significant.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.02.042.

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