CHAPTER-2

RATIONAL DESIGN, SYNTHESIS AND EVALUATION OF CHROMONE-INDOLE AND CHROMONE-PYRAZOLE BASED CONJUGATES FOR COX-2 INHIBITORY ACTIVITIES

Identification of metabolites\(^1\) and corresponding enzymes of arachidonic acid (AA) metabolism led to systematic treatment of inflammatory diseases by controlling the enzymatic activities of phospholipase, lipoxygenase and/or cyclooxygenase. House-keeping functions of cyclooxygenase-1 (COX-1) and the formation of inflammatory prostaglandins through cyclooxygenase-2 (COX-2) enzymatic activity are well documented.\(^2\) Succeeding COX-1/2 non-selective aspirin, ibuprofen, indomethacin; COXIBs were developed as selective COX-2 inhibitors.\(^3\)

Because of the specificity to the cellular target and compatibility to the biological system, a wide variety of natural products are being used in the form of drugs.\(^4\) Several compounds from terpenoid, alkaloid and flavonoid categories of natural products have been isolated\(^5\) and evaluated for their COX inhibitory activities amongst which chromone based compounds\(^5\) (1, 2; Chart 1) have shown promising inhibition of COX-2 enzymatic activity. Besides chromone; indole and pyrazole are the other naturally occurring moieties which are part of a number of clinically used drugs like indomethacin (3) and celecoxib (4).\(^6,7\)

![Chart 1. Natural product based COX-2 inhibitors](image)

Keeping in mind the higher efficacy of hybrid molecules,\(^8,9\) it was envisaged that the compounds obtained by the combination of chromone-indole and chromone-pyrazole may prove as better COX-2 inhibitors in comparison to their individual parent molecules. Also, being the part of natural compounds, the molecules obtained by the combination of chromone-indole and chromone-pyrazole moieties may exhibit poor toxicity to the host. The results of stepwise development of molecules by the combination of two and three
biologically relevant pharmacophores in a single molecule and their enzyme inhibitory activities are given below:

**Section 2A. Synthesis and evaluation of hybrid molecules obtained by the combination of two biologically significant moieties**

As reported earlier, the hybridization of two different molecules worked well in the development of cytotoxic agents. Chromone-indole based compound 5 (Chart 2) with appreciable anti-cancer activity (average 50% growth inhibitory concentration; GI\textsubscript{50} over 60 human tumor cell lines 3.2 µM) was taken for determining its COX-2 inhibitory activity. Similar to compound 5, compounds 6-10 (Chart 2) were synthesized and screened for the inhibition of enzymatic activity of COX-2, COX-1 and 5-LOX.

![Chart 2](image)

**Chart 2**

2.1. Chemistry

A simple experimental protocol was employed for the synthesis of compounds. Compound 5 was obtained by the reaction of chromone-3-carboxaldehyde (1 mmol) with indolinone (1-(2,6-dichlorophenyl)-2-indolinone) (1.2 mmol) under microwave irradiation. Similar reactions of 6-bromo/isopropyl-chromone-3-carboxaldehyde (1 mmol) yielded compounds 6 and 7 (Scheme 1). Using the same synthetic protocol, a finely ground mixture of chromone-3-carboxaldehyde (1 mmol) and oxindole (1.2 mmol) was irradiated under microwaves to obtain 8 while 9 was obtained by the reaction of 6-bromochromone-3-carboxaldehyde (1 mmol) with oxindole (1.2 mmol) (Scheme 1). With the limited availability of 6-fluoro-8-nitrochromone-3-carboxaldehyde (1 mmol) (Sigma-Aldrich stopped the supply), compound 10 was prepared via its condensation with 1-(3-chlorophenyl)-3-methyl-2-pyrazolin-5-one (1.2 mmol) (Scheme 1). Therefore, as per the design of the molecules and in accordance with the fact “the most fruitful basis for the discovery of a new drug is to start
with an old one”, the new compounds were synthesized, retaining the potential structural features of some old drugs.

2.2. Enzyme inhibition assays

Compounds 5-10 were screened for inhibition of catalytic activities of COX-2, COX-1 and 5-lipoxygenase (5-LOX); the enzymes participating in AA metabolism. All the enzyme inhibition assays were performed in triplicate. Compounds were tested at $10^{-5}$, $10^{-6}$, $10^{-7}$ and $10^{-8}$ M concentrations. The enzyme inhibition assay\textsuperscript{11,12} used here is based on COX-1/2 and 5-LOX catalyzed production of prostaglandins and leukotrienes, respectively during AA metabolism. Catalytic activities of the three enzymes were quantified by measuring the amount of prostaglandins/leukotrienes produced by each enzyme in presence of various concentrations of the test compounds and comparing with control experiments. Enzyme inhibition is inversely proportional to the concentration of prostaglandins or leukotrienes in each enzymatic reaction.

Compounds 5, 7, 8 and 9 showed considerable inhibition of COX-2 with IC\textsubscript{50} (50% inhibitory concentration) 1.3, 5.8, 29 and 20 nM, respectively while their respective IC\textsubscript{50} for COX-1 was 0.0047, 0.65, 1.35 and 6.75 µM. Since COX-1 and COX-2 are responsible for
the synthesis of different prostaglandins in AA pathway, preferred inhibition of catalytic activity of COX-2 is desirable for controlling inflammation. Compound 5 showed considerable inhibition of catalytic activities of both COX-2 and COX-1, exhibiting negligible selectivity for COX-2 over COX-1 (Table 1) and hence cannot be recognized as a proficient candidate for its use as COX-2 inhibitor. Compound 6 with IC\(_{50}\) 0.63 and 7.6 µM for COX-1 and COX-2, respectively also exhibited poor selectivity for COX-2 over COX-1. Compound 10 exhibited an IC\(_{50}\) value >10 µM for both COX-1 and COX-2. As per the results of present experiments, compounds 8 and 9 are identified with substantial inhibition of COX-2 as well as desirable selectivity for COX-2 over COX-1.

**Table 1. Inhibition of catalytic activity of COX-1 and COX-2 by compounds 5-10**

| Compound | COX-1 | | | COX-2 | | |
| --- | --- | | | --- | --- | --- |
| | % inhibition (Molar conc) | IC\(_{50}\) (µM) | | % inhibition (Molar conc) | IC\(_{50}\) (µM) | SI\(^*\) |
| 5 | 80.3 | 75 | 63 | 0.0047 | 78.1 | 11.6 | 64.9 | 51 | 0.0013 | 4 |
| 6 | 80.3 | 60 | 44.4 | 0.63 | - | 46.5 | 34.9 | 23.5 | 7.6 | 0.08 |
| 7 | 64.6 | 56 | 47 | 0.65 | 72.8 | - | 67.2 | 54 | 0.0058 | 112 |
| 8 | 51.6 | 43 | 35 | 1.35 | 96.7 | 73.3 | 57 | 52 | 0.029 | 46 |
| 9 | 40.5 | 30.4 | 20 | 6.75 | 70.4 | 66.3 | 61.7 | 57.4 | 0.020 | 337 |
| 10 | 27.9 | 18 | 15 | >10 | 18.6 | - | 36.0 | 15 | >10 | - |
| Wogonin | - | - | - | - | - | - | - | - | 46.0 | - |
| Indomethacin | - | - | - | 0.08 | - | - | - | - | 0.96 | 0.08 |
| Celecoxib | - | - | - | 15.0 | - | - | - | - | 0.04 | 375 |

\(^*\)selectivity index = IC\(_{50}\)(COX-1)/IC\(_{50}\)(COX-2)

Only compound 7 showed appreciable inhibition of 5-LOX with IC\(_{50}\) 20 nM (Table 2). For making comparison of COX-2 inhibitory activities of these hybrid compounds and chromone, indole and pyrazole based drugs; IC\(_{50}\) of wogonin (1),\(^{14}\) indomethacin (3),\(^{15}\) and celecoxib (4;\(^{16}\) Chart 1) were taken into consideration. 8 and 9 showed better COX-2 inhibitory activity and selectivity for COX-2 over COX-1 than that of wogonin and indomethacin which justifies the design of the molecules. IC\(_{50}\) of these two compounds for COX-2 was also comparable to that of celecoxib.
Table 2. Inhibition of catalytic activity of 5-LOX by compounds 5-10

<table>
<thead>
<tr>
<th>Compound</th>
<th>5-LOX % inhibition (Molar conc)</th>
<th>IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10$^{-5}$</td>
<td>10$^{-6}$</td>
</tr>
<tr>
<td>5</td>
<td>8.62</td>
<td>15.5</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>81</td>
</tr>
<tr>
<td>8</td>
<td>74.1</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>77.6</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>46.5</td>
</tr>
<tr>
<td>Wogonin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

2.3. NMR spectral studies and molecular modelling

$^1$H NMR spectrum of compound 8 (0.35 mM in DMSO-$d_6$) was recorded (Figure 1, 2) and the signals for various hydrogens were identified. D$_2$O exchange experiment identified NH of indole moiety at $\delta$ 10.7. Followed by addition of 12 µL (0.08 mM) of COX-2 (low concentration for observing signals of the compound only) to the solution of 8 in DMSO-$d_6$ (no D$_2$O), NMR spectra were recorded after every 30 min. until no change was detected.

It was observed that NH signal was shifted from $\delta$ 10.7 to 10.85 and its intensity was considerably reduced which indicated that the enzyme COX-2 might be interacting with NH group of 8.

Figure 1. $^1$H NMR spectra of compound 8 (black), in presence of D$_2$O (red), in combination with COX-2 (blue).
Figure 2. Region between 6.5 – 11.0 ppm of figure 1 expanded. $^1$H NMR spectra of compound 8 (black), in combination with D$_2$O (red), in combination with COX-2 (blue).

Looking for the possibility of interactions of compound 8 with COX-2 through its carbonyl oxygens, $^{13}$C NMR spectra of 8 alone as well as in presence of COX-2 (same concentration and solvent as for $^1$H NMR spectra) were recorded. Comparison of two $^{13}$C NMR spectra (Figure 3) clearly showed the downfield shift of some of the signals especially those belonging to carbonyl carbons of chromone and oxindole moieties at $\delta$ 174.9 and 167.5, respectively (Figure 4). Hence, there seems to be the possibility of interaction between carbonyl oxygen of the compound and amino acid residue of the enzyme.

Figure 3. An overlapping view of $^{13}$C NMR spectrum of 8 (in black) showing shifting of peaks on addition of 12 μL of enzyme COX-2 (in red).

Figure 4. $^{13}$C NMR spectrum of 8 expanded in the C=O region showing a significant shift in the peak at $\delta$ 174.9 (in black) to 175.10 (in red) due to carbonyl carbon of chromone. Shift in the peak at $\delta$ 167.5 due to carbonyl carbon of oxindole is also visible.
In order to support NMR spectral data and pin-point the interactions of NH and C=O group with amino acid residues, molecular docking\textsuperscript{17} of compound 8 in the active site of COX-2\textsuperscript{18} was performed. Hydrogen bonds having bond lengths of 2.88 and 2.90 Å between NH group of 8 and Arg120 (Arg120 forms salt bridge with AA during the metabolic phase of the enzyme) and chromone carbonyl oxygen and Ser530, respectively were observed. Carbonyl oxygen of indole moiety also showed interaction with Tyr355 (\textbf{Figure 5}).

\textbf{Figure 5}. Compound 8 (in pink) docked in the active site of COX-2 (PDB ID 6COX). Yellow dotted lines represent H-bonds with bond length in Å. Purple amino acids are positively charged, grey amino acids are hydrophobic while blue amino acids are hydrophilic in nature.

Therefore, supporting the results of NMR spectral investigations, molecular docking studies also showed the interactions of NH and carbonyl oxygens of compound 8 with the enzyme.

Similarly, interactions of compound 8 with COX-1 were also studied. \textsuperscript{1}H NMR spectrum of 8 (DMSO-\textit{d}_6) after the addition of COX-1 (0.08 mM) resulted into small shift and decrease in intensity of the NH signal of 8 (\textbf{Figure 6}).
Figure 6. $^1$H NMR spectrum of compound 8 showing small change in intensity of NH signal on addition of enzyme COX-1.

$^{13}$C NMR spectrum of 8 in presence of COX-1 showed the shifting of chromone carbonyl carbon from $\delta$ 175.03 to 175.20 (Figure 7). Docking of compound 8 in the active site of COX-1\(^\text{19}\) indicated presence of H-bond interactions between NH and oxindole carbonyl groups of 8 with Arg120 (Figure 8). However, comparing the results of NMR studies and molecular modelling, it seems that compound 8 exhibited better interactions with COX-2 than that of COX-1.

Figure 7. $^{13}$C NMR spectrum of compound 8 (black) and in combination with COX-1 (red) expanded in the region of 165-175 ppm showing downfield shift (arrowhead) in the carbonyl carbon of chromone
2.4. Mass Spectral Studies

In continuation with the results of foregoing experiments, binding of compounds 8 and 9 with COX-2 and COX-1 was also evaluated in terms of association constants (K_a). Since electrospray ionization (ESI) mode of mass spectrometry is a prompt and accurate technique to study non-covalent interactions \(^{20-26}\), association constants of compounds 8 and 9 with COX-1 and COX-2 were calculated from mass spectral data. In order to compare 8/9 – COX-1/2 interactions with AA – COX-1/2 interactions, association constants of AA with COX-2, COX-1 were also calculated. Mass spectra of COX-1 and COX-2 alone as well as in combination with AA, compound 8 and 9 were recorded (Figure 9, 10).
Figure 9. Deconvoluted mass spectra of (a) COX-1; (b) AA-COX-1 complex; (c) 8-COX-1 complex and (d) 9-COX-1 complex
Association constants indicated better interaction of compounds 8 and 9 with COX-2 in comparison to their interactions with COX-1. Moreover, $K_a$ of compounds 8 and 9 for COX-2 were higher than those exhibited by AA with this enzyme. On the other hand, their $K_a$ for COX-1 was smaller than that of COX-1 – AA (Table 3).

Normally, an effective COX-2 inhibitor occupies the active site of the enzyme in preference to AA (the substrate) and provides relief from inflammation by blocking the production of inflammatory prostaglandins. Since the association constants of compounds 8 and 9 for COX-2 are better than that of AA, they may bind with COX-2 in preference to AA and hence may be developed as highly efficacious COX-2 inhibitors.
Table 3. Association constants of compounds 8, 9, AA and drugs for COX-1 and COX-2

<table>
<thead>
<tr>
<th>entry</th>
<th>Ligand</th>
<th>*K_a (M^-1) = [EL]/[E]<em>{free} * [L]</em>{free}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>COX-1</td>
</tr>
<tr>
<td>1</td>
<td>Arachidonic acid</td>
<td>0.39 x 10^4</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>0.29 x 10^4</td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>0.35 x 10^4</td>
</tr>
<tr>
<td>4</td>
<td>Aspirin</td>
<td>0.93 x 10^4</td>
</tr>
<tr>
<td>5</td>
<td>Indomethacin</td>
<td>0.54 x 10^4</td>
</tr>
<tr>
<td>6</td>
<td>Celecoxib</td>
<td>--</td>
</tr>
</tbody>
</table>

*[EL] = conc of enzyme-ligand complex, [E]_{free} = conc of free enzyme, [L]_{free} = conc of free ligand

Moreover, comparing with aspirin, compounds 8 and 9 exhibited larger association constants for COX-2 and smaller association constants with COX-1 which indicated that 8 and 9 may be better and safer COX-2 inhibitors than aspirin. However, K_a of 8 – COX-2 was comparable to K_a of indomethacin – COX-2 while K_a of compounds 8/9 for COX-1 were less than that of indomethacin.

2.5. Analgesic activity of compound 8

Based upon the results of enzyme immunoassay, NMR, mass spectrometric and molecular docking studies; compound 8 was checked for its analgesic activity (algesia being the major symptom of inflammation). Swiss albino mice (25-35 g) of either sex were used in the present study. The animals were housed at 25 ± 2 °C under 12 h light/12 h dark cycle and free access to food and water in the central animal house at Guru Nanak Dev University, Amritsar, Punjab. Animals were acclimatized to the laboratory for at least 1h before testing and were used only once throughout the experiments. All the protocols have been duly approved by the Institutional Ethics Committee and are in accordance with guidelines of Committee for the purpose of Control and Supervision of Experiments on Animals (CPSCEA), Ministry of Environment and Forests, India.

With a few modifications, the method used for capsaicin-induced licking was similar to that described by Sakurada et al.\(^27\) Capsaicin (1.6 µg) was dissolved in 20 µL of water and injected under the plantar surface of the right hand paw of mice. Animals were observed individually for 10 min. after capsaicin administration and the time spent on paw licking and twitching was recorded as an indicator of algesia. Animals were divided into 3 groups of 6
each. All the treatments were administered intraperitoneally 30 min. before capsaicin injection. Group I was control wherein the animals were injected normal saline in a volume of 0.5 mL. In group II, animals were injected diclofenac at a dose of 25 mg Kg$^{-1}$; in group III animals were injected compound 8 at a dose of 5 mg Kg$^{-1}$ and 10 mg Kg$^{-1}$, respectively.

Treatment with diclofenac was found to produce a marked decrease in the number of paw lickings after capsaicin injection. A decrease of 68% in paw lickings was observed on using 25 mg Kg$^{-1}$ diclofenac. Surprisingly, compound 8 produced far better analgesic activity than diclofenac and it reduced the paw lickings by 76% at a dose of 5 mg Kg$^{-1}$. On increasing the dose to 10 mg Kg$^{-1}$, further enhancement in the analgesic activity was observed (Figure 11). Moreover, compound 8, showing considerable analgesic activity, is under further refinements for developing into an effective anti-inflammatory drug.

**Figure 11.** Effect of compound 8 on capsaicin induced pain in mice. All values are expressed as mean ± SEM (n = 6 per group). Statistical significance has been calculated using one way ANOVA followed by Tukey’s multiple range comparison test. *** p < 0.001 vs. control group.

As indole and chromone based compounds are known for moderate COX-2 inhibition$^{6,28}$ it may be the combined inherent selectivity of both the fragments which make compounds 8 and 9 more selective for COX-2. Hence, the remarkable specificity and selectivity exhibited by the natural systems could be a roadmap for developing more such compounds showing selective inhibition of a particular enzyme.

Further to check the physico-chemical properties of compounds 8 and 9, Lipinski parameters and solid state morphology of the compounds were determined. Both the compounds obeyed Lipinski’s rule of ‘5’ (Table 4).$^{29}$
Table 4. Lipinski values for compounds 8 and 9

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mol wt.</th>
<th>NH, OH donors</th>
<th>NH, OH acceptors</th>
<th>Total Polar surface area (Å)</th>
<th>LogP</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>291</td>
<td>1</td>
<td>4</td>
<td>59.16</td>
<td>2.92</td>
</tr>
<tr>
<td>9</td>
<td>370</td>
<td>1</td>
<td>4</td>
<td>59.16</td>
<td>3.71</td>
</tr>
</tbody>
</table>

Since solid state morphology of the drug has significant impact on its bioavailability and stability, the diffraction spectra of compounds 8 and 9 were recorded on Rigaku Miniflex II desktop X-ray diffractometer (XRD). Appearance of sharp peaks at 2θ equal to 11.96°, 15.04°, 16.84°, 17.32°, 18.08°, 23.62°, 24.26°, 28.04° and 14.18°, 14.6°, 19.78°, 21.78°, 22.52°, 26.6°, 27.4°, 31.22° for compounds 8 and 9, respectively indicated that compounds 8 and 9 are highly crystalline in nature (Figure 12, 13).

Figure 12. PXRD pattern of compound 8

Figure 13. PXRD pattern of compound 9

Moreover, scanning electron microscope (SEM) images (recorded on Field Emission Scanning Electron Microscope, Zeiss) of compounds 8 and 9 showed molecular arrangement of the two compounds in detail. Molecules of 8 are arranged in the form of rods having
diameter and the length of the rods is in the range of µm and mm, respectively (Figure 14). Compound 9 showed sheet like structures with breadth of the sheet in the range of 1 µm and length of the sheets goes to 10 µm (Figure 15). Supporting the naked eye observation; the crystalline, shining surface of compound 8 was quite evident from its XRD pattern and SEM image. Although compound 9 was not as crystalline as 8 but XRD pattern and SEM image indicated that it also exists in a quite organized form.

**Figure 14.** SEM image of compound 8.

**Figure 15.** SEM image of compound 9 (Inset: a part magnified 11K times)
Section 2B. Synthesis and evaluation of hybrid molecules obtained by the combination of three biologically significant moieties

As discussed in the previous section, hybrid molecules obtained by the combination of two bioactive molecules, exhibit significantly better biological activity in comparison to their individual fragments.\textsuperscript{10,30} Further, to improve the COX-2 inhibitory activities of the compounds, three biologically significant moieties including chrysin (11), indole (12) and pyrazole (13) as shown in Chart 3 are combined together and thereby compounds 14-19 have been designed. While chrysin is an important member of flavone class of natural products identified with anti-oxidant,\textsuperscript{31,32} anti-bacterial,\textsuperscript{33} anti-inflammatory,\textsuperscript{34,35} anti-cancer,\textsuperscript{36} anti-estrogenic,\textsuperscript{37} anxiolytic\textsuperscript{38} activities and a number of its analogues like apigenin,\textsuperscript{39} tectorigenin\textsuperscript{40} and wogonin\textsuperscript{41} show significant anti-inflammatory activities, the biological potential of indole and pyrazole is also well established.\textsuperscript{42-47} Therefore, it is envisaged that the combination of three biologically significant heterocycles may result in the development of more efficacious molecules.

\begin{center}
\textbf{Chart 3}
\end{center}
2.6. Chemistry

Compounds 20 and 21 were obtained via N-alkylation of 3-formyl indole (1 mmol) with 1,3-dibromopropane and 1,4-dibromobutane (1 mmol), in the presence of NaH (1.5 mmol) as base in dry DMF, respectively (Scheme 2).

Scheme 2

In the next step, chrysin, (11, 1 mmol) was taken in acetone and was refluxed overnight with compounds 20 and 21 in presence of potassium carbonate (1 mmol). The reaction was performed under N₂ atmosphere and was monitored using TLC. The crude products were obtained by filtering the reaction mixtures which were purified by column chromatography to procure compounds 22 and 23 (Scheme 3).

Scheme 3
Compound 14 was obtained via treatment of compound 22 (1 mmol) with 1-(3-chlorophenyl)-3-methyl-2-pyrazolin-5-one (1 mmol) at 160-180 °C for 10-20 min. Similarly, reaction of 23 (1 mmol) with 1-(3-chlorophenyl)-3-methyl-2-pyrazolin-5-one (1 mmol) resulted in the formation of 15. Under same reaction conditions, treatment of 22 and 23 with 1,3-dihydro-indol-2-one (oxindole) and 1-(2,6-dichlorophenyl)-1,3-dihydro-indol-2-one (indolinone) provided products 16, 17 and 18, 19, respectively (Scheme 4).

2.7. COX-1/2 Immunoassay studies

Compounds 14-19 were screened for inhibition of catalytic activities of COX-2 and COX-1. All the enzyme inhibition assays were performed in duplicate. Compounds were tested at 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} M concentrations for COX-1/2 inhibitory activities. Catalytic activities of COX-1/2 enzymes were quantified by measuring the amount of prostaglandins produced by each enzyme in presence of various concentrations of the test compounds and comparing with control experiments.

Compound 14 showed considerable inhibition of COX-2 enzymatic activity with IC_{50} (50% inhibitory concentration) 0.7 µM while its IC_{50} for COX-1 was found to be 118 µM.
Compound 15, 16 and 18 also showed good inhibition of COX-2 with an IC\textsubscript{50} 7.3 and 6.2 µM for COX-2 enzyme (Table 5). However, 17 and 19 did not exhibit inhibition of COX-1/2 at the four concentrations used here. All the compounds showed insignificant COX-1 inhibition which is the housekeeping enzyme for the production of useful prostaglandins.

Table 5. IC\textsubscript{50} (µM) of compounds 14-19 against COX-1, COX-2 and their selectivity index

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC\textsubscript{50} (µM)</th>
<th>Selectivity Index*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(COX-2)</td>
<td>(COX-1)</td>
</tr>
<tr>
<td>14</td>
<td>0.7</td>
<td>118</td>
</tr>
<tr>
<td>15</td>
<td>6.2</td>
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<tr>
<td>16</td>
<td>7.9</td>
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<tr>
<td>17</td>
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<td>-</td>
</tr>
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<td>18</td>
<td>7.3</td>
<td>115</td>
</tr>
<tr>
<td>19</td>
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<tr>
<td>Indomethacin</td>
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<td>0.08</td>
</tr>
<tr>
<td>diclofenac</td>
<td>0.02</td>
<td>0.07</td>
</tr>
<tr>
<td>celecoxib</td>
<td>0.04</td>
<td>15</td>
</tr>
<tr>
<td>chrysin</td>
<td>25.5</td>
<td>39.3</td>
</tr>
</tbody>
</table>

*IC\textsubscript{50}(COX-1)/IC\textsubscript{50}(COX-2)

As evident from Table 5, IC\textsubscript{50} of 14 for COX-2 is better than that of chrysin. Although IC\textsubscript{50} of 14 for COX-2 is comparable to indomethacin but its selectivity for COX-2 over COX-1 is better than that of indomethacin while IC\textsubscript{50} of 14 for COX-2 is higher than that of diclofenac and celecoxib but its selectivity for COX-2 over COX-1 is better than diclofenac and poorer than celecoxib. This may be a desirable feature of compound 14 because too poor and too higher selectivity of a drug for COX-2 over COX-1 leads to side effects (as it happens in case of aspirin and was a major limitation of rofecoxib). Therefore, better/comparable inhibition and desirable selectivity index of 14 for COX-2 than the drugs based on individual components of this compound justify the design of the molecules. Moreover, compound 14 may act as a lead molecule for further refinement to increase its efficacy for COX-2 inhibition.
2.8. Molecular docking studies

In order to come across the rationale behind the type of interactions and their extent between the compounds and the enzymes, molecular docking of compounds 14-19 in the active sites of COX-1 and COX-2 was performed. Compounds were built in the same way as discussed in section 2A. Crystal co-ordinates of COX-1 (PDB ID 1EQG and 3KK6)\(^{19,48}\) and COX-2 (PDB ID 6COX and 3MQE)\(^{18,49}\) were downloaded from protein data bank (www.rcsb.org).

Compound 14 was well docked in the active site of both the crystal coordinates of COX-2 viz. 6COX and 3MQE (Figure 16-18). It showed H-bond interactions of 1.59, 2.04, 2.35 and 2.92 Å between its pyrazolic nitrogens and Thr118 amino acid of the enzyme active site. It also showed a strong H-bond interaction of 1.66 Å through its indole nitrogen towards Arg120 (Figure 16), the amino acid residue forming salt bridge with AA during the turnover phase of the enzyme. Similarly, compound 14 fit in the hydrophobic cavity of another crystal structure of COX-2 (Figure 18).

![Figure 16](image_url)

**Figure 16.** Compound 14 (in pink) docked in the active site of enzyme COX-2 (pdb ID 6COX); Yellow dotted lines indicate H-bonds formed between the compound and the enzyme active site residues. Hs’are omitted for clarity.
Figure 17. Pymol view of compound 14 in the active site of enzyme COX-2 (pdb ID 6COX)

Figure 18. CPK model of the active site of COX-2 (pdb ID 3MQE) showing fitting of compound 14 in the active site
Compound 14 was also tried to dock in the active site of the housekeeping enzyme COX-1 (pdb ID 1EQG and 3KK6) but it did not enter into the active site of COX-1 and lies outside the active site of the enzyme (Figure 19).

![Figure 19](image)

**Figure 19.** CPK mode of compound 14 docked in the active site of COX-1 (pdb ID 1EQG).

Hence, the molecular docking results, in harmony with the enzyme inhibition assay results give evidence about the interaction as well as inhibition of COX-2 with compound 14. Molecular docking of compounds 15-19 was also performed in the active site of enzymes COX-1/COX-2. Similar results were obtained on docking compounds 15-19 in the active site of COX-2 and their compatibility in the active site varies from compound to compound (Table 6).

**Table 6.** Summary of H-bond interactions and docking score of compounds 14-19 docked in the active site of COX-2 (pdb ID 6COX)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Compound</th>
<th>Hydrogen bonds</th>
<th>Docking Energy (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14</td>
<td>1.66 Å with R120, 1.59, 2.04 and 2.35 Å with T118</td>
<td>-17.91</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>2.70 Å with Y115, 2.90 Å with S119</td>
<td>-15.17</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>2.72 Å with Y115</td>
<td>-14.71</td>
</tr>
<tr>
<td>4</td>
<td>17</td>
<td>2.80 Å with S530, 2.99 Å with H351</td>
<td>-10.17</td>
</tr>
<tr>
<td>5</td>
<td>18</td>
<td>-</td>
<td>-16.13</td>
</tr>
<tr>
<td>6</td>
<td>19</td>
<td>2.34 Å with Q192</td>
<td>-11.62</td>
</tr>
</tbody>
</table>
However, compounds 15-19 did not enter the active site of COX-1 enzyme and interacted very poorly with the amino acid residues of the active site.

2.9. Analgesic (anti-nociceptive) activity evaluation

Based on the results of enzyme immunoassays, compounds 14-16 and 18 were evaluated for their anti-nociceptive activities. Same procedure was used as discussed in the previous section.

Animals were divided into 10 groups of 6 each for studying the anti-nociceptive activity of compounds 14-16 and 18 using capsaicin induced paw licking. All the treatments were administered intraperitoneally 30 min. before capsaicin injection. Group I was control wherein the animals were injected normal saline in a volume of 0.5 mL. In group II, animals were injected diclofenac at a dose of 25 mg Kg⁻¹; in group III and IV, animals were injected 14 at a dose of 5 mg Kg⁻¹ and 10 mg Kg⁻¹ respectively. Similarly, in group V and VI, animals were injected 15 at a dose of 5 mg Kg⁻¹ and 10 mg Kg⁻¹ respectively; in group VII and VIII, with 16 and in group IX and X, with 18, respectively.

**Figure 20.** Effect of compounds 14-16 and 18 on capsaicin induced pain in mice. All values are expressed as mean ± SD (n = 6 per group). Statistical significance has been calculated using one way ANOVA followed by Tukey’s multiple range comparison test. * p < 0.001 vs. control group.

The treatment with diclofenac was found to produce a marked decrease in the number of paw lickings after capsaicin injection. Compounds 14 and 16 did not produce any decrease in capsaicin induced pain at a dose of 5 mg Kg⁻¹. However, 14 was found to produce a
significant analgesic effect at a dose of 10 mg Kg\(^{-1}\) whereas 16 did not attenuate the capsaicin induced algesia even at a dose of 10 mg Kg\(^{-1}\). The effect of compound 14 at a dose of 10 mg Kg\(^{-1}\) was comparable to the standard drug diclofenac with 25 mg Kg\(^{-1}\) dose. On the other hand, both the compounds 15 and 18, showed a remarkable analgesic effect at a dose of 5 mg Kg\(^{-1}\) while not much improvement was observed at increasing the dose to 10 mg Kg\(^{-1}\) (Figure 20). However, compound 15 at 5 mg Kg\(^{-1}\) dose was found to show analgesic activity comparable to the standard drug diclofenac (25 mg Kg\(^{-1}\) dose). Overall, compounds 14, 15 and 18 were identified for promising analgesic potential.

2.10. Conclusions

The enzyme specificity of naturally occurring compounds along with their medicinal potential was utilized for the development of leads for anti-inflammatory drugs. In comparison to chromone and indole based drugs, combination of chromone and oxindole in the form of compounds 8 and 9, resulted into considerable inhibition and selectivity for COX-2 over COX-1. These two compounds exhibited IC\(_{50}\) for COX-2 in the nM range and as observed during NMR, mass spectral studies and molecular modelling, they showed preferential interactions with COX-2 over COX-1. Interestingly, the association constants of compounds 8, 9 with COX-2 were higher than the association constants of AA and aspirin with COX-2. However, their association constants with COX-1 were smaller than those of AA, aspirin and indomethacin. Compound 8 at 5 mg Kg\(^{-1}\) concentration reduced the number of paw lickings due to capsaicin induced pain in albino mice by 76% in comparison to 68% reduction on using diclofenac at a dose of 25 mg Kg\(^{-1}\) which was an indicative of better analgesic activity of 8 than that of diclofenac. Adding to all, the desirable physico-chemical properties and morphology was shown by compounds 8 and 9. The results of present studies also indicated that the compounds obtained by the combination of chrysin, indole and pyrazole are more potent for COX-2 inhibition than the drugs based on these three moieties, individually as well as the molecules prepared by the hybridization of only two significant moieties. As per the results of enzyme immunoassays, it was found that on introduction of one more biologically relevant moiety, COX-1 inhibition was almost lost (for all the compounds under study) which performs the housekeeping functions. Compounds 14, 15 and 18 exhibited significant activity for inhibition of COX-2. Molecular modelling studies indicating considerable interactions of these compounds with COX-2 active site amino acids also favour the enzyme immunoassay results. Hence, compounds 8, 9, 14, 15 and 18 are identified with significant anti-inflammatory and anti-nociceptive activities.
2.11. Experimental protocols

2.11.1. General methods

Melting points were determined in capillaries and were uncorrected. $^1$H and $^{13}$C NMR spectra were recorded on JEOL 300 MHz and 75 MHz NMR spectrometer, respectively using CDCl$_3$ and/or DMSO-$d_6$ as solvent. Chemical shifts are given in ppm with TMS as an internal reference. $J$ values are given in Hertz. Signals are abbreviated as singlet, s; doublet, d; double-doublet, dd; triplet, t; multiplet, m; bridging hydrogen, =H. Elemental analysis was performed on Thermoelectron FLASH EA1112 CHN analyzer. Reactions under microwaves were performed using microwave oven (INALSA model 1MW17EG) with microwave power 700 W and operating frequency 2450 MHz. Mass spectra were recorded on Bruker micrOTOF QII Mass spectrometer. SEM images were recorded on Zeiss Supra 55 Scanning Electron Microscope. Powder XRD was performed on Rigaku Miniflex II desktop X-ray diffractometer (XRD).

2.11.2. General Procedure for the preparation of compounds 5-10

A finely ground mixture of chromone-3-carboxaldehyde (1 mmol) and oxindole / indolinone (1-(2,6-dichlorophenyl)-2-indolinone)/ 1-(3-chlorophenyl)-3-methyl-2-pyrazolin-5-one (1.2 mmol) was irradiated in microwave oven for 2-10 min. and completion of the reaction was monitored with TLC. Reaction mixture was washed with diethylether and then purified by column chromatography and recrystallization to get pure compounds 5-10.

2.11.3. 1-(2,6-Dichlorophenyl)-3-(4-oxo-4H-chromen-3-ylmethylene)-1,3-dihydroindol-2-one (5)

Yellow solid, 87% yield, mp 242 °C; IR $\nu_{\text{max}}$ (KBr, cm$^{-1}$): 1720, 1680 (C=O); $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ ppm: 6.44 (d, $J = 7.8$ Hz, 1H, ArH), 7.16 (t, $J = 7.5$ Hz, 1H, ArH), 7.21-7.26 (m, 1H, ArH), 7.37-7.42 (m, 1H, ArH), 7.54 (d, $J = 8.4$ Hz, 3H, ArH), 7.67-7.77 (m, 3H, ArH), 8.17 (s, 1H, =H), 8.30-8.33 (dd, $J = 1.5$ Hz, $J = 8.1$ Hz, 1H, ArH), 10.27 (s, 1H, indole 2-H); $^{13}$C NMR (75 MHz, CDCl$_3$ + DMSO-$d_6$) $\delta$ ppm: 108.6, 117.3, 118.4, 120.0, 122.8, 125.0, 125.5, 125.9, 127.2, 129.0, 129.3, 129.5, 131.6, 134.4, 134.6, 139.7, 155.4, 160.1, 164.5 (C=O), 174.6 (C=O); HRMS (ESI) Calcd for [C$_{24}$H$_{13}$Cl$_2$O$_3$N + Na]$^+$: 456.0165, Found: 456.0169 [M+Na]$^+$; Anal. For C$_{24}$H$_{13}$Cl$_2$O$_3$N Calcd%: C 66.38, H 3.02, N 3.23; Found%: C 63.73, H 3.24, N 3.75.
2.11.4. 3-(6-Bromo-4-oxo-4H-chromen-3-ylmethylene)-1-(2,6-dichlorophenyl)-1,3-dihydroindol-2-one (6)

Yellow solid, 87% yield, mp 229 °C; IR ν_max (KBr, cm⁻¹): 1722, 1655 (C=O); ¹H NMR (300 MHz, CDCl₃) δ ppm: 6.43 (d, J = 7.8 Hz, ArH), 7.16 (t, J = 7.5 Hz, 1H, ArH), 7.22-7.25 (m, 1H, ArH), 7.37-7.43 (m, 2H, ArH), 7.52-7.55 (m, 2H, ArH), 7.75-7.80 (m, 2H, ArH), 8.10 (s, 1H, =H), 8.44 (s, 1H, ArH), 10.25 (s, 1H, indole 2-H); ¹³C NMR (75 MHz, CDCl₃ + DMSO-d₆) δ ppm: 108.5, 117.6, 118.5, 119.6, 120.3, 122.5, 124.3, 125.3, 125.9, 127.8, 128.5, 129.0, 130.7, 134.7, 136.6, 139.7, 154.1, 160.1, 164.6 (C=O), 173.6 (C=O); HRMS (ESI) Calcd for [C₂₄H₁₂Br₂Cl₂O₃N + Na]⁺: 533.9270, 535.9249, 537.9221, Found: 533.9205, 535.9185, 537.9165 [M+Na]⁺; Anal. For C₂₄H₁₂Br₂Cl₂O₃N Calcd% C 56.17, H 2.36, N 2.73; Found%: C 56.17, H 2.34, N 2.75.

2.11.5. 1-(2,6-Dichlorophenyl)-3-(6-isopropyl-4-oxo-4H-chromen-3-ylmethylene)-1,3-dihydroindol-2-one (7)

Yellow solid, 91% yield, mp 232 °C; IR ν_max (KBr, cm⁻¹): 1730, 1675 (C=O); ¹H NMR (300 MHz, CDCl₃) δ ppm: 1.32 (d, J = 6.6 Hz, 6H, 2×CH₃), 3.02-3.11 (m, 1H, CH), 6.44 (d, J = 7.5 Hz, 1H, ArH), 7.14-7.24 (m, 2H, ArH), 7.43 (t, J = 7.5 Hz, 2H, ArH), 7.52-7.59 (m, 3H, ArH), 7.78 (d, J = 7.5 Hz, 1H, ArH), 8.15 (s, 1H, =H), 8.19 (s, 1H, ArH), 10.28 (s, 1H, indole 2-H); ¹³C NMR (75 MHz, CDCl₃) δ ppm: 24.2 (CH₃), 28.8 (CH₃), 116.6, 117.2, 118.2, 118.7, 118.8, 119.2, 123.6, 124.9, 126.4, 126.5, 127.3, 129.8, 130.4, 134.5, 136.6, 139.2, 151.5, 156.0, 162.5, 164.1 (C=O), 175.3 (C=O); HRMS (ESI) Calcd for [C₂₇H₁₂BrCl₂O₃N + Na]⁺: 498.0634, Found: 498.0647 [M+Na]⁺; Anal. For C₂₇H₁₂BrCl₂O₃N Calcd% C 69.08, H 4.02, N 2.94; Found%: C 69.11, H 4.03, N 2.91.

2.11.6. 3-(4-Oxo-4H-chromen-3-ylmethylene)-1,3-dihydroindol-2-one (8)

Yellow solid, 78% yield, mp 230 °C; ¹H NMR (300 MHz, DMSO-d₆) δ ppm: 6.79 (d, J = 7.8 Hz, 1H, ArH), 6.90 (t, J = 7.5 Hz, 1H, ArH), 7.11 (t, J = 7.5 Hz, 1H, ArH), 7.33-7.48 (m, 1H, ArH), 7.56 (d, J = 7.5 Hz, 1H, ArH), 7.70-7.74 (m, 2H, ArH), 7.91 (s, 1H, =H), 8.01-8.10 (dd, J = 1.2 Hz, J = 7.2 Hz, 1H, ArH), 10.03 (s, 1H, indole 2-H), 10.48 (s, 1H, NH, D₂O exchange); ¹³C NMR (75 MHz, DMSO-d₆) δ ppm: 109.6, 117.3, 118.6, 119.8, 121.4, 123.1, 124.0, 124.9, 125.4, 126.0, 127.6, 129.4, 134.6, 140.7, 155.4, 159.9, 167.5 (C=O), 174.9 (C=O); HRMS (ESI) Calcd for [C₁₈H₁₁O₃N + Na]⁺: 312.0631, Found: 312.0626 [M+Na]⁺; Anal. For C₁₈H₁₁O₃N Calcd% C 74.73, H 3.83, N 4.84; Found%: C 74.65, H 3.64, N 4.98.
2.11.7. 3-(6-Bromo-4-oxo-4H-chromen-3-ylmethylene)-1,3-dihydroindol-2-one (9)

Yellow solid, 75% yield, mp >300 °C; \(^1\)H NMR (300 MHz, DMSO-\(d_6\)) \(\delta\) ppm: 6.84 (d, \(J = 7.8\) Hz, 1H, ArH), 6.99 (t, \(J = 7.5\) Hz, 1H, ArH), 7.23 (t, \(J = 7.8\) Hz, 1H, ArH), 7.64 (d, \(J = 7.5\) Hz, 1H, ArH), 7.70 (s, 1H, =H), 7.73 (d, \(J = 9.0\) Hz, 1H, ArH), 7.99-8.03 (dd, \(J = 2.7\) Hz, \(J = 9.0\) Hz, 1H, ArH), 8.20 (d, \(J = 2.4\) Hz, 1H, ArH), 9.91 (s, 1H, indole 2-H), 10.71 (s, 1H, NH, D\(_2\)O exchange); \(^1^3\)C NMR (75 MHz, DMSO-\(d_6\)) \(\delta\) ppm: 109.7, 117.5, 118.5, 120.1, 121.5, 123.9, 124.6, 125.5, 127.5, 128.1, 129.6, 130.3, 137.2, 140.8, 154.5, 160.1, 164.7 (C=O), 173.9 (C=O); HRMS (ESI) Calcd for \([C_{18}H_{10}BrO_3N + H]^+\): 367.9917, 369.9897, Found: 367.9905, 369.9889 [M+H]\(^+\); Anal. For C\(_{18}\)H\(_{10}\)BrO\(_3\)N Calcd%: C 58.72, H 2.74, N 3.80; Found%: C 58.70, H 2.79, N 3.55.

2.11.8. 2-(3-Chlorophenyl)-4-(6-fluoro-8-nitro-4-oxo-4H-chromen-3-ylmethylene)-5-methyl-2,4-dihydropyrazol-3-one (10)

Orange solid, 89% yield, mp 230 °C; IR \(\nu_{\text{max}}\) (KBr, cm\(^{-1}\)): 1730, 1653 (C=O); \(^1\)H NMR (300 MHz, DMSO-\(d_6\)) \(\delta\) ppm: 2.35 (s, 3H, CH\(_3\)), 7.26 (d, \(J = 7.8\) Hz, 1H, ArH), 7.39-7.49 (m, 1H, ArH), 7.79-7.96 (m, 2H, ArH), 8.07 (s, 1H, =H), 8.24-8.27 (dd, \(J = 3.0\) Hz, \(J = 7.5\) Hz, 1H, ArH), 8.62-8.65 (dd, \(J = 3.0\) Hz, \(J = 7.5\) Hz, 1H, ArH), 10.30 (s, 1H, indole 2-H); \(^1^3\)C NMR (75 MHz, CDCl\(_3\) + DMSO-\(d_6\)) \(\delta\) ppm: 12.7 (CH\(_3\)), 116.2, 116.9, 117.1, 117.4, 118.6, 119.8, 124.4, 125.2, 127.9, 130.5, 133.3, 135.4, 147.0, 151.6, 161.7, 162.3, 172.3 (C=O), 177.7 (C=O); HRMS (ESI) calcd for \([C_{20}H_{12}ClFO_5N_3 + H]^+\): 428.0444, 430.0416, Found: 428.0450, 430.0450 [M+H]\(^+\); Anal. For C\(_{20}\)H\(_{12}\)ClFO\(_5\)N\(_3\) Calcd%: C 56.15, H 2.59, N 9.82, Found%: C 56.14, H 2.60, N 9.83.

2.11.9. General Procedure for the synthesis of compounds 14-19

Compounds 14-19 were prepared through the condensation of compounds 22 and 23 (1 mmol) with active methylene compounds including 1-(3-chlorophenyl)-3-methyl-2-pyrazolin-5-one, oxindole and indolinone (1 mmol). The reactants were heated at 160-180 °C for about 10-20 min. in oil bath and the completion of the reaction was monitored using TLC. The crude products were solidified by triturating with diethyl ether and purified by recrystallization and column chromatography.
2.11.10. 4-{1-[3-(5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)-propyl]-1H-indol-3-ylmethylene}-5-methyl-2-phenyl-2,4-dihydro-pyrazol-3-one (14)

Yellow solid, 87% yield, mp 202 °C; \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) ppm: 2.38 (s, 3H, CH\(_3\)), 2.46 (t, \(J = 6\) Hz, 2H, CH\(_2\)), 4.05 (t, \(J = 5.4\) Hz, 2H, CH\(_2\)), 4.51 (t, \(J = 6.3\) Hz, 2H, CH\(_2\)), 6.28 (s, 1H, ArH), 6.44 (s, 1H, ArH), 6.60 (s, 1H, ArH), 7.07 (d, \(J = 7.8\) Hz, 1H, ArH), 7.14-7.36 (m, 4H, ArH), 8.05 (s, 1H, ArH), 9.86 (s, 1H, ArH), 12.69 (s, 1H, OH); \(^1\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) ppm: 13.1 (CH\(_3\)), 29.3 (CH\(_2\)), 44.2 (CH\(_2\)), 64.9 (CH\(_2\)), 93.1, 98.6, 105.8, 110.6, 112.2, 116.6, 118.2, 118.7, 119.6, 122.8, 123.9, 124.1, 126.2, 129.0, 129.1, 129.6, 131.7, 134.3, 135.2, 136.7, 140.3, 143.9, 150.8, 157.7, 162.1, 163.3, 163.9, 164.2, 182.3; HRMS Calcd for C\(_{37}\)H\(_{28}\)ClO\(_5\)N\(_3\): 652.1610. Found: \(m/z\) 652.1615 [M+Na]\(^+\).

2.11.11. 4-{1-[4-(5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)-butyl]-1H-indol-3-ylmethylene}-5-methyl-2-phenyl-2,4-dihydro-pyrazol-3-one (15)

Yellow solid, 60% yield, mp 200 °C; \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) ppm: 1.89-1.94 (m, 2H, CH\(_2\)), 2.18-2.23 (m, 2H, CH\(_2\)), 2.40 (s, 3H, CH\(_3\)), 4.04 (t, \(J = 6\) Hz, 2H, CH\(_2\)), 4.39 (t, \(J = 6\) Hz, 2H, CH\(_2\)), 6.25 (s, 1H, ArH), 6.45 (s, 1H, ArH), 6.60 (s, 1H, ArH), 7.23 (d, \(J = 7.2\) Hz, 1H, ArH), 7.79-7.85 (m, 5H, ArH), 8.09 (s, 1H, ArH), 9.89 (s, 1H, ArH), 12.69 (s, 1H, OH); \(^1\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) ppm: 13.0 (CH\(_3\)), 29.1 (CH\(_2\)), 44.1 (CH\(_2\)), 64.6 (CH\(_2\)), 93.0, 98.5, 105.7, 110.5, 112.1, 116.5, 118.1, 118.6, 119.5, 122.7, 123.8, 124.0, 126.1, 128.9, 129.0, 129.5, 131.1, 131.6, 135.1, 136.6, 140.2, 150.7, 157.6, 162.0, 163.1, 163.9, 164.2, 182.4; HRMS Calcd for C\(_{38}\)H\(_{30}\)ClO\(_5\)N\(_3\): 644.1947. Found: \(m/z\) 644.1932 [M+H]\(^+\).

2.11.12. 3-{1-[3-(5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)-propyl]-1H-indol-3-ylmethylene}-1,3-dihydro-indol-2-one (16)

Yellow solid, 60% yield, mp 225 °C; \(^1\)H NMR (300 MHz, DMSO-\(d_6\)) \(\delta\) ppm: 2.31-2.48 (m, 2H, CH\(_2\)), 4.11 (t, \(J = 5.1\) Hz, 2H, CH\(_2\)), 4.52 (t, \(J = 7.2\) Hz, 2H, CH\(_2\)), 6.35-6.39 (m, 1H, ArH), 6.71 (d, \(J = 6\) Hz, 1H, ArH), 6.81-6.84 (m, 1H, ArH), 6.96 (s, 1H, ArH), 7.06-7.13 (q, 1H, ArH), 7.19-7.30 (m, 2H, ArH), 7.53-7.74 (m, 6H, ArH), 7.84 (d, \(J = 6\) Hz, 1H, ArH), 8.01-8.29 (m, 4H, ArH), 9.47 (s, 1H, ArH), 10.46 (d, \(J = 11.4\) Hz, 1H, ArH); \(^1\)C NMR (75 MHz, DMSO-\(d_6\)) \(\delta\) ppm: 26.1 (CH\(_2\)), 46.9 (CH\(_2\)), 67.1 (CH\(_2\)), 92.7, 93.3, 98.7, 105.8, 108.6, 111.6, 118.2, 118.3, 121.7, 122.2, 122.4, 123.1, 126.2, 126.9, 127.9, 129.0, 129.1, 129.4,
135.9, 136.0, 136.2, 157.7, 162.3, 164.0, 164.1, 164.4, 182.2; HRMS Calcd for C\textsubscript{35}H\textsubscript{26}O\textsubscript{5}N\textsubscript{2}: 555.1914. Found: m/z 555.1918 [M+H]\textsuperscript{+}.

2.11.13. 3-{1-[4-(5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)-butyl]-1H-indol-3-ylmethylene}-1,3-dihydro-indol-2-one (17)

Yellow solid, 78% yield, mp 156 °C; \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) δ ppm: 1.86-1.89 (m, 2H, CH\textsubscript{2}), 2.15-2.18 (m, 2H, CH\textsubscript{2}), 4.02 (t, J = 5.7 Hz, 2H, CH\textsubscript{2}), 4.33 (t, J = 6.3 Hz, 2H, CH\textsubscript{2}), 6.28-6.33 (m, 1H, ArH), 6.42 (s, 1H, ArH), 6.61-6.65 (m, 1H, ArH), 6.84-6.91 (m, 2H, ArH), 7.05 (t, J = 7.2 Hz, 1H, ArH), 7.16 (t, J = 6.6 Hz, 1H, ArH), 7.29-7.60 (m, 6H, ArH), 7.80-7.98 (m, 4H, ArH), 8.10 (s, 1H, ArH), 9.49 (s, 1H, OH); \textsuperscript{13}C NMR (75 MHz, CDCl\textsubscript{3}) δ ppm: 29.3 (CH\textsubscript{2}), 29.5 (CH\textsubscript{2}), 43.6 (CH\textsubscript{2}), 65.8 (CH\textsubscript{2}), 92.8, 93.0, 98.5, 105.8, 108.6, 111.5, 118.1, 118.2, 121.6, 122.1, 122.2, 123.0, 126.2, 126.8, 127.8, 128.2, 128.9, 129.0, 129.1, 135.8, 135.9, 136.2, 157.6, 162.1, 163.9, 164.2, 164.3, 182.4; HRMS Calcd for C\textsubscript{36}H\textsubscript{28}O\textsubscript{5}N\textsubscript{2}: 591.1890. Found: m/z 591.1880 [M+Na]\textsuperscript{+}.

2.11.14. 1-(2,6-dichloro-phenyl)-3-{1-[3-(5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)-propyl]-1H-indol-3-ylmethylene}-1,3-dihydro-indol-2-one (18)

Yellow solid, 69% yield, mp 146 °C; \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) δ ppm: 2.12-2.17 (m, 2H, CH\textsubscript{2}), 3.98 (t, J = 6 Hz, 2H, CH\textsubscript{2}), 4.31 (t, J = 6.6 Hz, 2H, CH\textsubscript{2}), 6.30 (d, J = 15 Hz, 1H, ArH), 6.40-6.42 (m, 1H, ArH), 6.62 (d, J = 15 Hz, 1H, ArH), 6.84-6.91 (m, 2H, ArH), 7.04 (t, J = 7.2 Hz, 1H, ArH), 7.15 (t, J = 7.5 Hz, 1H, ArH), 7.28-7.59 (m, 7H, ArH), 7.77-7.93 (m, 5H, ArH), 8.11 (d, J = 9.3 Hz, 1H, ArH), 9.48 (s, 1H, ArH), 12.67 (s, 1H, OH); \textsuperscript{13}C NMR (75 MHz, CDCl\textsubscript{3}) δ ppm: 26.4 (CH\textsubscript{2}), 46.8 (CH\textsubscript{2}), 67.2 (CH\textsubscript{2}), 93.2, 98.7, 105.9, 108.8, 110.4, 118.4, 121.6, 121.7, 122.3, 123.1, 126.4, 126.9, 128.0, 129.1, 129.2, 130.6, 131.4, 131.9, 136.1, 136.2, 137.2, 139.0, 157.9, 162.3, 164.1, 164.2, 164.9, 182.5; HRMS Calcd for C\textsubscript{41}H\textsubscript{28}Cl\textsubscript{2}O\textsubscript{5}N\textsubscript{2}: 699.1448. Found: m/z 699.1421 [M+H]\textsuperscript{+}.

2.11.15. 1-(2,6-dichloro-phenyl)-3-{1-[4-(5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)-butyl]-1H-indol-3-ylmethylene}-1,3-dihydro-indol-2-one (19)

Yellow solid, 67% yield, mp 120 °C; \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) δ ppm: 1.62-1.89 (m, 2H, CH\textsubscript{2}), 2.09-2.16 (m, 2H, CH\textsubscript{2}), 3.98-4.06 (dt, 2H, CH\textsubscript{2}), 4.26-4.36 (dt, 2H, CH\textsubscript{2}), 6.29-6.39 (m, 1H, ArH), 6.41-6.46 (m, 2H, ArH), 6.64 (d, J = 3.3 Hz, 1H, ArH), 7.14-7.53 (m, 9H, ArH), 7.83-7.97 (m, 5H, ArH), 8.09 (s, 1H, ArH), 8.24 (s, 1H, ArH), 9.50 (s, 1H, ArH), 12.66 (s, 1H, OH); \textsuperscript{13}C NMR (75 MHz, CDCl\textsubscript{3}) δ ppm: 26.8 (CH\textsubscript{2}), 27.1 (CH\textsubscript{2}), 47.3 (CH\textsubscript{2}), 68.1
(CH₂), 93.4, 98.9, 106.1, 109.0, 110.6, 111.7, 117.8, 118.4, 118.6, 121.9, 122.6, 123.0, 123.3, 126.6, 126.7, 127.2, 128.2, 129.3, 129.4, 129.5, 130.8, 131.7, 132.1, 136.3, 136.4, 137.4, 139.2, 157.5, 162.5, 163.7, 163.8, 165.1, 182.7; HRMS Calcd for C₄₂H₃₀Cl₂O₅N₂: 735.1424. Found: m/z 735.1408 [M+Na]+.

### 2.12. Docking procedure

Compounds were built using the builder tool kit of the software package Argus Lab 4.0.1 and energy minimized with semi-empirical quantum mechanical method PM3.

![Figure 21](image.png)

**Figure 21.** Celecoxib (purple) docked in the active site of COX-2 (pdb ID 6COX) overlaps with native (part of enzyme crystal) celecoxib (pink), validating the docking procedure

Crystal co-ordinates of COX-1 (PDB ID 1EQG, 3KK6) and COX-2 (PDB ID 6COX, 3MQE) were downloaded from protein data bank and in the molecule tree view of the software, the monomeric structure of the crystal co-ordinate was selected and the active site was defined as 15 Å around the ligand. Validation of the docking programme was checked by docking celecoxib in the binding site of COX-2 (Figure 21).

The molecule to be docked in the active site of the enzyme was pasted in the work space carrying the structure of the enzyme. The docking programme implements an efficient
grid based docking algorithm which approximates an exhaustive search within the free volume of the binding site cavity. The conformational space was explored by the geometry optimization of the flexible ligand (rings were treated as rigid) in combination with the incremental construction of the ligand torsions. Thus, docking occurs between the flexible ligand parts of the compound and enzyme. The docking was repeated several times (approx. 10000 iterations) until no change in the position of the ligand and a constant value of the binding energy was observed. The ligand orientation was determined by a shape scoring function based on Ascore and the final positions were ranked by lowest interaction energy values. Finally, H-bonds and hydrophobic interactions between the respective compound and enzyme were explored.

2.13. Procedure for COX-1/2 inhibitory immunoassay

For studying the COX-1, COX-2 inhibitory activities of the compounds, various reagents were prepared as per the protocol of the assay. The background samples were prepared for both COX-1 (ovine) and COX-2 (human recombinant) by taking 20 µL of each enzyme in separate test tubes and keeping them in boiling water for 3 minutes. The inactivated enzymes were used to generate background values. In two test tubes named background COX-1 and background COX-2, 970 µL of reaction buffer, 10 µL of heme and 10 µL of inactive COX-1 or COX-2 were added. 100% initial activity tubes were prepared for both COX-1 and COX-2 by adding 950 µL of reaction buffer, 10 µL of heme and 10 µL of COX-1 or COX-2. Inhibitor tubes were prepared for various compounds. In each sample tube, 950 µL reaction buffer, 10 µL of heme, 10 µL of COX-1 or COX-2 enzyme and 20 µL of the inhibitor solution was added. All the solutions were incubated for 10 minutes at 37 °C. After incubation, 10 µL of AA was added to all the test tubes and vortex. They were again incubated for another 2 minutes. Afterwards, 50 µL of 1M HCl was added to each test tube to stop the reaction. Then 100 µL of stannous chloride solution was added to each test tube and vortex. Incubated for another 5 minutes and kept at 0-4 °C.

Prostaglandin screening standards were prepared as test tubes S1- S8. 800 µL of EIA buffer was added to S1 and 500 µL of the same was added to S2-S7. Then 200 µL of bulk standard (10 ng/mL) was added to tube S1 and mixed thoroughly. The standards were diluted serially by removing 500 µL from tube S1 and placing it in tube S2 and mixed thoroughly. Same process was repeated from S2-S3, S3-S4 upto S7-S8.
To make dilutions for COX reactions, two test tubes named BC1 and BC2 were prepared. To each test tube was added, 990 µL of EIA (enzyme immunoassay) buffer and 10 µL of background COX-1 or COX-2 and mixed thoroughly. COX 100% initial activity samples were prepared as three test tubes for COX-1 and COX-2 both and numbered as IA1-IA3. For each sample, aliquot 990 µL of EIA buffer to IA1, 950 µL of EIA buffer to IA2 and 500 µL of EIA buffer to IA3. 10 µL of COX-1 or COX-2 100% initial activity sample was added to IA1 and mixed thoroughly. Aliquot, 50 µL of tube IA1 and added to tube IA2 and mixed thoroughly. Again aliquot 500 µL from test tube IA2 and added to test tube IA3 and mixed well. In the same manner, COX inhibitor samples were prepared by further dilutions and named C1-C3 for each concentration.

After preparing all the dilutions, they were introduced on 96 well plate. The wells were distributed as blank-1A, NSB (Non-specific binding)-1B and B₀ (Maximum binding)-1C. Well 1H was named as TA (Total activity well). Wells 2A-2H were used for S1-S8 and 3A-3H were used for S1-S8 duplicate. Wells 4A and 5A were prepared as BC1 and its duplicate. Similarly, for BC2 wells 4B and 5B were prepared. Remaining wells were used for inhibitor samples for COX-1 and COX-2.

Addition of the reagents on 96-well plate was performed as follows:

100 µL EIA buffer was added to NSB well and 50 µL of EIA buffer was added to B₀ well. 50 µL of Prostaglandin screening standard was added to the respective wells S1-S8 from their respective test tubes S1-S8 and duplicated. 50 µL of BC1 and BC2 were added per well and in duplicate. 50 µL of 100% initial activity samples were added per well and only IA2 and IA3 were assayed in duplicate for both COX-1 and COX-2. 50 µL of COX inhibitor sample was added per well from their respective dilutions (only C2 and C3 were assayed). 50 µL of PG screening AchE tracer was added to each well except TA and Blank well. At last, 50 µL of PG screening EIA antiserum was added to each well except TA, NSB and blank wells. The plate was then covered with plastic film and was incubated for 18 hours at room temperature. After incubation, the plate was developed by emptying the wells and rinsing the wells with wash buffer for five times. After washing the wells, 200 µL of Ellman’s reagent was added to each well and 5 µL of tracer was added to Total activity well. The plate was covered with plastic film and it was kept for 60-90 minutes. Before reading the plate, it was wiped from bottom to remove any fingerprints and finally read at 420 nm.
Calculation of %age inhibition and IC\textsubscript{50} values

%B/B\textsubscript{o} value for each sample (at different concentrations used) was determined from the absorbance values attained after reading the 96 well plate at 420 nm according to the calculation strategy provided in the manufacturer’s protocol for inhibition assay.

From the values of %B/B\textsubscript{o}, concentration of prostaglandins formed during the enzymatic reaction were calculated for all the compounds at all concentrations with the help of the standard curve. After having the prostaglandin concentration at each tested concentration for all the compounds, percentage inhibition values were calculated at all the tested concentration according to the prescribed protocol.

Finally, the percentage inhibitions at all the concentrations were plotted against the respective tested concentration for each compound using Graph Pad Prism version 6.01 for calculating IC\textsubscript{50} (50% inhibitory concentration).

2.14. Procedure for 5-LOX inhibitory immunoassay

The reagents were prepared according to the standard protocol (Lipoxygenase inhibitor screening assay kit, Item No. 760700, Cayman Chemicals Co). Stock solutions of the compounds were prepared so as to obtain concentration of 10\textsuperscript{-4}, 10\textsuperscript{-5}, 10\textsuperscript{-6}, 10\textsuperscript{-7}, 10\textsuperscript{-8} and 10\textsuperscript{-9} M in the respective wells. The prepared solutions were then introduced on 96 well plate where the cells were distributed as blank-1A-2A-1D (triplicate), positive control- 1B-2B (duplicate), 100% initial activity wells- 1C-2C-2D (triplicate). Remaining wells were designated to the inhibitor solutions in duplicate/triplicate. The addition of the reagents was done according to the standard protocol, according to which, 100 µL of assay buffer was added to the blank wells, 90 µL of lipoxygenase (5-LOX, potato) enzyme and 10 µL of assay buffer was added to +ve control wells. To 100% initial activity wells, 90 µL of lipoxygenase enzyme and 10 µL of solvent (DMSO) was added. All the inhibitor (compound) wells were charged with 90 µL of lipoxygenase enzyme and 10 µL of respective stock (compound) solution. The reaction was initiated by adding 10 µL of the substrate (AA) to all the wells. The plate was then shaken for five minutes on an orbital shaker. Ultimately, 100 µL of chromogen solution (prepared according to standard protocol) was added to each well to stop enzyme catalysis. The plate was incubated for half an hour and was read at 500 nm.
2.15. Procedure for recording mass spectra

All the mass spectra were recorded on Bruker Micro TOF QII machine. Solutions of 11 μM (concentration calculated from UV spectra) of COX-1 and COX-2 in ACN-H₂O (acetonitrile-water) (7:3) in presence of 10 mM ammonium acetate buffer were prepared. Solutions of AA and compounds with the enzyme were prepared with four different concentrations of the ligand viz. 20, 40, 60 and 80 μM. The mass spectra of these solutions were recorded in +ve and –ve modes at different source temperatures (100-180 °C) and collision energies (2-12 eV). Most consistent results were obtained in +ve mode at source temperature 120 °C and collision energy 5-7 eV. Therefore, all the mass spectra were recorded under these conditions and deconvoluted to get m/z.

The association constants were calculated using equation 1.

\[ K_a = \frac{[EL]}{[E]_{free} \times [L]_{free}} \]

(1)

where \( I_{EL} \) and \( I_E \) are the intensities of mass peaks of EL complex and enzyme, respectively.

The concentrations of free enzyme ([E]_{free}) and free ligand ([L]_{free}) were calculated by subtracting the concentration of EL complex ([EL]) from the total concentration of enzyme and ligand, respectively. As apparent from equation 1, concentration of free enzyme and free ligand were taken into account after the complete complexation occurred and hence instead of stepwise complexation, overall binding constant, \( K_a \) were calculated from this equation irrespective of the stoichiometry of enzyme – ligand complex.

2.16. Procedure for recording NMR spectra

\(^1\)H NMR spectra of pure compounds (0.35 mM) were recorded in DMSO-\(d_6\) and deuterium exchange (using \(D_2O\)) was performed to identify the NH signals. 12 μL (0.08 mM) of COX-2/ COX-1 was added to the compound solutions in DMSO-\(d_6\) and the contents of NMR tube were incubated at 37 °C and NMR spectra were recorded after each 30 min.

2.17. REFERENCES


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