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Synthesis of new ibuprofen derivatives with their *in silico* and *in vitro* cyclooxygenase-2 inhibitions

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ABSTRACT

Cyclooxygenase-2 (COX-2) is one of the important targets for treatment of inflammation related diseases. In the literature, most of drug candidates are first synthesized and then their COX-2 inhibitory activities are tested by *in vitro* and *in vivo* experiments. However, synthesis of dozens of drug analogues without any interpretations on their inhibitory activity can result in loss of time and chemicals. Therefore, synthetic drug designs with molecular modeling are of importance to synthesize selective drug candidates against inflammatory diseases. The synthesis of the novel ibuprofen derivatives through their *in silico* and *in vitro* COX-2 inhibitory activities were investigated in the present study. Starting from ibuprofen, ibuprofen amide and ibuprofen acyl hydrazone derivatives were synthesized. According to the results of the *in silico* molecular docking and *in vitro* enzyme inhibition studies, the synthesized novel ibuprofen derivatives have selective COX-2 inhibition, and molecule 3a and 3c were showed higher inhibition compared to ibuprofen. In conclusion, the newly synthesized ibuprofen derivatives can be used in model *in vivo* studies.

1. Introduction

Prostaglandin H synthase (PGHS) catalyses the first step in the cyclic pathway of eicosanoid metabolism. PGHS have two different catalytic activities, a cyclooxygenase (COX) and a peroxidase activity. Radical mediated addition of two molecules of oxygen through Tyr385 residue to arachidonic acid is catalyzed by COX. The formed product is called as PGG_2. Peroxidase activity of PGHS transforms PGG_2 into PGH_2 that is precursor of some important bio-molecules such as prostaglandins, prostacyclins and thromboxanes [1–3]. The COX subunit within the PGHS is found on the opposite side of the heme and it is located at the end of a long narrow hydrophobic channel. Arg120, Tyr385 and Ser530 are important residues of the active site of COXs. Acetylsalicylic acid (aspirin) based studies revealed that Ser530 is not responsible for catalytic activities of COXs. Acetylation of Ser530 by aspirin prevents arachidonic acid to reach to the Tyr385 in the active site of COX. Arg120 is of also great importance for inhibition of COX according to a flurbiprofen based study [1,4]. Carboxyl group of flurbiprofen interacts with Arg120, and this interaction also closes the channel of PGHS that is responsible for entrance of substrate to the COX's active site [1,4]. COX has two well known isoforms, COX-1 and COX-2 [1,2,5]. When they are aligned (Fig. 1), it is seen that they have quite similar sequence and structural identity. Although COX-1 is expressed in many organs, COX-2 is expressed in some tissues for forming a response against inflammatory stimuli. Since most of the developed non-steroidal inflammatory drugs (NSAIDs) are not specific for COXs, they affect both isoforms [1]. Therefore, there is a great need for development of selective inhibitors for COX-2. Molecular dockings is a powerful approach to study structure based drug design. This method provides a big contribution to the development of novel selective drugs for COX-2. Organic synthesis strategies of novel ibuprofen derivatives were described and COX-2 inhibitory activities of novel ibuprofen derivatives were supported with *in silico* and *in vitro* molecular docking analysis in the present study.

2. Results

Molecular docking studies showed that the interaction of the inhibitor with Arg120 through a strong hydrogen bond, hydrophobic interactions between the substrate binding site of COX-2 and hydrophobic tale of the inhibitor are key factors in designing of NSAIDs. Docking studies with LeadIT revealed that among synthesized COX-2 inhibitors, molecules 2a, 2b, 2c, 3b and 3d were imitated the arachidonic acid better than classical NSAIDs (Table 1).

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1    MSRSLLLWFLLFLLLLPPLPVLLADPGAPTPVNPCCYYPCQHQGICVRFGLDRYQCDCTR  60  P23219   PGH1_HUMAN
1    --------------MLARALLLCAVLALSHTANPCCSHPCQNRGVCMSVGFDQYKCDCTR  46  P35354   PGH2_HUMAN
                  :*    :* *  .    .**** :***.:*:*: .*:*:*:*****

61   TGYSGPNCTIPGLWTWLRNSLRPSPSFTHFLLTHGRWFWEFVN-ATFIREMLMRLVLTVR 119  P23219   PGH1_HUMAN
47   TGFYGENCSTPEFLTRIKLFLKPTPNTVHYILTHFKGFWNVVNNIPFLRNAIMSYVLTSR 106  P35354   PGH2_HUMAN
     **: * **: * : * ::  *:*:*. .*::*** : **:.**   *:*: :*  *** *

120  SNLIPSPPTYNSAHDYISWESFSNVSYYTRILPSVPKDCPTPMGTKGKKQLPDAQLLARR 179  P23219   PGH1_HUMAN
107  SHLIDSPPTYNADYGYKSWEAFSNLSYYTRALPPVPDDCPTPLGVKGKKQLPDSNEIVEK 166  P35354   PGH2_HUMAN
     *.** ******: : * ***:***:***** ** **.*****:*.********:: :..:

180  FLLRRKFIPDPQGTNLMFAFFAQHFTHQFFKTSGKMGPGFTKALGHGVDLGHIYGDNLER 239  P23219   PGH1_HUMAN
167  LLLRRKFIPDPQGSNMMFAFFAQHFTHQFFKTDHKRGPAFTNGLGHGVDLNHIYGETLAR 226  P35354   PGH2_HUMAN
     :************:*:*****************. * **.**:.******* ****:.* *

240  QYQLRLFKDGKLKYQVLDGEMYPPSVEEAPVLMHYPRGIPPQSQMAVGQEVFGLLPGLML 299  P23219   PGH1_HUMAN
227  QRKLRLFKDGKMKYQIIDGEMYPPTVKDTQAEMIYPPQVPEHLRFAVGQEVFGLVPGLMM 286  P35354   PGH2_HUMAN
     * :********:***::*******:*::: . * **  :* : ::**********:****:

300  YATLWLREHNRVCDLLKAEHPTWGDEQLFQTTRLILIGETIKIVIEEYVQQLSGYFLQLK 359  P23219   PGH1_HUMAN
287  YATIWLREHNRVCDVLKQEHPEWGDEQLFQTSRLILIGETIKIVIEDYVQHLSGYHFKLK 346  P35354   PGH2_HUMAN
     ***:**********:** *** *********:***************:***:****.::**

360  FDPELLFGVQFQYRNRIAMEFNHLYHWHPLMPDSFKVGSQEYSYEQFLFNTSMLVDYGVE 419  P23219   PGH1_HUMAN
347  FDPELLFNKQFQYQNRIAAEFNTLYHWHPLLPDTFQIHDQKYNYQQFIYNNSILLEHGIT 406  P35354   PGH2_HUMAN
     *******  ****:**** *** *******:**:*:: .*:*.*:**::*.*:*:::*:

420  ALVDAFSRQIAGRIGGGRNMDHHILHVAVDVIRESREMRLQPFNEYRKRFGMKPYTSFQE 479  P23219   PGH1_HUMAN
407  QFVESFTRQIAGRVAGGRNVPPAVQKVSQASIDQSRQMKYQSFNEYRKRFMLKPYESFEE 466  P35354   PGH2_HUMAN
      :*::*:******:.****:   : :*:   * :**:*: * ******** :*** **:*

480  LVGEKEMAAELEELYGDIDALEFYPGLLLEKCHPNSIFGESMIEIGAPFSLKGLLGNPIC 539  P23219   PGH1_HUMAN
467  LTGEKEMSAELEALYGDIDAVELYPALLVEKPRPDAIFGETMVEVGAPFSLKGLMGNVIC 526  P35354   PGH2_HUMAN
     *.*****:**** *******:*:**.**:** :*::****:*:*:*********:** **

540  SPEYWKPSTFGGEVGFNIVKTATLKKLVCLNTKTCPYVSFRVPDASQDDGPAVERPSTEL 599  P23219   PGH1_HUMAN
527  SPAYWKPSTFGGEVGFQIINTASIQSLICNNVKGCPFTSFSVPDPELIKTVTINASSSRS 586  P35354   PGH2_HUMAN
     ** **************:*::**:::.*:* *.* **:.** *** .  .  :::  *:.

600  ------------------  599  P23219   PGH1_HUMAN
587  GLDDINPTVLLKERSTEL  604  P35354   PGH2_HUMAN
```

Fig. 1. Alignment of COX-1 (UniProt ID: P23219) and COX-2 (UniProt ID: P35354).

Table 1
Molecular docking results obtained with LeadIT 2.0.2 for newly synthesized potential COX-2 inhibitors and commercial inhibitors on COX-2 crystal structures (PDB ID: 1CVU, 1CX2 and 1PXX).

COX-2 inhibitor	COX-2 structure						Mean score
	PDB ID: 1CVU		PDB ID: 1CX2		PDB ID: 1PXX		
	HYDE score (kJ mol^{-1})	H-bonded residues	HYDE score (kJ mol^{-1})	H-bonded residues	HYDE score (kJ mol^{-1})	H-bonded residues	
Arachidonic acid	−74	Arg120	−50	Arg513, Tyr355	−68	Arg120	−64
Celecoxib	−39	Arg120, Val116	−37	Arg120	−42	Tyr385, Ser530	−39
Curcumin	−36	Arg120, Tyr385, Ser530	−34	Arg120, Arg513, Asp515	−32	Arg120, Met522	−34
Diclofenac	−41	Arg120	−37	Val523	−32	Tyr385, Val523	−37
Etoricoxib	−34	Arg120	−31	Arg513	−38	Tyr385	−34
Meloxicam	−27	Arg120, Ser530	−19	Arg120, Val523	−35	Val523, Ser530	−27
Naproxen	−36	Arg120	−35	Arg120, Tyr385	−40	Tyr385, Ser530	−37
Rofecoxib	−34	Arg120	−34	Arg120, Tyr385	−34	Ser530	−34
S58	−32	Arg120, Tyr355	−25	Gln192, Arg513	−43	Tyr385, Ser530	−33
Valdecoxib	−38	Arg120, Tyr355	−30	Arg120	−36	Arg120	−35
Molecule 2a	−50	Arg120	−46	Arg120, Tyr355	−42	Arg120	−46
Molecule 2b	−54	Arg120	−52	Arg120	−49	Arg120	−52
Molecule 2c	−65	Arg120	−46	Arg120, Arg513	−49	Arg120, Val523	−53
Molecule 2d	−40	Val523	−31	Arg120	−38	Tyr385, Ser530	−36
Molecule 3a	−44	Arg120	−34	Arg120	−40	Tyr385, Ser530	−39
Molecule 3b	−50	Arg120	−35	Arg120	−45	Tyr385, Ser530	−43
Molecule 3c	−42	Val523	−30	Arg120, Tyr355, Glu524	−40	Tyr385, Ser530	−37
Molecule 3d	−59	Arg120	−40	Arg120, Tyr355	−41	Tyr385, Ser530	−47

The highest binding affinity was found as −65 kJ mol^{-1} for molecule 2c and this score was very close to that of arachidonic acid on 1CVU crystal structure. Among novel ibuprofen derivatives, higher binding affinities were observed when the inhibitor interacts with Arg120. On the other hand, the binding affinities of diclofenac and celecoxib were not very high. As can be seen from Fig. 2, molecule 2c interacts with active site of COX-2 similar to arachidonic acid; however, diclofenac was not able to interact with

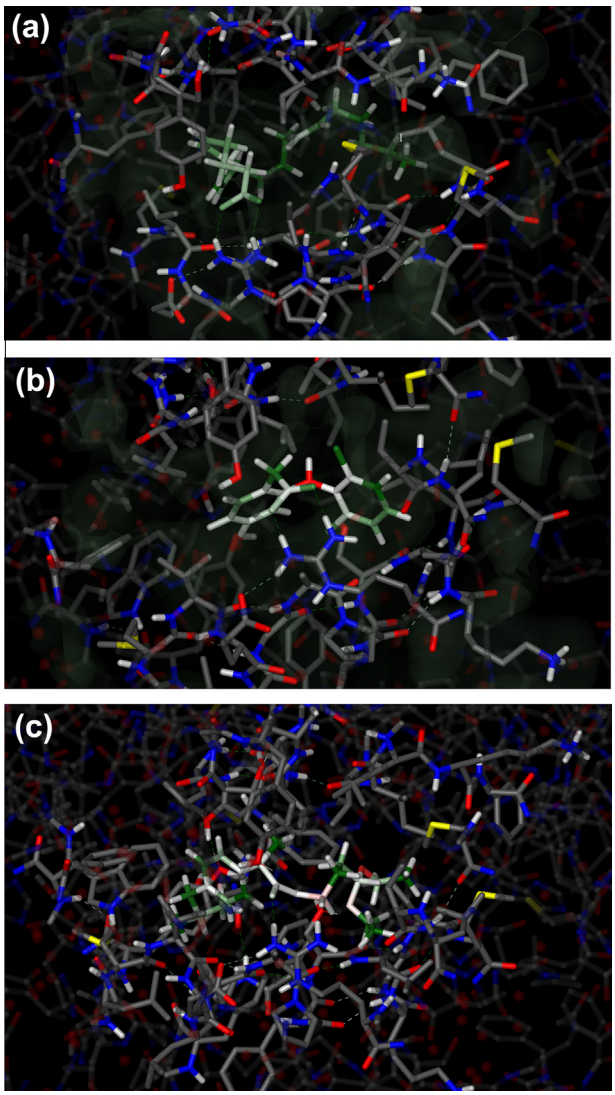


Fig. 2. 3D representation of docked molecules and their interactions on active site of COX-2 (PDB ID: 1CVU). (a) Arachidonic acid, (b) Diclofenac, and (c) Molecule 2c.

same number of amino acids. 2D representations of enzyme-inhibitor interactions showed that arachidonic acid and molecule 2c were able to interact with active site through their long hydrophobic tales (Fig. 3). Binding affinity results obtained from AutoDock Vina were not parallel to results of LeadIT. According to the results of AutoDock Vina, the molecules 2d, 2a and 2b had greater mean binding affinities compared to other inhibitors (Table 2). When compared to the docking result of ibuprofen, only molecule 3d has lower binding affinity against COX-2. The *in silico* binding affinities of synthesized COX-2 inhibitors were also found for COX-1 to compare their selectivity theoretically. According to the AutoDock Vina results, the binding affinities of the synthesized ibuprofen analogues against COX-1 were close to that of COX-2 (Table 3). However, binding affinity of the ibuprofen against COX-1 was found higher compared to COX-2.

The *in vitro* inhibition studies on COX-2 showed that molecule 3a and 3c were the most effective inhibitors among other studied ibuprofen derivatives at 50 μM of final inhibitor concentration (Table 4). The inhibition percentage of ibuprofen (positive control) was 9.7%. Molecule 2a and 3d were the least effective compounds compared to ibuprofen and their binding energies (or docking scores) were also low according to the *in silico* analysis.

3. Discussion and conclusion

Before the synthesis of COX-2 inhibitors in wet lab conditions, the evaluation of molecular interactions between enzyme and inhibitor is an important step for successful design of selective COX-2 inhibitors. Also, the structural differences between the active sites of COX-1 and COX-2 should be investigated extensively for designing selective COX-2 inhibitors. The major difference between COX-1 and COX-2 is the larger volume (about 20%) of the active site channel of COX-2 [1]. Thus, the design of an inhibitor which is able to bind to the active site of COX-2 by preventing the entrance of the arachidonic acid is very important. On the other hand, arachidonic acid binds to the active site from Arg120 with its carboxyl oxygens and Tyr385 is involved in the radicalic transformation of arachidonic acid to further intermediates. Therefore, both steric hinderance and enzyme-inhibitor side chain interactions are of great importance for successful drug design. Most of the classical NSAIDs are known to inhibit both COX-1 and COX-2 non-selectively. Since some NSAIDs have negative side effects on human metabolism such as cardiovascular and gastrointestinal system problems, some of them (e.g., rofecoxib, valdecoxib) were withdrawn from the market [6–8]. Ibuprofen is one of the NSAIDs that inhibit COXs non-selectively. It was found to interact with Arg120 and Tyr355 in the active site of COX-1 [9]. Modification of ibuprofen from its carboxyl group has been studied in the literature [10–19] and improvements in the selectivity and anti-inflammatory activity were achieved. Hegazy and Ali [10] have reported reduced ulcerogenic potential for NSAIDs that modified from their carboxyl group in addition to their anti-inflmmatory and analgesic acitivities. Also, NSAIDs esterified from their carboxyl group showed higher binding affinity and good selectivity for COX-2 in molecular docking studies [10]. Raval et al. [11] have observed good anti-inflammatory and analgesic activities with reduced ulcerogenic effect for ibuprofen derivatives, synthesized with cyclization of carboxyl group and bearing thiadiazolo[3,2-a][1,3,5]triazine-5-thione nucleus. Amides of arylpropionic acids have been studied in numerous researches, but only few of them were related with ibuprofen amide derivatives [20,21]. It is well known that amide derivatives of arylpropionic acids may retain the activity of the parent acids and decrease their gastrointestinal toxicity [22,23]. In this study, it was aimed at synthesizing selective ibuprofen analogues that differ in functional group reactivity and steric hindrance. Also, inhibitory activities of synthesized molecules were estimated by *in silico* molecular docking and *in vitro* enzyme inhibition studies. Among synthesized molecules, binding affinities of molecules 2a, 2b and 2c were close to that of arachidonic acid and also higher than commercial COX-2 inhibitors. This theoretical result indicates the effect of aliphatic hydrocarbon chain that is fixed to carboxyl group of ibuprofen by amide bond when compared to other synthesized molecules. This linear hydrocarbon chain could be able to mimic the arachidonic acid by its hydrophobic nature and it might prevent the entrance of substrate by closing the active site channel around Arg120. However, molecule 3a and 3c were found more effective than ibuprofen and other synthesized ibuprofen derivatives in *in vitro* analyses. The predicted binding affinity of molecule 3a was closer to reflect the *in vitro* % inhibition of COX-2. In addition, the results of LeadIT did not confirm the findings of AutoDock Vina. Spearman's rank correlation coefficient ($\rho = -0.8$) between the results of LeadIT and AutoDock Vina can confirm this controversial result. It can be explained by the different theoretical backgrounds of both softwares. Similar controversial results were also obtained by Maldonado-Rojas and Olivero-Verbel for AutoDock Vina, GOLD and Surflex-Dock

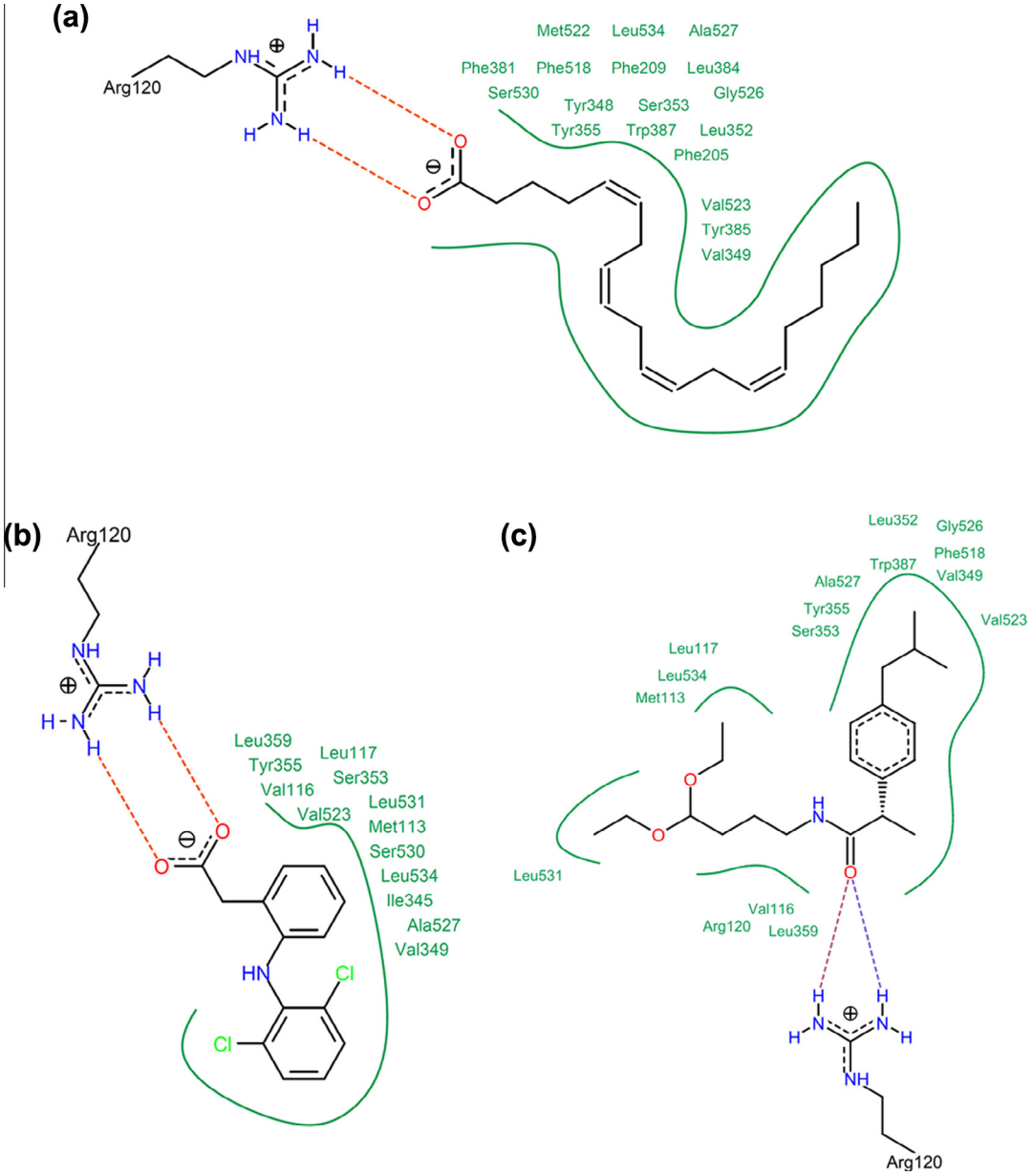


Fig. 3. 2D representation of interactions between docked molecules and active site of COX-2. (a) Arachidonic acid, (b) Diclofenac, and (c) Molecule 2c.

[24]. Therefore, the differences observed between results of *in silico* and *in vitro* analyses could be due to the rigid prediction methods used in molecular docking softwares that do not include instant conformational changes in the protein or inhibitor, and close prediction results arising from similarity of functional groups in the inhibitors [25–39]. Moreover, the controversies between *in vivo* and *in vitro* experiments were also reported in the literature [40–43]. There are increasing numbers of studies that use computer aided drug design approaches including molecular docking to predict interactions of novel inhibitor molecules [44–59]. Also, theoretical results such as binding affinity are reported to be well in line with experimental results in the great number of molecular docking studies [24,53,60,61]. Since Spearman's rank correlation coefficient was found as 0.6 between mean scores (Table 2) and percent inhibition (Table 4), the molecular docking calculations in our study could be considered as reliable. In conclusion, the newly synthesized ibuprofen derivatives mentioned in the present paper can be used in model *in vitro* and *in vivo* studies.

4. Experimental section

4.1. Molecular docking studies

Crystal structures of COX-1 (PDB ID: 1CQE and 1EQH) and COX-2 (PDB ID: 1CVU, 1CX2, and 1PXX) were retrieved from Protein Data Bank [62]. 3D structures of commercial COX-1 and COX-2 inhibitors were obtained from PubMed Compound Database in SDF file format. 2D structures of novel ibuprofen analogues were drawn with Accelrys Draw 4.0 and saved as MOL file format. Prepared 2D coordinates of inhibitors were converted to 3D, optimized and explicit hydrogens were removed with CHEMSKETCH 12.01 (freeware) and saved as MOL file format. 3D coordinates in MOL format were converted to MOL2 file format with YASARA 11.11.2 [63]. PyRx 0.8 was used for energy minimization calculations and MOL to PDBQT file format conversion [64]. Molecular docking calculations were comparatively performed with LeadIT v2.0.2 and AutoDock Vina 1.1.2 [65,66]. LeadIT is a molecular drug design platform for docking applications. In LeadIT, possible

Table 2
Molecular docking results obtained with AutoDock Vina 1.1.2 for newly synthesized potential COX-2 inhibitors and commercial inhibitors on COX-2 crystal structures (PDB ID: 1CVU, 1CX2 and 1PXX).

COX-2 inhibitor	COX-2 structure			
	PDB ID: 1CVU Vina score (kcal mol^{-1})	PDB ID: 1CX2 Vina score (kcal mol^{-1})	PDB ID: 1PXX Vina score (kcal mol^{-1})	Mean score
Arachidonic acid	−6.43 ± 0.21	−6.43 ± 0.15	−5.87 ± 0.21	−6.24
Celecoxib	−5.93 ± 0.06	−7.13 ± 0.23	0.03 ± 2.02	−4.34
Curcumin	−7.23 ± 0.06	−7.53 ± 0.23	−4.20 ± 2.17	−6.32
Diclofenac	−6.00 ± 0.35	−5.80 ± 0.00	−5.50 ± 0.10	−5.77
Etoricoxib	−6.03 ± 0.11	−6.60 ± 0.00	0.60 ± 2.36	−4.01
Meloxicam	−7.30 ± 0.00	−7.73 ± 0.06	−7.37 ± 0.06	−7.47
Naproxen	−6.67 ± 0.31	−6.67 ± 0.23	−6.07 ± 0.06	−6.47
Rofecoxib	−6.23 ± 0.23	−7.47 ± 0.06	−5.30 ± 0.70	−6.33
S58	−5.90 ± 0.17	−6.60 ± 0.00	−1.76 ± 2.14	−4.75
Valdecoxib	−6.77 ± 0.11	−7.07 ± 0.32	−5.57 ± 0.49	−6.47
Ibuprofen	−6.60 ± 0.00	−5.93 ± 0.12	−5.50 ± 0.00	−6.01
Molecule 2a	−6.57 ± 0.06	−6.73 ± 0.15	−5.30 ± 0.10	−6.20
Molecule 2b	−5.80 ± 0.35	−6.90 ± 0.00	−5.70 ± 0.20	−6.13
Molecule 2c	−5.67 ± 0.15	−7.07 ± 0.15	−5.63 ± 0.55	−6.12
Molecule 2d	−6.87 ± 0.06	−8.30 ± 0.00	−4.03 ± 2.48	−6.40
Molecule 3a	−6.57 ± 0.06	−7.47 ± 0.06	−5.53 ± 1.16	−6.52
Molecule 3b	−6.40 ± 0.10	−7.57 ± 0.06	−5.73 ± 0.23	−6.57
Molecule 3c	−7.53 ± 0.06	−8.30 ± 0.26	−2.63 ± 4.34	−6.15
Molecule 3d	−6.50 ± 0.52	−7.83 ± 0.15	−0.10 ± 3.29	−4.81

Table 3
Molecular docking results obtained with AutoDock Vina 1.1.2 for newly synthesized ibuprofen derivatives and commercial inhibitors on COX-1 crystal structures (PDB ID: 1CQE and 1EQH).

COX-1 inhibitor	COX-1 structure		
	PDB ID: 1CQE Vina score (kcal mol^{-1})	PDB ID: 1EQH Vina score (kcal mol^{-1})	Mean score (kcal mol^{-1})
Arachidonic acid	−7.13 ± 0.06	−7.53 ± 0.06	−7.33
Aspirin	−6.70 ± 0.00	−6.80 ± 0.00	−6.75
Celecoxib	−4.70 ± 0.00	−3.70 ± 0.00	−4.20
Flurbiprofen	−9.00 ± 0.00	−9.67 ± 0.06	−9.34
Ibuprofen	−7.80 ± 0.00	−7.80 ± 0.00	−7.80
Indomethacin	−4.90 ± 0.00	−4.20 ± 0.00	−4.55
Mofezolac	−5.97 ± 0.23	−6.40 ± 0.00	−6.19
Molecule 2a	−6.23 ± 0.06	−6.80 ± 0.00	−6.51
Molecule 2b	−6.83 ± 0.58	−5.86 ± 0.12	−6.34
Molecule 2c	−7.53 ± 0.06	−6.00 ± 0.00	−6.77
Molecule 2d	−6.63 ± 0.55	−6.23 ± 0.58	−6.43
Molecule 3a	−6.73 ± 0.21	−6.83 ± 0.06	−6.78
Molecule 3b	−5.87 ± 0.81	−5.40 ± 0.00	−5.64
Molecule 3c	−5.43 ± 0.06	−6.80 ± 0.69	−6.12
Molecule 3d	−5.63 ± 0.84	−4.20 ± 0.10	−4.92
SC 560	−5.87 ± 0.06	−4.76 ± 0.06	−5.32

Table 4
Percent inhibition of COX-2 by the synthesized ibuprofen derivatives. Data is obtained from *in vitro* inhibition assay of COX-2 and the study was performed as three replicates.

Inhibitor	% Inhibition of COX-2
Molecule 2a	8.3
Molecule 2b	12.4
Molecule 2c	11.4
Molecule 2d	15.6
Molecule 3a	22.9
Molecule 3b	12.4
Molecule 3c	21.5
Molecule 3d	5.0
Ibuprofen	9.7

binding regions of inhibitors were selected around amino acid residues which were 6.5 Å away from the binding site of arachidonic acid in spherical geometry. Binding affinities of inhibitors were calculated with HYDE function in LeadIT. Interactions of inhibitors with COX-2 were viewed in 2D with PoseView program in LeadIT [67]. Molecular docking calculations with AutoDock Vina were performed according to Maldonado-Rojas and Olivero-Verbel [24]. Briefly, AutoDock Vina performs calculations by using coordinates belong to the binding area of native ligand as a template in cubic geometry. Thus, each dimension of the cubic area was set to 24 Å and Cartesian coordinates (*X*, *Y* and *Z*) of the center of cubic area were selected for each crystal structures as follows: 25.277, 22.358 and 49.308 for 1CVU; 25.374, 21.657 and 17.292 for 1CX2; 27.058, 24.431 and 15.437 for 1PXX; 25.3693, 33.9205 and 207.8642 for 1CQE; 27.6342, 33.7218 and 199.0601 for 1EQH, respectively. Average binding affinities of inhibitors were calculated after three runs of the software by selecting the value of best pose in each run. The chiral centers of all the designed compounds were at S-configuration for *in silico* analysis.

4.2. Organic synthesis of novel ibuprofen derivatives

Molecular structures of synthesized ibuprofen amide and ibuprofen acyl hydrazone derivatives were given in Fig. 4.

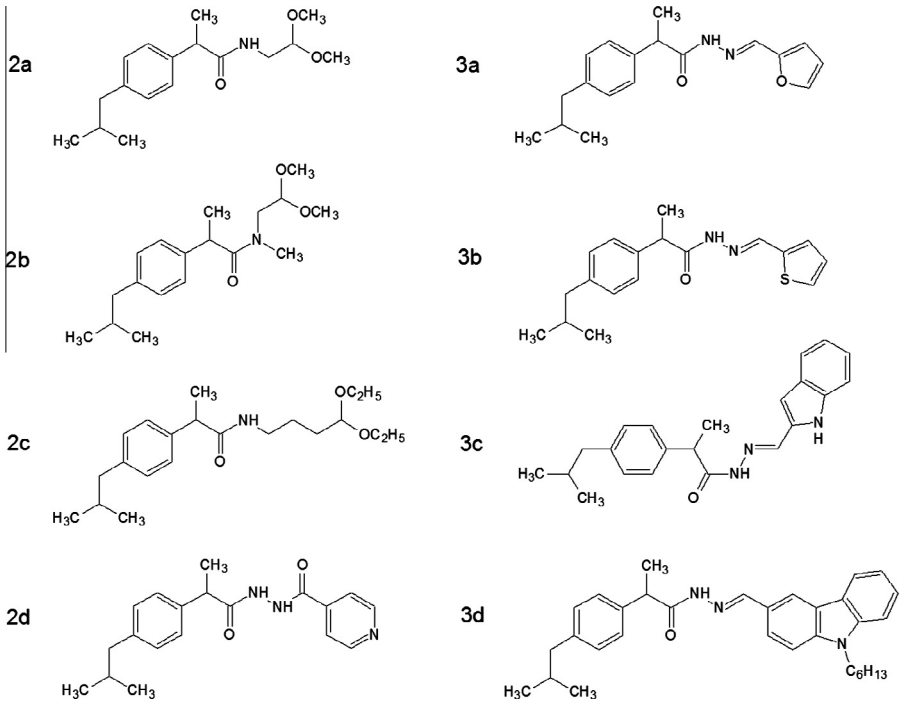
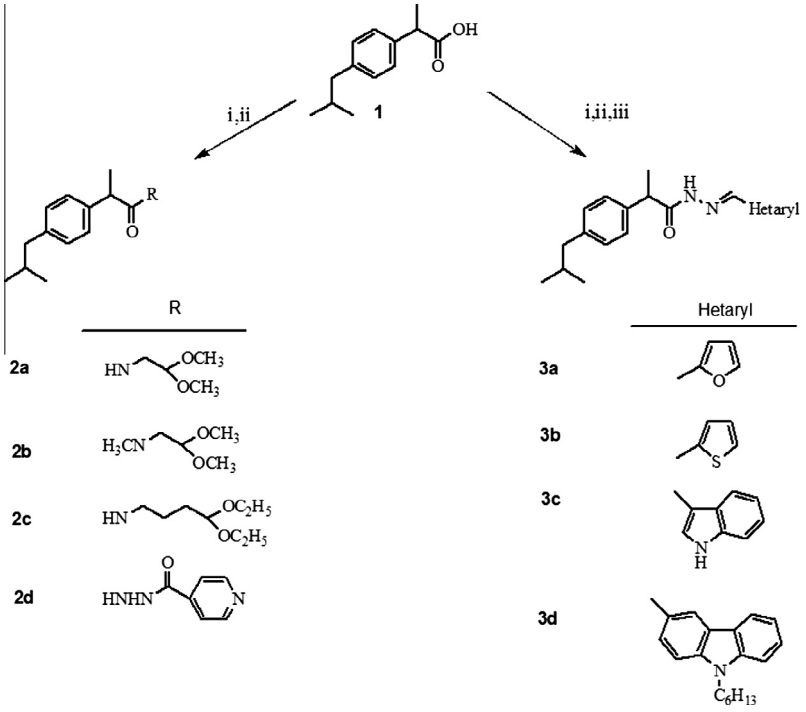


Fig. 4. Molecular structures of novel ibuprofen derivatives as potential COX-2 inhibitors.



Scheme 1. Overall scheme of reagents and reaction conditions: (i) $SOCl_2$, reflux, 2 h; (ii) THF, triethyl amine, amine, 70 °C, 6 h; (iii) HetArCHO, C_2H_5OH, AcOH, reflux, 4 h. Molecule 1 is ibuprofen.

4.3. General procedure for the synthesis of ibuprofen amide derivatives

A mixture of ibuprofen (1 in Scheme 1) (5 mmoles) and thionyl chloride (5.5 mmoles) was refluxed on an oil bath for 2 h. The excess thionyl chloride was removed under reduced pressure to produce ibuprofen acyl chloride as yellow oil. Then, ibuprofen acyl chloride (5 mmoles) was dissolved in tetrahydrofuran (20 mL). Triethylamine (5 mmoles) and amine derivatives or hydrazide (5 mmoles) were added into this solution and then the solution was heated at 70 °C for 6 h. The reaction mixture was filtered and the solvent was evaporated. The residue was chromatographed over silica gel with ethyl acetate-hexane (1:1). The solvent was evaporated under reduced pressure to obtain ibuprofen amide derivatives (Scheme 1).

4.4. General procedure for the synthesis of ibuprofen acyl hydrazone derivatives

Ibuprofen acyl chloride (5 mmoles) was dissolved in tetrahydrofuran (20 mL). Triethylamine (5 mmoles) and hydrazine hydrate (5 mmoles) were added into this solution and then the solution was heated at 70 °C for 6 h. The reaction mixture was poured into the cold water. Then the precipitate was filtered and washed with water. The crude ibuprofen hydrazide was refluxed with heteroaryl aldehydes in the presence of glacial acetic acid in ethanol for 4 h. Then the reaction mixture was cooled and precipitate was filtered. The crude product was re-crystallized from ethanol yielded ibuprofen acyl hydrazone derivatives (Scheme 1).

4.5. N-(2,2-dimethoxyethyl)-2-(4-isobutylphenyl)propanamide (molecule 2a)

Oily product (50%). IR (KBr): ν 3308 (NH), 2952 (CH), 1651 (C═O), 1131 (C—O) cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 0.89 (d, 6H, J = 6.4 Hz, 2xCH_3), 1.51 (d, 3H, J = 7.2 Hz, CH_3), 1.84 (m, 1H, CH), 2.45 (d, 2H, J = 7.2 Hz, CH_2), 3.27 (d, 2H, J = 1.6 Hz, CH_2), 3.32 (s, 6H, 2xOCH_3), 3.53 (q, 1H, J = 6.8 Hz, CH), 4.27 (t, 1H, J = 10.8 Hz, CH), 5.57 (s, 1H, NH), 7.11 (d, 2H, J = 8 Hz, ArH), 7.19 (d, 2H, J = 8.4 Hz, ArH). Anal. Calcd. for $C_{17}H_{27}NO_3$: 69.59C; 9.28H; 4.77N. Found: 69.45C; 9.34H; 4.81N.

4.6. N-(2,2-dimethoxyethyl)-2-(4-isobutylphenyl)-N-methylpropanamide (molecule 2b)

Oily product (45%). IR (KBr): ν 2954 (CH), 1649 (C═O), 1124 (C—O) cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 0.89 (d, 6H, J = 14 Hz, 2xCH_3), 1.26 (t, 1H, J = 14 Hz, CH), 1.42 (d, 3H, J = 7.2 Hz, CH_3), 1.84 (m, 1H, CH), 2.44 (d, 2H, J = 6.8 Hz, CH_2), 2.93 (s, 3H, NCH_3), 3.30 (s, 6H, 2xOCH_3), 3.35 (d, 2H, J = 4.4 Hz, CH_2), 3.85 (q, 1H, J = 6.8 Hz, CH), 7.08 (d, 2H, J = 8 Hz, ArH), 7.16 (d, 2H, J = 8.4 Hz, ArH). Anal. Calcd. for $C_{18}H_{29}NO_3$: 70.32C; 9.51H; 4.56N. Found: 70.22C; 9.63H; 4.62N.

4.7. N-(4,4-diethoxybutyl)-2-(4-isobutylphenyl)propanamide (molecule 2c)

Oily product (48%). IR (KBr): ν 3320 (NH), 2930 (CH), 1650 (C═O), 1128 (C—O) cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 0.89 (d, 3H, J = 6.8 Hz, CH_3), 0.91 (d, 3H, J = 6.8 Hz, CH_3), 1.16 (t, 3H, J = 6.8 Hz, CH_3),1.20 (t, 3H, J = 5.6 Hz, CH_3), 1.47 (d, 3H, J = 6.8 Hz CH_3), 1.47–1.53 (m, 4H, 2x CH_2), 1.85 (m, 1H, CH), 2.45 (d, 2H, J = 7.6 Hz, CH_2), 3.20 (m, 2H, CH_2), 3.40–3.53 (m, 4H, 2xCH_2), 3.6 (m, 1H, CH), 4.12 (t, 1H, J = 7.2 Hz CH), 5.49 (s, 1H, NH), 7.09 (d, 2H, J = 8 Hz, ArH), 7.18 (d, 2H, J = 8 Hz, ArH). Anal. Calcd. for $C_{21}H_{35}NO_3$: 72.17C; 10.09H; 4.01N. Found: 72.25C; 10.22H; 4.08N.

4.8. N′-(2-(4-isobutylphenyl)propanoyl)isonicotinohydrazide (molecule 2d)

Oily product (76%). IR (KBr): ν 3445 (NH), 3232 (NH), 2953 (CH), 1695 (C═O), 1651 (C═O) cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 0.90 (d, 6H, J = 6.8 Hz, 2xCH_3), 1.50 (d, 3H, J = 6.8 Hz, CH_3), 1.81 (m, 1H, CH), 2.41 (d, 2H, J = 7.2 Hz, CH_2), 3.76 (q, 1H, J = 6.8 Hz, CH), 7.07 (d, 2H, J = 7.6 Hz, ArH), 7.21 (d, 2H, J = 7.6 Hz, ArH), 7.57 (d, 2H, J = 7.6 Hz, ArH), 8.60 (d, 2H, J = 6.8 Hz, ArH), 9.36 (s, 1H, NH), 10.47 (s, 1H, NH). Anal. Calcd. for $C_{19}H_{23}N_3O_2$: 70.13C; 7.12H; 12.91N. Found: 70.02C; 7.20H; 12.95N.

4.9. N′-(furan-2-ylmethylene)-2-(4-isobutylphenyl)propanehydrazide (molecule 3a)

Light brown solid (74%); mp 148 °C. This compound was synthesized previously by our group [68], and also Nakka et al. [69].

4.10. 2-(4-isobutylphenyl)-N′-(thiophen-2-ylmethylene)propanehydrazide (molecule 3b)

Light brown solid (72%). mp: 141–142 °C. IR (KBr): ν 3447 (NH), 2953 (CH), 1667 (C═O) cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 0.88 (d, 6H, J = 6.9 Hz, 2xCH_3), 1.53 (d, 3H, J = 6.8 Hz, CH_3), 1.75–1.94 (m, 1H, CH), 2.38 (d, 2H, J = 7.1 Hz, CH_2), 4.66 (q, 1H, J = 7.0 Hz, CH), 6.96 (d, 2H, J = 4.0 Hz, Thiophene-H), 7.06 (d, 2H, J = 7.6 Hz, ArH), 7.14 (d, 1H, J = 3.6 Hz, Thiophene-H), 7.22 (t, 1H, J = 4.6 Hz, Thiophene-H), 7.33 (d, 2H, J = 8.0 Hz, ArH), 7.91 (s, 1H, N═CH), 10.20 (s, 1H, NH). Anal. Calcd. for $C_{18}H_{22}N_2SO$: 68.75C; 7.05H; 8.91N; 10.20S. Found: 68.83C; 7.13H; 8.82N; 10.22S.

4.11. N′-((1H-indol-3-yl)methylene)-2-(4-isobutylphenyl)propanehydrazide (molecule 3c)

White solid (71%). mp: 165–166 °C. IR (KBr): ν 3447 (NH), 3395 (NH), 2975 (CH), 1662 (C═O) cm^{-1}. ^1H NMR (400 MHz, d6-DMSO): δ 0.82 (d, 6H, J = 7.1 Hz, 2xCH_3), 1.41 (d, 3H, J = 6.9 Hz, CH_3), 1.73–1.78 (m, 1H, CH), 2.36 (d, 2H, J = 7.1 Hz, CH_2), 4.74 (q, 1H, J = 7.2 Hz, CH), 7.03–7.42 (m, 8H, ArH and indole-H), 7.72 (d, 1H, J = 13.2 Hz, indole-H), 8.18 (s, 1H, N═CH), 11.13 (s, 1H, NH), 11.49 (s, 1H, NH). Anal. Calcd. for $C_{22}H_{25}N_3O$: 76.05C; 7.25H; 12.09N. Found: 75.91C; 7.33H; 12.14N.

4.12. N′-((9-hexyl-9H-carbazol-3-yl)methylene)-2-(4-isobutylphenyl)propanehydrazide (molecule 3d)

Pale yellow solid (62%). mp: 177–178 °C. IR (KBr): ν 3420 (NH), 2982 (CH), 1664 (C═O) cm^{-1}. ^1H NMR (400 MHz, d6-DMSO): δ 0.75 (t, 3H, J = 6.8 Hz, CH_3), 0.81 (d, 6H, J = 6.9 Hz, 2xCH_3), 1.16–1.22 (m, 6H, CH_2), 1.40 (d, 3H, J = 7.0 Hz, CH_3), 1.71–1.87 (m, 3H, CH and CH_2), 2.38 (d, 2H, J = 7.3 Hz, CH_2), 4.32 (t, 2H, J = 6.8 Hz, NCH_2), 4.72 (q, 1H, J = 6.8 Hz, CH), 7.02–7.59 (m, 7H, ArH), 7.79 (m, 1H, ArH), 8.09 (s, 1H, N═CH), 8.13–8.39 (m, 3H, ArH), 11.41 (s, 1H, NH). Anal. Calcd. for $C_{32}H_{39}N_3O$: 79.79C; 8.16H; 8.72N. Found: 80.16C; 8.24H; 8.64N.

4.13. In vitro evaluation of cyclooxygenase-2 enzyme activity inhibition

The inhibition of COX-2 enzyme activity was studied by using the colorimetric ovine inhibitor screening assay kit (Cayman Chemical Company, catalogue no 760111). The assay buffer (0.1 M Tris–HCl, pH 8.0), heme and the (ovine) COX-2 were prepared according to the instructions of the producer and added into a 96-well plate. The arachidonic acid was prepared at 0.55 mM before its use to achieve 50 μM final substrate concentration in the wells. Then, the inhibitors were dissolved in dimethyl sulfoxide and added into the wells at a final concentration of 50 μM. Following the five minutes of incubation at 25 °C, 20 μL of the colorimetric substrate (TMPD) and the arachidonic acid were added into the wells. After a second incubation for five minutes at 25 °C, the absorbances of the wells were measured at 590 nm by using a microplate reader (BioTek Instruments, USA, ELx800). Ibuprofen was used as positive control and the results are obtained from three independent measurements (n = 3). Inhibiton percentage (% Inhibition) of the COX-2 enzyme activity was calculated as below:

$$\%\,\text{Inhibition} = \frac{(A_{100\%} - A_{Bl}) - (A_{sample} - A_{Bl})}{(A_{100\%} - A_{Bl})} \times 100 \quad (1)$$

where $A_{100\%}$ is the mean absorbance value of 100% enzyme activity wells, A_{Bl} is the mean absorbance of blank wells, and A_{sample} is the mean absorbance value of the wells including the inhibitors.

4.14. Statistical analysis

Spearman's rank correlation coefficients between *in silico* and *in vitro* data were calculated by using Minitab 16.

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