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Design, synthesis, biological evaluation, and comparative Cox1 and Cox2 docking of *p*-substituted benzylidenamino phenyl esters of ibuprofenic and mefenamic acids

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ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs) are frequently associated with gastric mucosal and renal adverse reactions, related to inhibition of cyclooxygenase1 (Cox1) in tissues where prostaglandins exert physiological effects. This led us to develop a set of ibuprofenic acid and mefenamic acid esters, namely: 4-((4-substituted benzylidene)amino)phenyl 2-(4-isobutylphenyl)propanoate and 4-((4-substituted benzylidene)amino)phenyl 2-((2,4-dimethylphenyl)amino)benzoate analogs, which were synthesized by condensation of the corresponding acids with Schiff's bases [4-(4-substituted benzylideneamino)phenols] involving dicyclohexyl carbodiimmide (DCC) as mild dehydrating agent. The main objective is to reduce the GIT toxicity associated with acute and chronic NSAIDs use. Anti-inflammatory, analgesic as well as ulcerogenic activities of the prepared esters were evaluated in vivo and compared with that of ibuprofen as reference standard in all screenings, involving the carrageenan induced paw oedema model and hot plate method. Most of the synthesized esters showed remarkable analgesic and anti-inflammatory activities. Interestingly, all of the compounds were found to be non-ulcerogenic under the tested conditions. This evidence have suggested that modification of the carboxyl function of representative NSAIDs results in retained or enhanced anti-inflammatory and analgesic activities with reduced ulcerogenic potential. Additionally, a comparative AutoDock study into Cox 1 and Cox2 has been done involving both of rigid and flexible docking for potential selectivity of our compounds within different Cox enzymes and to find out the binding orientation of these novel esters into their binding site. Some of the newly prepared aforementioned compounds showed considerable more Cox2 over Cox1 binding affinities by flexible docking better than rigid one.

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely used drugs worldwide and represent a mainstay in the therapy of acute and chronic pain. However, their use is frequently associated with a broad spectrum of adverse effects, which are related to inhibiting prostaglandin synthesis in tissues where PGs are responsible for physiological homeostasis.[1] A lot of strategies have emerged to improve the therapeutic efficacy and tolerability of NSAIDs via defining novel targets in the complex picture of the inflammatory process or modifying classical NSAIDs by adding chemical moieties that release gastroprotective mediators. These trials include development of highly selective Cox2 inhibitors, referred to as 'coxibs', were rapidly introduced in the market and gained an impressive success.[2,3]

In recent years, however, serious cardiovascular effects of some selective Cox2 inhibitors emerged from clinical studies and pharmacosurveillance, forcing the drug companies to withdraw from the market rofecoxib and, soon afterwards, valdecoxib.[4,5] Following the withdrawal of rofecoxib, which has been considered the most serious disaster after thalidomide, another strategy involved the development of dual inhibitors of Cox and 5-lypooxygenase (5-LOX) by blocking the formation of both prostaglandins and leucotrienes but do not affect lipoxin formation. Such combined inhibition avoids some of the disadvantages of selective Cox2 inhibitors and spares the gastrointestinal mucosa.[6] An alternative strategy to limit the risk of GI damage induced by NSAIDs is to enhance the protective mechanisms of the gastric mucosa. This can be pursued by two gaseous mediators; nitric oxide (NO) and hydrogen sulfide (H_2S) which exert protective effects on gastric mucosa. The inhibitory effects of NO on nonsteroidal anti-inflammatory drugs-induced leukocyte adherence have been exploited in the development of NO-releasing nonsteroidal anti-inflammatory drugs known as Cox-inhibiting NO donors (CINODs). This class

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of anti-inflammatory agents reduces systemic blood pressure and might have enhanced cardiovascular safety than coxibs, while causing less gastrointestinal damage than its parent drug.[7] H_2S-releasing NSAID derivatives have been recently developed based on the observed ability of this gaseous mediator to cause vasodilatation and to prevent leukocyte adherence. In preclinical settings, H_2S-releasing NSAIDs produce less gastric damage as compared to the parent drugs[8].

Another way to avoid GIT complications of NSAIDs involves synthesis of the ester form of the corresponding irritant parent compounds. Where, phenylcarbamoylmethyl ester of ibuprofen, naproxen, and *N*-acetylanthranilic acid were recently synthesized and tested for their anti-inflammatory activity. Further the ulcerogenic liability and PGE2 inhibitory properties for the most active compounds were determined. Results showed that all the tested compounds exhibited promising anti-inflammatory activity, compared to ibuprofen and naproxen, with marked decreases in the ulcerogenic side effects. Moreover, esterification of both ibuprofen and naproxen derivatives led to increases in the anti-inflammatory activity, compared to the parent drugs, and this was enhanced in the case of the 4-methoxyphenylcarbamoyl methyl ester and the phenylcarbamoylmethyl ester of ibuprofen and naproxen, respectively.[9] Ester and amide prodrugs of ibuprofen and naproxen were synthesized and evaluated for anti-inflammatory activity and gastrointestinal toxicity. All prodrugs, except the glycine amide, were significantly less irritating to the gastric mucosa and exhibited significantly better activity (p <0.01) than the parent compounds.[10] In addition esterification increases Cox2 selectivity in esters rather than the acids.[11]

Gastroprotective agents are commonly co-prescribed with NSAIDs to protect against these side effects such as misoprostol, Cox2 specific and selective NSAIDs, and probably proton pump inhibitors significantly reduce the risk of symptomatic ulcers.[12] In spite of the unprecedented advances in drug discovery, developing a safe, effective and economical therapy for treating inflammatory conditions still presents a major challenge. Various Computer Aided Drug Design (CADD) approaches were introduced to develop Cyclooxygenase based anti-inflammatory and anticancer drugs.[13] Flexible docking was carried out for structurally diverse Cox2 inhibitors. The obtained docking score was correlated with the biological activities. The detailed analysis of the resulted Cox2–ligand complexes may improve our knowledge in understanding the binding interactions in detail. Moreover, a discovery of a phenothiazine derivative as a new cyclooxygenase-2 lead compound was done through 3-D database searching and combinatorial chemistry to serve as a lead compound for a potentially novel series of anti-inflammatory compounds.[14] Pathway reconstruction and kinetic modeling are two approaches of computational systems biology were applied involving classical biochemistry, genomics, proteomics and metabolomics. These models quantitatively describe the changes in dynamics and regulations of the pathways caused by the following NSAIDs: aspirin, celecoxib, diclofenac, naproxen, indomethacin, and ibuprofen.[15]

Schiff's bases have a wide range of biological activities such as antimalarial,[16] anticancer,[17] antibacterial,[18] antifungal,[19] antitubercular,[20] anti-inflammatory, antimicrobial,[21] and antiviral,[22] etc. They also serve as a back bone for the synthesis of various heterocyclic compounds.

The above circumstances led us to seek a convenient synthetic route for replacing the carboxylic acid group of ibuprofenic and mefenamic acids with less acidic *p*-substituted benzylidenamino phenyl esters. In our attempt to accentuate potency and reduce GI toxicities associated with the parent ibuprofenic and mefenamic acids due to their free –COOH group. These compounds were investigated for their biological activities including in vitro acute anti-inflammatory effect, analgesic activity, as well as ulcerogenic liability. In fact, many of the designed compounds were found to possess much significant analgesic and anti-inflammatory profile with significant reduction in potential for ulcerogenic toxicities. Additionally, comparative docking study was carried out by differential binding of the synthesized compounds into the active sites of Cox1 and Cox2. Where, a series of computations were performed for the prediction of mode of their binding affinities using flexible AutoDock 4.2.

2. Results and discussion

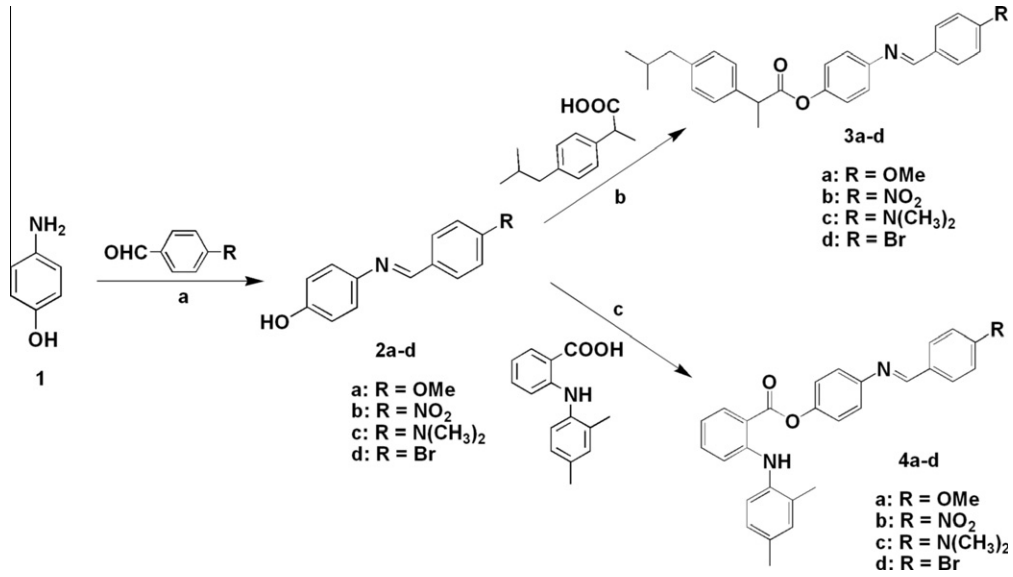
2.1. Chemistry

Synthetic approaches based upon chemical modification of NSAIDs have been taken with the aim of improving safety profile and in turn therapeutic window of the NSAIDs in this study, namely ibuprofen and mefenamic acid. Where several studies have described the derivatization of the carboxylate function[23–25] of NSAIDs with less acidic analogs which resulted in an increased anti-inflammatory activity with reduced ulcerogenicity. The requisite starting intermediate Schiff's bases, namely 4-(4-substituted benzylidene-amino)phenols (**2a–d**), which were used as precursors for synthesis of 4-(4-substituted benzylideneamino)phenyl 2-(4-isobutylphenyl)propanoate (**3a–d**) and 4-(4-substituted benzylideneamino)phenyl 2-(2,4-dimethylphenylamino) benzoate (**4a–d**), were synthesized by treatment of equimole of *p*-amino phenol (**1**) and *p*-substituted aromatic aldehyde in ethanol under reflux for 6–8 h as shown in Scheme 1 to afford yellow needles recrystallized from ethanol in 70–85% yields. On the other hand, The prodrugs, 4-(4-substituted benzylideneamino)phenyl 2-(4-isobutylphenyl)propanoate (**3a–d**) and 4-(4-substituted benzylideneamino) phenyl 2-(2,4-dimethylphenylamino)benzoate (**4a–d**), were prepared by condensation reaction of the corresponding anti-inflammatory acid (ibuprofen or mefenamic acid) with the intermediate Schiff's bases, 4-(4-substituted benzylideneamino)phenols (**2a–d**) in THF/dichloromethan at room temperature overnight, involving dicyclohexyl carbodiimmide (DCC) as mild dehydrating agent to avoid the hydrolysis of Schiff's base, where the products were obtained as yellow needles in 52–84% yields.

IR, ^{1}H NMR spectra, and elemental analyses, were used for determination and identification of the newly assigned structures. The structures of the ibuprofen esters **3a–d** were confirmed in particular by the presence of an equivalent proton resonance of ylideneamino moiety (–CH═N) as a singlet signal at δ_H 8.36–8.62 in ^{1}H NMR spectra. Also the mefenamic acid esters **4a–d** showed that characteristic singlet signal at δ_H 8.37–8.57. It is implying that ylideneamino proton (–CH═N) is the most electron-deficient nucleophile in the lowest magnetic field. The 4-(4-substituted benzylideneamino)phenyl 2-(4-isobutylphenyl)propanoate (**3a**–**d**) and 4-(4-substituted benzylideneamino)phenyl 2-(2,4-dimethylphenylamino) benzoate (**4a**–**d**) were differentiated from the intermediate Schiff's base, 4-(4-substituted benzylideneamino)phenols (**2a–d**) by the absence of the singlet signal of the aromatic C–OH proton in their ^{1}H NMR spectra which is obviously shown in the starting precursors **2a–d** and disappeared on esterification with the appearance of additional aromatic protons as multiplets at δ_H 6.72–8.02 and 6.88–8.02 in ibuprofen esters **3a–d** and mefenamic acid esters **4a–d**, respectively. Moreover, mefenamic acid esters **4a–d** exhibit singlet NH proton, exchangeable with D_2O at δ_H 5.46–5.50.

2.2. Biological evaluation

In the pharmacological study, we have investigated anti-inflammatory and analgesic activities as well as the acute ulcerogenicity of both *p*-substituted benzylidenamino phenyl esters of ibuprofe-



Scheme 1. General method for the preparation of 4-(4-substituted benzylideneamino)phenols (**2a–d**), 4-(4-substituted benzylideneamino)phenyl 2-(4-isobutylphenyl)propanoate (**3a–d**), and 4-(4-substituted benzylideneamino)phenyl 2-(2,4-dimethylphenylamino) benzoate (**4a–d**). *Reagent and conditions:* (**a**) Ar-CHO, AcOH, EtOH, reflux, 6–8 h; (**b**) ibuprofen, Schiff's base, THF/dichloromethan, dicyclohexyl carbodiimmide (DCC), rt, overnight; (**c**) mefenamic acid, Schiff's base, THF/dichloromethan, dicyclohexyl carbodiimmide (DCC), rt, overnight.

nic acid (**3a–d**) and *p*-substituted benzylidenamino phenyl esters of mefenamic acid (**4a–d**), utilizing the carrageenan-induced rat anti-inflammatory testing, hot plate analgesia testing, and influence on the gastric irritation, respectively. Ibuprofen, one of the parent compounds, was used as a reference standard control, and the control group animals were given saline with few drops of carboxymethyl cellulose (CMC) 0.5% (0.3 ml/100 g rat).

2.2.1. Acute anti-inflammatory effect (carrageenan-induced paw oedema)

Carrageenan-induced paw oedema bioassay in rats was involved as a suitable experimental animal model for evaluating an anti-inflammatory effect of the newly synthesized compounds: **3a–d** and **4a–d**. Results were expressed as mean ± S.E. Difference between vehicle control and treatment groups were statistically tested using repeated measures one way ANOVA, followed by least significant test for multiple comparisons. Methods of statistical analysis were done according to Armitage et al.[32]

To demonstrate the validity of the carrageenan-induced paw oedema test, rats were administered ibuprofen orally as a positive control 100 mg/kg bwt, then compared to rats which were administered various synthesized compounds **3a–d** and **4a–d** 100 mg/kg bwt. Both of reference and the test compounds were orally administered 1 h before carrageenan administration. Obviously, as cited in Table 1 and Figure 1, all the involved esters of ibuprofenic and mefenamic acids at 100 mg/kg bwt, decreased the paw volume significantly (p <0.05) after all times of its administration at 1, 2, 3, and 4 h (**3a**–**d**, and **4a–d**). Where 4-(4-nitro benzylideneamino)phenyl 2-(4-isobutylphenyl)propanoate (**3b**) inhibited paw oedema significantly after all times of its administration especially after 1 h, where it decreased the inflamed paw volume significantly (p <0.05) to 29.85% more than that of ibuprofen (30.03%). This indicates that this *p*-nitro ester derivative of ibuprofen has potential anti-inflammatory activity slightly higher than its corresponding acid analog.

To a better extent, 4-(4-bromo benzylideneamino)phenyl 2-(4-isobutylphenyl)propanoate (**3d**), significantly (p <0.05) decreased the paw volume at 1, 2, 3 and 4 h after carrageenan administration more than compound **3b** at 2, 3 and 4 h being of 40.59%, 44.72%, and 49.30%, respectively. On the other hand, many of 4-(4-substituted benzylideneamino)phenyl 2-(2,4-dimethylphenylamino)benzoate derivatives (**4a**–**d**) significantly reduce the inflamed paw volume especially compounds 4-(4-nitro benzylideneamino)phenyl 2-(2,4-dimethylphenylamino)benzoate (**4b**), and 4-(4-bromo benzylideneamino)phenyl 2-(2,4-dimethylphenylamino)benzoate (**4d**), where the latter one revealed the highest reduction of paw volume at all. It is clear to compare all compounds **3a–d** and **4a–d** used in this study with ibuprofen as reference standard as shown in Figure 1, where all of them except compounds **3a** and **3c** showed significant reduction of paw oedema after carrageenan injection.

Our entitled compounds can be compared by involving cumulative manner for their % inhibitions of oedema volume (anti-inflammatory effects) at all times of 1, 2, 3, and 4 h. Where ibuprofen exhibited cumulative % inhibitions of 152.01. Whereas, the other derivatives revealing the closest activities to ibuprofen depending on their cumulative manner in descending anti-inflammatory order are **4d**, **3d**, **4b**, and **3b** being of 168.61, 171.5, 174.26, and 178.35, respectively, as cited in Table 1.

Table 1
Acute anti-inflammatory effect of the tested ester derivatives (**3a**–**d** and **4a**–**d**) in comparison to ibuprofen involving carrageenan-induced paw oedema technique

Group	Oedema volume (% inhibition)			
	1 h	2 h	3 h	4 h
Control	55.76 ± 7.84*	68.60 ± 7.14*	74.63 ± 7.13*	78.14 ± 7.10*
3a	53.96 ± 7.03*	56.17 ± 6.43*	61.25 ± 6.05*	64.68 ± 7.10*
3b	29.85 ± 6.15*	44.40 ± 7.51*	50.32 ± 8.91*	53.78 ± 8.86*
3c	71.69 ± 3.65*	96.14 ± 6.89*	103.75 ± 5.62*	107.35 ± 6.81*
3d	36.54 ± 2.63*	40.59 ± 1.94*	44.72 ± 3.46*	49.30 ± 3.96*
4a	35.18 ± 4.13*	49.44 ± 7.06*	57.03 ± 7.30*	62.11 ± 7.80*
4b	36.90 ± 6.59*	41.11 ± 5.53*	43.78 ± 6.38*	52.47 ± 8.43*
4c	41.64 ± 3.74*	45.60 ± 4.38*	53.43 ± 5.58*	63.45 ± 5.09*
4d	31.99 ± 5.25*	38.47 ± 5.78*	46.45 ± 6.54*	51.70 ± 7.03*
Ibuprofen	30.03 ± 4.11*	36.18 ± 5.04*	41.84 ± 6.04*	43.96 ± 6.06*

Values represent the mean ± S.E. of six rats for each group.
Control group animals were given saline with few drops of carboxymethyl cellulose 0.5%.
* Statistically significant (P <0.05) from the control normal inflamed group at the corresponding time, using one way ANOVA.

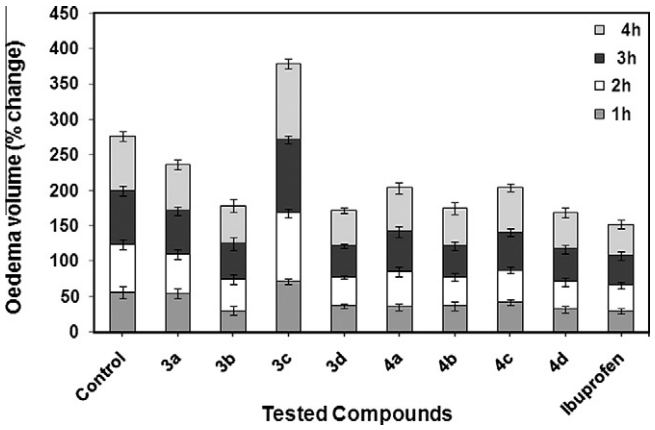


Figure 1. Acute anti-inflammatory effect of the tested ester derivatives (**3a–d**, and **4a–d**) on carrageenan-induced oedema, in comparison with 100 mg/kg bwt, ibuprofen at 1hr before initiation of inflammation with carrageenan. Oedema was measured at 1, 2, and 3 h after carrageenan injection. Data shown are means ($n = 6$) ± S.E., $p < 0.05$.

This results suggest that *p*-substituted benzylidenamino phenyl esters of ibuprofenic and mefenamic acids produces an anti-oedematous effect during the first phase, similarly to ibuprofen. The anti-oedematous effect of low-dose these esters had a prompt onset (1 h). This phenomenon may partly be due to the high systemic bioavailability of *p*-substituted benzylidenamino phenyl esters following oral dosing, due to their higher and efficient absorptivity.

2.2.2. Analgesic activity

The analgesic activity of the synthesized derivatives was evaluated by applying hot plate test,[33,34] using 10 groups each of five rats, each were given vehicle and/or the different compounds, where ibuprofen was used as a standard reference. Results were expressed as mean ± S.E. Difference between vehicle control and treatment groups were tested using one way ANOVA followed by the least significant difference (L.S.D.).

In the classical hot plate test, mice react by licking their paws and/or jumping. However, tests relying on the unilateral application of thermal radiant heat (UHP) to the plantar side of the hind paw have become popular in recent years since unilateral changes in nociceptive sensitivity can be detected. Ibuprofen increases latencies; hence the last group received ibuprofen (250 mg/kg bwt) 30 min prior to testing. Latency to lick a hind paw or jumping was recorded sequentially before and at 0.5, 1.0, and 1.5 h post treatment.

The results revealed that all tested compounds (**3a–d** and **4a–d**) at the doses of (250 mg/kg bwt) produced significant anti-nociception in the hot plate test. According to Table 2, almost all these ester derivatives showed significant analgesic activity higher than that obtained by ibuprofen at all time post administration. Where compounds **3a–d** exhibited the most potent analgesic effects, where they increased latency time significantly ($p < 0.05$) after all times of their administration. Where the most pronounced compounds of analgesic activities are compounds **3b** and **4c** at 0.5 h, compounds **3a** and **3b** at 1.0 h, and compounds **3a** and **3d** at 1.5 h post administration as cited in Table 2.

Thus, it can be concluded that, compounds **3a–d** have significant analgesic activity and are the most potent analogs as illustrated in Figure 2. Substances that produce strong inhibitory effects in the hot plate test can inhibit centrally induced pain and they act as strong analgesics.[35,36]

The observed activities of the aforementioned derivatives in the hot plate test, therefore suggests that they have strong analgesic activities.

Table 2
Analgesic effect of the tested ester derivatives (**3a–d** and **4a–d**) in comparison to ibuprofen using hot plate method

Group	Time after induction (s)			
	Basal	0.5 h	1 h	1.5 h
Control	12.54 ± 1.12	11.16 ± 1.66	14.3 ± 0.99	16.8 ± 2.12
3a	10.52 ± 0.75	20.18 ± 1.03*	35.14 ± 2.92*	28.1 ± 3.85*
3b	9.24 ± 1.29	23.52 ± 4.82*	34.62 ± 3.13*	24.98 ± 0.76*
3c	12.64 ± 2.08	22.22 ± 1.93*	32.14 ± 2.29*	23.0 ± 1.01*
3d	11.74 ± 1.27	19.62 ± 1.08*	33.44 ± 2.36*	26.12 ± 2.30*
4a	11.42 ± 2.10	21.68 ± 4.19*	25.08 ± 3.32*	18.46 ± 0.82
4b	11.18 ± 1.40	18.04 ± 2.57	25.98 ± 1.27*	20.82 ± 0.59
4c	9.60 ± 1.23	23.16 ± 2.67*	24.40 ± 2.99*	15.96 ± 1.63
4d	11.84 ± 1.67	20.72 ± 2.87*	21.22 ± 4.36	19.38 ± 2.37
Ibuprofen	13.56 ± 1.42	17.84 ± 3.57	16.10 ± 0.94	19.0 ± 3.20

Values represent the mean ± S.E. of five mice for each group.
Control group animals were given saline with few drops of carboxymethyl cellulose 0.5%.
* Statistically significant ($P < 0.05$) from the control normal group at the corresponding time, using one way ANOVA.

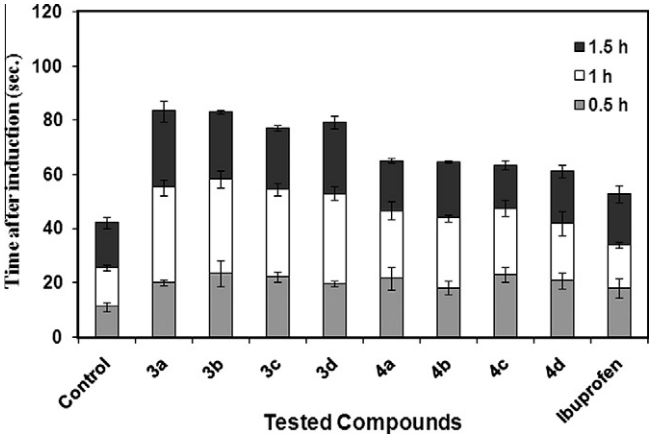


Figure 2. The analgesic effect of compounds (**3a–d**, and **4a–d**; 250 mg/Kg bwt) involving hot plate method, in comparison with ibuprofen. Latency was measured at 0.5, 1.0, and 1.5 h after oral drug administration. Data shown are means ($n = 5$) ± S.E., $p < 0.05$.

2.2.3. Ulcerogenic liability

Being many of the compounds under investigation having pronounced anti-inflammatory and analgesic activity in comparison to ibuprofen, therefore the ulcerogenic liability for all of them was evaluated in albino rats following the reported method.[37,38] From the data obtained (Table 3), it has been surprisingly observed that all the synthesized *p*-substituted benzylidenamino phenyl esters of ibuprofenic and mefenamic acids exhibited no ulcerogenic effect in all of the experimental animals, compared to that of the standard ibuprofen (ulcer index of 4.03 ± 0.79). Therefore, the potential medicinal value of these compounds as anti-inflammatory and analgesic agents is that they have highly better safety margin on gastric mucosa than ibuprofen.

This promising ulcer protective properties of the designed and synthesized esters is greatly support our main objective to avoid ibuprofen and mefenamic acid limitation of gastric injuries caused by their free carboxylic group. Therefore, this successful results support the present work to develop these novel series of ibuprofen and mefenamic acid esters to mask their drawbacks.

2.3. Molecular docking study

Molecular docking is a frequently used tool in computer-aided structure-based rational drug design. It evaluates how small mole-

Table 3
Ulcerogenic effect of compounds (**3a–d**, and **4a–d**) on the gastric mucosa of six rat groups indicating number and severity after compounds after 5 h of injection

Group	Ulcer index	
	No. of ulcer	Severity of ulcer
Control	0	0
3a	0	0
3b	0	0
3c	0	0
3d	0	0
4a	0	0
4b	0	0
4c	0	0
4d	0	0
Ibuprofen	4.03 ± 0.79*	9.5 ± 1.78*

Statistical analysis was carried out using Kruskal–Wallis non-parametric one way ANOVA.
Values represent the mean ± S.E. of six rats for each group.
* Statistically significant from the control normal $P < 0.05$.

cule (substrate, inhibitor, drug or drug candidate) and the target macromolecule (receptor, enzyme or nucleic acid) fit together. This can be useful for developing better drug candidates and also for the understanding the nature of the binding. Herein in silico comparative Cox1 and Cox2 docking study aims to rationalize the obtained biological data and to explain the possible interactions of the tested derivatives into the crystal structure of Cox1 and Cox2 enzymes. The main interactions of the native co-crystallized ligands namely, ibuprofen and S58 with Cox1 and Cox2, respectively, were investigated. The carboxylate group of ibuprofen exhibited three hydrogen bond with the key amino acids of Cox1: Tyr 355 (*p*-OH group) and Arg120 (guanidine $-NH_2$ group). Also other residues in the binding pocket can be involved in such interactions include Val349, Met522, Ile523, Gly526, and Ala527 as shown in Figure 3. Whereas, the main amino acids involved in Cox2 interaction with the native ligand S58 are His90, Arg120, Val349, Tyr355, Arg53, Phe518, Gly526, and Ala527. Where His90, Arg120, and Arg513 form four hydrogen bonds with native ligand S58.

Computer simulated automated docking study was performed using the widely distributed molecular grid-based docking program. AutoDock3.05[39] for docking of flexible ligand within rigid protein and AutoDock4.2[40] for docking of flexible ligand within flexible protein, where flexibility of the target protein is taken into account in the later type and ignored in the former one.

AutoDock scans the active site for low energy binding models and for orientations of the probe molecule, using a modified genetic algorism that employs a local search (GALS) and pre-computed grids for the evaluation of the interaction energy. Both of the target Cox1 (PDB code: 1EQG) and Cox2 (PDB code: 1CX2) proteins were handled by using Accelyrs Discovery Studio visualize v2.5 software [Accelrys Inc., San Diego, CA (2005)] and the representative amino acids of the ligand-binding site were selected within 5 Å neighborhood surrounding the embedded ligand; ibuprofen. The results of 10 randomly seeded runs were analyzed for each of the docked inhibitors. The docked inhibitors were assigned to a cluster if the atomic coordinates of the docked inhibitors exhibited a root mean square deviation (RMSD) of less than 0.5 Å difference from each other (RMSD-tolerance of 0.5 Å). The analysis was carried out for the top 10 docking clusters. The clusters were ranked from the averaged lowest energy obtained for members of the cluster to the highest. Each of the clusters that exhibited significant negative interaction energies were examined by Accelyrs Discovery Studio visualize modeling program to determine their binding orientations.

2.3.1. Validation of the accuracy and performance of AutoDock

As cited in literature,[41] if the RMSD (root mean square deviation) of the best docked conformation of the native ligand is $\leq$2.0 Å from the experimental one, the used scoring function is successful. Therefore the validation of the docking accuracy was investigated by docking of the native co-crystallized ligands to inspect how closely the best docked conformation resembles the bound ligand in the biological method.[42,43] The obtained success rates of AutoDock was highly excellent. Where the co-crystallized ligands of Cox1 and Cox2, namely ibuprofen and S58, respectively seem exactly superimposed on the native bound ones as shown in Figure 3. The RMSD of the docked ibuprofen into Cox1 were 0.91 and 2.51 Å, and that of the docked S58 into Cox2 were 0.40 and 0.34 Å by rigid and flexible docking for each receptor, respectively.

Moreover, flexible docking involving AutoDock 4.2 seems to be more accurate, being of smaller RMSD values, to be of more resemblance to the biological co-crystallization.

2.3.2. AutoDock binding affinities of the synthesized compounds into Cox1 and Cox2 enzymes

The binding affinity was evaluated by the binding free energies (ΔG_b, kcal/mol), inhibition constants (K_i), hydrogen bonding, and RMSD values in comparison to the native co-crystallized ligands. All of these data into the target macromolecule are represented in Tables 4–7.

The compounds which commonly revealed the highest binding affinities, that is, lowest binding free energies, and the hydrogen bond interactions into Cox1 and Cox2 include; to a higher extent; compounds: 4-(4-nitro benzylideneamino)phenyl 2-(4-isobutylphenyl)propanoate (**3b**), 4-(4-bromobenzylideneamino) phenyl 2-(2,4-dimethylphenylamino)benzoate (**4d**), compounds: 4-(4-nitro benzylideneamino)phenyl 2-(2,4-dimethylphenylamino)benzoate (**4b**), 4-(4-methoxy benzylideneamino)phenyl 2-(4-isobutylphenyl)propanoate (**3a**), and 4-(4-bromo benzylideneamino)phenyl 2-(4-isobutylphenyl)propanoate (**3d**).

These derivatives exhibited up to three hydrogen bonds between their carboxylate or imide group with the key amino acids of Cox1: His90–NH, Arg120–NH_2, Tyr 355–OH, Tyr385–OH, and Arg513–NH_2, within highly acceptable RMSD being 1.90–4.72 Å as cited in Tables 4–7. Compound 4-(4-nitro benzylideneamino)phenyl 2-(4-isobutylphenyl)propanoate (**3b**) on applying its flexible docking into Cox1 macromolecule, it revealed the highest binding affinity elucidated by its lowest binding free energies being, (ΔG_b: −14.32 kcal/mol) as cited in Table 5. This predicts its promising anti-inflammatory activity and higher selectivity towards Cox1 more than Cox2 receptor. Moreover, **3b** exhibited one and three hydrogen bonds with Tyr355 and Arg120 by flexible and rigid docking within Cox1 receptor sites, respectively (Tables 4 and 5). Figure 4 illustrates differential binding affinities of **3b** and **4b** analogs into Cox1 target site, where, compound **3b** (ΔG_b: −10.07 kcal/mol) exhibited three hydrogen bonds between its –COO moiety and Arg120 within RMSD: 4.72 Å, whereas, compound **4b** (ΔG_b: −10.35 kcal/mol) exhibited two hydrogen bonds between its NO_2 moiety and Tyr385 within RMSD: 4.05 Å as cited in Table 4.

To investigate the potential Cox2 inhibitory activity, compounds **4c** (ΔG_b: −12.76 kcal/mol) and **4d** (ΔG_b: −13.07 kcal/mol) exhibited selective binding affinities towards Cox2 more than Cox1, as cited in Table 7. This high binding affinity was supported more by their close superimposing onto the native co-crystallized ligand (S58) being their RMSD 1.90 and 2.68 Å, respectively, providing better RMSD values than other derivatives. These aforementioned docking results of **4c** and **4d** indicate that these compounds are expected to be a reasonable candidate for Cox2 inhibition.

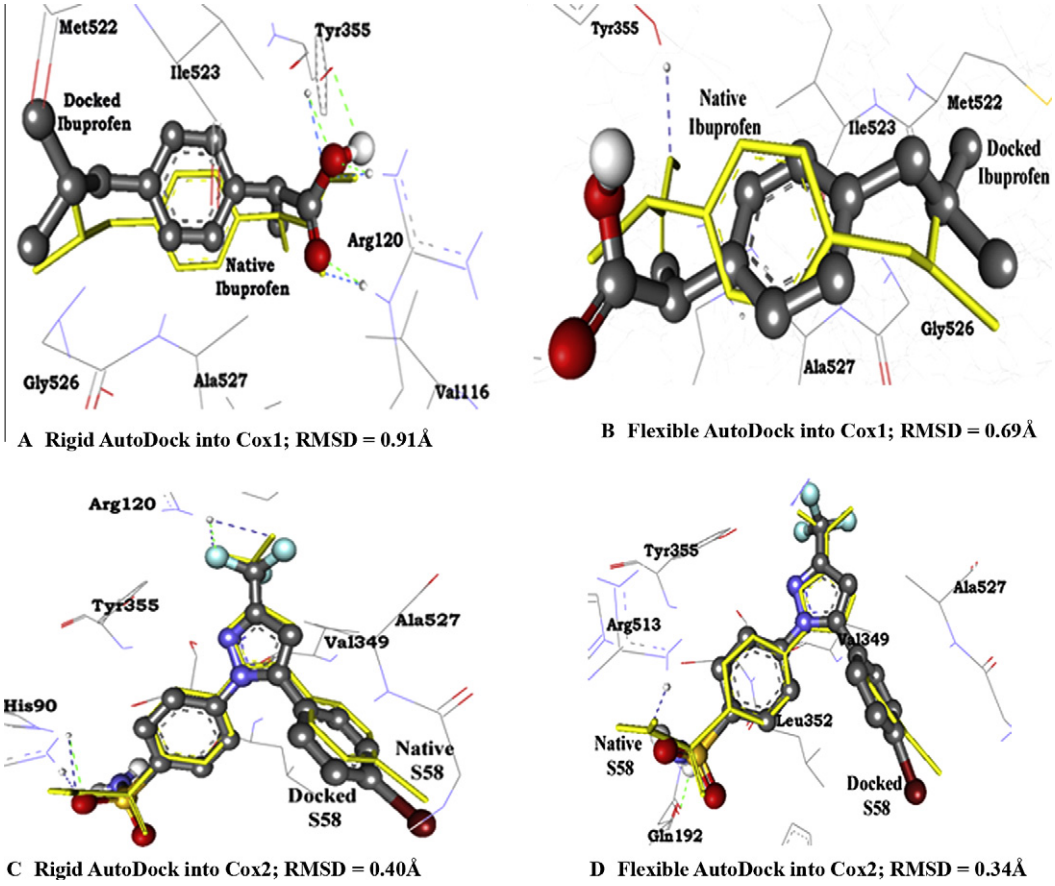


Figure 3. Differential validation of AutoDock 3.05 (in **A**, **C**) and AutoDock 4.2 (in **B**, **D**) programs by docking of the native ligands of Cox1 and Cox2 into their binding sites. The native co-crystallized ibuprofen and S58 are shown in yellow sticks, while the docked ligands are shown in balls and sticks, colored by element. The hydrogen bonds are shown as blue and green dotted lines, respectively. The docked ligands seem exactly superimposed on the native ones.

Table 4
The rigid docking results (AutoDock 3.05), regarding the binding free energies (ΔG_b) and inhibition constants (K_i) of compounds **3a**–**d**, and **4a**–**d** docked into ovine Cox1(1EQG), the distances and angles of hydrogen bonds between compounds and amino acids involved in Cox1, and RMSD from the co-crystallized ibuprofen

Compd	ΔG_b [a] (kcal/mol)	K_i [b]	Hydrogen bonds between atoms of compounds and amino acids of ovine Cox1				RMSD[c] (Å)
			Atom of compd	Amino acid	Distance (Å)	Angle (°)	
3a	−9.40	1.28E-07	*p*-C=O	HN (HE) of Arg120	2.37	155.2	4.31
			p-COO	HN (HE) of Arg120	2.43	147.7	
			p-COO	HN (HH21) of Arg120	2.20	156.0	
3b	−10.07	4.23E-08	*p*-C=O	HN (HE) of Arg120	2.43	127.7	4.72
			p-C=O	HN (HH21) of Arg120	1.80	142.3	
			p-COO	HN (HE) of Arg120	2.18	164.0	
3c	−9.06	2.28E-07	*p*-COO	HN (HE) of Arg120	1.71	151.1	3.93
3d	−9.37	1.36E-07	*p*-C=O	HN (HH21) of Arg120	1.68	170.5	4.71
			p-C=O	HN (HE) of Arg120	2.34	111.7	
4a	−8.44	6.46E-07	d				4.60
4b	−10.35	2.57E-08	*p*-N=O	*p*-HO of Tyr385	1.85	135.1	4.05
			p-N-O	*p*-HO of Tyr385	1.94	143.2	
4c	−9.21	1.79E-07	C=N	HN (HE) of Arg120	1.82	147.6	7.96
			C=N	HN (HH21) of Arg120	2.10	136.7	
4d	−9.65	8.38E-08	C=N	HN (HE) of Arg120	2.35	144.5	7.22
			C=N	HN (HH21) of Arg120	2.10	152.4	
			H-N	HN (HH22) of Arg83	2.20	155.9	
Ibuprofen	−8.79	3.08E-14	*p*-C=O	HN (HE) of Arg120	1.75	162.0	0.91
			p-COO	HN (HH21) of Arg120	1.60	170.3	
			p-COO	*p*-HO of Tyr355	1.91	109.3	
			p-COOH	*p*-OH of Tyr35	2.20	109.6	

[a] Binding free energy.
[b] Inhibition constant.
[c] Root mean square deviation.
[d] No hydrogen bond detected.

Table 5
The flexible docking results (AutoDock 4.2), regarding the binding free energies (ΔG_b) and inhibition constants (K_i) of compounds **3a–d**, and **4a–d** docked into ovine Cox1(1EQG), the distances and angles of hydrogen bonds between compounds and amino acids involved in Cox1, and RMSD from the co-crystallized ibuprofen

Compd	ΔG_b [a] (kcal/mol)	K_i [b]	Hydrogen bonds between atoms of compounds and amino acids of Cox1				RMSD[c] (Å)
			Atom of compd	Amino acid	Distance (Å)	Angle (°)	
3a	−11.78	2.32 nM	*p*-C=O	*p*-HO of Tyr355	2.32	155.0	1.77
3b	−14.32	32.0 pM	*p*-COO	*p*-HO of Tyr355	2.02	153.8	3.92
3c	−9.46	116.5 nM	d				3.77
3d	−13.36	159.9 pM	*p*-C=O	HN (HH12) of Arg120	2.04	131.3	3.12
4a	−10.81	11.83 nM	*p*-C=O	HN (HE) of Arg120	1.94	135.8	4.39
			p-MeO	*p*-HO of Tyr355	2.01	144.6	
4b	−9.71	76.7 nM	C=N	*p*-HO of Tyr355	2.07	123.7	5.77
4c	−8.74	393.5 nM	C=O	HN (HH22) of Arg120	2.08	170.7	5.59
4d	−10.61	16.81 nM	C=O	HN (HE) of Arg120	2.10	135.4	5.58
Ibuprofen	−11.14	6.83 nM	*p*-COO	*p*-HO of Tyr355	1.99	173.2	2.51
			p-COOH	*p*-OH of Ser530	2.49	169.8	

[a] Binding free energy.
[b] Inhibition constant.
[c] Root mean square deviation.
[d] No hydrogen bond detected.

Table 6
The rigid docking results (AutoDock 3.05), regarding the binding free energies (ΔG_b) and inhibition constants (K_i) of compounds **3a–d**, and **4a–d** docked into Cox2 (1CX2), the distances and angles of hydrogen bonds between compounds and amino acids involved in Cox2, and RMSD from the co-crystallized S58 ligand

Compd	ΔG_b [a] (kcal/mol)	K_i [b]	Hydrogen bonds between atoms of compounds and amino acids of Cox2				RMSD[c] (Å)
			Atom of compd	Amino acid	Distance (Å)	Angle (°)	
3a	−10.53	1.93E-08	*p*-C=O	HN (HE21) of Gln192	2.02	129.7	6.84
			p-COO	HN of His90	1.91	136.5	
3b	−11.63	2.96E-09	*p*-C=O	HN (HH11) of Arg513	1.71	130.9	2.78
			p-COO	HN of His90	2.44	148.3	
3c	−10.51	1.98E-08	*p*-C=O	*p*-HO of Tyr355	2.20	128.0	1.32
3d	−10.17	3.50E-08	*p*-C=O	HN of Ile517	1.86	149.7	5.94
4a	−10.23	3.18E-08	–C=N	HN (HE22) of Gln192	2.47	157.6	10.10
4b	−9.00	2.54E-07	HN	HN of Asp515	2.28	150.3	10.37
4c	−10.78	1.25E-08	*p*-COO	HN of Asp515	2.42	124.9	7.51
4d	−11.81	2.19E-09	–C=N	HN of His90	1.86	143.6	1.65
S58[d]	−11.53	3.55E-09	*C–F*	HN (HH11) of Arg120	2.26	120.4	0.40
			S=O	HN of His90	2.50	113.2	

[a] Binding free energy.
[b] Inhibition constant.
[c] Root mean square deviation.
[d] 1-Phenylsulfonamido-3-trifluoromethyl-5-(*p*-bromophenyl) pyrazole.

Table 7
The flexible docking results (AutoDock 4.2), regarding the binding free energies (ΔG_b) and inhibition constants (K_i) of compounds docked into Cox2 (1CX2), the distances and angles of hydrogen bonds between compounds and amino acids involved in Cox2, and RMSD from the co-crystallized S58 ligand

Compd	ΔG_b [a] (kcal/mol)	K_i [b]	Hydrogen bonds between atoms of compounds and amino acids of Cox2				RMSD[c] (Å)
			Atom of compd	Amino acid	Distance (Å)	Angle (°)	
3a	−12.01	1.57 nM	C=O	*p*-HO of Tyr355	2.03	136.8	3.77
3b	−10.94	9.56 nM	C=O	*p*-HO of Tyr355	2.01	152.3	4.32
			C=N	HN(HH2) of Arg120	2.40	113.4	
3c	−11.02	8.82 nM	C=O	HN (HH11) of Arg513	1.80	141.9	5.29
3d	−10.43	22.46 nM	C=O	HN (HE) of Arg120	2.08	134.2	7.07
			C=O	HN (HH2) of Arg120	2.10	134.7	
			COO	*p*-HO of Tyr355	2.01	139.7	
4a	−6.64	13.50 mM	C=N	HN (HH1) of Arg120	2.02	166.5	3.07
4b	−11.61	3.10 nM	d				3.07
4c	−12.76	446.8 pM	C=N	HN (HE2) of His90	1.85	138.9	1.90
4d	−13.07	261.0 pM	C=N	*p*-HO of Tyr355	2.31	177.8	2.68
S58[e]	−9.47	115.1 nM	S–NH	O=C of Gln192	2.00	109.2	0.34

[a] Binding free energy.
[b] Inhibition constant.
[c] Root mean square deviation.
[d] No hydrogen bond detected.
[e] 1-Phenylsulfonamido-3-trifluoromethyl-5-(*p*-bromophenyl)pyrazole.

Figure 5 illustrates data represented docking of compound **4d** (ΔG_b: −13.07 kcal/mol) which is almost superimposed on the native ligand (RMSD: 2.68 Å), exhibiting one hydrogen bond between C=N and *p*-OH of Tyr355. Relatively, compound **3d** (ΔG_b:

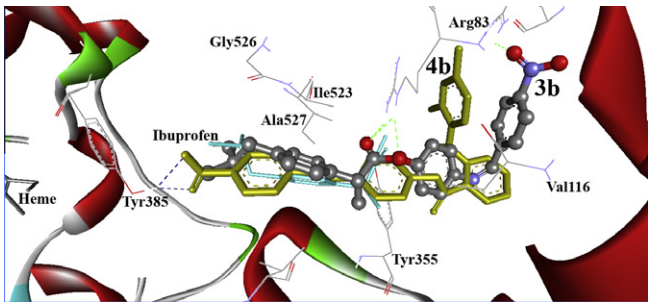


Figure 4. Relative docking mode of compounds **3b**; ball and stick, colored by element, and **4b**; yellow sticks involving rigid docking into Cox1. They exhibit three and two hydrogen bonds shown as dashed lines with Arg120 and Tyr385, respectively. The binding site of the Cox1 is shown with labeled amino acids and native co-crystallized ibuprofen is shown as cyan stick.

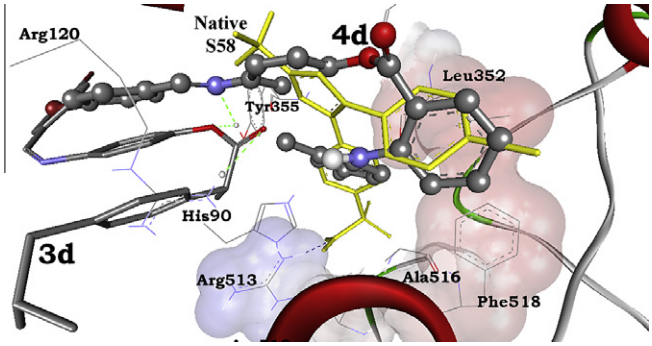


Figure 5. The comparative binding affinities of compounds **4d**; ball and stick, colored by element, and **3d**; sticks, colored by element, involving flexible docking into Cox2. Compound **4d** (ΔG_b: −13.07 kcal/mol; RMSD: 2.68 Å) is superimposed onto the S58 native ligand; yellow sticks, and it exhibited one hydrogen bond with Tyr355. Compound **3d** exhibited lower binding affinity (ΔG_b: −10.43 kcal/mol, RMSD: 7.07 Å), and it exhibited three hydrogen bond with Arg120 and Tyr355. Cox2 is shown as solid backbone ribbon for protein and its amino acids are shown as labeled lines, and the binding site is shown with solid surface.

−10.43 kcal/mol) is docked deviated from the binding site (RMSD: 7.07 Å), bound to Arg120 and Tyr355 amino acids by its C=O moiety. This different binding mode of these compounds may explain their different biological anti-inflammatory activities of compounds **3d** and **4d** after 1 and 2 h, where **4d (31.99 ± 5.25%, and 38.47 ± 5.78%)** revealed higher anti-inflammatory effect than compound **3d (36.54 ± 2.63% and 40.59 ± 1.94%)** as shown in Table 1 and Figure 1.

It is clearly noticed that compounds **4d, 4c, 3a, 4b**, and **3c**, respectively revealed more selective Cox2 binding over Cox1 by flexible docking way as cited in Table 7, whereas, compound **3b** exhibited more selective Cox1 fitting.

In the analysis of docking results we can easily find an overall excellent correlation between the biological results (anti-inflammatory and analgesic activities) and docking studies. Where compounds **3b**, **3d**, **4b**, and **4d** which has the highest acute anti-inflammatory effect (Table 1 and Fig. 1), have also the lowest binding free energies in both of rigid and flexible docking into Cox1 and Cox2 with the nearest RMSD values to that of ibuprofen and S58 as illustrated in Tables 4–7 of the docking results. Additionally, compounds **3a–d** revealed the best analgesic activities (Table 2 and Fig. 2), especially after 0.5 and 1.0 h. These results are directly correlated with compounds **3a, 3b**, and **3d** which have the lowest binding free energies as cited in Table 5.

3. Conclusion

Oral dosage forms of ibuprofen and mefenamic acid, though popular, suffer from the limitation of gastric injuries caused by their free carboxylic group. Therefore, in this study we considered it interesting to modify NSAIDS structure in such a way that it would lead to molecules with greatly reduced acidic character by developing a series of ibuprofen and mefenamic acid esters to mask the free carboxylic groups. These prodrugs were synthesized by conventional method of esterification by replacing the carboxylic acid group of ibuprofenic and mefenamic acids with less acidic *p*-substituted benzylidenamino phenyl esters. Where the intermediate **Schiff's bases [4-(4-substituted benzylideneamino) phenols; **2a–d**] were synthesized by reaction of *p*-amino phenol and *p*-substituted aromatic aldehyde, then subjected to condensation reaction with ibuprofen or mefenamic acid involving dicyclohexyl carbodiimmide (DCC) as mild dehydrating agent to afford the prodrugs; 4-(4-substituted benzylideneamino)phenyl 2-(4-isobutylphenyl)propanoate (**3a–d**) and 4-(4-substituted benzylideneamino)phenyl 2-(2,4-dimethylphenylamino) benzoate (**4a–d**). The structures of the newly synthesized compounds were elucidated by microanalytical and spectral (IR, ^{1}H NMR, mass) data.

In addition to design and synthesis, this work covered testing of eight drug candidates for their analgesic, anti-inflammatory potencies, in addition to ulcerogenic effects. The anti-inflammatory activity of the synthesized compounds was evaluated by carrageenan induced rat paw oedema model, where significant (p <0.05) reduction of rat paw oedema was observed by most of the test compounds at 1–3 h compared to control group (given saline with few drops of CMC).

Moreover, esterification of ibuprofen led to pronounced increase of its analgesic activity, which was estimated by increasing the latency time, means strongly inhibited the peripheral pain response in the mice compared to the control animals. In addition, most of the compounds were found to be more active in comparison with parent drugs, indicating that esterification of these NSAIDs improved their analgesic activity.

Being almost all of these compounds exhibit significant anti-inflammatory and analgesic activities, they were subjected to ulcerogenicity potential test at (100 mg/kg) of 12 times the therapeutic doses. A thorough examination of the results of histopathological studies indicated absence of the disruption of gastric epithelial morphology and absence of ulcers/erosion in test group animals compared to reference standard, ibuprofen, and control group animals. These results are attributed to the acquired acid protective design which reduced their acidic characters of the synthesized esters compared to the parent acids.

From close inspection of the aforementioned results of in vivo ulcerogenicity studies, we can conclude that the esterification of carboxylate moiety of ibuprofenic and mefenamic acids may lead to the development of novel, useful anti-inflammatory, analgesic, and cytoprotective pharmacomolecules, with potentially important therapeutic applications.

Moreover, we have successfully carried out comparative rigid and flexible docking for eight compounds. The obtained binding free energies predicted by AutoDock 3.05 and 4.2 were highly correlated to the biological activities, especially by flexible docking, where a lot of compounds of pronounced biological activities revealed lowest binding free energies with smallest RMSD and reasonable number of hydrogen bonds into the binding site. The results are encouraging to improve our knowledge in understanding the binding interactions in detail. Where, flexible docking into the cyclooxygenase receptor revealed more selective Cox2 binding affinities of compounds **3a, 3c, 4b, 4c** and **4d** over Cox1 one, whereas, compounds **3b** and **3d** exhibited more selective Cox1 fitting.

4. Experimental

4.1. Chemistry

Melting points were obtained on a Yanagimoto micro melting point apparatus and are uncorrected. Microanalyses were measured by Yanaco CHN Corder MT-5 apparatus. IR spectra were recorded on a JASCO FT/IR-200 spectrophotometer as Nujol mulls. ^{1}H NMR spectra and ^{1}H and ^{13}C NMR spectra were obtained using a Varian VXR 300 MHz and 75 MHz spectrophotometer, respectively, and chemical shift values were expressed in δ values (ppm) relative to tetramethylsilane (TMS) as internal standard. Coupling constants are given in Hz. Coupling constants are given in Hz. All NH and OH protons were exchangeable with D_2O. Microanalysis was carried out at microanalytical center, Faculty of Science, Cairo University, and the mass spectra were recorded on GCMC-QP 1000 EX Shimadzo Gas Chromatography MS spectrometer, Japan E.I.70 ev. All reagents were of commercial quality and were used without further purification. Organic solvents were dried in the presence of an appropriate drying agent and were stored over suitable molecular sieves. Reaction progress was monitored by analytical thin layer chromatography (TLC) on pre-coated glass plates silica gel 60F254-plate-Merck) and the products were visualized by UV light. Ibuprofen and mefenamic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA) in the form of racemic mixtures.

4.1.1. General procedure for the preparation of the intermediate Schiff's bases: 4-(4-substituted benzylideneamino)phenol (2a–d)[26–31]

Each reactant of *p*-aminophenol (**1**, 10.9 g; 0.1 mol) and *p*-substituted aromatic aldehyde (0.1 mol), was dissolved in a minimum amount of absolute ethanol (20 ml), then mixed together and followed by addition of 0.2 ml of glacial acetic acid. The reaction mixture was refluxed for 6–8 h. After completion of the reaction (monitored by TLC), the resulting clear solution was concentrated in vacuo. The obtained yellow residue was treated with ice water and the precipitate powdery crystals were filtered off, washed well with water, dried and recrystallized from ethanol to afford the corresponding product as yellow needles in 70–85% yields.

4.1.2. General procedure for the preparation of 4-(4-substituted benzylideneamino)phenyl 2-(4-isobutylphenyl)propanoate (3a–d) and 4-(4-substituted benzylideneamino)phenyl 2-(2,4-dimethylphenyl amino) benzoate (4a–d)

N,*N*′-Dicyclohexyl carbodiimmide (DCC) (2.48 g, 12 mmol) was added to the corresponding acid (ibuprofen, 2.06 g; 10 mmol) or (mefenamic acid, 2.41 g; 10 mmol) in 10 ml dichloromethane/THF (1:1) on cold. The resulting suspension was vigorously stirred for 15 min at room temperature. Subsequently, was added a solution of the appropriate Schiff's base (10 mmol) in 10 ml dichloromethane/THF (1:1). The mixture was left stirring overnight at room temperature. The mixture was then filtered and evaporated in vacuo. The crude residue was obtained and crystallized from methanol to afford the corresponding product as yellow needles in 52–84% yields.

4.1.2.1. 4-(4-Methoxy benzylideneamino)phenyl 2-(4-isobutylphenyl)propanoate (3a). Yield, (3.28 g, 79%); mp 210 °C (from MeOH); IR (ν_{max}/cm^{-1}): 1656 (C=O); ^{1}H NMR [$CDCl_3$]: δ 1.50 (6H, d, J = 7.2 Hz, 4-CH_2–CH–(CH_3)$_2$, 1.83 (3H, d, J = 7.2 Hz, α-CH–CH_3), 2.15 (1H, m, 4-CH_2–CH–(CH_3)$_2$, 2.30 (2H, d, J = 7.2 Hz, 4-CH_2–CH–(CH_3)$_2$, 3.45 (3H, s, *p*-OCH_3), 3.84 (1H, m, α-CH–CH_3, 6.90–7.25 (12H, m, Ar-H), 8.37 (1H, s, CH=N); ^{13}C NMR [$CDCl_3$]: δ_C 20.11 (CH_3), 23.31 (isobut. 2,2-CH_3), 29.55 (isobut. CH), 44.0 (isobut. CH_2), 48.6 (CH), 55.03 (OCH_3), 121.06 (C1, C2′, C3′, C5′ and C6′), 124.10 (C2, C3, C5 and C6), 137.50 (C2″, C3″, C5″ and C6″), 138.60 (C4 and C1″), 141.2 (C1′ and C4′), 143.4 (C4″), 153.0 (C=N), 171.9 (C=O); Anal. Calcd for $C_{27}H_{29}NO_3$: C, 78.04; H, 7.03; N, 3.37. Found: C, 77.87; H, 6.84; N, 3.00.

4.1.2.2. 4-(4-Nitro benzylideneamino)phenyl 2-(4-isobutylphenyl)propanoate (3b). Yield, (3.61 g, 84%); mp 172 °C (from MeOH); IR (ν_{max}/cm^{-1}): 1620 (C=O); ^{1}H NMR [$CDCl_3$]: δ 1.06 (6H, d, J = 6.1 Hz, 4-CH_2–CH–(CH_3)$_2$), 1.43 (3H, d, J = 6.0 Hz, α-CH–CH_3), 2.43 (1H, m, 4-CH_2–CH–(CH_3)$_2$), 2.45 (2H, d, J = 6.0 Hz, 4-CH_2–CH–(CH_3)$_2$), 3.90 (1H, m, α-CH–CH_3), 7.06–7.25 (8H, m, Ar-H), 8.02 (2H, dd, $J_{2,3}$ = 9.0 Hz, $J_{3,5}$ = 3.0 Hz, Ar *m*-H), 8.28 (2H, dd, $J_{2,3}$ = 9.0 Hz, $J_{2,6}$ = 3.0 Hz, Ar *o*-H), 8.56 (1H, s, CH=N); ^{13}C NMR [$CDCl_3$]: δ_C 21.3 (CH_3), 23.7 (isobut. 2,2-CH_3), 29.4 (isobut. CH), 44.0 (isobut. CH_2), 44.7 (CH), 125.9 (C1, C2′, C3′, C5′ and C6′), 128.6 (C2, C3, C5 and C6), 137.8 (C2″, C3″, C5″ and C6″), 139.5 (C4 and C1″), 142.0 (C1′ and C4′), 146.4 (C4″), 153.2 (C=N), 174.3 (C=O); Anal. Calcd for $C_{26}H_{26}N_2O_4$: C, 72.54; H, 6.09; N, 6.51. Found: C, 72.57; H, 6.29; N, 6.85. MS, *m*/*z* (%): (M+1) at 431.

4.1.2.3. 4-[4-(Dimethylamino)benzylideneamino]phenyl 2-(4-isobutylphenyl)propanoate (3c). Yield, (2.96 g, 69%); mp 260 °C (from MeOH); IR (ν_{max}/cm^{-1}): 1680 (C=O); ^{1}H NMR [DMSO-d_6]: δ 0.81 (6H, d, J = 6.1 Hz, 4-CH_2–CH–(CH_3)$_2$), 1.24 (3H, d, J = 6.1 Hz, α-CH–CH_3), 1.67 (1H, m, 4-CH_2–CH–(CH_3)$_2$), 2.48 (2H, d, J = 6.1 Hz, 4-CH_2–CH–(CH_3)$_2$), 2.97 (6H, s, NCH_3)$_2$), 3.36 (1H, m, α-CH–CH_3), 6.72–7.69 (12H, m, Ar-H), 8.36 (1H, s, CH=N); ^{13}C NMR [$CDCl_3$]: δ_C 21.0 (CH_3), 23.4 (isobut. 2,2-CH_3), 28.89 (isobut. CH), 32.66 (*p*-N$(CH_3)_2$), 43.67 (isobut. CH_2), 44.35 (CH), 125.58 (C2′, C3′, C5′ and C6′), 125.99 (C1), 128.26 (C2, C3, C5 and C6), 128.34 (C2″, C3″, C5″ and C6″), 137.6 (C4 and C1″), 139.2(C1′ and C4′), 145.5 (C4″), 152.8 (C=N), 173.0 (C=O); Anal. Calcd for $C_{28}H_{32}N_2O_2$: C, 78.47; H, 7.53; N, 6.54. Found: C, 78.31; H, 7.42; N, 6.88. MS, *m*/*z* (%): (M+1) at 429.

4.1.2.4. 4-(4-Bromo benzylideneamino)phenyl 2-(4-isobutylphenyl)propanoate (3d). Yield, (3.81 g, 82%); mp 180 °C (from MeOH); IR (ν_{max}/cm^{-1}): 1650 (C=O); ^{1}H NMR [DMSO-d_6]: δ 0.82 (6H, d, J = 8.7 Hz, 4-CH_2–CH–(CH_3)$_2$), 1.22 (3H, d, J = 8.7 Hz, α-CH–CH_3), 2.40 (1H, m, 4-CH_2–CH–(CH_3)$_2$), 2.50 (2H, d, J = 8.7 Hz, 4-CH_2–CH–(CH_3)$_2$), 3.16 (1H, m, α-CH–CH_3), 7.14–7.81(12H, m, Ar-H), 8.62 (1H, s, CH=N); ^{13}C NMR [$CDCl_3$]: δ_C 21.2 (CH_3), 23.6 (isobut. 2,2-CH_3), 29.94 (isobut. CH), 43.9 (isobut. CH_2), 44.55 (CH), 125.8 (C1, C2′, C3′, C5′ and C6′), 126.14 (C2, C3, C5 and C6), 128.6 (C2″, C3″, C5″ and C6″), 130.9 (C4″), 137.8 (C4 and C1″), 139.1(C1′ and C4′), 153.1 (C=N), 173.8 (C=O); Anal. Calcd for $C_{26}H_{26}BrNO_2$: C, 67.24; H, 5.64; N, 3.02. Found: C, 67.34; H, 5.89; N, 3.40.

4.1.2.5. 4-(4-Methoxy benzylideneamino)phenyl 2-(2,4-dimethylphenylamino)benzoate (4a). Yield, (2.52 g, 56%); mp 165 °C (from MeOH); IR (ν_{max}/cm^{-1}): 3500 (NH), 1658 (C=O); ^{1}H NMR [$CDCl_3$]: δ 2.16 (3H, s, *p*-CH_3), 2.31 (3H, s, *o*-CH_3), 3.87 (3H, s, *p*-OCH_3), 5.47 (1H, br s, NH, exchangeable with D_2O), 6.91–7.83 (15H, m, Ar-H), 8.38 (1H, s, CH=N); ^{13}C NMR [$CDCl_3$]: δ_C 19.8 (2‴-CH_3), 24.4 (4‴-CH_3), 55.2 (OCH_3), 114.8 (C1), 117.8 (C3″ and C5″), 118.5 (C3, and C6‴), 121.6 (C5), 124.3 (C3′and C5′), 124.4 (C2′ and C6′), 125.1 (C1″), 126.8 (C5‴), 127.0 (C2), 128.5 (C2‴), 128.6 (C4‴), 129.4 (C3‴), 130.8 (C2″ and C6″), 137.4 (C4), 138.8 (C1‴), 143.4 (C6 and C1′), 144.3 (C4′), 153.4 (C=N), 157.3 (C4″), 169.8 (C=O); Anal. calcd for $C_{29}H_{26}N_2O_3$: C, 77.31; H, 5.82; N, 6.22. Found: C, 77.20; H, 6.0; N, 6.55. MS, *m*/*z* (%): (M) at 450.

4.1.2.6. 4-(4-Nitro benzylideneamino)phenyl 2-(2,4-dimethylphenylamino)benzoate (4b). Yield, (2.42 g, 52%); mp 190 °C (from MeOH); IR (ν_{max}/cm^{-1}): 3550 (NH), 1657 (C=O); ^{1}H NMR [$CDCl_3$]: δ 2.16 (3H, s, *p*-CH_3), 2.31 (3H, s, *o*-CH_3), 4.30 (1H, br s, NH, exchangeable with D_2O), 6.88–7.26 (11H, m, Ar-H), 8.02 (2H, dd, $J_{2,3}$ = 8.9 Hz, $J_{3,5}$ = 3.0 Hz, Ar *m*-H), 8.28 (2H, dd, $J_{2,3}$ = 8.9 Hz, $J_{2,6}$ = 3.0 Hz, Ar *o*-H), 8.57 (1H, s, CH=N); ^{13}C NMR [$CDCl_3$]: δ_C ^{13}C NMR [$CDCl_3$]: δ_C 19.41 (2‴-CH_3), 24.03 (4‴-CH_3), 114.92 (C1), 117.48 (C3″ and C5″), 118.22 (C3, and C6‴), 121.51 (C5), 124.11 (C3′and C5′), 124.73 (C2′ and C6′), 126.68 (C2, C1″ and C5‴), 127.78 (C2‴, C3‴ and C4‴), 130.4 (C2″ and C6″), 137.02 (C4), 138.34 (C1‴), 140.73 (C6), 141.87 (C1′), 143.03 (C4′), 148.5 (C4″), 153.2 (C=N), 169.55 (C=O); Anal. Calcd for $C_{28}H_{23}N_3O_4$: C, 72.24; H, 4.98; N, 9.03. Found: C, 72.18; H, 5.08; N, 9.30.

4.1.2.7. 4-(4-(Dimethylamino)benzylideneamino)phenyl 2-(2,4-dimethylphenylamino)benzoate (4c). Yield, (3.85 g, 83%); mp 155 °C (from MeOH); IR (ν_{max}/cm^{-1}): 3500 (NH), 1650 (C=O); ^{1}H NMR [DMSO-d_6]: δ 2.20 (3H, s, *p*-CH_3), 2.40 (3H, s, *o*-CH_3), 2.98 (6H, s, NCH_3)$_2$), 6.72–7.93 (15H, m, Ar-H), 8.37 (1H, s, CH=N), 9.32 (1H, br s, NH, exchangeable with D_2O); ^{13}C NMR [$CDCl_3$]: δ_C 19.66 (2‴-CH_3), 23.90 (4‴-CH_3), 32.20 (*p*-N(CH_3)$_2$), 113.84 (C1), 115.72 (C3″ and C5″), 119.50 (C3, and C6‴), 119.71 (C5), 124.36 (C3′and C5′), 124.64 (C2′ and C6′), 124.93 (C2, C1″ and C5‴), 126.20 (C2‴, C3‴ and C4‴), 131.02 (C2″ and C6″), 137.00 (C4), 138.61 (C1‴), 143.30 (C1′ and C6), 145.90 (C4′ andC4″), 153.26 (C=N), 167.86 (C=O); Anal. Calcd for $C_{30}H_{29}N_3O_2$: C, 77.73; H, 6.31; N, 9.06. Found: C, 77.49; H, 6.43; N, 9.36.

4.1.2.8. 4-(4-Bromo benzylideneamino)phenyl 2-(2,4-dimethylphenylamino)benzoate (4d). Yield, (3.90 g, 78%); mp 160 °C (from MeOH); IR (ν_{max}/cm^{-1}): 3540 (NH), 1640 (C=O); ^{1}H NMR [$CDCl_3$]: δ 2.16 (3H, s, *p*-CH_3), 2.31 (3H, s, *o*-CH_3), 4.29 (1H, br s, NH, exchangeable with D_2O), 6.91–7.37 (15H, m, Ar-H), 8.41 (1H, s, CH=N); ^{13}C NMR [$CDCl_3$]: δ_C ^{13}C NMR [$CDCl_3$]: δ_C 19.45 (2‴-CH_3), 24.11 (4‴-CH_3), 114.47 (C1), 117.52 (C3″ and C5″), 118.20 (C3, and C6‴), 121.64 (C5), 124.13 (C3′and C5′), 124.80 (C2′ and C6′), 126.75 (C2, C1″ and C5‴), 128.19 (C2‴, C3‴ and C4‴), 130.8 (C2″ and C6″), 137.09 (C4 and C1‴), 138.47 (C6 and C1′), 143.08 (C4′ and C4″), 153.18 (C=N), 169.61 (C=O); Anal. Calcd for $C_{28}H_{23}BrN_2O_2$: C, 67.34; H, 4.64; N, 5.61. Found: C, 67.11; H, 4.56; N, 5.98.

4.2. Biological screening

4.2.1. Materials and methods

4.2.1.1. Animals. Adult rats of both sexes weighing 150–200 g and adult mice weighing 20–25 g were used in this study. They were housed and bred under standardized conditions for light, temperature, ventilation and free access to feeds (mouse cubes) and water receiving standard rat chow and libitum. Animals were randomly assigned to different experimental groups, each kept in a separate cage. Laboratory investigations on the animals were carried out in accordance with the ethical guidelines stipulated by ethical committee of the National Research Center and in accordance with the recommendations for the proper care and use of laboratory animals (NIH publication No. 85-23, revised 1985) with the internationally accepted principles for laboratory animal use and care.

4.2.1.2. Drugs and chemicals. Carrageenan Iambda Sigma–Aldrich chemical company (USA), ibuprofen Khahira Pharmaceutical and Chemical Company (Cairo, Egypt) and 7280- Hot-plate module, Ugo Basile, Comerio, Italy was used in analgesia testing.

4.2.1.3. Analysis of data. The results are expressed as standard error of the mean ± S.E.M. Differences in mean values between vehicle control and treatment groups were analyzed by a one-way analysis of variance (ANOVA). Statistical significance was assessed as p <0.05. Methods of statistical analysis were done according to Armitage et al.[32]

4.2.2. Acute anti-inflammatory effect (carrageenan-induced paw oedema test)

Anti-inflammatory activity against carrageenan-induced rat paw oedema was assayed in adult male Wistar CF rats weighing 180–220 g according to the method of Winter et al.,[44] with slight modifications. The tested compounds (**3a–d** and **4a–d**; 100 mg/kg bwt), were orally administered into eight groups of six rats, 1 h before injection of 0.1 ml of a 1% suspension of carrageenan lambda saline into the subcutaneous tissue of the right hind paw. The left hind paw was injected in the same way with 0.05 ml of a saline solution. Rats were fasted 24 h before the experiment and water (1.5 ml per 100 g body weight) was orally administered twice (2 and 4 h) before injections. One hour before induction of oedema saline with few drops of CMC 0.5% (0.3 ml/100 g rat) was administered orally to a group of animals and served as control. Rats were kept in the same experimental conditions. Carrageenan caused visible redness and pronounced swelling that was well developed by 4 h and persisted for more than 48 h. The volume of both hind paws of control and treated animals were measured immediately before and after carrageenan and test compounds injections at selected times (1, 2, 3 and 4 h) using a planimeter.[45,46] The inhibition percentage of the inflammatory reaction was determined for each animal by comparison with controls, and calculated by the formula:

$$I(\%) = 100 \times (1 - dt/dc)$$

Where dt is the difference in paw volume in the drug-treated group and dc the difference in paw volume in the control group. The reference standard drug; ibuprofen 100 mg/kg bwt; was administered to a group of rats that served as a positive control. Statistically significant from the control normal inflamed group at the corresponding time: P <0.05. Statistical analysis was carried out using repeated measures one way ANOVA followed by least significant test for multiple comparisons.[47]

4.2.3. Hot plate analgesia testing

The Central analgesia of the eight synthesized derivatives was evaluated by applying hot plate test[33,34] Mice weighing 20–25 g were divided into 10 groups (n = 5). The control group animals were given saline with few drops of carboxymethyl cellulose (CMC) 0.5% (0.3 ml/100 g rat). The positive control group animals were given ibuprofen (250 mg/kg bwt) as a standard reference analgesic. Mice were introduced to electronically controlled hot-plate surface adjusted to 55 ± 0.1 °C (7280- Hot-plate module, Ugo Basile, Comerio, Italy). A cut-off time of 45 s was selected to avoid tissue damage. The latency (licking or jumping) of nociceptive responses was recorded at 0, 0.5, 1 and 1.5 h after oral administration of the eight test compounds (250 mg/kg bwt). Time required for mice to lick paw/jump was recorded using built-in digital timer and designated as withdrawal latency (WDL).

4.2.4. Ulcerogenic liability

The experiments were performed on albino rats of Wistar strain of either sex, following the previously reported standard method.[37,38] Rats weighing 120–140 g maintained at 25 ± 2 °C, 50 ± 5% relative humidity and 12 h light/dark cycle, were divided into ten groups of six animals each. Pregnant female rats were excluded. The animals were fasted 18 h before drug administration. Ibuprofen (reference standard) and the tested compounds were sus-

pended in 1% aqueous carboxymethyl cellulose (CMC) solution, and were administered orally once daily for three consecutive days to fasted rats in doses which exhibit high activity being (100 mg/kg). The control group animals were given saline with few drops of carboxymethyl cellulose (CMC). On the fourth day the animals were sacrificed by cervical dislocation and the stomachs were quickly and carefully dissected out. A longitudinal incision along the greater curvature was made with fine scissor, then stomach was inverted over the index finger and the presence or the absence of gastric irritation was examined using a hand held microscope for the presence of gastric irritation. Ulcers were scored according to the arbitrary scale used by Singh et al.[48] Where 0 = no lesion, 0.5 = hyperaemia, 1 = one or two slight lesions, 3 = very severe lesions and 4 = mucosa full of lesions. Ulcer index was calculated as mean ulcer scores (Tan et al.),[49] where the presence of a single or multiple lesions (erosion, ulcer or perforation) is considered to be positive.[50]

4.3. Molecular docking

Using Auto Dock Tools (ADT), you will perform a docking study of anti-inflammatory compounds, a known inhibitor of the cyclooxygenase enzyme (Cox) to calculate the position of docked ligand and flexible residues moved in the process of interaction. Your task is to compare the energies of the interaction in different conformations and determine the best fit.

4.3.1. Procedure

AutoDock tool (ADT) consists of two main programs: AutoGrid pre-calculates these grids. AutoDock performs the docking of the ligand to a set of grids describing the target protein; and Working with ADT includes major three steps:

4.3.1.1. Preparation of target protein and ligand files. *4.3.1.1.1. Preparation of the protein.* Two different Cox target enzymes were investigated. These include ovine Cox-1 complexed with ibuprofen (PDB code: 1EQG) and Cox2 complexed with a selective inhibitor, SC-558 (PDB code: 1CX2). Those were retrieved from the Protein Data Bank, http://www.rcsb.org/pdb/home/home.do. For each docking target, crucial amino acids of the active site were identified using data in PDBsum, http://www.ebi.ac.uk/pdbsum/. In this step we need to place back all hydrogens for ADT calculations. The other required action is to remove water molecules from the surface of the protein. This is necessary because the extra water molecules will mask the protein surface from the ligand.

4.3.1.1.2. Preparation of the ligand. The ligands are originally drawn with a widely used chemical structure drawing software. The three-dimensional structures of the aforementioned compounds were constructed using Chem3D Ultra 8.0 software [Chemical Structure Drawing Standard; Cambridge Soft corporation, USA (2003)] to obtain standard 3D structures (pdb format)., then they were energetically minimized by using MOPAC with 100 iterations and minimum RMS gradient of 0.10. It is recommended to confirm whether all hydrogen atoms are in the file before working with ADT. After opening the ligand, it can be visualized and ADT now automatically computes Gasteiger charges (empirical atomic partial charges) and distinguishes between hybridization state and type of each atom. As a part of preparation, the program determines rotatable bonds of the ligand to be able to generate different conformers for the docking.

4.3.1.1.3. Preparation of the flexible residue file. A unique property of the program is its ability to take into account the flexibility not only of the ligand but also the enzyme during docking process. It means that ADT is able to model not only how the ligand docks to the protein but also the position of flexible residues. In order to use this advantage, the flexible residues must be chosen and the rotatable bonds must be found. Flexible residues are amino acids in the binding site region of the protein that are able to alter their position via conformational change upon ligand binding. They are found by comparison of different crystallized structures or by molecular dynamic simulations. According to the literature, the flexible residues are Arg120 and Tyr355 in our system. Rotatable bonds are used by the program to generate rotational isomers of amino acids and to present enzyme structures with those conformers.

4.3.1.2. Calculation of affinity maps by using a 3D-grid embracing the protein and ligand. A part of ADT, the AutoGrid calculates the energy of the non-covalent interactions between the protein and probe atoms that are located in the different grid points of a lattice that defines the area of interest (i.e., the area of the macromolecule where the possibility of ligand binding is studied). AutoGrid builds as many files as the number of probe atoms used. There are about 30 different types of grid maps. Each one shows the interaction energies for a particular atom type, such as aliphatic carbons, aromatic carbons, hydrogen bonding oxygens, etc. The grid itself is a box with determined dimensions that is located at the site on the surface of the protein where we expect the interaction with the ligand. In other words, the grid is our field of study. The created three-dimensional grids of $60 \times 60 \times 60$ Å size (x,y,z) with a spacing of 0.375 Å centered at 26.64, 32.60, and 200.23 Å that encompassed the active site where the co-crystallized ligand; ibuprofen; was embedded, was used to guide the docked inhibitors within Cox1 receptor. Whereas, S58 native ligand of Cox2 receptor centered at 23.95, 21.58, and 15.44 Å, respectively. In this part of modeling, we need to determine the area where the ligand interacts with the enzyme on its surface, size of that area and particular types of atoms participating in the interaction of both a ligand and an enzyme. The first two parameters are determined by size and position of the grid box; the third parameter is given by map type. Once those parameters were set in one file, AutoGrid calculates grid parameter files for each type of atom within a given area.

4.3.1.3. Defining the docking parameters and running the docking simulation. When the preparation of the input files (ligand and protein) and the calculation of the affinity maps are properly performed, AutoDock will carry out the docking automatically using the newest docking algorithm (Lamarckian Genetic Algorithm).

4.3.1.3.1. Preparation of the docking parameter file (.dpf). Once all files are ready, we need to specify for the program what particular ligand, protein, flexible protein and maps we want to work with and also what algorithm we want to use, how many iterations are required and so on. That information is kept usually in docking parameter file.

4.3.1.3.2. Running Autodock 4 and viewing the docking results. As a result of AutoDock calculations we obtain the output file with in our case ten conformers of the protein–ligand complex with flexible residues and the ligand located within the binding pocket. Each structure are scored and ranked by the program by the calculated interaction energy.

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