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Rationally designed hybrid molecules with appreciable COX-2 inhibitory and anti-nociceptive activities

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ABSTRACT

Six molecules were obtained by the combination of three biologically and medicinally significant moieties—indole, chrysin and pyrazole. Bio-evaluation of these hybrid molecules showed significant inhibition of COX-2 enzymatic activity over that of COX-1 and appreciable anti-nociceptive activity, checked at swiss albino mice.

Inflammation is an innate immunity response against harmful stimulus which leads to the activation of arachidonic acid (AA) metabolism and hence more production of prostaglandins and leukotrienes.[1] These metabolites trigger the signals for induction of inflammation-symptomised as pain, redness, fever, etc. COX-2 is the enzyme associated with production of inflammatory metabolites during arachidonic acid metabolism[2] and hence a primary target of anti-inflammatory drugs.[3] However, the process of blockage of COX-2 enzyme through the use of various inhibitors suffers from inhibition of COX-1 which is structurally an isoform of COX-2 but associated with production of desirable prostaglandins in arachidonic acid pathway.[4] Starting with the use of aspirin as COX-1/2 nonselective drug,[5] treatment of inflammation travelled a long journey up to the use of selective COX-2 inhibitors, the COXIBS.[6] However, the side effects associated with the use of COXIBS and withdrawal of some of them from the market[7] necessitated the search for their substitutes.

The hybrid molecules, obtained by the combination of two bioactive molecules, exhibit significantly higher biological activity in comparison to their individual fragments,[8,9] a number of such molecules containing indole–pyrazole, indole–pyrimidine, indole–chromone are reported by our group for anti-cancer and COX-2 inhibitory activities.[10–12] For the present studies, another biologically significant moiety chrysin (**1**, Chart 1) was combined with indole (**2**, Chart 1) and pyrazole (**3**, Chart 1) and through appropriate combination of these three moieties, compounds **4**–**9** were procured. While chrysin is an important member of flavone class of natural products identified with anti-oxidant,[13,14] anti-bacterial,[15] anti-inflammatory,[16,17] anti-cancer,[18] antiestrogenic,[19] anxiolytic[20] activities and a number of its analogues like apigenin,[21] tectorigenin[22] and wogonin[23] show significant anti-inflammatory activities, the biological potential of indole and pyrazole is also well established.[24–29] Therefore, it is envisaged that the combination of three biologically significant heterocycles may result in the development of more efficacious molecules.

N-alkylation of 3-formyl indole (1 mmol) with 1,3-dibromopropane (1 mmol) and 1,4-dibromobutane (1 mmol) provided compounds **10** and **11** (Scheme 1).

In the next step, chrysin, (**1**, 1 mmol) was refluxed with compounds **10** and **11** in presence of potassium carbonate (1 mmol) in acetone. The reaction was performed under N_2 atmosphere to procure compounds **12** and **13** (Scheme 2).

Compound **12** (100 mg, 1 mmol) on treatment with 1-(3-chlorophenyl)-3-methyl-2-pyrazolin-5-one (1 mmol) at 160–180 °C for 10–20 min provided compound **4**. Similarly, reaction of compound **13** (100 mg, 1 mmol) with 1-(3-chlorophenyl)-3-methyl-2-pyrazolin-5-one gave product **5**. Under same reaction conditions, treatment of compounds **12** and **13** with 1,3-dihydro-indol-2-one (oxindole) and 1-(2,6-dichlorophenyl)-1,3-dihydro-indol-2-one (indolinone) provided products **6**–**9**, respectively (Scheme 3).[30]

Compounds **4**–**9** were screened for inhibition of catalytic activities of COX-2 and COX-1, the enzymes associated with arachidonic

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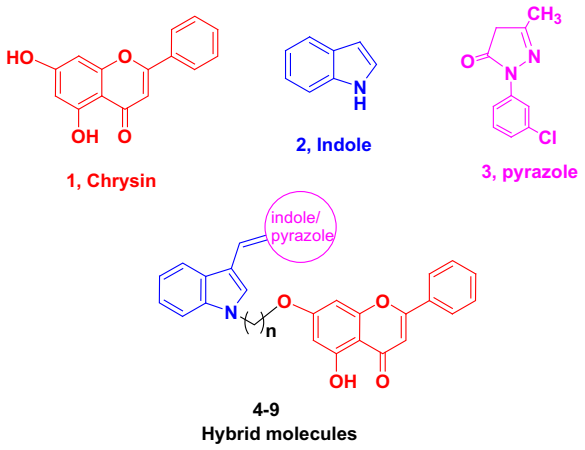
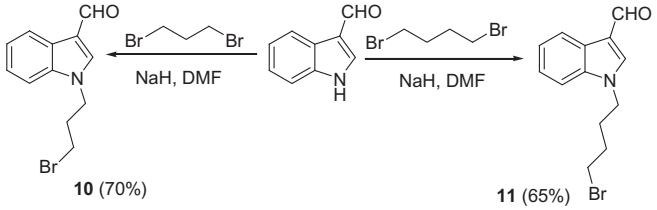


Chart 1.



Scheme 1.

acid metabolism producing prostaglandins. All the enzyme inhibition assays were performed in duplicate as per the manufacturer's protocol available along with the enzyme immunoassay kits[31] and the difference between two readings was 3–5%. Compounds were tested at 10^{-5} M, 10^{-6} M, 10^{-7} M and 10^{-8} M concentrations for COX-1/2 inhibitory activities and based on these results, compounds were further screened at 10^{-3} M, 10^{-4} M, 10^{-5} M and 10^{-6} M concentrations for COX-1 inhibition. 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.

In general, inhibition of enzymatic activity by test compound $\propto$ 1/conc of prostaglandins produced during enzymatic reaction.

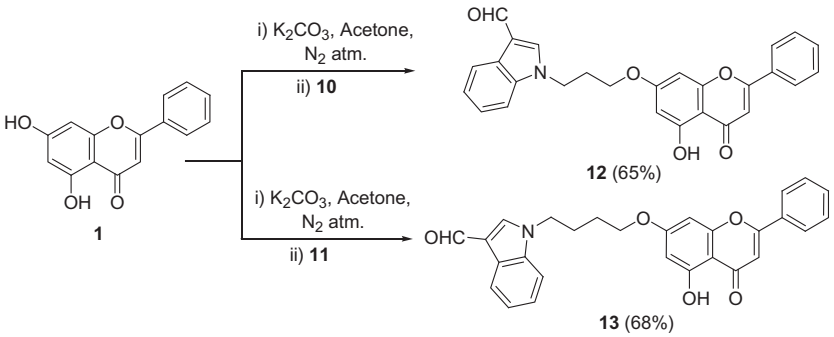
Compound **4** showed considerable inhibition of COX-2 enzymatic activity with IC_{50} (50% inhibitory conc) 0.7 μM while its IC_{50} for COX-1 was found to be 118 μM. Compound **5**, **6** and **8** also showed good inhibition of COX-2 with IC_{50} 7.9, 7.3 and 6.2 μM for COX-2 enzyme (Table 1). Compounds **7** and **9** did not exhibit inhibition of COX-1/2 at four molar concentrations used here. All the compounds showed insignificant COX-1 inhibition which is the housekeeping enzyme for the production of useful prostaglandins.

IC_{50} of compound **4** for COX-2 is better than that of chrysin. Although IC_{50} of compound **4** for COX-2 is comparable to indomethacin but its selectivity for COX-2 over COX-1 is better than that of indomethacin. While IC_{50} of compound **4** 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 **4** 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 compound **4** for COX-2 than the drugs based on individual components of this compound justify the design of the molecules. Compound **4** may act as a lead molecule for further refinement to increase its efficacy for COX-2 inhibition.

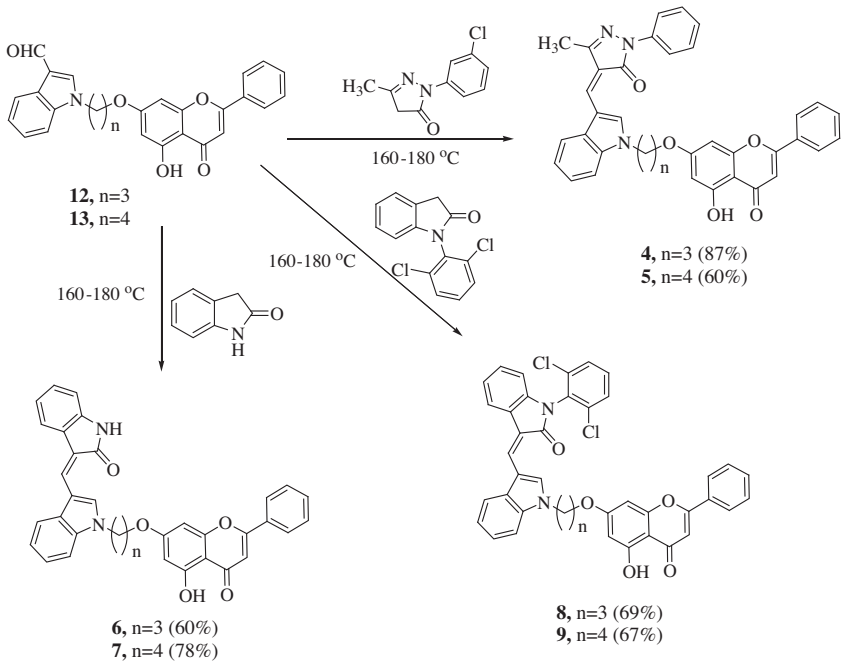
In order to come across the rationale behind the type of interactions and their extent between the compounds and the enzymes, molecular docking of compounds **4**–**9** in the active sites of COX-1 and COX-2 was performed. Compounds were built using the builder tool kit of the software package Argus Lab 4.0.1[32] and energy minimized with semi-empirical quantum mechanical method PM3. Crystal co-ordinates of COX-1 (PDB ID 1EQG and 3KK6)[33,34] and COX-2 (PDB ID 6COX and 3MQE)[35,36] were downloaded from protein data bank (www.rcsb.org) and in the molecule tree view of the software, the monomeric structures of the crystal coordinate was selected and the active site was defined as 15 Å around the ligand.

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. Docking was repeated several times (approx. 10,000 iterations) until no change in position of the ligand and a constant value of binding energy was observed. The ligand orientation was determined by a shape scoring function based on Ascore and final positions were ranked by lowest interaction energy values. H-bonds and hydrophobic interactions between the respective compound and enzyme were explored.

Compound **4** was well docked in the active site of both the crystal coordinates of COX-2 viz. 6COX and 3MQE (Fig. 1a and b). It showed H-bond interactions of 1.59, 2.04, 2.35 and 2.92 Å between its pyrazolic nitrogens and T118 amino acid of the enzyme active



Scheme 2.



Scheme 3.

site. It also showed a strong H-bond interaction of 1.66 Å through its indole nitrogen towards R120 (Fig. 1a), the amino acid residue forming salt bridge with AA during the turn over phase of the enzyme. Similarly, compound **4** fit in the hydrophobic cavity of another crystal structure of COX-2 (Fig. 1b and c).

Compound **4** 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 (Supporting information). 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 **4**. Molecular docking of compounds **5**–**9** was also performed in the active site of enzymes COX-1/COX-2. Similar results were obtained on docking compounds **5**–**9** in the active site of COX-2 and their compatibility in the active site varies from compound to compound. However, compounds **5**–**9** did not enter the active site of COX-1 enzyme (Supporting information).

Based on the results of enzyme immunoassays, compounds **4**–**6** and **8** were checked for their anti-nociceptive activities. 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 1 h 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.

The method used for capsaicin-induced licking was similar to that described by Sakurada et al.[37] with a few modifications. Briefly, 20 μL of capsaicin (1.6 μg) was injected into the plantar surface of the right hand paw. Animals were observed individually for 10 min after capsaicin administration and the time spent paw licking and twitching was recorded as an indicator of nociception.

Animals were divided into 10 groups of 6 each for studying the anti-nociceptive activity of compounds **4**–**6** and **8** using capsaicin induced paw licking. All the treatments were administered intraperetoneally 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 compound **4** at a dose of 5 and 10 mg Kg^{-1}, respectively. Similarly, in group V and VI, animals were injected compound **5** at a dose of 5 and 10 mg Kg^{-1}, respectively; in group VII and VIII, with compound **6** and in group IX and X, with compound **8**, respectively.

The treatment with diclofenac was found to produce a marked decrease in the number of paw lickings after capsaicin injection. Compounds **4** and **6** did not produce any decrease in capsaicin induced pain at a dose of 5 mg Kg^{-1}. However, compound **4** was found to produce a significant analgesic effect at a dose of 10 mg Kg^{-1} whereas compound **6** did not attenuate the capsaicin induced algesia even at a dose of 10 mg Kg^{-1}. The effect of compound **4** 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 **5** and **8**, 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} (Fig. 2). However, compound **5** at 5 mg Kg^{-1} dose was found to show analgesic activity comparable to the standard drug diclofenac (25 mg Kg^{-1} dose). Overall,

Table 1
IC_{50} (μM) of compounds **4**–**9** against COX-1, COX-2 and their selectivity index

Compound	IC_{50}		Selectivity index*
	(COX-2)	(COX-1)	
4	0.7	118	168.5
5	6.2	90	14.5
6	7.9	102	12.9
7	–	–	–
8	7.3	115	15.7
9	–	–	–
Indomethacin	0.96	0.08	0.08
Diclofenac	0.02	0.07	3.5
Celecoxib	0.04	15	375
Chrysin	25.5	39.3	1.5

* IC_{50} (COX-1)/IC_{50} (COX-2).

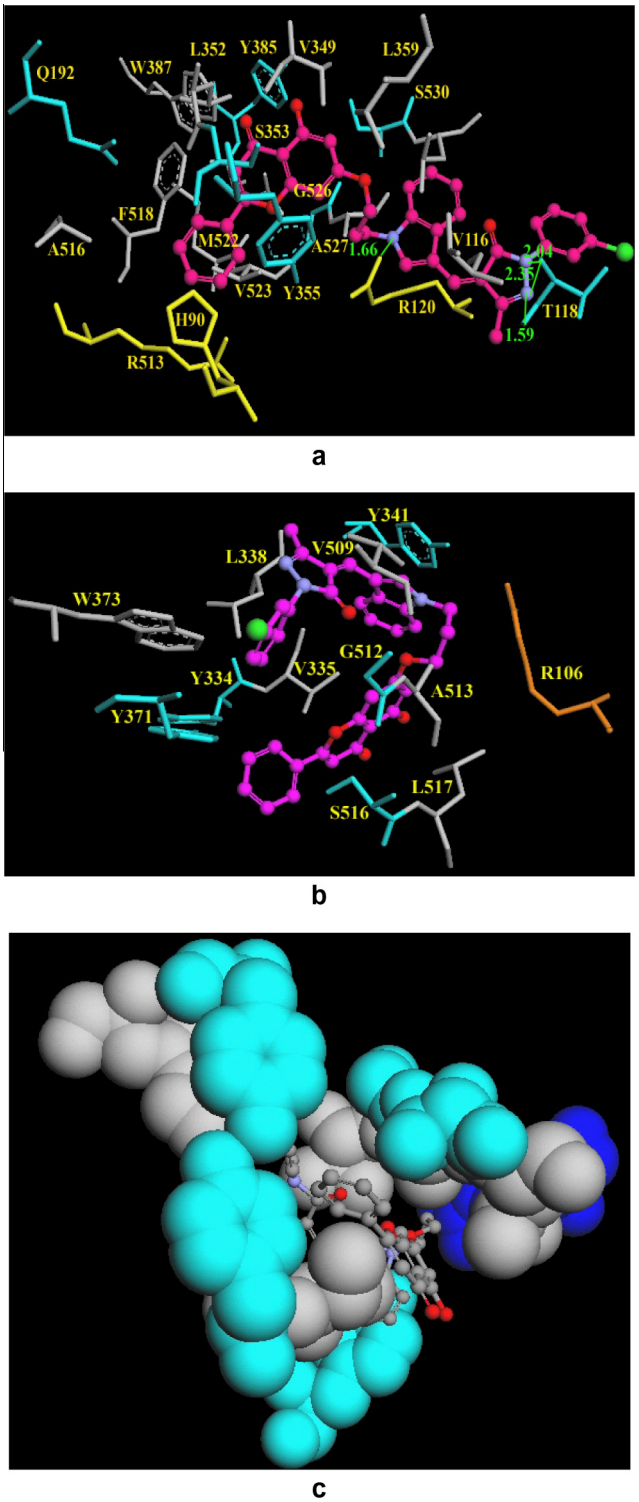


Figure 1. (a) Compound **4** docked in the active site of enzyme COX-2 (pdb ID 6COX). Green lines indicate H-bonds formed between the compound and the enzyme active site residues. Hs′ are omitted for clarity; (b) compound **4** docked in the active site of enzyme COX-2 (pdb ID 3MQE); (c) CPK model of the active site of COX-2 (pdb ID 3MQE) showing fitting of compound **4** in the active site.

compounds **4**, **5** and **8** were identified for promising analgesic potential.

In conclusion, the results of present studies indicate that the compounds obtained by the combination of chrysin, indole and pyrazole are more potent for COX-2 inhibition than the drugs

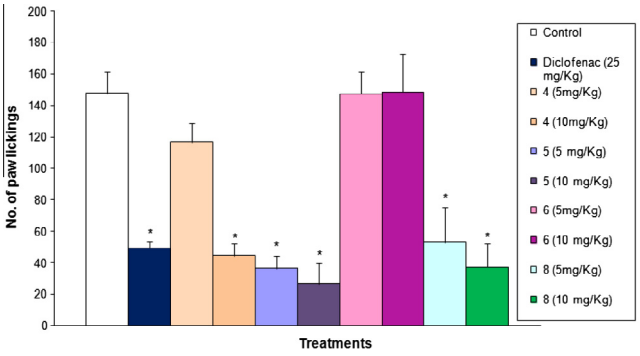


Figure 2. Effect of compounds **4**–**6** and **8** on capsaicin induced pain in mice. All values are expressed as mean ± SD ($n = 6$ per group). Statistical significance has been calculated using one way ANOVA followed by Tukey's multiple range comparison test. $^{*}p < 0.001$ versus control group.

based on these three individual moieties. As per the results of enzyme immunoassays, compounds **4**, **5** and **8** exhibit significant activity for inhibition of COX-2. Molecular modeling studies indicating considerable interactions of these compounds with COX-2 active site amino acids also favor the enzyme immunoassay results. Compounds **4**, **5** and **8** are identified with significant antinociceptive potential comparable with the standard drug diclofenac.

Acknowledgments

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Supplementary data

Supplementary data (docking structures, NMR and mass spectra) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.11.080.

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30. Melting points were determined in capillaries and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on JEOL 300 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. Mass spectra were recorded on Bruker micrOTOF Q II Mass spectrometer.

Compounds **4**–**9** were prepared through knoevenagel condensation of compounds **12** and **13** (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.

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 (***4***): Compound **4** was synthesized according to the synthetic procedure given above as a yellow solid, in a yield of 87%, mp 202 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 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), 7.46–7.53 (m, 4H, ArH), 7.70–7.94 (m, 4H, ArH), 8.05 (s, 1H, ArH), 9.86 (s, 1H, ArH), 12.69 (s, 1H, OH); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 13.10 (CH_3), 29.33 (CH_2), 44.24 (CH_2), 64.99 (CH_2), 93.12, 98.62, 105.82, 110.67, 112.26, 116.60, 118.21, 118.72, 119.60, 122.83, 123.95, 124.11, 126.24, 129.02, 129.17, 129.68, 131.79, 134.31, 135.27, 136.74, 140.39, 143.93, 150.82, 157.78, 162.19, 163.34, 163.96, 164.22, 182.37; HRMS Calcd for $C_{37}H_{28}ClO_5N_3$: 652.1610. Found: *m*/*z* 652.1615 $(M+Na)^+$.

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 (***5***): Compound **5** was synthesized according to the synthetic procedure given above as a yellow solid, in a yield of 60%, mp 200 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 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), 6.65 (s, 1H, ArH), 7.23 (d, *J* = 7.2 Hz, 1H, ArH), 7.24–7.51 (m, 8H, ArH), 7.79-7.85 (m, 5H, ArH), 8.09 (s, 1H, ArH), 9.89 (s, 1H, ArH), 12.69 (s, 1H, OH); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 13.00 (CH_3), 29.10 (CH_2), 29.23 (CH_2), 44.14 (CH_2), 64.69 (CH_2), 93.02, 98.52, 105.72, 110.57, 112.16, 116.50, 118.11, 118.62, 119.50, 122.73, 123.85, 124.01, 126.14, 128.92, 129.07, 129.58, 131.14, 131.69, 135.17, 136.64, 140.29, 150.72, 157.66, 162.09, 163.19, 163.92, 164.12, 182.41; HRMS Calcd for $C_{38}H_{30}ClO_5N_3$: 644.1947. Found: *m*/*z* 644.1932 $(M+H)^+$.

3-{1-[3-(5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)-propyl]-1H-indol-3-ylmethylene}-1,3-dihydro-indol-2-one (***6***): Compound **6** was synthesized according to the synthetic procedure given above as a yellow solid, in a yield of 60%, mp 225 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 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); ^{13}C NMR (75 MHz, DMSO-d_6) δ ppm: 26.10 (CH_2), 46.97 (CH_2), 67.12 (CH_2), 92.77, 93.35, 98.78, 105.84, 108.68, 111.64, 118.22, 118.37, 121.72, 122.26, 122.41, 123.18, 126.26, 126.97, 127.93, 129.02, 129.15, 129.48, 135.93, 136.00, 136.25, 157.75, 162.31, 164.05, 164.14, 164.47, 182.26; HRMS Calcd for $C_{35}H_{26}O_5N_2$: 555.1914. Found: *m*/*z* 555.1918 $(M+H)^+$.

3-{1-[4-(5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)-butyl]-1H-indol-3-ylmethylene}-1,3-dihydro-indol-2-one (***7***): Compound **7** was synthesized according to the synthetic procedure given above as a yellow solid, in a yield of 78%, mp 156 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.86–1.89 (m, 2H, CH_2), 2.15–2.18 (m, 2H, CH_2), 4.02 (t, *J* = 5.7 Hz, 2H, CH_2), 4.33 (t, *J* = 6.3 Hz, 2H, CH_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, ArH), 12.68 (s, 1H, OH); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 29.30 (CH_2), 29.58 (CH_2), 43.62 (CH_2), 65.82 (CH_2), 92.85, 93.03, 98.56, 105.82, 108.66, 111.52, 118.10, 118.15, 121.60, 122.14, 122.19, 123.06, 126.24, 126.85, 127.81, 128.20, 128.90, 129.03, 129.16, 135.81, 135.88, 136.23, 157.63, 162.19, 163.93, 164.24, 164.35, 182.43; HRMS Calcd for $C_{36}H_{28}O_5N_2$: 591.1890. Found: *m*/*z* 598.1880 $(M+Na)^+$.

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 (***8***): Compound **8** was synthesized according to the synthetic procedure given above as a yellow solid, in a yield of 69%, mp 146 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.12–2.17 (m, 2H, CH_2), 3.98 (t, *J* = 6 Hz, 2H, CH_2), 4.31 (t, *J* = 6.6 Hz, 2H, CH_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); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 26.40 (CH_2), 46.80 (CH_2), 67.23 (CH_2), 93.23, 98.76, 105.98, 108.87, 110.44, 118.40, 121.66, 121.76, 122.38, 123.13, 126.44, 126.99, 128.08, 129.13, 129.21, 130.63, 131.48, 131.93, 136.11, 136.19, 137.20, 139.07, 157.90, 162.31, 164.10, 164.16, 164.90, 182.58; HRMS Calcd for $C_{41}H_{28}Cl2O_5N_2$: 699.1448. Found: *m*/*z* 699.1421 $(M+H)^+$.

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 (***9***): Compound **9** was synthesized according to the synthetic procedure given above as a yellow solid, in a yield of 67%, mp 120 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.62–1.89 (m, 2H, CH_2), 2.09–2.16 (m, 2H, CH_2), 3.98–4.06 (dt, 2H, CH_2), 4.26–4.36 (dt, 2H, CH_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); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 26.83 (CH_2), 27.11 (CH_2), 47.34 (CH_2), 68.18 (CH_2), 93.44, 98.97, 106.19, 109.08, 110.65, 111.72, 117.87, 118.49, 118.61, 121.97, 122.60, 123.01, 123.35, 126.61, 126.65, 127.20, 128.29, 129.34, 129.42, 129.50, 130.85, 131.70, 132.14, 136.32, 136.40, 137.41, 139.28, 157.52, 162.52, 163.71, 163.87, 165.12, 182.78; HRMS Calcd for $C_{42}H_{30}Cl_2O_5N_2$: 735.1424. Found: *m*/*z* 735.1408 $(M+Na)^+$.

31. COX inhibitor screening assay kit, Item No. 560131. Cayman Chemical Co.

General 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 assays. 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 min. 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 compounds **4**–**9**. 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 min 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 min. Afterwards, 50 μl of 1 M 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 min 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 up to 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, theywere introduced on 96 well plate. The wells were distributed as blank-1A, NSB (nonspecific binding)-1B and B_o (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.

The 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 B_o 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 h 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 min. Before reading the plate, it was wiped from bottom to remove any fingerprints and finally read at 420 nm.

Calculation of % inhibition and IC_{50} values: $\%B/B_o$ value for each sample (all compounds **4**–**9** at 10^{-3} M, 10^{-4} M, 10^{-5} M, 10^{-6} M, 10^{-7} M and 10^{-8} M conc each) 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.[31]

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

Finally, the percentage inhibitions at all the conc were plotted against the respective tested conc for each compound using GraphPad Prism version 6.01 for calculating IC_{50} (50% inhibitory conc).

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