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FULL PAPER

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Synthesis and Biological Evaluation of 1,3,5-Trisubstituted 2-Pyrazolines as Novel Cyclooxygenase-2 Inhibitors with Antiproliferative Activity

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A new series of 1,3,5-trisubstituted 2-pyrazolines for the inhibition of cyclooxygenase-2 (COX-2) were synthesized. The designed structures include a COX-2 pharmacophore SO_2CH_3 at the *para*-position of the phenyl ring located at C-5 of a pyrazoline scaffold. The synthesized compounds were tested for *in vitro* COX-1/COX-2 inhibition and cell toxicity against human colorectal adenocarcinoma cell lines HT-29. The lead compound (4-chlorophenyl){5-[4-(methanesulfonyl)phenyl]-3-phenyl-4,5-dihydro-1*H*-pyrazol-1-yl}methanone (**16**) showed significant COX-2 inhibition ($IC_{50} = 0.05 \pm 0.01$ μM), and antiproliferative activity ($IC_{50} = 5.46 \pm 4.71$ μM). Molecular docking studies showed that new pyrazoline-based compounds interact *via* multiple hydrophobic and hydrogen-bond interactions with key binding site residues of the COX-2 enzyme.

Keywords: COX-2 inhibitors, 2-pyrazolines, anti-inflammatory agents, anticancer drugs.

1. Introduction

The biochemical origin of inflammation is linked with the metabolic conversion of arachidonic acid (AA) to a set of prostanoids, including prostaglandins (PGs) and thromboxane.[1–4] Prostanoids metabolism is involved in several physiological and pathological processes such as inflammation, ovulation, renal protection, thrombosis, fever, and angiogenesis.[5]

Cyclooxygenases (COXs) are homodimeric, rate-limiting enzymes for the metabolism of AA to yield various prostanoids. COXs are known to exist in three isoforms, COX-1, COX-2, and COX-3.[6–8] Among these, COX-1 isoform is constitutively expressed and linked with the production of PGs to maintain homeostasis.[8] COX-2 is the inducible isoform and is known to be expressed mainly at inflammation sites and during the progression of several types of cancers.[9–13] COX-3 is described as a different COX-1 splice variant.[14] Both COX-1 and COX-2 have a degree of close structural and sequence similarity. However, these isoforms differ by the existence of an additional sub-pocket (known as secondary pocket) in the COX-2 binding site, making its active site approximately 25% larger than that of COX-1.[4] The ulcerogenic and gastrointestinal side effects of classical non-steroidal anti-inflammatory drugs (aspirin, ibuprofen, and naproxen) are believed to be linked with non-selective inhibition of both COX-1 and COX-2 isoforms. Therefore, selective COX-2

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inhibitors (celecoxib, rofecoxib, valdecoxib) were developed to overcome the side effects associated with the use of classical non-steroidal anti-inflammatory drugs. Unfortunately, some of the selective COX-2 inhibitors, like rofecoxib, were removed from the market due to an increased risk of cardiovascular complications. In recent years, several new classes of COX-2 inhibitors have been developed, including the modification of known COX inhibitors to nitric oxide-releasing prodrugs for eliminating cardiovascular complications.[15,16] The different roles of COX enzymes in the development and progression of cancer also prompted the use of COX inhibitors as anticancer drugs.[17]

Selective COX-2 inhibitors are frequently comprised of a vicinal diaryl system with a central heterocyclic ring, and the presence of either SO_2CH_3 or SO_2NH_2 groups, which play a crucial role for selective binding to the secondary pocket of the COX-2 isoform.[15,18] Differing from this general chemical arrangement, several other classes of selective COX-2 inhibitors have also been developed (*Figure 1*). As shown in the Figure, the central five-membered ring can be exemplified by different structures such as pyrazole, 2-furanone, 2-pyrazoline, 1,2,3-triazole, and 2-pyranone present in celecoxib (**1**), rofecoxib (**2**), compounds **3**, **4**, and **5**, respectively. The variations on the central ring have also been achieved by introducing substitution other than the diaryl groups. For example, Rathish et al. developed a series of compounds having extra substituted phenyl moiety on the central ring (Compound **3**) with potent anti-COX-2 activity and *in vivo* anti-inflammatory effects.[19] Wuest and co-workers prepared a series of diaryl-substituted triazoles, where the linker between the arylsulfonamide group and central triazole ring was modified (Compound **4**).[20] The similarity of these structures to that of the renowned selective COX-2 inhibitors is evident, as illustrated in *Figure 1*.

Several classes of biologically active 2-pyrazolines (or 4,5-dihydropyrazoles) have been developed with anti-inflammatory,[21] anticancer,[22,23] antidepressant,[24] anticonvulsant,[25] antibacterial,[26,27] and antimalarial activities.[27] There are also some studies showing that compounds with 2-pyrazoline scaffold have selective COX-2 inhibitory effects.[19,28–31]

The suggested compounds in this study were designed by introducing variations in the template structures in order to improve the inhibitory effects through increased interaction with the COX-2 enzyme. The variations included modification of the linker between the aryl and central 2-pyrazoline ring, the introduction of the third aryl group on the central

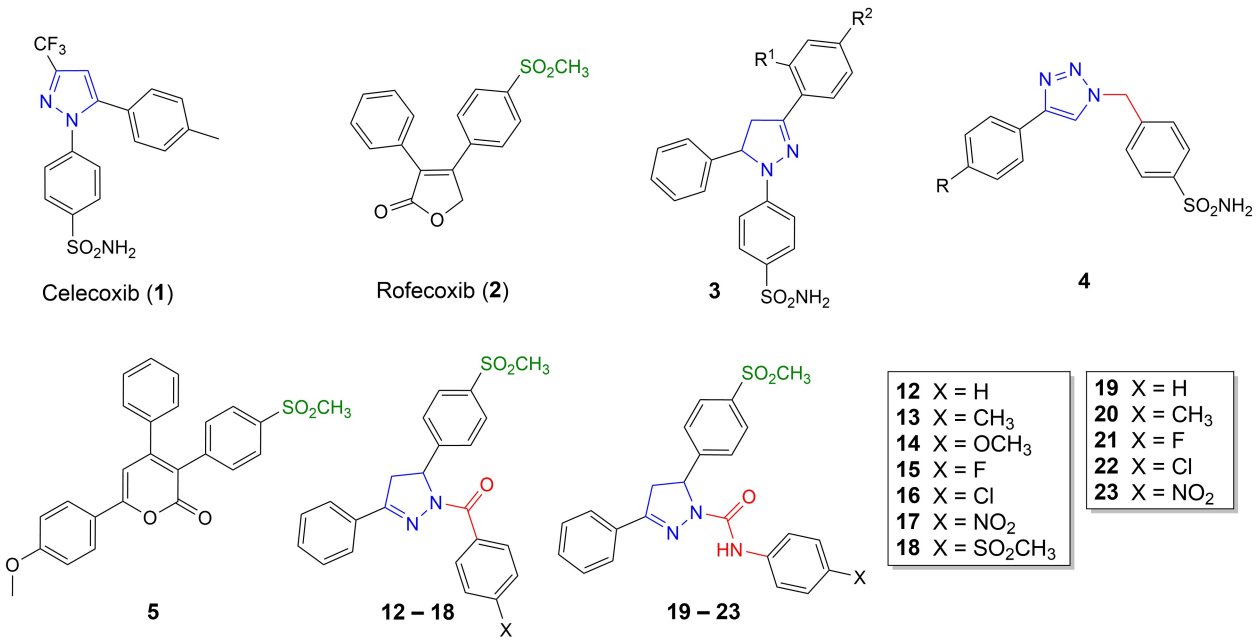


Figure 1. Structure of selective COX-2 inhibitors and novel pyrazoline-based compounds. The title compounds were designed based on celecoxib, rofecoxib, and structures 3,[19] 4,[20] and 5[32] with known selective COX-2 inhibitory effect. The pharmacophores are color coded for easy tracking the origin of the different structural elements in the designed scaffold.

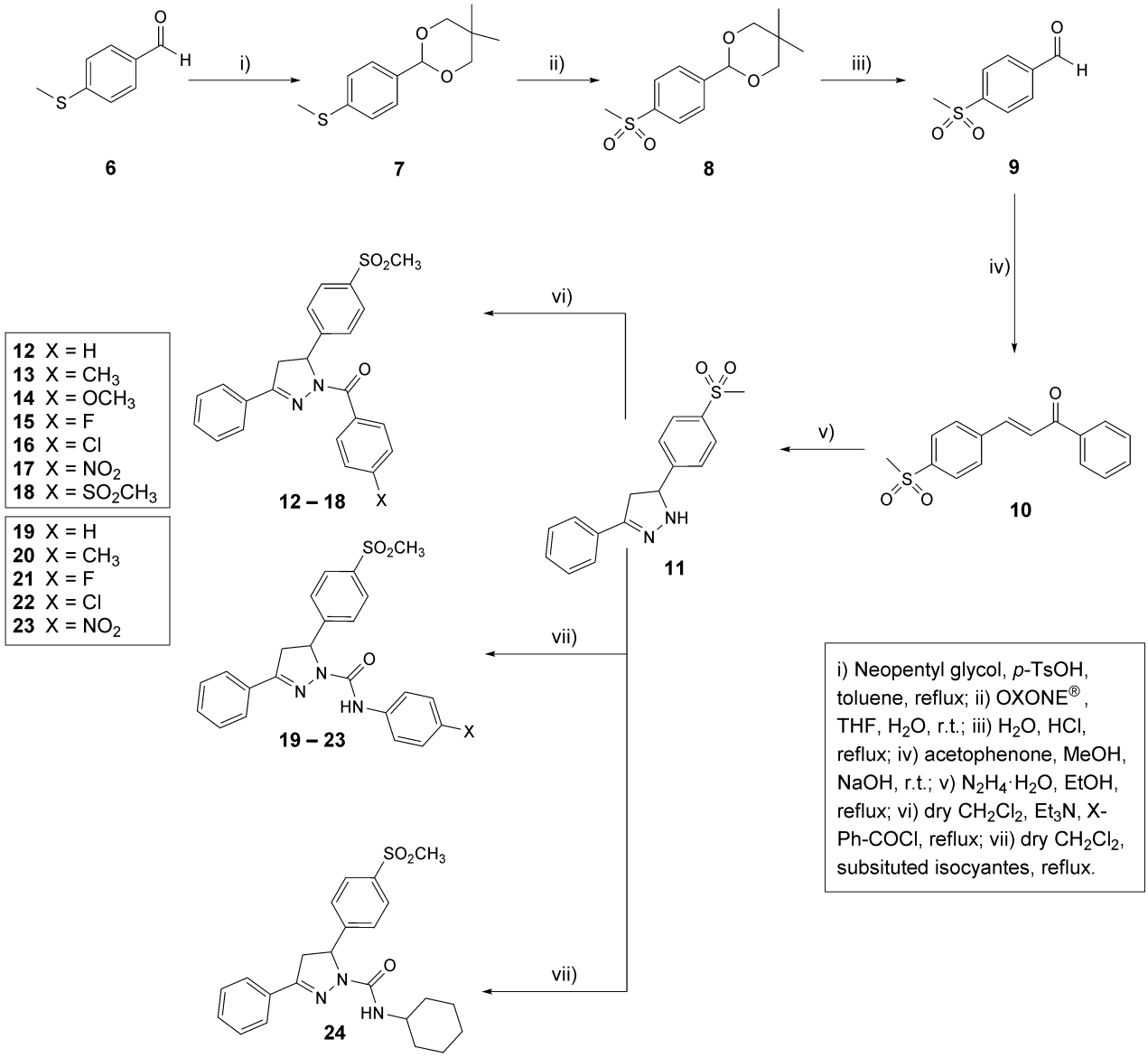
ring, and rearrangement of the substitutions on the central five-membered ring (*Figure 1*).

We prepared a series of tri-aryl/alicyclic substituted 2-pyrazoline-based compounds as novel tandem anti-inflammatory and anticancer drugs (compounds **12**–**24**). All compounds were tested for the COX-1/2 inhibitory potency and their antiproliferative activity in human colorectal adenocarcinoma cells.

2. Results and Discussion

2.1. Chemistry

The synthetic route for the preparation of pyrazoline-based compounds is depicted in *Scheme 1*. Briefly, starting from compound **6**, compounds **7** and **8** were synthesized sequentially by using protection and OXONE-mediated oxidation, respectively. Then the key building block 4-(methylsulfonyl)benzaldehyde (**9**) was prepared by deprotection of **8** under acidic reaction conditions. Next, Claisen-Schmidt reaction between 4-(methylsulfonyl)benzaldehyde (**9**) and acetophenone gave compound **10**, which was refluxed with hydrazine hydrate to afford pyrazoline compound **11**.



Scheme 1. Synthesis route for the preparation of pyrazoline-based compounds **12**–**24**.

Pyrazoline compound **11** was reacted with a series of substituted benzoyl chlorides and isocyanates to prepare tri-aryl/alicyclic substituted 2-pyrazolines **12**–**24**. The spectroscopic data of compounds **12**–**24** can be found in Supporting Information.

2.2. In Vitro *Cyclooxygenase* (*COX*) *Inhibition Studies*

COX-1 and COX-2 inhibitory potencies of compounds **11**–**24** were determined in triplicate using a COX inhibitor screening assay kit. The results are summarized in *Table 1*. Among all tested pyrazolines **11**–**24**, (4-chlorophenyl){5-[4-(methanesulfonyl)phenyl]-3-phenyl-4,5-dihydro-1*H*-pyrazol-1-yl}methanone (**16**) showed very good COX-2 inhibitory potency (IC_{50} = 0.05 μM), which is in the same range of the reference compound celecoxib (IC_{50} = 0.01 μM). However, compound **16** also displayed considerable inhibition against COX-1 isoform (IC_{50} = 0.16 μM). Among first series of compounds **12**–**18** with carbonyl linker, compound **14** (X=OCH_3) also showed appreciable inhibition of both COX-1 and COX-2 isoforms (COX-2 IC_{50} = 0.21 μM, COX-1 IC_{50} = 0.70 μM). Compounds **12** (X=H) and **13** (X=CH_3) displayed moderate COX-2 inhibition of 0.81 and 0.78 μM, and a COX-2 selectivity index (SI) of **12** and **23**, respectively. Although SO_2CH_3 group is known to support binding to the COX-2 isoform, compound **18** with two SO_2CH_3 groups showed only micromolar inhibitory potency (8.6 μM). Evaluation of the second series of compounds **19**–**24** carrying an amide linker revealed that the incorporation of a *para*-substituent on one of the phenyl rings resulted in an overall improvement in COX-2 inhibition activity and selectivity profile. Within this series, the 5-[4-(methylsulfonyl)phenyl]-3-phenyl-N-(*p*-tolyl)-4,5-dihydro-1*H*-pyrazole-1-carboxamide (**20**) showed appreciable submicromolar COX-2 inhibition and a good selectivity profile over COX-1 (COX-2 IC_{50} = 0.80 μM, SI = 116). Interestingly, replacement of the phenyl ring with a cyclohexyl group in compound **24** led to a comparable submicromolar COX-2 inhibitory (IC_{50} = 0.78 μM) and selectivity profile (SI > 128).

A closer evaluation revealed that the physicochemical features of *para*-substitutions such as electron-withdrawing (Hammett σ constant),[33] lipophilicity (Hansch π constant),[34] and steric (Taft E_s constant and vdW volume) properties influence the COX-2 inhibition potency of the first series of compounds **12** to **18**.[35] The results showed that COX-2 activity increases as the lipophilicity of the *para*-substitution increases (R^2 = 0.82, *Table S1* and *Figure S71*, Supporting Information). However, the activity decreases as the electron withdrawing effect and size of the substituent increase (*Table S1* and *Figure S71*, Support-

Table 1. *In vitro* COX-1 and COX-2 enzyme inhibition and cytotoxicity data of compounds **11**–**24**.

Compd.	IC_{50} (μM)[a]		COX-2 SI[b]	Cytotoxicity[c] (IC_{50}, μM)
	COX-1	COX-2		
11	2.44	0.36	6.78	219.00 ± 26.77
12	9.82	0.81	12.12	22.64 ± 12.87
13	17.76	0.78	22.77	9.53 ± 3.28
14	0.70	0.21	3.33	61.71 ± 26.46
15	1.21	3.44	0.35	344.40 ± 127.85
16	0.16	0.05	3.20	5.46 ± 4.71
17	> 100	3.02	> 33.11	314.70 ± 44.87
18	> 100	8.58	> 11.66	72.94 ± 24.23
19	8.90	9.40	0.95	23.79 ± 12.42
20	93.39	0.80	116.74	57.41 ± 12.01
21	> 100	1.95	> 51.28	117.90 ± 23.01
22	3.17	0.69	4.59	121.20 ± 33.57
23	3.06	0.58	5.28	> 500
24	> 100	0.78	> 128.21	44.18 ± 9.05
Celecoxib	> 100	0.01	> 10000.00	40.59 ± 1.74
Cisplatin	–	–	–	19.85 ± 2.79

[a] Assays were conducted as described in the Methods section. IC_{50}, half-maximal inhibitory concentration. The *in vitro* test compound concentration required to produce 50% inhibition of ovine COX-1 or human recombinant COX-2. The result (IC_{50}, μM) is the mean of three determinations acquired using the enzyme assay kit (Cayman Chemical, Ann Arbor, USA; item number: 700100) and the deviation from the mean is < 10% of the mean value. [b] COX-2 SI, *in vitro* COX-2 selectivity index: [(COX-1 IC_{50})/(COX-2 IC_{50})]. [c] In vitro cytotoxicity MTT assay. IC_{50} values are presented as mean ± SD of three independent experiments and the values greater than 100 μM are extrapolations obtained from curve fitting method.

ing Information). It seems that *para*-Cl group has appropriate lipophilicity, electronic effect and size required for the observed high activity. For instance, the higher COX-2 inhibitory potency of *para*-Cl compound **16** than that of *para*-F **15** and *para*-NO_2 **17** could be attributed to its higher lipophilicity. Moreover, its steric and electron-withdrawing effects are smaller than those of *para*-NO_2, which may also indicate its higher potency. Although compared to *para*-F group, *para*-Cl has unfavorable steric and electron withdrawing effects, its ultimate influence on COX-2 activity is positive, which may have brought about due to the summed impact of all properties. The medium activity of *para*-CH_3 of **18** and low activity of *para*-SO_2CH_3 of **18** derivatives could also be rationalized based on the above drawn structure-activity relationship (SAR). However, relatively good anit-COX-2 activity of *para*-OCH_3 **14** cannot be explained by its low hydrophobicity and relatively bigger size. It seems that the more electron donating the *para*-substitution, the higher the activity (*Table S1* and *Figure S71*, Supporting Information). Considering that *para*-OCH_3 is an electron donating group, and its size is not that big, the observed sub-micro molar IC_{50} for COX-2 inhibition may be justified.

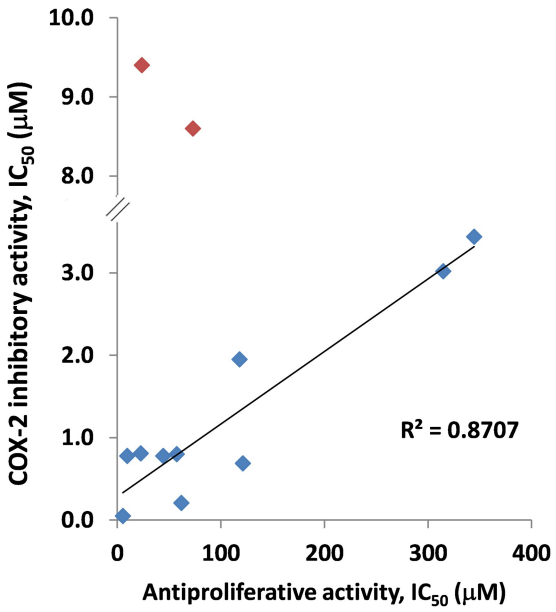


Figure 2. Correlation between COX-2 inhibitory potency and antiproliferative activity against HT-29 cells for synthesized compounds **11**–**24**. Compounds **18** and **19** are outliers, and compound **23** was not included in the correlation due to lack of cytotoxicity at the used concentrations.

2.3. In Vitro *Cell Toxicity Effect*

To investigate the cytotoxic effect of compounds **11**–**24**, the MTT assay was conducted using human colorectal adenocarcinoma cells HT-29. The cytotoxicity effect of the compounds, celecoxib, and cisplatin is summarized in *Table 1*. Among all tested compounds, the most potent COX-2 inhibitor (4-chlorophenyl){5-[4-(methanesulfonyl)phenyl]-3-phenyl-4,5-dihydro-1*H*-pyrazol-1-yl}methanone (**16**) showed the highest cytotoxicity activity against HT-29 cells (IC_{50} = 5.46 μM). The observed cytotoxicity effect of compound **16** was higher than that of celecoxib and cisplatin.

A correlation between the COX-2 inhibition activity and cytotoxicity against HT-29 cells is presented in *Figure 2*. Except for compounds **18**, **19**, and **23**, all tested compounds showed a good correlation.

2.4. Molecular Docking

To predict the mode of interactions between the synthesized compounds and COX-2 isoenzyme, molecular docking studies were conducted using GOLD program. Both (*R*)- and (*S*)-enantiomers of the studied compounds were docked into the binding site of human COX-2 structure (PDB code: 5KIR). The analysis of the obtained results demonstrated that (*S*)-enantiomers were docked with much better consistency into the active site of COX-2 enzyme when compared to (*R*)-enantiomers. For example, the results of docking calculations showed that the *para*-methylsulfonyl moiety of the phenyl ring on pyrazoline C5 position as the predominant pharmacophore of the studied compounds enters the secondary pocket (also named side pocket). The interactions of all (*S*)-enantiomers docked into the active site of COX-2 were analyzed using the LigPlot$^+$ (Version v.2.1) and the most commonly observed interactions were identified as follows: a hydrogen bond between SO_2CH_3 group of the studied compounds and side chain of Arg^{513} residue as well as hydrophobic contacts between the compounds and His^{90}, Val^{116}, Gln^{192}, Val^{349}, Leu^{352}, Ser^{353}, Tyr^{385}, Trp^{387}, Ala^{516}, Phe^{518}, Met^{522}, Val^{523}, Gly^{526}, Ser^{530}, and Leu^{531}. *Figure 3* shows the predicted interactions between compound **16** (the most potent derivative) and COX-2 enzyme. *In vitro* COX-2 inhibition assay showed that compound **16** with a *para*-Cl substitution is the most active derivative. The activity enhancing effect of this substitution was related to its optimum physico-chemical properties explained above. Furthermore, the results of docking analyses

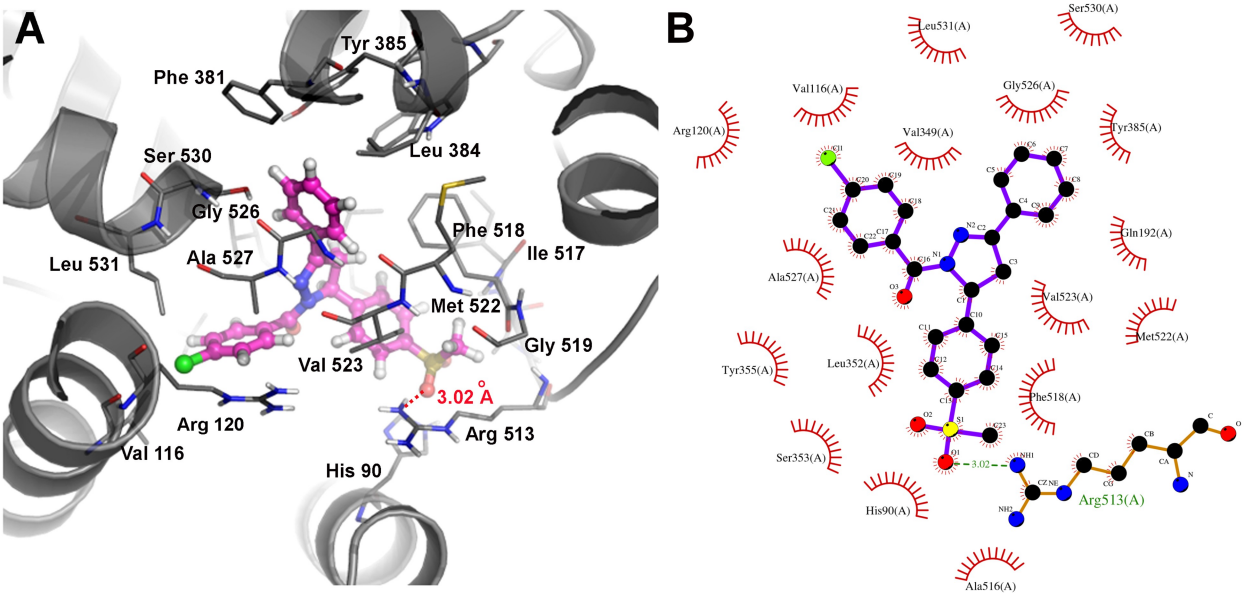


Figure 3. Molecular docking of lead compound **16** in COX-2. (A) The side chains of important residues of the enzyme were shown as lines, while **16** was shown as ball-and-stick representation. The H-bond between side chain guanidine N atom of Arg^{513} (donor) and oxygen atom (acceptor) of SO_2CH_3 group of **16** was highlighted with red dashed line. The measured distance between these heavy atoms involved H-bond was 3.02 Å. (B) The two-dimensional schematic LigPlot view of the interaction between **16** and COX-2 obtained by docking.

were also in agreement with the *in vitro* COX-2 assay. Investigation of docking results showed that residues in the binding pocket of COX-2 enzyme surrounding the *para*-substituted benzoyl moiety of the synthesized compounds are Val^{116}, Arg^{120}, Val^{349}, Tyr^{355}, Leu^{359}, Ala^{527} and Leu^{531} with predominantly hydrophobic side chains forming mainly hydrophobic interactions with the *para*-substituted benzoyl part of the compounds. This hydrophobic subsite perfectly accommodates the hydrophobic and medium-size Cl group of compound **16**, but less hydrophobic and relatively small F group (compound **15**) may not suitably form appropriate interaction, and hence show less activity. Substitution of *para*-NO_2 group (compound **17**) with much bigger size and very hydrophilic nature relative to Cl has led to decreased COX-2 inhibition activity most probably due to steric hindrance and inability to form favorable hydrophobic interaction. Presence of *para*-SO_2CH_3 group in **18** instead of *para*-Cl as in **16** on benzoyl moiety diminished COX-2 inhibition potency most likely due to its bigger size and hydrophilic properties. The compound **13** with *para*-CH_3 group demonstrated an activity less than that of **16** (*para*-Cl), but higher than those shown for **15** (*para*-F), **17** (*para*-NO_2) and **18** (*para*-SO_2CH_3). Lipophilicity of the methyl functional group is less than Cl group and hence may involve in a weaker hydrophobic interaction with the enzyme. On the other hand, it size is bigger than Cl and once again may not be accommodated in the binding subsite as perfectly as does the Cl group. However, the differences between CH_3 and Cl groups are not as substantial as those of F, NO_2 and SO_2CH_3 relative to Cl group. This might be the reason for sub-micromolar IC_{50} value of compound **13** in COX-2 inhibition assay. Collectively, one may conclude that presence of a *para*-substitution on benzoyl moiety of the synthesized compounds with appropriate size capable of forming hydrophobic interaction with COX-2 enzyme may lead to stronger inhibition.

3. Conclusions

In this study, we synthesized two series of 2-pyrazoline-based derivatives with COX-2 inhibitory activity, among which compounds **16** was identified as the most potent COX-2 inhibitor. The binding interactions were analyzed using molecular docking studies. In addition, cytotoxic effects of the studied compounds were evaluated on colorectal adenocarcinoma cells and the findings revealed that compound **16** is also a

potent cytotoxic agent. The results suggest that **16** can serve as a dual anti-inflammatory and antiproliferative agent.

Experimental Section

Materials and Measurements

All chemicals and solvents were purchased from the Sigma-Aldrich or Merck companies. Solvents were dried and purified according to the literature if required. The progress of the reactions was monitored by thin-layer chromatography (TLC) using pre-coated plates of silica gel 60 F_{254} (Merck) and visualized under UV light (254 nm). Melting points were determined using Electrothermal melting point apparatus (Cat. No. IA 9200). Infrared spectra were recorded on a Shimadzu FT-IR-8400S spectrophotometer using KBr disk sample preparation method. The ^{1}H- and ^{13}C-NMR spectra were recorded on Bruker operating at 400 MHz and 100 MHz, respectively, using (D_6)DMSO as solvent and TMS as internal reference. Coupling constant (*J*) values are estimated in hertz (Hz) and spin multiples are given as s (singlet), d (double), t (triplet), q (quartet), m (multiplet), and br. (broad). The elemental analyses were obtained using Costech Elemental Combustion System CHNS–O (ECS 4010). The purity of the compounds was determined using HPTLC (CAMAG system, Switzerland) and reported as percentages.

Synthesis of 4-(Methylsulfonyl)benzaldehyde (**9**)

Compound **9** was synthesized through three steps including protection, oxidation, and deprotection as outlined below.

(a) Protection Step

4-(Methylthio)benzaldehyde (**6**; 10 g, 65.69 mmol), neopentyl glycol (7 g, 67.21 mmol), *p*-toluenesulfonic acid monohydrate (120 mg, 0.63 mmol, as catalyst), and 100 mL dry toluene were mixed in a round bottom flask equipped with a dean-stark trap and a reflux condenser. Reaction mixture was refluxed for azeotropic dehydration. After completion of dehydration, reaction mixture was cooled and washed with $NaHCO_3$ aqueous solution. The organic phase was dried over Na_2SO_4 and then, the organic solvent was removed by evaporator under reduced pressure at 40 °C. The crude solid residue was crystallized from hexane to obtain white crystals of compound **7** (5,5-dimethyl-2-(4-(methylthio)phenyl)-1,3-dioxane). White crystal. Yield: 93 %; M.p. 74–75 °C.

(b) Oxidation Step

Compound **7** (10 g, 41.95 mmol) was dissolved in tetrahydrofuran (THF). A solution of OXONE® (MW = 307.38) (31 g, 0.1 mol) in distilled water (130 mL) was then added dropwise and the reaction mixture was stirred at room temperature for 6 h. After completion of the reaction, determined by TLC, the acidic environment of the reaction mixture was neutralized with Na_2CO_3 followed by removing of solvent mixture by a rotary evaporator under reduced pressure at room temperature. The solid residue was dissolved in a mixture of water and ethyl acetate, and transferred to a separating funnel. The organic ethyl acetate phase was separated and dried with Na_2SO_4, and subsequently filtered and evaporated to afford the crude product **8** (5,5-dimethyl-2-(4-(methylsulfonyl)phenyl)-1,3-dioxane). White crystal. Yield: 98 %. M.p. 143–144 °C.

(c) Deprotection Step

Compound **8** (3 g, 11.10 mmol) was mixed with 100 mL water and then, 2 mL concentrated HCl (37 %) was added to the mixture dropwise followed by reflux for 1 h. After that, the reaction mixture was neutralized by adding K_2CO_3 crystals while monitoring the pH using pH paper. The mixture was cooled to room temperature to obtain white crystals of compound **9**, which was then collected on a sintered glass filter under vacuum and rinsed with distilled water. White crystal; Yield: 91 %. M.p. 160–161 °C.

Synthesis of (E)-3-(4-(Methylsulfonyl)phenyl)-1-phenylprop-2-en-1-one (**10**)

Compound **9** (4-(methylsulfonyl)benzaldehyde; 2 g, 10.86 mmol) was mixed with acetophenone (1.3 g, 10.86 mmol) in 60 mL alkaline methanol (12 mmol NaOH) and stirred at room temperature for 6 h to prepare compound **10**. About one half of the solvent was evaporated under reduced pressure and the precipitate of **10** was filtered and rinsed with cold methanol. The crude product was recrystallized in ethanol. Yellow solid. Yield: 85 %. M.p. 122–124 °C. IR (KBr, cm^{-1}): 1143 (S=O, symmetric stretching), 1298 (S=O, asymmetric stretching), 1602 (C=C, stretching), 1670 (C=O, stretching).

*Synthesis of 5-(4-(Methylsulfonyl)phenyl)-3-phenyl-4,5-dihydro-1*H*-pyrazole* (**11**)

To the chalconic compound **10** (3 g, 10.49 mmol) dissolved in boiling ethanol in a round bottom flask equipped with a reflux condenser was added hydrazine hydrate 80% (2 equiv.) and the reflux was continued. After completion of the reaction after 1 h, monitored by TLC, the mixture was cooled down to room temperature and the precipitate was collected on a sintered glass filter and washed with cold ethanol. The crude product was recrystallized in ethanol. White crystal. Yield: 90%. M.p. 190–192 °C. IR (KBr, cm^{-1}): 1145 (S=O, symmetric stretching), 1299, (S=O, asymmetric stretching), 1591 (C=N, stretching), 3346 (N–H, stretching)

*General Synthesis of (5-(4-(Methylsulfonyl)phenyl)-3-phenyl-4,5-dihydro-1*H*-pyrazol-1-yl)(aryl)methanone* (**12**–**18**)

Pyrazoline **11** and triethylamine were dissolved in dry dichloromethane and then, appropriate acyl chloride was added. The reaction mixture was refluxed and monitored by TLC to ensure the absence of significant amount of pyrazoline **11**. After cooling, the reaction mixture was transferred to separating funnel and washed with water solution of sodium carbonate. The organic layer was separated, dried with sodium sulfate, and evaporated. The crude product was crystallized in ethyl acetate. The pyrazoline ring in these structures contain three protons giving rise to three doublet of doublet (dd) peaks in NMR spectra known for the ABC system. Two germinal protons located at the C4 position are either in *cis* or *trans* geometry relative to the vicinal proton at C5 position of the pyrazoline ring. These protons are referred to as 4-H_{cis}, 4-H_{trans}, and 5-H in the description of NMR spectra, respectively. In some compounds, the peaks for 4-H_{trans} were hidden under the peak for methylsulfonyl protons.

*General Synthesis of 5-(4-(Methylsulfonyl)phenyl)-3-phenyl-*N*-(aryl/cyclohexyl)-4,5-dihydro-1*H*-pyrazole-1-carboxamide* (**19**–**24**)

Pyrazoline **11** and appropriate isocyanate were dissolved in dry dichloromethane in round bottom flask equipped with a reflux condenser. The reaction mixture was refluxed until significant amount of pyrazoline **11** disappeared in TLC. After cooling, the reaction mixture was transferred to separating funnel and washed with water. The organic layer was separated, dried with sodium sulfate, and evaporated. The crude product was crystallized in ethyl acetate.

COX Inhibition Assay

The inhibition activity of test compounds against ovine COX-1 and recombinant human COX-2 was determined using a COX inhibitor assay (Cayman Chemical, Ann Arbor, USA; item number: 700100) following the manufacturer's protocol. Each compound was assayed in triplicate, and PRISM5 software was used to calculate IC_{50} values. In addition to celecoxib, both Dup-697 (potent COX-2 inhibitor) and SC-560 (potent COX-1 inhibitor) were used as internal controls during the screening of test compounds. All statistical analyses were performed by Microsoft Excel.

MTT Assay

The antiproliferative activities of the synthesized pyrazoline-based derivatives against HT29 (human colorectal adenocarcinoma cells) were evaluated using colorimetric MTT assay. Cisplatin and celecoxib were used as positive controls. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 100 u/mL penicillin, and 100 μg/L streptomycin at 37 °C and 5% CO_2 atmosphere in a humidified incubator. Then, cells were seeded into sterile 96-well plates (7000 cells/well) and incubated overnight at 37 °C. All of compounds and controls were prepared in DMSO with stock concentration of 20 mM. Different concentrations of compounds were prepared using serum free DMEM media. Cells were treated with compounds in final concentrations ranging from 0.032–100 μM. After 48 h treatment, MTT solution was added to the wells with a final concentration of 0.5 mg/mL and incubated for 4 h. Following the removal of medium from wells, for solubilization of formed formazan crystals, 100 μL of DMSO was added and incubated for 30 min at room temperature. The absorbance was then measured at 570 nm using an ELISA microplate reader. The cell viability was compared to negative controls where cells were only exposed to the medium. The maximum concentration of DMSO in the cell culture media was less than 0.5%. All experiments were performed in triplicates and the data were analyzed using PRISM software to calculate IC_{50} values. Microsoft Excel was used to calculate mean IC_{50} and standard deviations.

Molecular Docking Studies

The 3D structures of compounds were generated by HyperChem software (version 8.0). The initial structures were energy minimized using molecular mechanics MM+ force field (ref) followed by fully optimization via AM1 semi-empirical method available in HyperChem. The optimized structures were converted to SYBYL mol2 file format using OpenBabel 2.0.2 version to be used as an acceptable format for docking procedure. Flexible docking of the studied compounds into the active site of the COX-2 isoenzyme was performed using GOLD program (version 5.0) running under LINUX operating system. For this purpose, the crystal structure of COX-2 (PDB code: 5KIR) was obtained from Protein Data Bank. Prior to molecular docking, hydrogen atoms were added to the protein in GOLD program. The active site of the enzyme was determined based on the position of rofecoxib co-crystallized with COX-2. To this end, geometric center of the residues involved in the binding to the co-crystallized inhibitor molecule (i.e., rofecoxib) was calculated and set as the center of the binding site. Following the optimization of the docking parameters by re-docking the rofecoxib into the active site of the enzyme, the optimized structures were docked into the COX-2. The best solutions in terms of scoring function were selected and used for analysis of interactions using LigPlot+ and PyMOL program.

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Author Contribution Statement

T.V. performed all the experiments. J.K. performed the COX inhibition assays. S.H. supervised the synthetic procedure. M.H. helped in molecular docking and data analysis. A.A.A. assisted in MTT assay. F.W. helped in COX inhibition assay and data analysis. S.D. designed and supervised this project. All authors have contributed to the final version and approved the final manuscript.

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