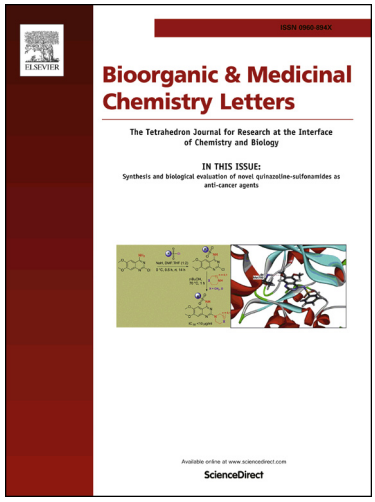

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Novel Pyrazoles and Pyrazolo[1,2-a]pyridazines as Selective COX-2 Inhibitors; Ultrasound-assisted Synthesis, Biological Evaluation, and DFT Calculations

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Keywords: Pyrazole; pyrazolo[1,2-a]pyridazine; COX-2; anti-inflammatory; Ultrasound-assisted reactions; DFT calculations

Abstract

COX-2 is an inducible enzyme mediating inflammatory responses. Selective targeting of COX-2 is useful for developing anti-inflammatory agents devoid of ulcerogenic activity. Herein, we report the design and synthesis of a series of Pyrazoles and pyrazolo[1,2-a]pyridazines with selective COX-2 inhibitory activity and *in-vivo* anti-inflammatory effect. Both series were accessed through acid-catalyzed ultrasound-assisted reactions. The most active compounds in this study are two novel molecules, **11** and **16**, showing promising selectivity and decent IC_{50} of 16.2 and 20.1 nM, respectively. These compounds were also docked into the crystal structure of COX-2 enzyme (PDB ID: 3LN1) to understand their mode of binding. Finally, Mulliken charges and electrostatic surface potential were calculated for both compound **11** and celecoxib using DFT method to get insights into the molecular determinants of activity of this compound. These results could lead to the development of novel COX-2 inhibitors with improved selectivity.

Non-steroidal anti-inflammatory drugs (NSAIDs) represent one of the most widely used classes of medicinal agents for the treatment of pain, inflammation, and fever associated with some diseases. NSAIDs are generally indicated for the symptomatic relief of a variety of conditions including rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, acute gout, dysmenorrhoea, headache, and migraine.[1] They produce their pharmacological effects by the common ability to inhibit cyclooxygenase (COX), a key enzyme that catalyzes the conversion of arachidonic acid to prostaglandin H2 (PGH2), the immediate precursor to prostaglandins, thromboxane A2 and prostacyclin.[2] Sir John Vane proved in 1971 that aspirin and other NSAIDs

exert their action by preventing the synthesis of prostaglandins.[3, 4] In 1997, Needleman and Isakson proposed two isoenzymes of cyclooxygenase; "House Keeping" enzyme, COX-1, which is responsible for a basic level of PGs for the maintenance of physiological homeostasis, such as gastrointestinal integrity, and an "Inducible" enzyme, COX-2, which is activated by different stimuli mediating inflammatory reactions. Therefore, the design of novel NSAIDs with selective targeting of COX-2 over COX-1 is useful for the treatment of inflammatory disorders with reduced gastrointestinal toxicities when compared with the traditional non-selective NSAIDs.[5, 6] Generally, reported COX-2 selective inhibitors can be classified into three major classes; diarylheterocycles including celecoxib and roficoxib,[7, 8] sulfonanilide derivatives such as nimesulide,[9, 10] and compounds obtained via modification of traditional NSAIDs (**Fig. 1**). [11-13] Over the past few years, several attempts have been made to exploit traditional NSAIDs as starting points for the design of novel COX-2 selective inhibitors, including aspirin, meloxicam, meclofenamic acid, ketoprofen, and indomethacin.[8, 11, 13-15]

Salicylamide is a non-prescription drug with analgesic and antipyretic properties comparable to those of aspirin. It can be considered as the simplest anti-inflammatory agent and its derivatives were reported for their ability to ease aches, pains and reduce fever since ancient times.[16] In this study, we designed novel COX-2 inhibitors, as hybrid molecules, by merging salicylamide with the pyrazole ring of celecoxib to obtain a molecular architecture similar to that of the diarylheterocycles COX-2 inhibitors. We report herein the design and synthesis of two series of compounds, pyrazole and pyrazolo[1,2-a]pyridazine derivatives, and the biological evaluation of their analgesic and anti-inflammatory activities using enzyme assay as well as common rodent pain and inflammatory models (**Fig. 1**). The most active compounds were also docked into the crystal structure of COX-2 to gain insights into the binding mode of this series of inhibitors. We used ultrasound-assisted reactions for the synthesis of both pyrazole and pyrazolo[1,2-a]pyridazine derivatives. This utilizes acoustic activation that abolishes the attractive forces of molecules in the solution phase.[17] This technique allowed us to get access to a diverse library of pyrazoles in a one-step reaction under mild conditions.

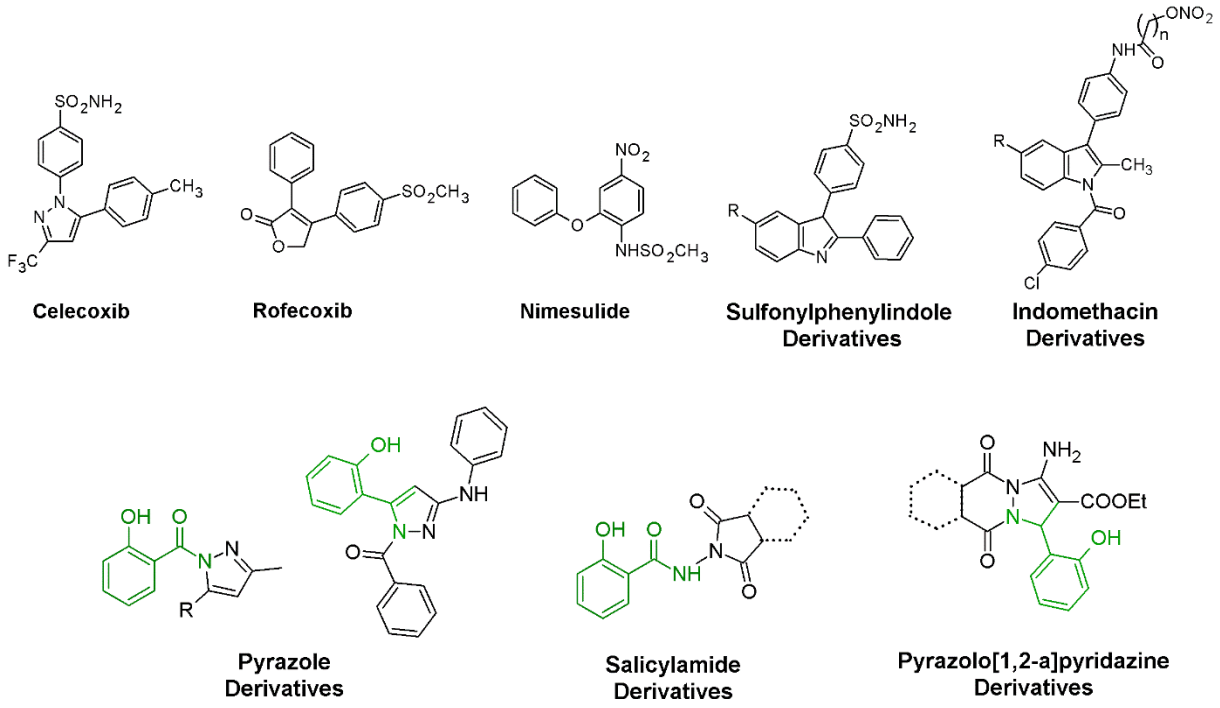


Figure 1: Selected reported COX-2 inhibitors and, below, the hybrid-molecules designed in the current study. Substructures in green represent the structural elements derived from salicylamide.

Following the FDA approval of celecoxin in 1998, several pyrazole-bsed selective COX-2 inhibitors have been reported (compounds **A-D**, **Fig. 2**). The majority of these compounds were, similar to celecoxib, diarylpyrazoles, with the central pyrazole ring carrying a trifluoromethyl moiety or an isostere of it such as methyl or methoxy groups.[18-22] Representative examples of these ligands are given in **Figure 2**. Most of them showed decent inhibitory activities for COX-2. However, selectivity and/or pharmacokinetic profiles hampered the introduction of real competitors to celecoxin into the market. In addition, few pyrazolopyridazines were reported in only two studies in 2004 and 2012 (compounds **E-G**, **Fig. 2**).[23, 24] To the best of our knowledge, the pyrazolo[1,2-a]pyridazine scaffold has not yet been explored for COX-2 inhibition.

As mentioned above, several research groups have made use of the traditional NSAIDs as a starting point for the design of novel molecules with selective COX-2 inhibitory activity. For example, Hu and co-workers utilized indole ring as a scaffold for the design of new selective COX-2 inhibitors.[25] This study led to the discovery of a series of sulfonylphenylindole derivatives with higher COX-2 inhibitory activity than celecoxib by replacing the central pyrazole ring of the latter with indole. Few years later, Wey and coworkers reported a series of

nitric oxide-donating COX-2 selective inhibitors, obtained through modifications of the acidic moiety of indomethacin (**Fig. 1**).[13] Inspired by the previous observations, we decided to build a hybrid scaffold through the combination of the structural elements of salicylamide and celecoxib (**Fig. 1**) and to introduce diversity at the heterocyclic ring. We were encouraged by the synthetic accessibility of pyrazole derivatives via acid catalyzed cyclization of hydrazine derivatives.[26]

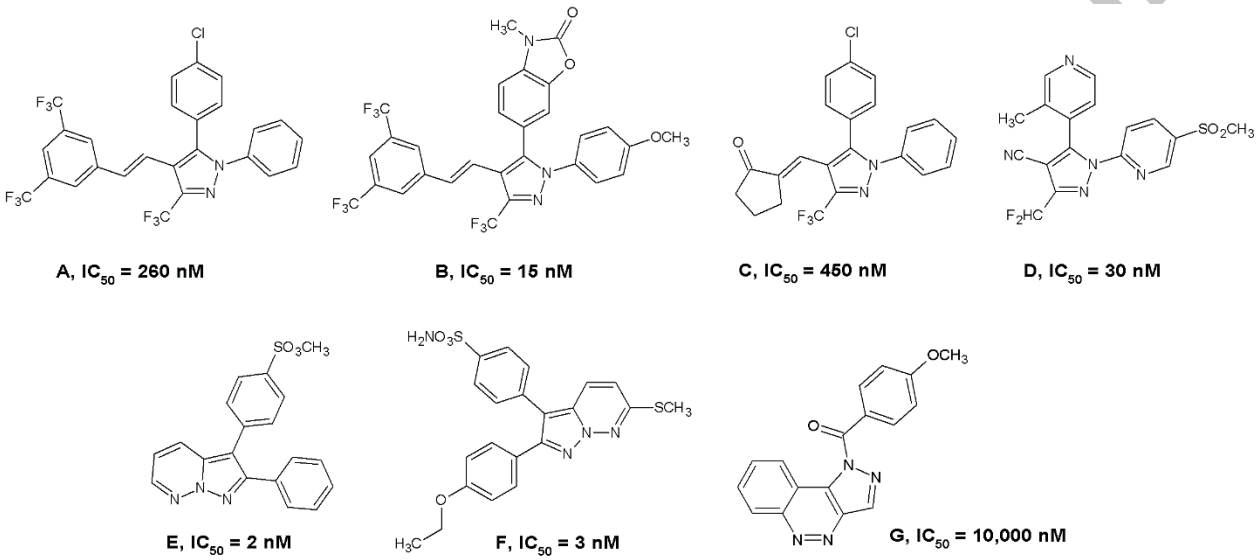
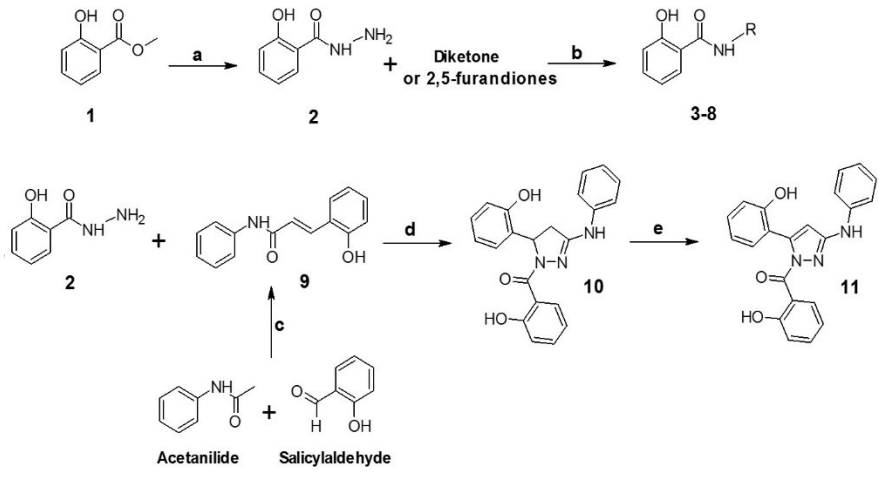


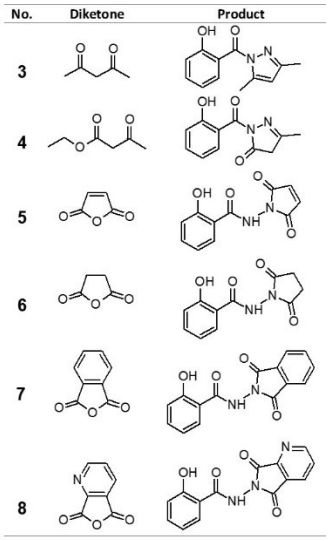
Figure 2: Selected examples of reported pyrazoles and pyrazolopyridazines COX-2 inhibitors.

The crystal structure of the inhibitor-bound COX-2, PDB ID: 3LN1, shows that the catalytic binding site is a long hydrophobic channel.[27, 28] At the entrance of the binding site, Arg106 forms a salt bridge with the carboxylic acid end of the traditional NSAIDs. However, this interaction is not essential for COX-2 inhibition. On the other hand, many Cox-2 inhibitors were noticed to form an H bond with Tyr341 at the entrance of the active site. It is worth noting that in COX-1, Ile523 blocks the diarylheterocycle-type selective inhibitors from entering the catalytic site. However, a side-pocket is created in COX-2 because of the shorter side chain of Val509. This results in a 17% increase in size next to the main catalytic binding site which becomes accessible for the diarylheterocycle inhibitors. This side pocket, lined by His75, Glu178, and Arg499, is referred to as the "selectivity binding site" as traditional NSAIDs do not use it, while the sulfonamide group of celecoxib fills it forming a strong H bond with Arg499 (**Fig. 3a**).[28, 29] In the designed compounds, we merged the salicylamide moiety with a substituted pyrazole ring to obtain a heterocyclic core with two aryl arms, an arrangement similar to that of celecoxib. In

addition, we have investigated the effect of fusing one of the aryl groups with the pyrazole ring to form a pyrazolo[1,2-a]pyridazine nucleus. Moreover, various hydrogen bonding groups, such as amino, hydroxyl, and carbonyl, were installed on the aryl rings in order to form hydrogen bonding interactions with the polar selectivity pocket of COX-2 catalytic site or the critical Tyr341 at the entrance.

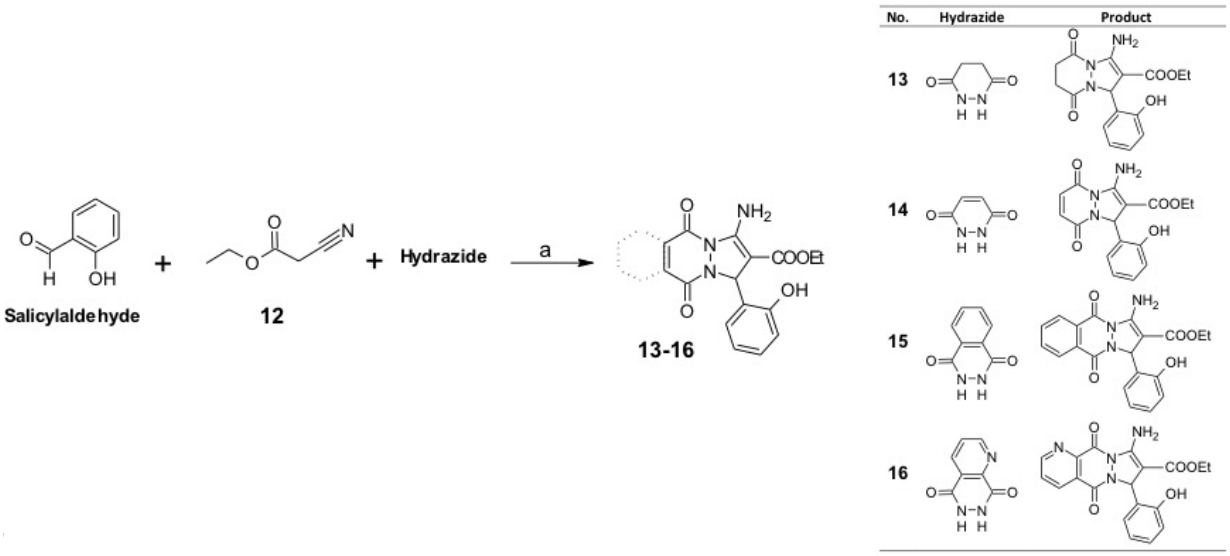
Retrosynthetic analysis of our first series of compounds showed that the substituted pyrazoles (**3** and **4**) and the salicylamide derivatives (**5-8**) could be obtained via the reaction of 2-hydroxybenzohydrazide (**2**) with β-diketones and Dihydro-2,5-furandiones, respectively. The hydrazide key intermediate 2-hydroxybenzohydrazide **(2)** was prepared by the reaction of methyl 2-hydroxybenzoate (**1**) with excess hydrazine hydrate.[30] The hydrazide **2** was cyclized with *β*-diketones such as pentane-2,4-dione or ethyl 3-oxobutanoate by applying sonication or heat on equimolar amounts of the reactants in presence of acetic acid to afford the novel pyrazole derivatives **3** and **4** in high yields.[31] Interestingly, sonication gave us better yields under mild conditions with comparable reaction times (discussed later). Similarly, hydrazide **2** was condensed with a series of Dihydro-2,5-furandione derivatives in acidic medium to give the salicylamide derivatives **5-8**.[32] In addition, 2-Hydroxybenzohydrazide **2** was condensed with chalcone **9** in ethanol, also under sonication, to give the dihydropyrazole derivative **10**. The chalcone **9** could be easily accessed through the reaction of acetanilide and salicyladehyde in basic medium.[33] The target pyrazole **11** was then obtained after oxidation of **10** with bromine water. (**Scheme 1**).





Scheme 1: (**a**) Hydrazine hydrate, EtOH, reflux, 6 h; (**b**) EtOH, ACOH, sonication or reflux, 1 h; (**c**) EtOH, NaOH, rt, 16 h; (**d**) EtOH, ACOH, sonication or reflux, 4 h; (**e**) Br_2, H_2O, rt, 2 h.

After the completion of series one, we turned our attention to our second series, the pyrazolo[1,2-a]pyridazine derivatives (**13-16**). This heterocyclic scaffold could be accessed via the condensation of hydrazine with tetrabromo-*o*-xylene.[34] Then, the products could be condensed with the appropriate diketone in acidic medium. However, due to the low yield of this multi-step procedure, we opted to use the recently reported ultrasound-assisted, one-pot, three-component method. The target compounds (**13-16**) were obtained via one-pot reaction of hydrazide, salicyladehyde, and ethyl cyanoacetate in ethanol under ultrasonic irradiation (**Scheme 2**).[35] The use of ultrasound irradiation gave significantly improved yield and straightforward purification. It has been reported that ultrasound could effectively enhance chemical reactivity by as much as a million-fold.[36] It catalyzes the reaction by exciting the molecular vibrational, rotational, and translational modes. Solid reactants usually undergo extensive degradation by the energy released from the bubbles created by cavitation leading to a great increase of their surface area as well as the observed rate of reaction.[37]



Scheme 2: (**a**) Et_3N, EtOH, sonication, 50 °C, 1 h.

The aim of this study is to design novel and selective COX-2 inhibitors with *in-vivo* activity. Therefore, before performing the enzyme assay, we screened the prepared compounds for analgesic and anti-inflammatory activities using mild animal models, namely, tail-immersion test

and hind paw oedema, respectively.[38] Compounds **4**, **7**, **11, 13, 14**, and **16** showed acceptable analgesic and anti-inflammatory activities comparable to the standard diclofenac sodium (Tables **S1** and **S2**, Supporting Information). To validate the mode of action and the selectivity of these compounds, *in-vitro* assay for COX-1 and COX-2 enzymes inhibition was conducted. The most active compounds were screened using enzyme-immuno assay (EIA) kit (Cayman Chemical Company, Ann Arbor, MI 48108, USA).[39] IC_{50} values for the most active compounds were determined using celecoxib as the reference drug. Furthermore, selectivity index for COX-1/COX-2 inhibition was calculated for each compound. From the screening results, compounds **11** and **16** showed the best inhibitory activity as well as selectivity for COX-2 with IC_{50} of 16.2 nM and 20.1 nM, respectively. The IC_{50} values for COX-1 and COX-2 inhibition are listed in Table 1. It is obvious that simply merging salicylamide with pyrazole gave a compound that is too small to fill the relatively large catalytic site of COX-2 (compound **4**). Merging salicylamide with phthalimide ring in compound **7** led to a slight improvement in COX-2 inhibitory activity. The best activity and selectivity was noticed in compound **11** which possesses a very similar special arrangement to celecoxib with an additional aryl group. The pyrazolo[1,2-a]pyridazines series showed a gradual improvement of activity with increasing the molecular size and/or rigidity. Compound **16** is the most active in this series with more than a 3-fold selectivity for COX-2, perhaps, due to formation of additional hydrophobic and hydrogen bonding interactions with the enzyme (discussed later). Interestingly, compound **15**, which is very similar to **16**, lacks the COX-2 inhibitory activity. This might be attributed to the pyridine moiety of compound **16** with a nitrogen atom that can act as an H bond acceptor.

Table 1: *In-vitro* enzyme assay against COX-1 and COX-2 with selectivity ratios.*

Compound	COX-1 IC_{50} (nM)	COX-2 IC_{50} (nM)	Selectivity (COX-1/COX-2)
Celecoxib	125	4.8	**26.0**
3	NA	NA	--
4	82.1	151.4	0.54
5	NA	NA	--
6	NA	NA	--
7	92.2	130.4	0.71
8	NA	NA	--
11	**70.4**	**16.2**	**4.35**
13	88.1	181.0	0.47

14	74.4	90.1	0.83
15	NA	NA	--
16	**66.7**	**20.1**	**3.32**

* Enzyme-immuno assay, NA: Not Active

The two most active compounds **11** and **16** were docked into the crystal structure of the celecoxib-bound COX-2 enzyme (PDB ID: 3LN1).[40] Molecular docking investigations were performed using Glide software within Schrodinger Molecular Modeling package and the standard parameters.[41] The designed inhibitors were docked into the same binding site of the reference drug celecoxib (**fig. 3**). As mentioned earlier, the COX-2 catalytic site is lined with polar residues at the entry with His75, Glu178, and Arg499 forming the "selectivity pocket". Deeper, there is a wider hydrophobic pocket, formed by Leu338, Leu345, Val509, which accommodates the central aromatic part of celecoxib. The bottom of the catalytic site is mostly aromatic with Tyr371 and Trp373 making contacts with most co-crystallized NSAIDs.

Redocking of celecoxib resulted in a docking pose very similar to the crystallized ligand with RMSD value of 1.51 Å and a docking score of -9.12 KJ/mol. Also, the characteristic H bond with Arg499 in the selectivity pocket was maintained (**fig. 3a**). The synthesized ligands **11** and **16** achieved lower docking scores of -8.02 KJ/mol and -6.10 KJ/mol, with binding poses very similar to that of celecoxib and RMSD values of 1.9Å and 2.2 Å, respectively. **Figure 3b** shows the alignment of these compounds with celecoxib in the active site of COX-2. Compound **11** docked vertically inside the pocket to avoid steric clashes and, as expected, formed a strong hydrogen bonding interaction with the critical TYR341 using its NH group (**fig. 3c**). The aniline moiety at position 3 of the pyrazole nucleus docked smoothly deep inside the selectivity pocket avoiding implausible clashes with polar amino acids populated inside this domain. The lower salicylamide moiety was found to be flanked by Leu345 and Leu517 making preferable hydrophobic interactions without forming H bonds with Arg106. Meanwhile, the third arm of

this compound, the phenolic group at position 5 of the pyrazole, showed contacts with Tyr371 at the bottom of the catalytic site. Compound **16** docked in a similar manner to compound **11** showing interactions with the three aforementioned pockets. However, its ethyl ester group pointed downwards forming a hydrogen bond with Arg106. This could rationalize, at least in part, the reduced selectivity of this compound as most NSAIDs that form polar interactions with Arg106 usually suffer from low selectivity for COX-2.[42, 43] Also, the rigid tricyclic ring system of **16** approached the selectivity pocket, unfortunately, without forming any π-stacking interactions or H bonds with His75 or Arg499. Again, its phenyl moiety made contacts with Tyr371 and Trp373 deep inside the pocket. Generally speaking, by comparing the prepared compounds with celecoxib, it was noticed that the presence of H bonding groups on the para position of one of the aryl arms or on the central pyrazole ring (such as the NH group of the aniline moiety of **11**) could enhance the COX-2 inhibitory activity. This is mediated by interaction with residues of the selectivity pocket or Tyr341, respectively. These observations showed that salicylamide-pyrazole hybrids could offer a good starting point for the design of novel COX-2 inhibitors if substituted with aryl groups of suitable size and H bonding groups in the appropriate positions.

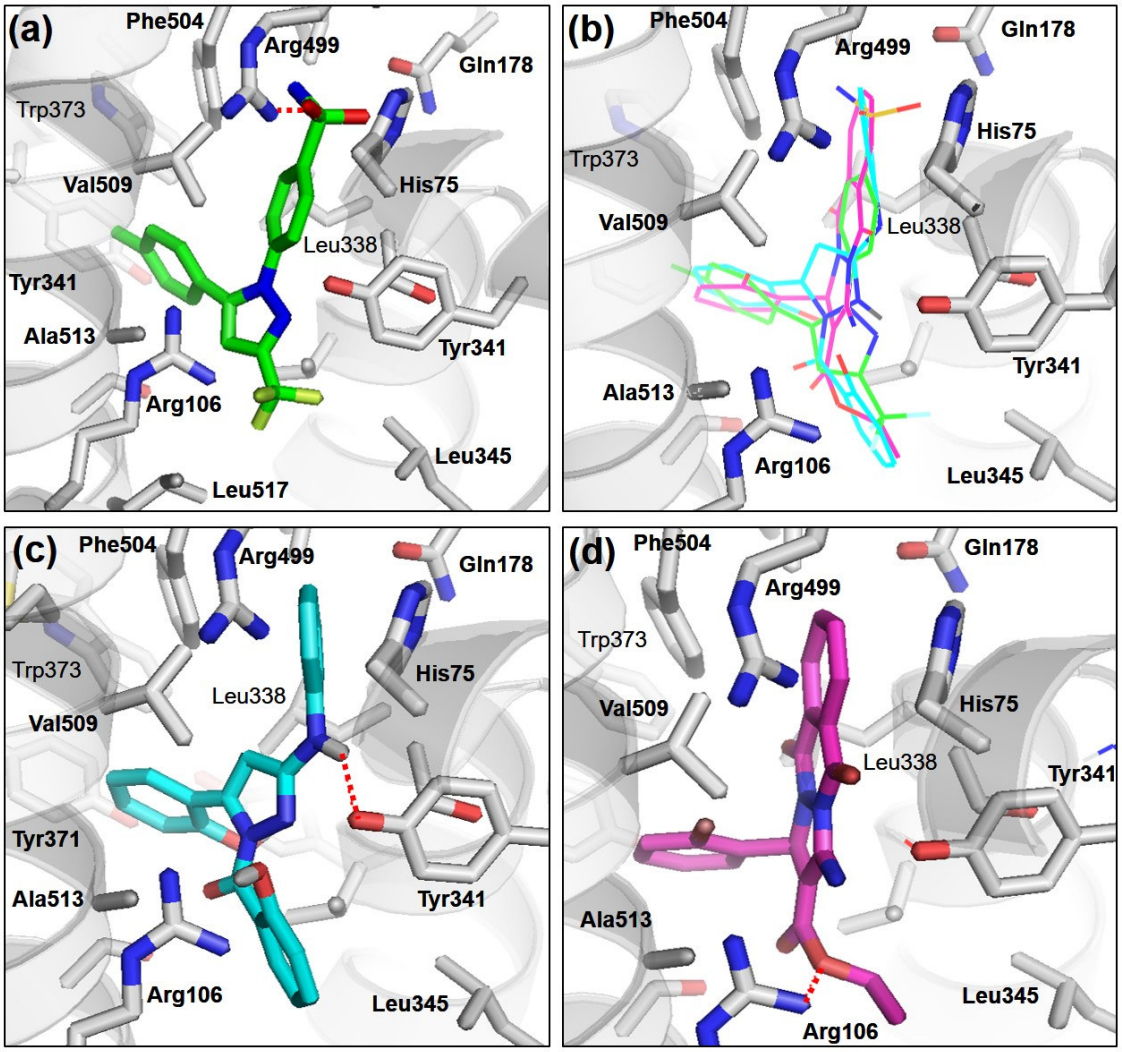


Figure 3: (**a**) Docked pose of celecoxib in the COX-2 crystal structure (PDB: 3LN1), the compound adopts a Y-shaped conformation and forms an H bond with the Arg499. (**b**) Alignment of the docked poses of the most active compounds **11** (cyan) and **16** (magenta) on the crystallized ligand celecoxib (green). (**c**) Proposed binding mode of **11** in COX-2 crystal structure forming an H bond with Tyr341. (**d**) Proposed binding mode of **16** in COX-2 crystal structure showing an H bond with Arg106. Protein is shown as a grey cartoon with the important binding site residues displayed as sticks.

To further investigate and verify the mode of binding of the most active compound in this study, compound **11**, we decided to examine the hydrophobic/aromatic interactions as they represent the major interaction forces in case of both celecoxib and this compound. Visual examination of both complexes reveals the absence of any aromatic (π/π) interactions. On the other hand, many hydrophobic contacts with Val509, Ala513, Tyr341, and Leu345 could be

noticed. Interestingly, the orientation of several amino acids within the active site suggests a CH/π interaction, a weak molecular attraction force occurring between a CH group and an electron-rich π-system. This attractive force is generally weak and difficult to distinguish from the non-directional van der Waals interaction. We assume that the existence of several CH/π interactions could play a critical role in the binding of celecoxib that has been overlooked in the previous studies. To examine the presence of this type of interaction quantitatively, we used the CHPi module as implemented in the Biostation Viewer software. As expected, both molecules showed an extensive network of CH/π interactions (**Fig. 4**). The right phenyl arm of celecoxib is sandwiched between Ser339 and Val509, while the other phenyl ring and pyrazole forms contacts with Ala513 and Val335, respectively. Compound **11** shows similar CH/π bonds with Val509 on the right side, while the lower benzoyl group exhibits more interactions of this type with Val335 and Ala513, analogous to celecoxib. These results give more emphasis on the importance of the aforementioned CH/π contacts for ligand binding in the hydrophobic pocket of COX2 enzyme.

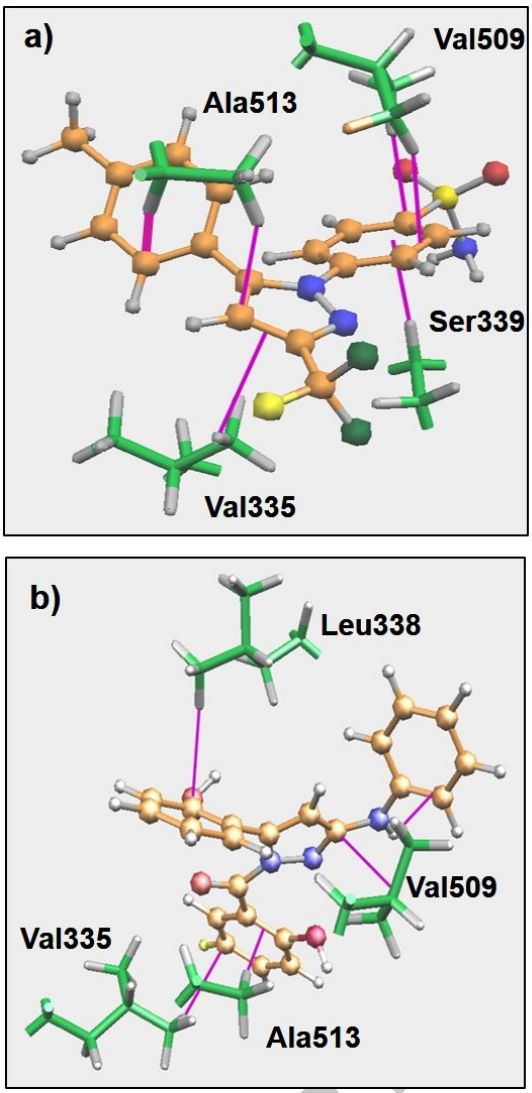


Figure 4: CH/π interactions as calculated using the CHPi module as implemented in the Biostation Viewer software for celecoxib (**a**) and compound **11** (**b**). Both compounds are shown as yellow balls and sticks, protein side chains are displayed as green sticks, and the predicted interactions are illustrated as red lines.

To visualize the charge distribution on both celecoxib and Compound **11** and to better estimate the contribution of polar interactions in their binding, we ran an energy calculation in Guassian 09 software using Density Functional Theory (DFT) method on the conformation of crystallized celecoxib as well as the docked conformation of compound **11**.[44, 45] The B3LYP functional was employed with 6-311++G (d, p) basis set to calculate the Mulliken charge distribution for all the atoms as well as the Electrostatic Surface Potential (ESP) for both molecules.[46] **Figure 5** shows the distribution of Mulliken atomic charges. It is obvious that both

compounds possess relatively negative phenyl moieties which indicates a high electron density responsible for the CH/π interactions. Interestingly, the calculated atomic charge of the celecoxib sulfonamide oxygen interacting with Arg499 was found to be – 0.541 (**Fig**. **S1,** Supporting Information). This suggests an ionic bond nature of this interaction rather than a traditional H bond and highlights the importance of interaction with the residues of the selectivity pocket for celecoxib binding. The ESP of both compounds (**Fig. 5**) reveals a very similar shape and size with mostly hydrophobic three arms. The only striking difference is the strong negative charge localized on the apex of the right arm of celecoxib. This highly negative surface represents the sulfonamide group of celecoxib which interacts with the polar residues of the selectivity pocket, especially Arg499. This may rationalize, to some extent, the higher inhibitory activity of celecoxib compared to compound **11**. It is worth noting that, for ease of comparison, we placed the compounds in **figure 5** in a similar orientation to their docked poses in **figure 3**.

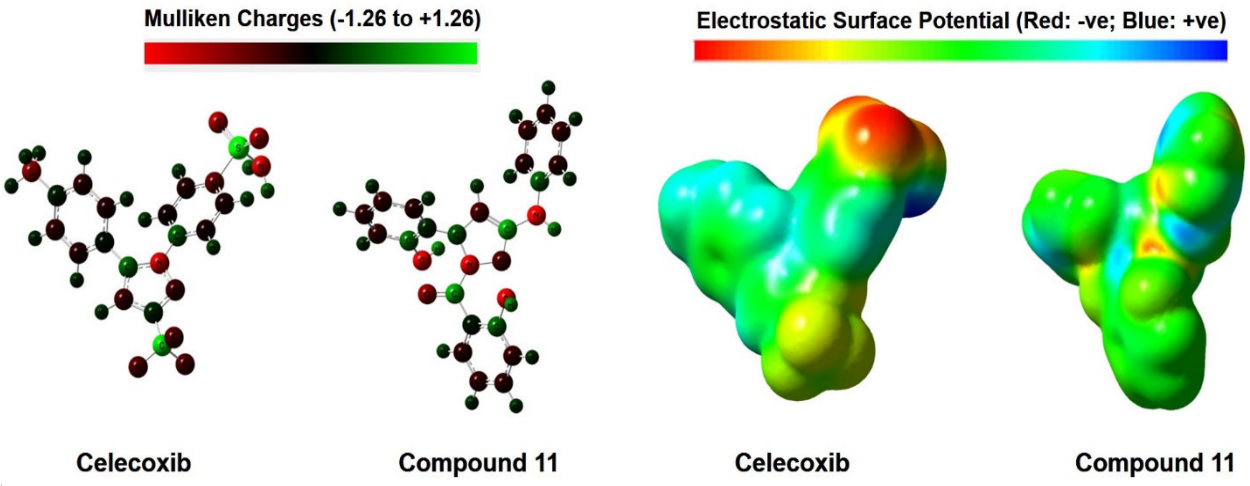


Figure 5: Mulliken charges (left) as calculated using DFT method with B3LYP functional and 6-311++G (d, p) basis set (color-coded from red to green). Electrostatic Surface Potential (ESP) calculated using the same methodology and mapped onto both compounds (color-coded from red to blue).

With the extensive use of anti-inflammatory medications, there is still a need for the development of novel COX-2 selective inhibitors with improved pharmacokinetics and safety margin. In this study, we reported the design and synthesis of some salicylamide-pyrazole hybrid molecules; two of them with promising selectivity. Docking studies and DFT calculations suggests that these molecules possess a suitable size and complementarity to the COX-2 active site. According to our results, the ideal selective COX-2 inhibitor should have three aryl arms to form the required hydrophobic or CH/π interactions with the hydrophobic residues in the active site such as Ala513 and Val509. In addition, the molecule should possess two polar regions; one in the center to interact with Tyr341 and the other at the upper aryl arm to fill the selectivity pocket interacting with Arg499 in a similar manner to celecoxib. Further studies are undergoing in our laboratory to improve the activity of our lead compounds **11** and **16** according to these proposed guidelines and to fine-tune their selectivity towards COX-2 enzyme.

Acknowledgments

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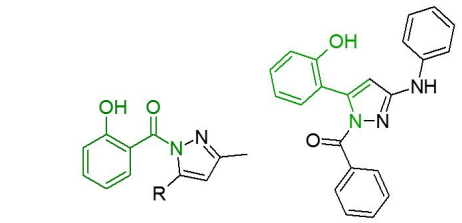
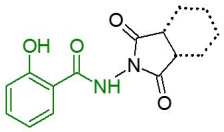


Pyrazole Derivatives



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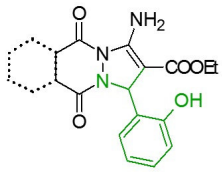
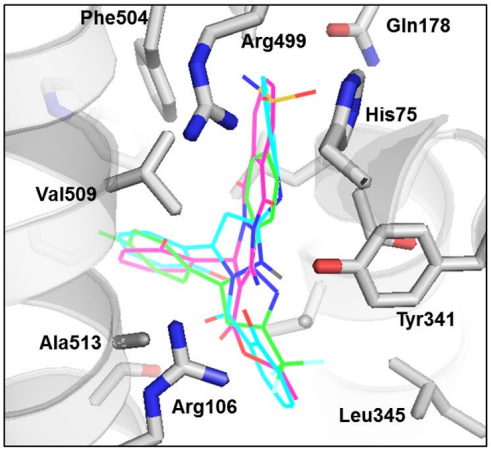


Pyrazolo[1,2-a]pyridazine Derivatives



COX-2 Active Site