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New indomethacin analogs as selective COX-2 inhibitors: Synthesis, COX-1/2 inhibitory activity, anti-inflammatory, ulcerogenicity, histopathological, and docking studies

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Abstract

New indomethacin analogs **4a–g**, **5**, **6**, **8a**, and **8b** were synthesized to overcome the nonselectivity and ulcer liability of indomethacin. All newly synthesized compounds were more potent against cyclooxygenase 2 (COX-2; IC_{50} value range: 0.09–0.4 μM) as compared with celecoxib (IC_{50} = 0.89 μM). Compounds **4a**, **4b**, **4d**, **5**, and **6** showed the highest COX-2 selectivity index (SI range = 4.07–6.33) as compared with indomethacin (SI = 1.14) and celecoxib (SI = 3.52). Additionally, **4a**, **4b**, **4d**, **5**, and **7** showed good anti-inflammatory activity with edema inhibition (79.36–88.8%), relative to celecoxib (78.96%) and indomethacin (90.43%), after 5 h. Also, ulcerogenic effects and histopathological examination were assessed for the most potent analogs, **4b**, **4d**, **5**, and **6**, to determine their safety. The results can shed light on indomethacin analog **5** as a remarkable anti-inflammatory lead compound with a good safety profile (ulcer index = 10.62) close to the nonulcerogenic drug celecoxib (ulcer index = 10.53) and better than indomethacin (ulcer index = 18.50). Docking studies were performed in the COX-2 active site for the most active compounds, to test their selectivity and to confirm their mechanism of action.

KEYWORDS

anti-inflammatory activity, COX-1, COX-2, histopathology, indomethacin

1 | INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) produce their pharmacological effect through inhibiting the action of cyclooxygenase enzymes (COX-1 and COX-2),[1] thus preventing the catalytic biotransformation of arachidonic acid into the corresponding proinflammatory prostaglandins and thromboxanes (TXs).[2] It was established that there are two distinct isoforms for cyclooxygenase enzyme: COX-1 isoenzyme, which is the constitutive form produced in different tissues and responsible for maintenance of normal physiological functions such as gastric cytoprotection, platelet aggregation, vascular homeostasis[3]; and COX-2 isoenzyme, which is the induced form responsible for fever, pain, and other inflammatory symptoms.[4] The structures of both COX-1 and COX-2 are closely similar to each other. However, the COX-2 active site is slightly larger, because it contains a secondary internal side pocket that could accommodate bigger structures and also its central channel is wider by approximately 17%.[5]

The long-term use of traditional nonsteroidal anti-inflammatory drugs, like aspirin, ibuprofen, indomethacin (**1**), and naproxen (**I**) as free carboxylate derivatives, could produce gastroduodenal mucosal injury, renal failure, and bleeding.[6] The ulcerogenicity for traditional NSAIDs was attributed to the direct effect of the COOH moiety and indirectly to nonselective inhibition of both COX enzymes. Therefore, the researchers synthesized many amide derivatives of these traditional NSAIDs that lacked a free COOH moiety. For example,

transformation of the acidic COOH group in indomethacin and formation of amide linkage resulted in producing compound **II** (Figure 1), which was 10 times more potent as a COX-2 inhibitor than fluorocoxibs.[7] Also, naproxen derivatives (**III–VI**) (Figure 1)[8–10] were synthesized in which the ulcer effect was reduced as compared with the standard drug. Furthermore, selective COX-2 inhibitors (coxibs) as celecoxib, rofecoxib, and valdecoxib were developed to overcome the nonselective drawbacks, but unfortunately, rofecoxib and valdecoxib were found to cause myocardial infarction and high blood pressure; therefore, their clinical use was terminated.[11,12] Regardless of the nonselectivity of the traditional NSAIDs, they did not pose a cardiac risk as coxibs.[13] Researchers have reconsidered the use of NSAIDs in a way aiming to improve their anti-inflammatory activity and decrease their ulcerogenic effect. According to these findings and as a continuation of our work on the development of anti-inflammatory drugs with a good safety profile,[14–21] we now report the synthesis of new amide indomethacin derivatives in which the acidic COOH group in position 3 of indomethacin was replaced with different groups to afford different thiourea derivatives **4a–g**, 2-cyanoacetyl derivative **5**, 2-pyridinone derivative **6**, and 3-cyanopyridone derivatives **8a** and **8b** (Figure 2). The synthesized derivatives are free of the acidic character responsible for the direct gastroduodenal ulcer. Also, their bulkiness was increased, causing them to be too large to fit the small COX-1 active site, increasing the selectivity for the COX-2 enzyme,[22] and consequently decreasing the gastroduodenal ulcer. The synthesized derivatives were evaluated for their COX-1/COX-2 inhibitory activity. Furthermore, the carrageenan-induced rat paw edema model and histopathological study were accomplished to evaluate their anti-inflammatory activity and their gastric safety. The compounds were docked into the COX-2 active site to reveal their possible mechanism of action. The handling of animals or tissues was preceded according to the institutional ethical committee instructions.

2 | RESULTS AND DISCUSSION

2.1 | Chemistry

To synthesize the new indomethacin analogs, indomethacin **1** was converted to the acid chloride **2** through reaction with thionyl

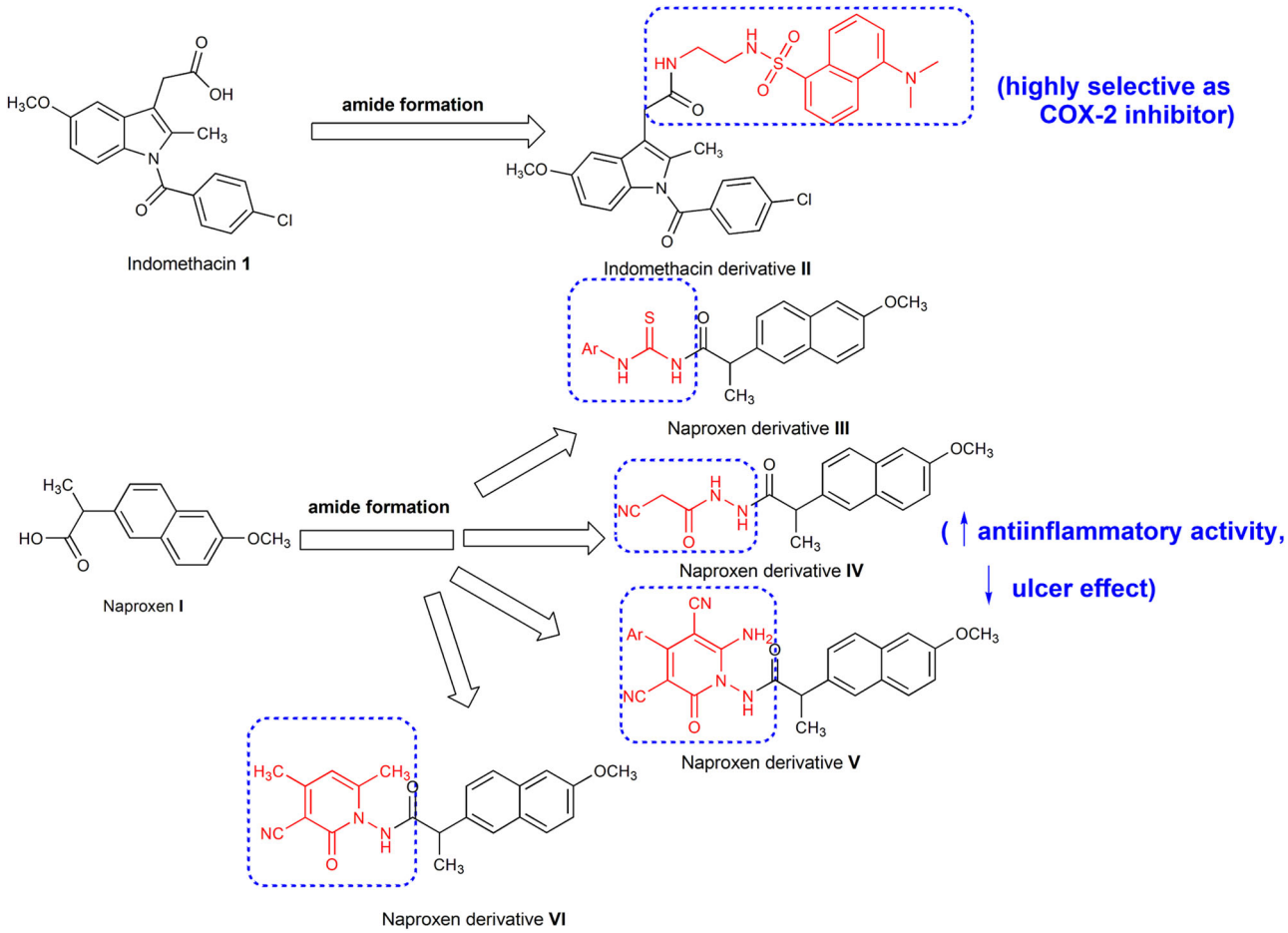


FIGURE 1 Chemical structures of traditional NSAIDs (indomethacin **1** and naproxen **I**) and some reported modified indomethacin and naproxen amide derivatives (**II–VI**)[7–10] as selective COX-2 inhibitors

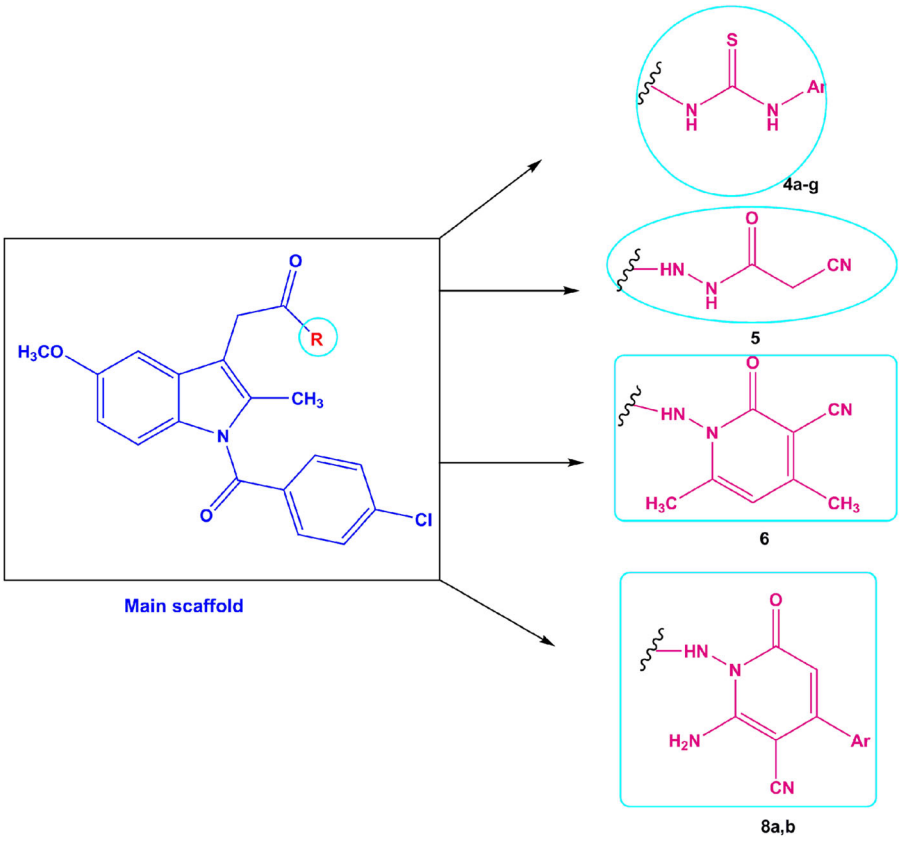
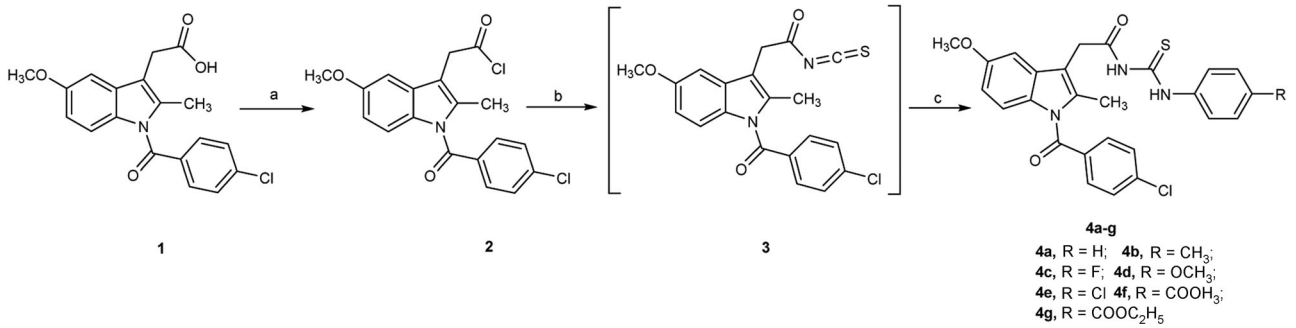
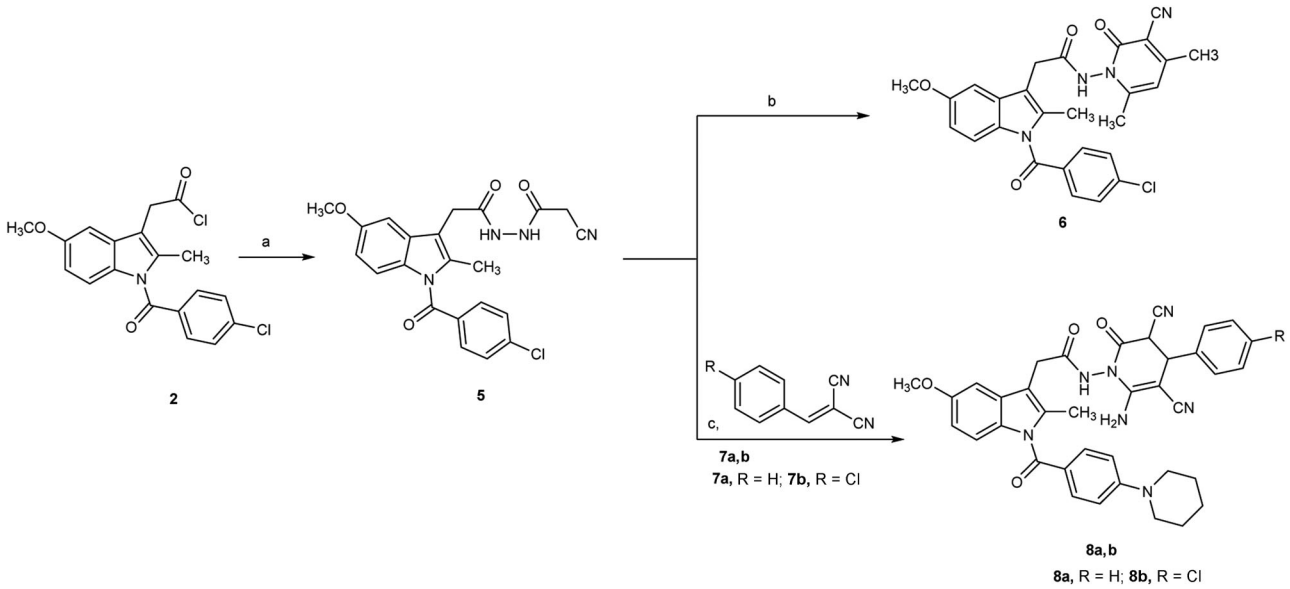


FIGURE 2 The strategy for the design of the new indomethacin analogs **4–8**

chloride, as reported.[23] The active acid chloride **2** was reacted with ammonium thiocyanate to give the intermediate indomethacin isothiocyanate **3**, which was not isolated and heated under reflux condition with various aromatic amines to yield a series of new thiourea–indomethacin hybrid analogs **4a–g** (Scheme 1). However, compound **2** was reacted with cyanoacetic acid hydrazide[24] to afford 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl)-*N*′-(2-cyanoacetyl)acetohydrazide **5** (Scheme 2), which upon reaction with acetylacetone furnished compound **6**. The cyclized product **6** was postulated to be formed via a nucleophilic attack of **5** on acetylacetone to form a Michael adduct, which spontaneously was cyclized by losing a molecule of water, giving **6**. Finally, compound **5** was heated under reflux with the appropriate arylidenes **7a** and **7b**[25,26] in the presence of piperidine to afford the respective pyridine derivatives **8a** and **8b** (Scheme 2). The infrared (IR) spectrum of compounds **4a–g** showed absorption bands at 3170.97– 3618.46, referring to the NH group, and bands at 1666.50–1743.65, indicating the presence of the C═O group. The ^{1}H NMR (nuclear magnetic resonance) spectrum revealed two singlet signals at δ 10.35–12.38 ppm for two NH groups that are exchangeable with D_2O. The IR spectrum of compound **5** showed an absorption band at 3197.98, indicating the presence of two NH groups, and another band at 2256.71 for the C≡N group. The ^{1}H NMR analysis showed a signal of two protons at δ 3.54 ppm, referring to CH_2–CN, a singlet signal at δ 10.28 ppm, indicating the presence of two NH groups exchangeable with D_2O. The ^{1}H NMR spectrum of compound **6**



SCHEME 1 The synthesis of compounds **4a–g**. Reagents and conditions: (a) Thionyl chloride, benzene, reflux 2 h; (b) ammonium thiocyanate, reflux 2 h; (c) suitable amine, reflux 3 h



SCHEME 2 The synthesis of compounds **5**, **6**, **8a**, and **8b**. Reagents and conditions: (a) Cyanoacetic acid hydrazide, benzene, RT 24 h; (b) acetylacetone, absolute ethanol, piperidine, reflux 6 h; (c) absolute ethanol, piperidine, reflux 2 h

showed two singlet signals at δ 2.16 and 2.27 ppm, indicating the presence of two CH_3 groups of the oxopyridine ring. Unexpectedly, during the reaction of **5** with **7a** and **7b**, the piperidine replaced the Cl atom of the *para*-chlorobenzoyl moiety of **5**, which was confirmed by the presence of a multiplet signal at δ 1.55–1.64 ppm and a triplet signal at δ 2.99–3.01 ppm in the ^{1}H NMR spectra and the presence of three signals in ^{13}C NMR spectra at δ 22.07–22.17 ppm (piperidine C-4), 22.69–22.83 ppm (piperidine C-3, C-5), and 44.26–44.34 ppm (piperidine C-2, C-6).

2.2 | Pharmacology/biology

The target indomethacin analogs were assayed against COX-1 and COX-2 using *N*, *N*, *N*′,*N*′-tetramethyl-*p*-phenylenediamine at 590 nm and the enzyme immunoassay (EIA) kit.[27] The potency of the synthesized compounds was determined as the concentration causing 50% inhibition (IC_{50}). Also, the COX-2 selectivity indexes (SI) were calculated as IC_{50} (COX-1)/IC_{50} (COX-2) and then compared with the SI of the standard drugs indomethacin as a nonselective COX inhibitor and celecoxib as a selective COX-2 inhibitor. The results obtained from the evaluation of all compounds, **4a**–**g**, **5**, **6**, **8a**, and **8b**, are listed in Table 1.

All compounds, **4a**–**g**, **5**, **6**, **8a**, and **8b**, inhibited the COX-1 isozyme at higher doses with an IC_{50} range of 0.19–1.82 μM. This range is more than that of indomethacin (IC_{50} = 0.10 μM), which means that the prepared derivatives have lower potency against COX-1 and consequently would be safer. Regarding COX-2, all compounds, **4a**–**g**, **5**, **6**, **8a**, and **8b**, showed an IC_{50} inhibitory range of 0.08–0.4 μM, which is more potent than celecoxib itself (IC_{50} = 0.89 μM). The SI calculation result showed that compound **4c** showed a good SI = 3.33, which is closer to that of celecoxib (SI = 3.52), whereas compounds **4e**, **4f**, **4g**, **8a**, and **8b** showed a moderate SI range (2.11–3.22). However, compounds **4a**, **4b**, **4d**, **5**, and **6** showed the highest SI range (4.07–6.33), which appeared to be more selective than celecoxib (SI = 3.52) and indomethacin (SI = 1.14). After determination of SIs for all prepared compounds, **4a**–**g**, **5**, **6**, **8a**, and **8b**, the anti-inflammatory (AI) activity

TABLE 1 In vitro IC_{50} values of COX-1 and COX-2 and selectivity indices (SI) of the tested compounds, indomethacin, and celecoxib

Compounds	COX-1 IC_{50} (μM)[a]	COX-2 IC_{50} (μM)[a]	COX-2 SI[b]
4a	1.10	0.27	4.07
4b	0.57	0.09	6.33
4c	0.30	0.09	3.33
4d	0.63	0.11	5.72
4e	0.29	0.09	3.22
4f	0.27	0.09	3
4g	0.22	0.09	2.44
5	1.82	0.4	4.55
6	1.54	0.31	4.96
8a	0.39	0.15	2.6
8b	0.19	0.09	2.11
Indomethacin	0.10	0.087	1.14
Celecoxib	3.14	0.89	3.52

[a]IC_{50}: The concentration causing 50% COX inhibition.
[b]Selectivity index (IC_{50} COX-1/IC_{50} COX-2).

TABLE 2 In vivo anti-inflammatory activity of the tested compounds **4a**, **4b**, **4d**, **5**, and **6**, indomethacin, and celecoxib against carrageenan-induced rat paw edema

Compounds	% of inhibition at 1 h	% of inhibition at 3 h	% of inhibition at 5 h
4a	30.48 ± 5.11[b]	73.03 ± 7.1[b]	79.56 ± 7.3
4b	21.36 ± 4.64	53.77 ± 5.25	88.52 ± 0.47
4d	76.76 ± 6.07[a,b]	81.06 ± 3.78[a,b]	88.8 ± 2.7
5	38.8 ± 3.32[a,b]	74.47 ± 11.19[b]	81.16 ± 2.45
6	23.57 ± 3.2	43.88 ± 5.22	79.36 ± 2.34
Indomethacin	25.9 ± 1.12	61.38 ± 1.08	90.43 ± 4.88
Celecoxib	18.37 ± 1.17	47.7 ± 2.82	78.96 ± 2.42

Note: Data are expressed as mean ± SEM (n = 6 rats). Statistical analysis was carried out using the one-way analysis of variance test, followed by the least significant difference post-hoc test.

[a] Significantly different from the indomethacin group at $p < .05$.

[b] Significantly different from the celecoxib group at $p < .05$.

of the most selective ones, **4a**, **4b**, **4d**, **5**, and **6**, against COX-2 was evaluated using the carrageenan-induced rat paw edema method.[28] The AI activity was calculated at 1, 3, and 5 h after carrageenan injection, as presented in Table 2.

It was noted that all compounds significantly decreased inflammation as compared with carrageenan at all time intervals. A comparative study of the anti-inflammatory activity of the test compounds, relative to indomethacin and celecoxib as reference drugs, at the different time intervals, showed that after 1 h, they had poor to good anti-inflammatory activity (AI = 21.36–76.76%) in comparison with indomethacin (AI = 25.9%) and celecoxib (AI = 18.37%). After 3 and 5 h, the anti-inflammatory activity was extremely increased and the target compounds showed AI = 43.88–81.06% and 79.36–88.8%, respectively, in comparison with indomethacin (AI = 61.38% and 90.43%, respectively) and celecoxib (AI = 47.7% and 78.96%, respectively). The methoxy derivative **4d** showed the highest AI activities at all time intervals (after 1 h, AI = 76.76%, after 3 h, AI = 81.06%, and after 5 h, AI = 88.8%). The ulcerogenic effect of the most COX-2 selective compounds, **4b**, **4d**, **5**, and **6**, which also showed a high percentage of edema inhibition, was evaluated according to a previous study (Table 3).[29]

The normal control group received only vehicles, the celecoxib group received celecoxib in a dose of 50 mg/kg, the indomethacin group received the drug at a dose of 50 mg/kg, and the other four groups received the prepared compounds **4b**, **4d**, **5**, and **6**, respectively, in a dose of 50 mg/kg for each. Compound **5** was the safest within all derivatives and showed the least ulcer index (UI = 10.62) when compared with indomethacin (UI = 18.50) and it was close to that of the nonulcerogenic reference drug celecoxib (UI = 10.53), whereas compounds **4b** and **4d** showed a lower ulcerogenic activity with ulcer index 14 and 17.25, respectively, as compared with indomethacin (UI = 18.50). However, compound **7** was the least safe drug, as it showed the highest ulcer index (UI = 24.87), which exceeded that of indomethacin and celecoxib.

Examination of microscopical lesions was carried out to evaluate the ulcerogenic effect of the in vivo active compounds on the rat's stomach including both glandular and nonglandular portions. Also, the severity of these lesions was compared with that induced by reference drugs indomethacin, celecoxib, and the control negative group (Table 4).

In the control negative group, a normal histological structure of both glandular and nonglandular stomach was observed. The glandular portion showed normal mucosal lining, submucosa, and mucosal layers (Figure 3a-i), associated with the normal histological structure of the nonglandular portion (Figure 3a-ii). For the indomethacin group, severe pathological lesions were observed in the form of degenerative changes and necrotic changes in the glandular and nonglandular stomach. The glandular stomach exhibited erosive and ulcerative changes with the presence of lymphocytic infiltration and congestive blood vessels accompanied by edema in the submucosal layer. Hyalinosis of the muscular layer and diffuse leukocytic infiltration are observed (Figure 3b-i). The nonglandular stomach exhibited focal erosive and ulcerative lesions and hyperkeratosis (Figure 3b-ii). However, in the celecoxib group, mild lesions could be detected in the glandular stomach. These lesions were in the form of degenerative changes of the mucosal lining and mild leukocytic infiltration in the submucosal layer (Figure 3c-i). The nonglandular stomach portion showed mild hyperkeratosis (Figure 3c-ii).

Regarding **4b** and **4d**, moderate pathological lesions in the form of multifocal areas of degeneration in the lining epithelium were observed. These lesions were associated with moderate necrotic changes. Also, moderate-to-severe submucosal congestion and leukocytic infiltration were found to be associated with mild hyalinosis in the muscular layer (Figure 4: **4b**-I and **4d**-I), and mild-to-moderate hyperkeratosis of the nonglandular stomach could be found without the presence of any erosive or ulcerative lesions (Figure 4: **4b**-II and **4d**-II). Administrations of **5** showed mild degenerative changes of the glandular mucosal lining, mild submucosal lymphocytic infiltration, and minimal necrotic changes (Figure 3: **5**-I), and the more or less

TABLE 3 Ulcerogenic liability for the most active derivatives, **4b**, **4d**, **5**, and **6**, and reference drugs indomethacin and celecoxib

Compounds	% of incidence	Average number of ulcers	Average severity	Ulcer index
4b	100	3	1	14
4d	100	6.25	1	17.25
5	75	2	1.12	10.62
6	100	13.75	1.12	24.87
Control	66	1.30	1	8.90
Indomethacin	100	6.30	2.20	18.50
Celecoxib	75	1.75	1.28	10.53

TABLE 4 Scoring of different pathological lesions caused by the tested compounds (**4b**, **4d**, **5**, **6**) on both glandular and nonglandular portions of the stomach as compared with those induced by celecoxib and indomethacin as standard drugs

Lesion	4b	4d	5	6	Celecoxib	Indomethacin	Negative
Glandular stomach							
Mucosa							
Degenerative changes	+	++	++	+++	++	+++	−/+
Nuclear pyknosis	+	+	−/+	+++	+	+++	−
Erosion	++	++	+	++	+	+++	−
Ulcer	+	+	−	+++	−	+++	−
Submucosa							
Congestion	++	++	+	+++	++	+++	−
Leukocytic infiltration	++	++	+	+++	+	+++	−
Edema	+	+	−/+	+++	+	+++	−
Musculosa							
Degenerative changes	+	+	+	+++	+	+++	−
Hyalinosis	−	+	+	++	+	+++	−
Leukocytic infiltration	−	−	−	++	+	+++	−
Nonglandular stomach							
Erosion	−	−	−	+	−	++	−
Ulcer	−	−	−	−	−	−/+	−
Hyperkeratosis	+	+	−	++	+	+++	−

Abbreviations: −/+, minimal, +, mild, ++, moderate, +++, severe.

normal histological structure could be found in the nonglandular stomach (Figure 3: **5**-II). Finally, administrations of **6** revealed the presence of severe degenerative changes and necrotic changes of the glandular stomach (Figure 4: **6**-I). Additionally, massive lymphocytic infiltration and congestion of the submucosal and muscular layers were observed. The latter showed moderate hyalinosis, moderate hyperkeratosis, and erosive and ulcerative changes, which could be detected in the glandular layer. The nonglandular portion showed focal areas of hyperkeratosis (Figure 4: **6**-II).

2.3 | Molecular docking

A docking study was performed for the most COX-2 selective compounds, **4a**, **4b**, **4d**, **5**, and **6**, using the MOE modeling version (2015.10) to illustrate the possible conformations that bound to the COX-2 receptor, which may reveal the possible mechanism of action for their anti-inflammatory activity toward the COX-2 active site. The X-ray coordinates were downloaded from the Protein Data Bank with code (PDB: 6BL4).[10] The dansyl moiety of the ligand was

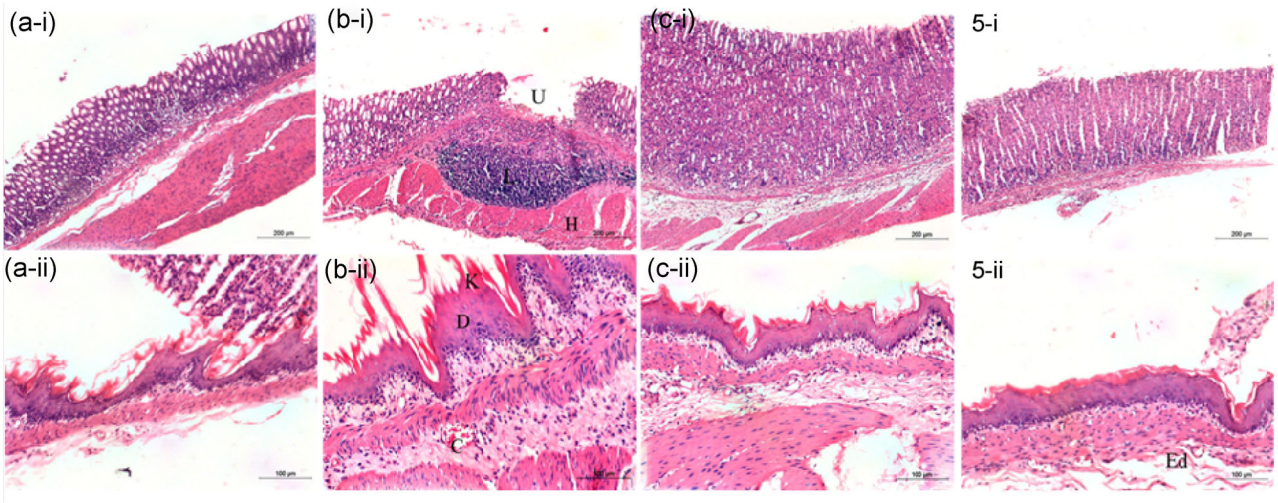


FIGURE 3 Histopathological alterations of the glandular stomach (first row) and nonglandular stomach (second row) in the control negative group (a-i, a-ii), indomethacin (b-i, b-ii), celecoxib (c-i, c-ii), and compound **5** (**5**-I, **5**-II)

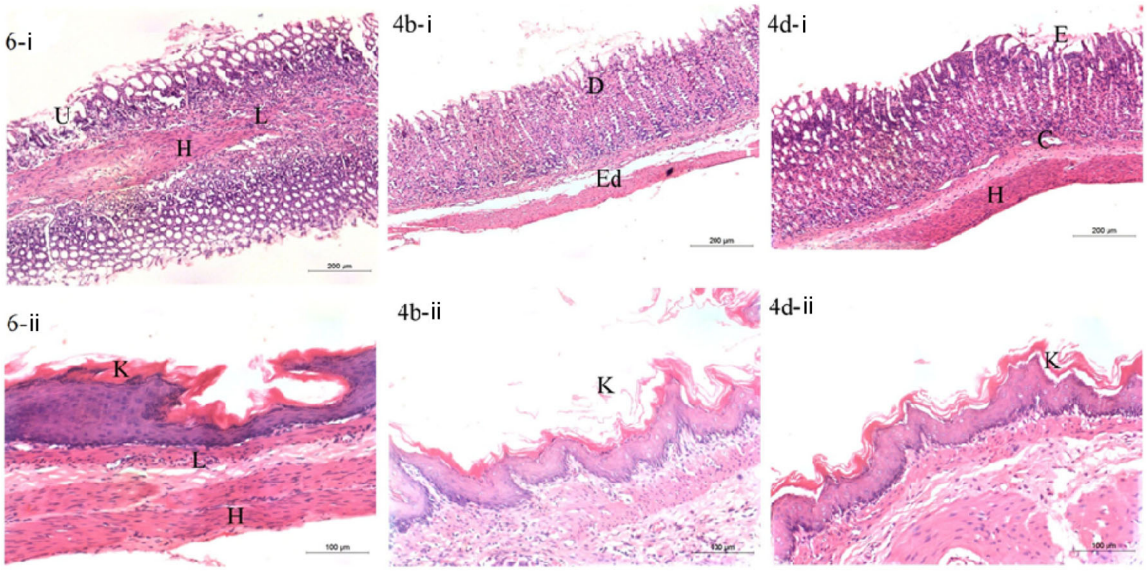


FIGURE 4 Histopathological alterations of the glandular stomach (first row, I) and nonglandular stomach (second row, II) in compound **6** (**6**-I, **6**-II), compound **4b** (**4b**-I, **4b**-II), and compound **4d** (**4d**-I, **4d**-II). C, congestion; D, degenerative changes; E, erosion; Ed, edema; H, hyalinosis; K, hyperkeratosis; L, leukocytic infiltration; U, ulcer

recognized to bind in the lobby region of the COX-2 active site. It was observed that a hydrogen bond was formed through the carbonyl oxygen between the phenyl and indole ring and Ser530 at a distance of 3.0 Å.[10] Tyr355 formed a hydrogen bond with the nitrogen atom of the amide at a distance of 2.8 Å. Arg120 formed a hydrogen bond with the carbonyl oxygen of the amide. The binding mode of the indomethacin–ethylenediamine–dansyl conjugate, a highly COX-2 selective inhibitor, inside the active site of the COX-2 isoenzyme is shown in Figure 5.

The ligand formed two hydrogen bonds of C═O linked to the indole ring and C═O of the amide group with Arg120 and Ser530 amino acids with a distance of 3.00 and 2.91 Å, respectively. The energy score for the ligand inside the COX-2 isoform was 17.43 kcal/mol. The most active compounds **4a**, **4b**, **4d**, **5**, and **6** fitted within the COX-2 active site with a binding energy score range of −12.82 to −16.08 kcal/mol, which is close to the ligand (−17.43 kcal/mol). It was observed that compound **4b** formed two hydrogen bond interactions: One of them was the interaction of C═O linked to the indole ring with Val116 amino acid with a distance of 3.20 Å and the other between C═O of the amide linkage and Arg120 amino acid at a distance of 2.97 Å with an energy score of −16.08 kcal/mol (Figure 6).

Also, compound **4d** formed one hydrogen bond interaction of benzoyl C═O with Ser530 amino acid with a distance of 2.95 Å with an energy score of −15.56 kcal/mol. Fortunately, compound **5** formed three hydrogen bonds of NH, amide C═O, and N of C≡N with Glu524, Arg120, and Lys83 at a distance of 2.88, 2.74, and 3.51 Å, respectively, with an energy score of −14.79 kcal/mol. All these interactions in addition to the presence of the C≡N group proved the

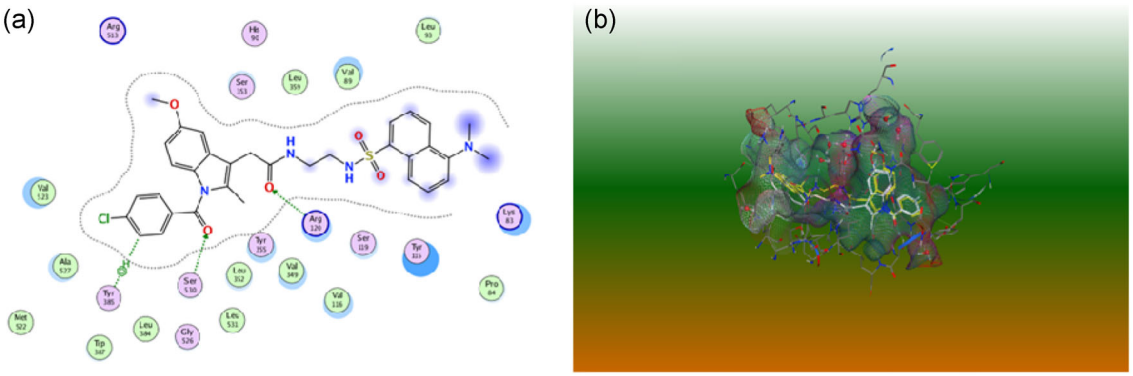


FIGURE 5 Binding of the ligand (indomethacin–ethylenediamine–dansyl conjugate) inside the COX-2 active site. (a) Two-dimensional interaction of the proposed binding mode of the ligand inside the active site of COX-2, which forms two H-bonds with Arg120 and Ser530 amino acids; (b) three-dimensional interaction of the ligand

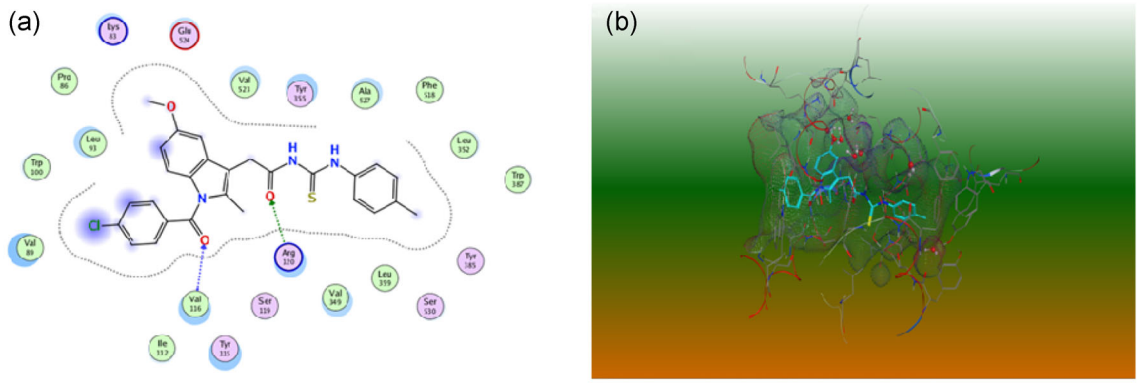


FIGURE 6 Binding of one of the most active compounds, **4b**, inside the COX-2 active site. (a) Two-dimensional interaction of the proposed binding mode of **4b**, which forms two hydrogen bonds with Val116 and Arg120 amino acids; (b) three-dimensional interaction of **4b** (cyan)

high selectivity of compound **5** toward the COX-2 isoenzyme (Figure 7).

Compound **6** showed two hydrogen bonds with Ser530 amino acid with a distance of 2.93 and 3.39 Å, respectively. Also, compound **4a** showed one hydrogen bond of C═S with Arg120 amino acid with a distance of 3.21 and formed a hydrophobic interaction with Tyr115 with a distance of 4.10 Å. Docking scores, number of hydrogen bonds, amino acid residues responsible for hydrogen bond formation, distances, and functional groups are listed in Table 5.

2.4 | Structure–activity correlation

Four series of indomethacin amide derivatives **4–8** were synthesized and evaluated as selective COX-2 inhibitors. It was found that the amidation of indomethacin increases the anti-inflammatory activity and selectivity toward the COX-2 enzyme and diminishes the ulcerogenic effect.

Thiourea derivatives **4a–g** have higher selectivity than indomethacin toward the COX-2 enzyme. From these derivatives, compound **4a** bearing the phenyl group has a higher SI and anti-inflammatory activity than celecoxib; however, the presence of an electron-donating group (CH_3 (**4b**) and OCH_3 (**4d**)) increases the SI and gives the highest anti-inflammatory activity at all time intervals and also decreases the ulcerogenic activity. On the contrary, the introduction of an electron-withdrawing group (F (**4c**) and Cl (**4e**), COOH (**4f**), $COOC_2H_5$ (**4g**)) decreases the SI.

Furthermore, 2-cyanoacetyl derivative **5** has a higher SI than celecoxib and promising anti-inflammatory activity with the least ulcer index (UI = 10.62), compared with indomethacin (UI = 18.50), which is very close to that of the nonulcerogenic reference drug celecoxib (UI = 10.53). For compound **6**, the presence of 3-cyano-4,6-dimethyl-2-oxopyridine moiety increases both the anti-inflammatory activity and the selectivity toward the COX-2 enzyme as compared to indomethacin and celecoxib. Finally, the introduction of a

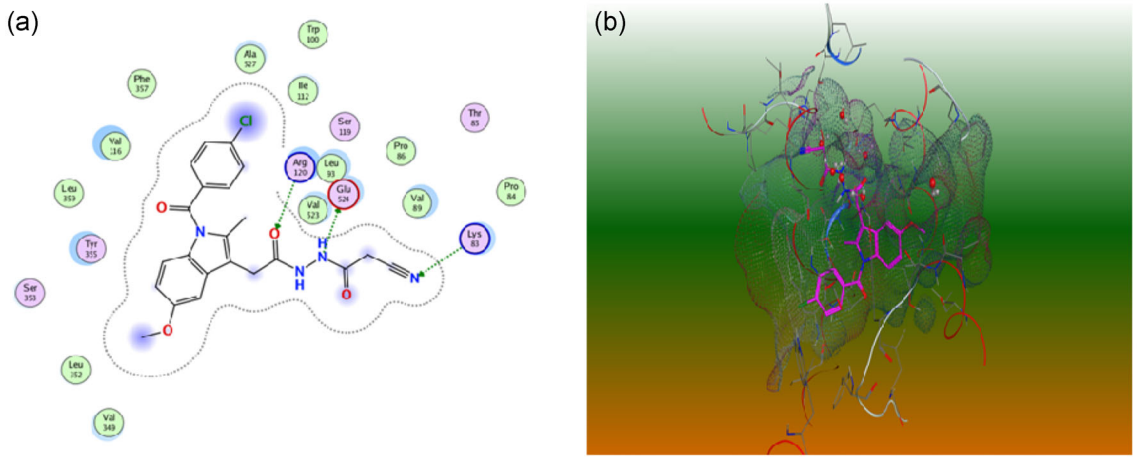


FIGURE 7 Binding of one of the most active compounds, **5**, inside the COX-2 active site. (a) Two-dimensional interaction of the proposed binding mode of **5**, which forms three hydrogen bonds with Glu524, Arg120, and Lys83 amino acids; (b) three-dimensional interaction of **5** (magenta)

TABLE 5 The molecular docking scores and binding interaction of compounds **4a**, **4b**, **4d**, **5**, **6**, and **8b**, and the indomethacin–dansyl conjugate (ligand) inside the COX-2 active site

Compounds	E-score (kcal/mol)	Number of hydrogen bonds	Hydrogen bonding residues	Distance (Å)	Functional groups
4a	−12.82	1	Arg120	3.21	C═S
4b	−16.08	1	Val116 Arg120	3.20 2.97	C═O indole C═O amide
4d	−15.56	1	Ser530	2.95	C═O amide
5	−14.79	3	Glu524 Arg120 Lys83	2.88 2.74 3.51	NH C═O amide C≡N
6	−13.93	2	Ser530 Ser530	2.93 3.39	O C≡N
Indomethacin–dansyl conjugate (ligand)	−17.43	2	Arg120 Ser530	3.00 2.91	C═O amide C═O indole
Indomethacin	−11.27	2	Arg120 Arg513	2.71 3.14	C═O indole C═O COOH
Celecoxib	−11.52	4	Tyr355 Arg513 Arg513 Arg513	3.10 3.11 3.03 2.81	S═O S═O S═O $SO_2\underline{NH_2}$

6-amino-3,5-dicyano-2-oxo-4-phenylpyridine moiety at position 3 in compounds **8a**,**b** does not improve the SI as compared with celecoxib.

3 | CONCLUSION

The goal of our study was to design a novel series of amide indomethacin derivatives as selective COX-2 inhibitors with minimal adverse effects on the gastric mucosa than indomethacin (one of the most ulcerogenic NSAIDs). Eleven indomethacin analogs, **4a–g**, **5**, **6**, **8a**, and **8b**, were synthesized and evaluated for their COX inhibitory activity, anti-inflammatory activity, and ulcerogenic liability. For these derivatives, the COOH group of indomethacin was replaced with bulky moieties via an amide linkage. All synthesized compounds showed 1.85- to 5.5-fold higher COX-2 selectivity index values than indomethacin, and most of them were even more COX-2 selective than the COX-2 selective reference drug celecoxib. Four derivatives, **4b**, **4d**, **5**, and **6**, showed good anti-inflammatory activity with a smaller number of ulcers than indomethacin. The histopathological study revealed that compound **5** was the best drug, showing mild lesions in the stomach, whereas compounds **4b** and **4d** showed moderate lesions. Molecular docking revealed that the selectivity could be attributed to the presence of C═O linked with the indole ring and C═O of the amide group that formed hydrogen bonds with Ser530 and Arg120 amino acids. Finally, we can conclude that modifications operated on the indomethacin structure to yield these novel derivatives achieved our main target to prepare anti-inflammatory agents as selective COX-2 inhibitors with minimal adverse effects on the gastric mucosa.

4 | EXPERIMENTAL

4.1 | Chemistry

4.1.1 | General

Melting points were determined by using the Griffin apparatus and were not corrected. IR spectra were recorded with a Shimadzu IR-435 spectrophotometer by using KBr disks and the data were represented in cm^{-1}. ^1H NMR spectra (at 400 MHz) and ^{13}C NMR spectra (at 100 MHz) were carried out by using a Bruker spectrophotometer at the Faculty of Pharmacy (Beni-Suef University, Egypt) in deuterated dimethyl sulfoxide (DMSO-d_6) and D_2O with tetramethylsilane as an internal standard. The coupling constant (J) values were estimated in Hertz (Hz), whereas the chemical shift was recorded in ppm on the δ scale. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; m, multiplet; dd, doublet of doublet. Mass spectra (MS) were determined by using a Hewlett Packard 5988 spectrophotometer. Microanalyses for C, H, and N (within ± 0.4% of the theoretical value) were carried out at the Regional Center for Mycology and Biotechnology (Al-Azhar University, Egypt). All reactions were observed with thin-layer chromatography using a UV lamp. All other reagents and solvents

were purchased from Aldrich Chemical Company and used without further purification. Indomethacin was purchased from Acros Chemical Company. A brown crystalline product **2** was obtained, as reported.[23,30]

The InChI codes of the investigated compounds, together with some biological activity data, are provided as Supporting Information.

4.1.2 | General procedure for the synthesis of compounds 4a–g

To a solution of **2** (0.01 mol, 3.80 g) in benzene (30 ml), ammonium thiocyanate (0.01 mol, 0.60 g) was added. The reaction mixture was refluxed for 2 h, cooled, and filtered. Then suitable aromatic amine (0.01 mol) was added to the filtrate. The mixture was heated under reflux for 3 h and then cooled and filtered. Next, the solid product was washed and crystallized from methanol to afford compounds **4a–g**. The physical and spectral data are listed below.

*2-[1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl]-*N*-(phenyl-carbamothioyl)acetamide (**4a**)*

Buff powder; yield, 75%; mp: 170–172°C; IR (KBr, cm^{-1}): 3178.69 (NH), 3032.10 (C–H aromatic), 2997.38, 2951.09, 2924.09 (C–H aliphatic), 1689.64 (C═O), and 1149.57 (C═S); ^{1}H NMR (DMSO-d_6): δ_H 2.29 (s, 3H, CH_3), 3.78 (s, 3H, OCH_3), 3.97 (s, 2H, CH_2), 6.73 (dd, J = 8.8 Hz, 2 Hz, 1H, indole H-6), 6.92 (d, J = 8.8 Hz, 1H, indole H-7), 7.21–7.26 (m, 2H, indole H-4, phenyl H-4), 7.38 (t, J = 8 Hz, 2H, phenyl H-3, H-5), 7.60 (d, J = 8 Hz, 2H, phenyl H-2, H-6), 7.67 (q_{AB}, J_{AB} = 8.4 Hz, 4H, benzoyl H-2, H-3, H-5, H-6), 11.76 (s, 1H, NH exchange with D_2O), and 12.38 (s, 1H, NH exchange with D_2O); ^{13}C NMR (DMSO-d_6): δ_C 13.80 (CH_3), 31.72 (CH_2), 55.97 (OCH_3), 102.44, 111.82, 113.02, 115.06, 124.68, 126.82, 129.14, 129.57, 130.69, 131.16, 131.66, 134.50, 136.43, 138.16, 138.20, 156.10, 168.38, 173.00, and 179.21; MS (*m/z*, %): 491.99 (M^+, 52.89%) and 493.93 (M^{+2}, 20.09%), and 335.09 (100%); Anal. calcd for $C_{26}H_{22}ClN_3O_3S$: C, 63.47; H, 4.51; N, 8.54. Found: C, 63.69; H, 4.73; N, 8.70.

*2-[1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl]-*N*-(p-tolyl-carbamothioyl)acetamide (**4b**)*

Brown powder; yield, 68%; mp: 162–164°C; IR KBr, cm^{-1}): 3178.69 (NH), 3032.10 (C–H aromatic), 2924.09 (C–H aliphatic), 1689.64 (C═O), and 1165.00 (C═S); ^{1}H NMR (DMSO-d_6): δ_H 2.29 (s, 6H, 2 CH_3), 3.86 (s, 3H, OCH_3), 3.96 (s, 2H, CH_2), 6.73 (dd, J = 8.8 Hz, 1.6 Hz, 1H, indole H-6), 6.92 (d, J = 8.8 Hz, 1H, indole H-7), 7.17–7.22 (m, 3H, indole H-4, tolyl H-2, H-6), 7.48 (d, J = 8 Hz, 2H, tolyl H-3, H-5), 7.67 (q_{AB}, J_{AB} = 8.4 Hz, 4H, benzoyl H-2, H-3, H-5, H-6), 11.73 (s, 1H, NH exchange with D_2O), and 12.31 (s, 1H, NH exchange with D_2O); ^{13}C NMR (DMSO-d_6): δ_C 13.79 (CH_3 indole), 21.02 (CH_3 tolyl), 31.71 (CH_2), 55.96 (OCH_3), 102.43, 111.81, 113.04, 115.05, 124.56, 129.56, 130.68, 131.16, 131.66, 134.49, 135.61, 136.18, 136.41, 138.20, 156.09, 168.38, 172.97, and 179.08; Anal. calcd for $C_{27}H_{24}ClN_3O_3S$: C, 64.09; H, 4.78; N, 8.30. Found: C, 63.79; H, 4.68; N, 8.10.

*2-[1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl]-*N*-(4-fluorophenyl-carbamothioyl)acetamide (**4c**)*

Brown powder; yield, 53%; mp: 170−172°C; IR (KBr, cm^{-1}): 3271.27 (2NH), 3047.53 (C–H aromatic), 2993.52, 2931.80 (C–H aliphatic), 1743.65, 1666.50 (C═O), and 1149.57 (C═S); ^{1}H NMR (DMSO-d_6): δ_H 2.29 (s, 3H, CH_3), 3.75 (s, 5H, CH_2, OCH_3), 6.72 (dd, J = 8.8 Hz, 2 Hz, 1H, indole H-6), 6.94 (d, J = 8.8 Hz, 1H, indole H-7), 7.12–7.19 (m, 3H, indole H-4, fluorobenzene H-3, H-5), 7.60–7.71 (m, 6H, fluorobenzene H-2, H-6, benzoyl H-2, H-3, H-5, H-6), and 10.26 (s, 1H, NH exchange with D_2O); ^{13}C NMR (DMSO-d_6): δ_C 13.89 (CH_3), 32.38 (CH_2), 55.89 (OCH_3), 102.38, 111.64, 114.47, 115.07, 115.67, 115.89 121.43, 121.51, 129.54, 130.71, 131.33, 131.65, 134.65, 135.94, 138.07, 156.02, 157.27, 159.65, 168.35, and 168.87; MS (*m/z*, %): 509.98 (M^+, 3.37%) and 111.11 (100%); Anal. calcd for $C_{26}H_{21}ClFN_3O_3S$: C, 61.23; H, 4.15; N, 8.24. Found: C, 61.25; H, 4.11; N, 8.14.

*2-[1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl]-*N*-(4-methoxyphenyl-carbamothioyl)acetamide (**4d**)*

Buff powder; yield, 76%; mp: 168–170°C; IR (KBr, cm^{-1}): 3170.97 (NH), 3032.10 (C–H aromatic), 2951.09, 2924.09, 2897.08 (C–H aliphatic), 1689.64, 1670.35 (C═O), and 1149.57 (C═S); ^{1}H NMR (DMSO-d_6): δ_H 2.28 (s, 3H, CH_3), 3.75 (s, 3H, OCH_3), 3.78 (s, 3H, OCH_3), 3.95 (s, 2H, CH_2), 6.73 (dd, J = 8.8 Hz, 2 Hz, 1H, indole H-6), 6.91–6.94 (m, 3H, methoxyphenyl H-3, H-5, indole H-7), 7.22 (s, 1H, indole H-4), 7.48 (d, J = 8.8 Hz, 1H, methoxyphenyl H-2, H-6), 7.67 (q_{AB}, J_{AB} = 8.4 Hz, 4H, benzoyl H-2, H-3, H-5, H-6), 11.71 (s, 1H, NH exchange with D_2O), and 12.21 (s, 1H, NH exchange with D_2O); ^{13}C NMR (DMSO-d_6): δ_C 13.80 (CH_3), 31.70 (CH_2), 55.74 (OCH_3), 55.96 (OCH_3), 102.45, 111.81, 113.08, 114.24, 115.06, 126.32, 129.57, 130.69, 131.04, 131.17, 131.66, 134.51, 136.40, 138.19, 156.10, 157.90, 168.37, 172.90, and 179.26; MS (*m/z*, %): 522.02 (M^+, 33.99%) and 357.15 (100%); Anal. calcd for $C_{27}H_{24}ClN_3O_4S$: C, 62.12; H, 4.63; N, 8.05. Found: C, 61.87; H, 4.89; N, 8.27.

*2-[1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl]-*N*-(4-chlorophenyl-carbamothioyl)acetamide (**4e**)*

Buff powder; yield, 83%; mp: 167–169°C; IR (KBr, cm^{-1}): 3271.27 (NH), 3109.25, 3039.81 (C–H aromatic), 2924.09 (C–H aliphatic), 1666.50 (C═O), and 1149.57 (C═S); ^{1}H NMR (DMSO-d_6): δ_H 2.28 (s, 3H, CH_3), 3.75 (s, 3H, OCH_3), 3.80 (s, 2H, CH_2), 6.72 (dd, J = 9.2 Hz, 2.4 Hz, 1H, indole H-6), 6.94 (d, J = 9.2 Hz, 1H, indole H-7), 7.18 (d, J = 2.4 Hz, 1H, indole H-4), 7.36 (d, J = 8.8 Hz, 2H, chlorophenyl H-2, H-6), 7.63–7.71 (m, 6H, chlorophenyl H-3, H-5, benzoyl H-2, H-3, H-5, H-6), and 10.35 (s, 1H, NH exchange with D_2O); ^{13}C NMR (DMSO-d_6): δ_C 13.85 (CH_3), 32.44 (CH_2), 55.91 (OCH_3), 102.39, 111.65, 114.35, 115.06, 121.29, 127.36, 129.12, 129.54, 130.73, 131.31, 131.62, 134.64, 135.93, 138.10, 138.48, 156.04, 168.37, and 169.15; MS (*m/z*, %): 526.43 (M^+, 18.58%) and 139.13 (100%); Anal.

calcd for $C_{26}H_{21}Cl_2N_3O_3S$: C, 59.32; H, 4.02; N, 7.98. Found: C, 59.42; H, 4.03; N, 7.94.

*4-3-{2-[1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl]-acetyl}thioureido)benzoic acid (**4f**)*

Brown powder; yield, 69%; mp: 175–177°C; IR (KBr, cm^{-1}): 3618.46 (NH), 3248.13 (O–H acidic), 3008.95 (C–H aromatic), 2931.80 (C–H aliphatic), 1681.93 (C═O), and 1172.72 (C═S); ^{1}H NMR (DMSO-d_6): δ_H2.29 (s, 3H, CH_3), 3.75 (s, 3H, OCH_3), 3.81 (s, 2H, CH_2), 6.72 (d, J = 8.8 Hz, 1H, indole H-6), 6.94 (d, J = 8.8 Hz, 1H, indole H-7), 7.20 (s, 1H, indole H-4), 7.65 (d, J = 8.4 Hz, 2H, benzoic acid H-3, H-5), 7.70 (d, J = 8.8 Hz, 2H, benzoyl, H-3, H-5), 7.74 (d, J = 8.8 Hz, 2H, benzoyl H-2, H-6), 7.90 (d, J = 8.4 Hz, 2H, benzoic acid H-2, H-6), and 10.57 (s, 1H, NH exchange with D_2O), OH acidic is not detected; ^{13}C NMR (DMSO-d_6): δ_C 13.89 (CH_3), 32.52 (CH_2), 55.90 (OCH_3), 102.37, 111.67, 114.27, 115.08, 118.93, 125.63, 129.54, 130.70, 130.90, 131.31, 131.66, 134.65, 135.98, 138.08, 143.61, 156.03, 167.37, 168.35, and 169.50; Anal. calcd for $C_{27}H_{22}ClN_3O_5S$: C, 60.50; H, 4.14; N, 7.84. Found: C, 60.6; H, 4.34; N, 7.54.

*Ethyl-4-(3-{2-[1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl]acetyl}thioureido)benzoate (**4g**)*

Buff powder; yield, 81%; mp: 150–152°C; IR (KBr, cm^{-1}): 3294.42 (NH), 3070.68 (C–H aromatic), 2931.80 (C–H aliphatic), 1712.79, 1666.50 (C═O), and 1149.57 (C═S); ^{1}H NMR (DMSO-d_6): δ_H 1.30 (t, J = 7.2 Hz, 3H, CH_3 ethyl), 2.29 (s, 3H, CH_3), 3.75 (s, 3H, OCH_3), 3.81 (s, 2H, CH_2), 4.28 (q, J = 7.2 Hz, 2H, CH_2 ethyl), 6.73 (dd, J = 8.8 Hz, 2.4 Hz, 1H, indole H-6), 6.94 (d, J = 8.8 Hz, 1H, indole H-7), 7.18 (d, J = 2.4 Hz, 1H, indole H-4), 7.67 (q_{AB}, J_{AB} = 8.4 Hz, 4H, benzoyl H-2, H-3, H-5, H-6), 7.75 (d, J = 8.4 Hz, 2H, ethylbenzoate H-3, H-5), 7.92 (d, J = 8.8 Hz, 2H, ethylbenzoate H-2, H-6), and 10.63 (s, 1H, NH exchange with D_2O); ^{13}C NMR (DMSO-d_6): δ_C 13.85 (CH_3 indole), 14.66 (CH_3 ethyl), 32.51 (CH_2 indole), 55.91 (OCH_3), 60.93 (CH_2 ethyl), 102.40, 111.66, 114.21, 115.07, 119.06, 124.78, 129.54, 130.72, 131.30, 131.63, 134.63, 136.00, 138.11, 143.88, 156.05, 165.80, 168.37, and 169.59; Anal. calcd for $C_{29}H_{26}ClN_3O_5S$: C, 61.75; H, 4.65; N, 7.45. Found: C, 61.95; H, 4.75; N, 7.35.

4.1.3 | General procedure for the synthesis of compound 5

To a solution of **2** (0.01 mol, 3.80 g) in benzene (20 ml), cyanoacetic acid hydrazide (0.01 mol, 0.99 g) was added and stirred for 24 h at room temperature. Then the reaction mixture was filtered and the precipitate was collected, washed, and crystallized from ethanol.

*2-[1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl]-N′-(2-cyano-acetyl)acetohydrazide (**5**)*

Yellowish green powder; yield, 82.5%; mp: 250–252°C; IR (KBr, cm^{-1}): 3197.98 (NH), 3051.39, 3008.95 (C–H aromatic), 2924.09 (C–H aliphatic), 2256.74 (CN), and 1678.07 (C═O); ^{1}H NMR (DMSO-d_6): δ_H 2.25 (s, 3H, CH_3), 3.54 (s, 2H, CH_2–CN), 3.73 (s, 2H, CH_2 indole), 3.78 (s, 3H, OCH_3), 6.71 (dd, J = 8.8 Hz, 2.4 Hz, 1H, indole H-6), 6.92 (d, J = 8.8 Hz, 1H, indole H-7), 7.16 (d, J = 2.4 Hz, 1H, indole H-4), 7.66 (q_{AB}, J_{AB} = 8.8 Hz, 4H, benzoyl H-2, H-3, H-5, H-6), and 10.28 (s, 2H, 2NH exchange with D_2O); ^{13}C NMR (DMSO-d_6): δ_C 13.79 (CH_3), 24.17 (CH_2–CN), 29.46 (CH_2–C═O), 55.88 (OCH_3), 102.34, 111.91, 113.88, 114.97, 116.07, 129.53, 130.69, 131.10, 131.64, 134.61, 135.85, 138.12, 156.03, 161.83, 168.36, and 168.92; MS (m/z, %): 438.86 (M^+, 50.42%) and 139.25 (100%); Anal. calcd for $C_{22}H_{19}ClN_4O_4$: C, 60.21; H, 4.36; N, 12.77. Found: C, 60.46; H, 4.49; N, 12.95.

4.1.4 | General procedure for the synthesis of compound 6

To a solution of **5** (0.01 mol, 4.38 g) in absolute ethanol (20 ml), acetylacetone (0.01 mol, 1.00 g) and three drops of piperidine were added, and the reaction was refluxed for 6 h. The reaction mixture was cooled, and the formed solid was filtered, dried, and crystallized from ethanol.

*2-[1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl]-N-(3-cyano-4,6-dimethyl-2-oxopyridin-1(2H)-yl)acetamide (**6**)*

Buff powder; yield, 55%; mp: 242–244°C; IR (KBr, cm-1): 3221.12 (NH), 3005.10 (C–H aromatic), 2924.09 (C–H aliphatic), 2368.59 (CN), and 1597.06 (C═O); ^{1}H NMR (DMSO-d_6): δ_H 2.16 (s, 3H, CH_3 oxopyridine), 2.27 (s, 3H, CH_3 oxopyridine), 2.36 (s, 3H, CH_3 indole), 3.80 (s, 3H, OCH_3), 3.85 (s, 2H, CH_2), 6.36 (s, 1H, oxopyridine H-5), 6.73 (dd, J = 8.8 Hz, 2.4 Hz, 1H, indole H-6), 6.98 (d, J = 8.8 Hz, 1H, indole H-7), 7.22 (d, J = 2.4 Hz, 1H, indole H-4), 7.68 (q_{AB}, J_{AB} = 8.4 Hz, 4H, benzoyl H-2, H-3, H-5, H-6), and 11.18 (s, 1H, NH exchange with D_2O); ^{13}C NMR (DMSO-d_6): δ_C 13.83 (CH_3 indole), 19.08 (CH_3 oxopyridine C-5), 21.22 (CH_3 oxopyridine C-3), 29.41 (CH_3), 55.94 (OCH_3), 100.41, 102.13, 108.66, 112.19, 113.10, 115.04, 115.84, 129.53, 130.74, 131.01, 131.70, 134.59, 136.17, 138.15, 154.64, 156.11, 158.65, 160.46, 168.36, and 169.92; MS (m/z, %): 502.96 (M^+, 4.27%), 504.50 (M^{+2}, 1.59%), and 139.15 (100%); Anal. calcd for $C_{27}H_{23}ClN_4O_4$: C, 64.48; H, 4.61; N, 11.14. Found: C, 64.16; H, 4.75; N, 11.43.

4.1.5 | General procedure for the synthesis of compounds 8a,b

A solution of **5** (0.01 mol, 4.38 g) in absolute ethanol (20 ml) was treated with 0.01 mol (1.54 g) of the appropriate arylidene **7a,b**[25,26] and five drops of piperidine; the mixture was heated under reflux for 2 h. The reaction mixture was filtered while hot, and the formed precipitate was dried and crystallized from ethanol.

*N-[6-Amino-3,5-dicyano-2-oxo-4-phenylpyridin-1(2H)-yl]-2-{5-methoxy-2-methyl-1-[4-(piperidin-1-yl)benzoyl]-1H-indol-3-yl}-acetamide (**8a**)*

Yellowish white powder; yield, 79%; mp: 275–277°C; IR (KBr, cm^{-1}): 3441.01 (NH, NH_2), 3101.54 (C–H aromatic), 2970.36, 2931.80,

2854.65 (C–H aliphatic), 2214.28 (CN), 1689.64 (C=O); ^{1}H NMR (DMSO-d_6): δ_H 1.55–1.64 (m, 6H, piperidine H-3, H-4, H-5), 2.33 (s, 3H, CH_3), 2.99 (t, J = 5.2 Hz, 4H, piperidine H-2, H-6), 3.75 (s, 3H, OCH_3), 4.17 (s, 2H, CH_2), 6.71 (dd, J = 8.8 Hz, 2.0 Hz, 1H, indole H-6), 6.94 (d, J = 8.8 Hz, 1H, indole H-7), 7.21 (d, J = 2.0 Hz, 1H, indole H-4), 7.46–7.50 (m, 5H, phenyl H-2, H-3, H-4, H-5, H-6), 7.65 (d_{AB}, J_{AB} = 8.4 Hz, 2H, benzoyl H-3, H-5), 7.71 (d_{AB}, J_{AB} = 8.4 Hz, 2H, benzoyl H-2, H-6); ^{13}C NMR (DMSO-d_6): δ_C 13.90 (CH_3), 22.17 (piperidine C-4), 22.83 (piperidine C-3, C-5), 24.04 (CH_2), 44.34 (piperidine C-2, C-6), 55.78 (OCH_3), 102.67, 111.69, 114.98, 116, 117, 118.86, 128.77, 129.03, 129.51, 129.75, 130.80, 131.17, 131.66, 134.77, 135.02, 136.48, 137.97, 155.12, 155.89, 156.65, 164.29, and 168.36; MS (*m/z*, %): 639.72 (M^+, 16.28%) and 110.68 (100%); Anal. calcd for $C_{37}H_{33}N_7O_4$: C, 69.47; H, 5.20; N, 15.33. Found: C, 69.57; H, 5.1; N, 15.36.

N-*[6-Amino-4-(4-chlorophenyl)-3,5-dicyano-2-oxopyridin-1(2* H*)-yl]-2-{5-methoxy-2-methyl-1-[4-(piperidin-1-yl)benzoyl]-1* H*-indol-3-yl}acetamide (**8b**)*

Yellow crystals; yield, 84%; mp: 280–282°C; IR (KBr, cm^{-1}): 3425.58 (NH, NH_2), 3101.54 (C–H aromatic), 2924.09, 2854.65 (C–H aliphatic), 2214.28 (CN), and 1689.64 (C=O); ^{1}H NMR (DMSO-d_6): δ_H1.55–1.64 (m, 6H, piperidine H-3, H-4, H-5), 2.33 (s, 3H, CH_3), 3.01 (t, J = 5.6 Hz, 4H, piperidine H-2, H-6), 3.73 (s, 3H, OCH_3), 4.18 (s, 2H, CH_2), 6.70 (dd, J = 8.8 Hz, 2 Hz, 1H, indole H-6), 6.94 (d, J = 8.8 Hz, 1H, indole H-7), 7.21 (d, J = 1.6 Hz, 1H, indole H-4), 7.52 (d, J = 8.4 Hz, 2H, chlorophenyl H-2, H-6), 7.60 (d, J = 8.4 Hz, 2H, chlorophenyl H-3, H-5), 7.65 (d_{AB}, J = 8.4 Hz, 2H, benzoyl H-3, H-5), 7.71 (d_{AB}, J_{AB} = 8 Hz, benzoyl H-2, H-6), and 8.23 (s, 1H, NH exchange with D_2O); ^{13}C NMR (DMSO-d_6): δ_C 13.89 (CH_3), 22.07 (piperidine C-4), 22.69 (piperidine C-3, C-5), 24.03 (CH_2), 44.26 (piperidine C-2, C-6), 55.79 (OCH_3), 102.66, 111.68, 114.98, 116.26, 117.31, 118.74, 128.94, 129.52, 130.80, 130.99, 131.15, 131.65, 134.65, 134.74, 135.05, 135.33, 138.00, 153.49, 153.86, 155.89, 156.55, 164.37, and 168.37; MS (*m/z*, %): 674.16 (M^+, 27.77%) and 359.93 (100%); Anal. calcd. for $C_{37}H_{32}ClN_7O_4$: C,65.92; H, 4.78; N, 14.54. Found: C, 65.91; H, 4.81; N, 14.58.

4.2 | Pharmacological/biological assays

The ability of the tested compounds listed in Table 1 to inhibit ovine COX-1 and COX-2 was measured with EIA[27] in which *N′*, *N′*, *N′*, *N′*-tetramethyl-*p*-phenylenediamine at 590 nm was used, as previously mentioned. Adult male Wistar albino rats, weighing 90–110 g, were housed in groups (each group of six rats) in the animal house of the Faculty of Pharmacy, Beni-Suef University, under controlled conditions (light period of 12 h/day and temperature 25 ± 2°C). Animals were fasted overnight with free access to water before the experiment. Before any drug administration, the thickness of the left hind paw of each rat was measured in millimeters. The anti-inflammatory activity of the synthesized compounds was determined in vivo by carrageenan-induced paw edema in rats. The anti-inflammatory activity of five compounds was evaluated by using the carrageenan-induced rat paw edema model.[28] First, five test compounds, **4a**, **4b**, **4d**, **5**, **6**, indomethacin, and celecoxib were administrated orally in a dose of 50 mg/kg dissolved in 1% Tween in saline, and the control group received vehicle only 1 h before subcutaneous injection of carrageenan. Paw edema was induced by subcutaneous injection of 1% carrageenan in saline (0.02 ml/rat) into the left hind paw of each rat. The hind paw thickness of each rat was measured after 1, 3, and 5 h of carrageenan injection, and then the change in thickness and % inhibition of paw edema were calculated according to the formula of Ratheesh and Helen[31] and Adedapo and Sofidiya[32] with two standard drugs celecoxib and indomethacin:

$$\%\ \text{of inhibition} = \frac{(V_0 - V_t)}{V_0} \times 100,$$

where V_0 is the volume of the paw of the control at time 0 and V_t is the volume of the paw of the drug-treated animal at time t.

The ulcerogenic liability of the most active compounds that showed an effective in vivo anti-inflammatory activity was evaluated according to a previous study.[29] Rats were fasted one day before drug administration and divided into eight separate groups (each group with six rats). All compounds were dissolved in 1% Tween in saline and administrated orally. The normal control group received only vehicle, the celecoxib group received celecoxib in a dose of 50 mg/kg, the indomethacin group received the drug in a dose of 50 mg/kg, and the other five groups received the tested compounds, respectively, in a dose of 50 mg/kg. Then animals were fed after 2 h. Rats were given the specified dose orally for three successive days. Rats were killed after 2 h of the last dose, and then the stomach of each rat was removed and opened along the greater curvature for determination of the ulcer number and ulcer index according to a previous study.[29] To examine the stomach, it was stretched by pins on a corkboard. The gastric mucosa was carefully inspected for the occurrence of ulcers with the aid of an illuminated magnifying lens (×10), and the ulcer index was calculated.

The number of mucosal damage that appeared as red spots was counted, and their severity was determined and graded from 0 to 4. The following parameters were calculated:

1. Percentage of incidence/10 = [number of rats showing ulcer of any grade/total number of rats in group × 100]/10
2. The average number of ulcers = number of ulcers in the group/total number of rats in the group
3. Average severity = ∑ [each ulcer multiplied by its score of severity/number of ulcers in the group
4. Ulcer index = the sum of the above three parameters

The stomach was collected and fixed in 10% buffered formalin for 72 h. Routine histological processing and paraffin embedding were carried out according to the method of Bancroft and Gamble.[33] Tissue sections (4–5 μm) were stained with hematoxylin and eosin stain.

4.3 | Molecular docking

In our docking study, Molecular Operating Environment (MOE) version 2015.10 modeling software was used to apply molecular docking for the most active compounds. First, we downloaded the crystal structure of indomethacin–ethylenediamine–dansyl conjugate bound to the COX-2 active site encoded (PDB: 6BL4)[10] from the Protein Data Bank. The ligand and the tested compounds must follow some steps before being docked including three-dimensional protonation and energy minimizing to get the lowest energy conformers. London dG and force field energy were used to perform docking study and refinement of results. Only one conformer was chosen according to its superposition with our ligand. Finally, we obtained results indicating energy scores of binding with the active site, amino acid interactions, and the length of the formed hydrogen bonds (Table 5).

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CONFLICTS OF INTERESTS

The authors declare that there are no conflicts of interests.

ETHICS STATEMENT

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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