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Synthesis, biological evaluation and molecular modeling of dihydro-pyrazolyl-thiazolinone derivatives as potential COX-2 inhibitors

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ABSTRACT

A series of dihydro-pyrazolyl-thiazolinone derivatives (**5a**–**5t**) have been synthesized and their biological activities were also evaluated as potential cyclooxygenase-2 (COX-2) inhibitors. Among these compounds, compound 2-(3-(3,4-dimethylphenyl)-5-phenyl-4,5-dihydro-1*H*-pyrazol-1-yl)thiazol-4(5*H*)-one (**5a**) displayed the most potent COX-2 inhibitory activity with IC_{50} of 0.5 μM, but weak to COX-1. Docking simulation was performed to position compound **5a** into the COX-2 active site to determine the probable binding model. Based on the preliminary results, compound **5a** with potent inhibitory activity and low toxicity would be a potential and selective anti-cyclooxygenase-2 agent.

1. Introduction

Inflammation is a normal response to any noxious stimulus that threatens the host and may vary from a localized response to a generalized one.[1] Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, ibuprofen and indomethacin are widely used in the treatment of acute and chronic inflammatory diseases.[2] However, the long-term clinical employment of NSAIDs is associated with significant side effects such as gastrointestinal lesions, bleeding and nephrotoxicity.[3,4] The clinical efficacy of most NSAIDs is closely to their inhibition of cyclooxygenase (COXs), which catalyze the bioconversion of arachidonic acid (AA) to prostaglandins (PGs).[5,6] COXs are membrane-bound hemeprotein which exist in three distinct isoforms: COX-1, COX-2 and COX-3.[7] COX-1 is constitutive form which is present in healthy tissues and responsible for the thrombogensis and the homeostasis. COX-2 is an inducible enzyme which is induced in response to the release of several pro-inflammatory mediators. COX-3 is located in the central nervous system and could be the pharmacological target of acetaminophen. Researches showed that the major side effect of NSAIDs was due to their inhibition of COX-1.[8–10] It was thought that selective COX-2 inhibitors would be effective anti-inflammatory agents with diminished side effects.[11,12] Therefore, the development of compounds that would inhibit COX-2 almost exclusively is an important target in order to reduce adverse side effects during non-steroidal anti-inflammatory treatment, thus improving therapeutic benefits.

The pyrazole scaffold represents a common motif in many pharmaceutical actively and remarkable compounds demonstrating a wide range of pharmacological activities; the most important activities are anti-tumor,[13] anti-bacterial,[14] anti-inflammatory,[15] the inhibition of cyclooxigenase-2[16] and CDK2/Cyclin A.[17] Literature survey revealed that many pyrazole derivatives had been found their clinical application as NSAIDs.[18] Among the highly marketed COX-2 inhibitors that comprise the pyrazole nucleus, celecoxib is the one which is treated as a safe anti-inflammatory and analgesic agent.[19]

As showed in Figure 1, the well-known COX-2 inhibitor celecoxib and SC-558 consist of a pyrazole core and coterminous substitutional benzene rings. Modeled on this structure, we have designed a series of novel compounds (Fig. 1) by sharing a similar template with celecoxib, with the presumption that the pyrazole group is a common nucleus of COX-2 inhibitors which inhibits COX-2 by binding to its active site.

On the other hand, thiazolinone is an important class of heterocyclic compounds, which exhibit anti-inflammatory, antiproliferative, anti-viral, and anti-bacterial ability.[20–22] Synthesis of the thiazolinones occupies an important place in the realm of synthetic pharmaceutical chemistry, due to their therapeutic and pharmacological properties. Athina A. Geronikaki et al. reported that 2-(thiazole-2-ylamino)-5-phenylidene-4-thiazolidinone derivatives (**5P–4T**) (Fig. 1) were potent inhibitors of COXs.[23] Maccari

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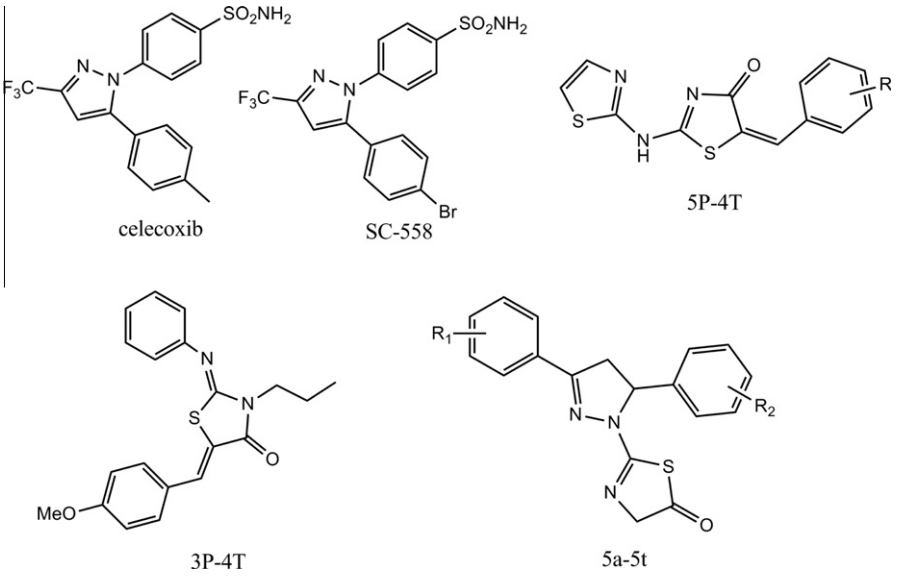


Figure 1. Chemical structures of some reported compounds.

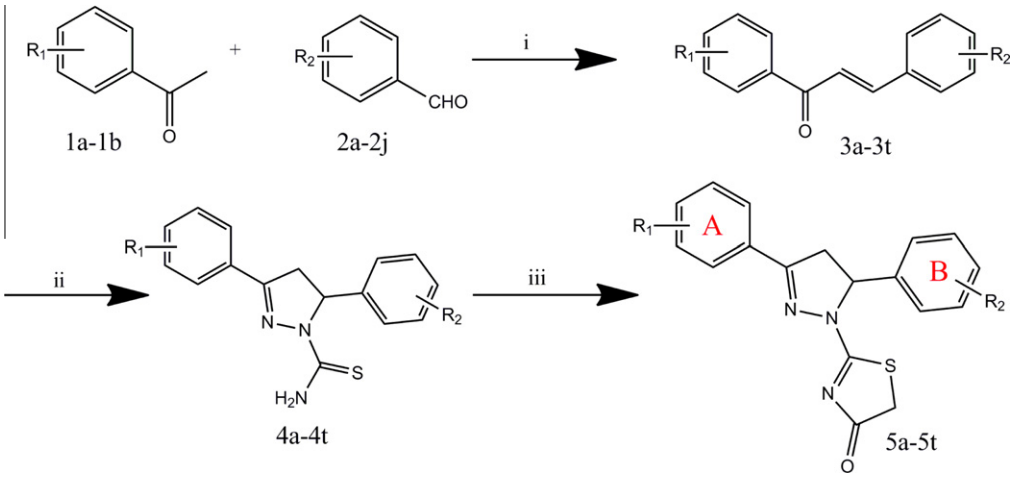
et al. reported that 5-(4-methoxyphenylidene)-2-phenylimino-3-propyl-4-thiazolidinone (**3P–4T**) (Fig. 1) had even showed better COX-2 selectivity than celecoxib and its docking result had given a proof of the auxiliary.[24]

On that basis, in this article, a thiazolinone heterocycle was introduced into the pyrazoline moiety to construct a 2-(3-(phenyl)-5-phenyl-4,5-dihydro-1*H*-pyrazol-1-yl) thiazolinone template with the reasoning that this template could integrate potently with the residues in COX-2 active domain through the hydrophobic interactions, strengthened by hydrogen bonds and π-cation bonds. In continuation to extend our research on anti-inflammatory compounds and screen for novel and powerful COX-2 inhibitors, it is supposed to be worthful to synthesize a series of dihydro-pyrazolyl-thiazolinone derivates (**5a**–**5t**) as a new class of COX-2 inhibitors combined with pyrazole and thiazolinone considering that they might exhibit synergistic effect in anti-inflammatory activities by the inhibition against COX-2. Herein, we reported in the present work the synthesis, biological evaluation and molecular modeling of dihydro-pyrazolyl-thiazolinone derivatives as potential COX-2 inhibitors.

2. Results and discussion

2.1. Chemistry

The synthesis of compounds **5a**–**5t** is followed the general pathway outlined in Scheme 1. Firstly, the chalcones were obtained by direct condensation by the aromatic aldehydes and the substituted acetophenone, using 40% potassium hydroxide as catalyst in ethanol. Secondly, cyclization of different chalcones with thiosemicarbazide under basic condition leads to the formation of pyrazole derivatives containing thiourea skeleton. Thirdly, dihydro-pyrazolyl-thiazolinone derivatives **5a**–**5t** were obtained by reacting compound **4** (1 equiv) with bromoacetic acid (1.2 equiv) in acetic acid and keeping under stirring at 80 °C for 6–8 h. Moreover, acetic anhydride (2 equiv) was added as dehydrating agent and sodium acetate (2 equiv) was used as acid-binding agent. All of the synthetic compounds are reported for the first time and gave satisfactory analytical and spectroscopic data, which were in full accordance with their depicted structures.



Scheme 1. General synthesis of dihydro-pyrazolyl-thiazolinone derivatives (**5a–5t**). Reagents and conditions: (i) 40% aqueous potassium hydroxide solution, ethanol, rt; (ii) thiosemicarbazide, KOH, ethanol, reflux; (iii) bromoacetic acid, acetic anhydride, sodium acetate, acetic acid, 80 °C, 6–8 h.

2.2. Cytotoxicity test

The inhibitory activity of the compounds is sometimes a result of their toxic effect and consequently might cause an erroneous conclusion, prior to the following bioactivity analyses, all the synthesized dihydro-pyrazolyl-thiazolinone derivatives were evaluated using LPS-activated murine macrophages RAW264.7 for cytotoxicity test.[25] The pharmacological results were summarized in Table 1. The data showed that most of dihydro-pyrazolyl-thiazolinone derivatives having a quite low toxicity.

Table 1
Cytotoxicity assay of the compounds in RAW264.7 cells

Compound	R_1	R_2	CC_{50} (μM)[a]	SD[b]
5a	3,4-2Me	4-H	>450	20.36
5b	3,4-2Me	4-F	246.24	20.43
5c	3,4-2Me	4-Cl	225.63	18.97
5d	3,4-2Me	4-Br	365.34	12.86
5e	3,4-2Me	4-Me	383.58	24.32
5f	3,4-2Me	4-OMe	387.46	19.52
5g	3,4-2Me	3-Cl	178.32	9.05
5h	3,4-2Me	3-Me	236.78	12.54
5i	3,4-2Me	2-Cl	227.30	11.37
5j	3,4-2Me	2-Me	316.65	15.57
5k	3,4-2Cl	4-H	295.17	14.69
5l	3,4-2Cl	4-F	138.98	8.34
5m	3,4-2Cl	4-Cl	187.24	10.92
5n	3,4-2Cl	4-Br	279.51	14.58
5o	3,4-2Cl	4-Me	279.35	14.17
5p	3,4-2Cl	4-OMe	264.43	13.27
5q	3,4-2Cl	3-Cl	165.22	15.32
5r	3,4-2Cl	3-Me	203.59	16.25
5s	3,4-2Cl	2-Cl	143.27	7.34
5t	3,4-2Cl	2-Me	178.63	8.93

The CC_{50} (cytoxicity) represents the concentration of drug that is required for 50% cell kill (cytoxicity) of the uninfected cells.

[a] The cytotoxicity of each compound was expressed as the concentration of compound that reduced cell viability to 50% (CC_{50}).

[b] The data represented the mean of three experiments in triplicate and were expressed as means ± SD. SD means The Standard deviation values.

Table 2
Date of the in vitro COX-1/COX-2 enzyme inhibition assay of the designed compounds

Compound	R_1	R_2	IC_{50} (μM)[a]		SI[b]
			COX-1	COX-2	
5a	3,4-2Me	4-H	42.4	0.5	84.8
5b	3,4-2Me	4-F	40.8	6.4	6.4
5c	3,4-2Me	4-Cl	48.6	4.5	10.8
5d	3,4-2Me	4-Br	>50	1.8	>27.8
5e	3,4-2Me	4-Me	45.3	1.2	37.8
5f	3,4-2Me	4-OMe	>50	2.9	>17.2
5g	3,4-2Me	3-Cl	>50	5.2	>9.6
5h	3,4-2Me	3-Me	>50	2.7	>18.5
5i	3,4-2Me	2-Cl	44.1	7.2	6.1
5j	3,4-2Me	2-Me	47.2	6.3	7.5
5k	3,4-2Cl	4-H	33.2	2.7	12.3
5l	3,4-2Cl	4-F	34.3	19.4	1.8
5m	3,4-2Cl	4-Cl	37.7	11.7	3.2
5n	3,4-2Cl	4-Br	48.5	7.6	6.4
5o	3,4-2Cl	4-Me	44.3	4.7	9.4
5p	3,4-2Cl	4-OMe	>50	6.8	>7.4
5q	3,4-2Cl	3-Cl	>50	16.1	>3.1
5r	3,4-2Cl	3-Me	>50	7.2	>6.9
5s	3,4-2Cl	2-Cl	40.2	18.4	2.2
5t	3,4-2Cl	2-Me	45.6	16.3	2.8
Celecoxib			10	0.1	100

[a] IC_{50} value is the compound concentration required to produce 50% inhibition of COX-1 or COX-2 for means of two determinations and deviation from the mean is <10% of the mean value.

[b] Selectivity index (SI = COX-1 IC_{50}/COX-2 IC_{50}).

2.3. In vitro enzyme inhibition activity

The synthesized derivatives were evaluated for their ability to inhibit the COX-1 and COX-2 isozyme using a solid-phase ELISA assay. The results were summarized in Table 2. It was observed that dihydro-pyrazolyl-thiazolinone derivatives containing thiazolinone and pyrazole rings showed fairly good inhibiting COX-2 activities displaying IC_{50} values from 0.5 to 19.4 μM, but weak to COX-1. Among them, compound **5a** revealed the most potent inhibitory activity (IC_{50} = 0.5 μM for COX-2) and best selectivity (SI = 84.8 for **5a** contrast to SI = 100 for celecoxib). The results of western blotting assay further confirmed that compound **5a** with the concentration above 1.25 μM could obviously inhibit lipopolysaccharide (LPS)-induced COX-2 expression in murine macrophage RAW 264.7 cell line (Fig. 2).

Subsequently SAR studies were performed by modification of the parent compound to determine how the substituents of the subunits affect the COX-2 inhibitory activities. Inspection of the chemical structures of compounds **5a–5t** suggested that they could be divided into two subunits: A and B rings (Scheme 1). As showed in Table 2, it was obvious that compounds with electron-donating substituents at *para*-position and *meta*-position at A-ring (3, 4-2Me, IC_{50} = 0.5–7.2 μM for COX-2) had better inhibitory activity than that with electron-withdrawing substituents at the same position (3, 4-2Cl, IC_{50} = 2.7–19.4 μM for COX-2). In the case of constant A ring substituents, the groups at B ring also have a great significance. Compounds **5a–5f**, **5k–5p** with *para* substituents on phenyl ring exhibited significant COX-2 inhibitory activities in the order of H > Br > Cl > F, H > Me > OMe. The results demonstrated that an electron-withdrawing halogen group and an electron donating methyl/methoxyl substituent may show some relatively negative effects. Faintish electron-withdrawing substituent (*para*-Br) (**5d**, **5n**) and faintish electron-donating substituent (*para*-Me) (**5e**, **5o**) had relatively minor effects compared with **5a**, whereas strong electron-withdrawing substituent (*para*-F/*para*-Cl) (**5b**, **5l**/**5c**, **5m**) or powerful electron-donating substituent (*para*-OMe) (**5f**, **5p**) led to a relatively poor result.

Considering the steric effect of substituents at B-ring, we designed and evaluated the compounds with *ortho*-Cl-phenyl (**5i**, **5s**), *ortho*-Me-phenyl (**5j**, **5t**), *meta*-Cl-phenyl (**5g**, **5q**) and *meta*-Me-phenyl (**5h**, **5r**) group substituents in the B-ring. From the activity datas from Table 2, it can be seen that compounds with substituents at the *para*-position (**5c**, **5m**) showed more potent activities than those with substituents at *meta*-position (**5g**, **5q**). Meanwhile, a significant loss of activity was observed when the halogen substituent was moved from the *para*-position (**4c**, **4m**) to the *ortho*-position (**5i**, **5s**) in the B-ring. The modification of methyl group in the B ring also apply to this rule with the order of *para* > *meta* > *ortho.*

2.4. Molecular docking study

To gain better understanding on the potency of the synthesized compounds and guide further structure–activity relationships

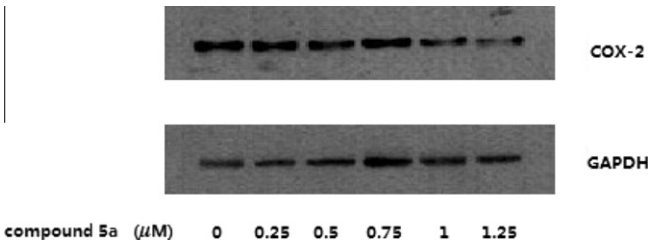


Figure 2. Inhibitory effect of compound **5a** on COX-2 protein expression.

(SAR) studies. The molecular docking was performed by putting inhibitor **5a** into binding site of COX-2, the same binding site of SC-558 (Fig. 1).[26] All docking runs were applied by Discovery Studio3.1 (DS. 3.1). The binding models of compound **5a** with COX-2 were depicted in Figure 3A and 3B. The amino acid residues which had interaction with **5a** in the active site were labeled. We can see that compound **5a** was nicely bound to the COX-2 via hydrophobic interaction and the binding was stabilized by two hydrogen bonds and a π-cation interaction. It was shown that the S and O atoms in the thiazolinone ring of compound **5a** exhibited hydrogen bonds with His 90 and Ile 517 in COX-2 active site (distance: N–H···S = 1.98 Å, 159.46° and N–H···O = 2.37 Å, 136.44°, respectively). In addition, a π-cation interaction (3.95 Å) was formed by Arg 120 with the benzene ring, which enhanced the binding action between receptor COX-2 and ligand compound **5a**. The electron-donating substituent amino-NH_2 strengthened the π-cation binding and the results indicated that it was primarily due to direct through-space interaction between the substituent and the cation. Overall, these results can provide a good explanation for the high potency of compound **5a** towards COX-2 protein.

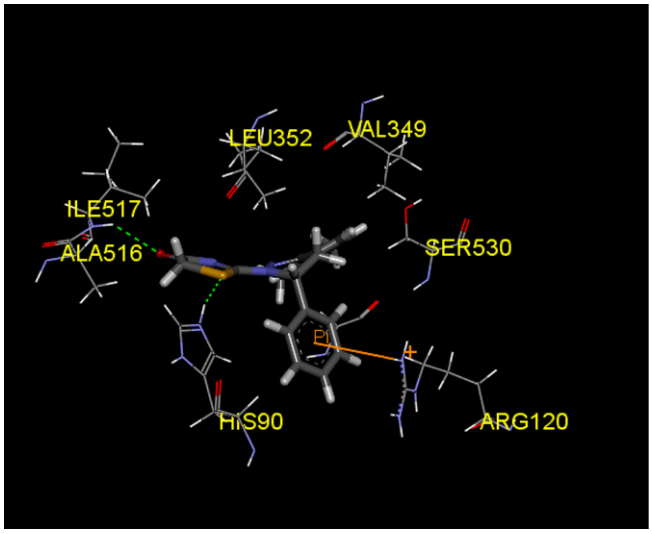


Figure 3A. Molecular docking 3D modeling of compound **5a** with COX-2: for clarity, only interacting residues are displayed. The H-bond (green lines) is displayed as dotted lines, and the π-cation interaction is shown as orange lines.

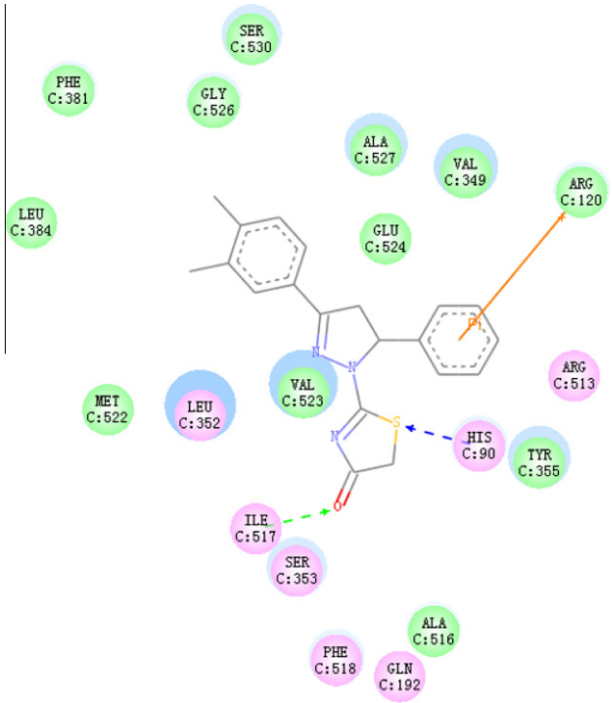


Figure 3B. Molecular docking 2D modeling of **5a** with COX-2: for clarity, only interacting residues are displayed. The H-bond (green lines and blue lines) is displayed as arrow dots, and the π-cation interaction is shown as orange lines.

It is known that the main difference between the two COX active sites is the replacement of Ile 523 in COX-1 by Val 523, a less bulky amino acid. This replacement makes COX-2 binding site 25% larger than the COX-1 and creates an adjunct pocket in the COX-2 active site which may be responsible for selectivity.[24]

Our docking study showed that compound **5a** was oriented so that its phenyl-pyrazoline ring and thiazolinone ring could fill this adjunct pocket. But when we docked **5a** to the active site of COX-1, the binding site of flurbiprofen, we found it's hard to combine (compound **5a** could not get into the active site of COX-1). This may explain why compound **5a** shows significant selective activity against COX-2 enzyme.

3. Conclusions

In this paper, we have prepared a series of dihydro-pyrazolyl-thiazolinone derivatives (**5a**–**5t**) and evaluated their biological activities. Preliminary results showed that most of the compounds displayed enhanced inhibitory activities and low toxicity. Among them, compound **5a** was most active (IC_{50} = 0.5 μM for COX-2), as it showed the half maximal inhibitory concentration (IC_{50}) most close to the positive control celecoxib (IC_{50} = 0.1 μM for COX-2). Moreover, its selectivity between COX-1/COX-2, which was represented by SI value was very close to celecoxib (SI = 84.8 for **5a** and 100 for celecoxib).

Molecular docking was further performed to study the inhibitor-COX-2 protein interactions. After analysis of the binding model of compound **5a** with COX-2, it was found that two hydrogen bonds and a π-cation interaction with the receptor protein residues in the active site might play a crucial role in its COX-2 inhibition and anti-inflammatory activities. Docking result also had showed that **5a** could not even bind into the same active site of COX-1, probable with the reason that the pocket of COX-1 is 25% smaller than that of COX-2 by the replacement of Ile 523 in COX-1 by Val 523, so originating a secondary internal pocket not accessible in COX- 1.[27]

All of this told us that compound **5a** was a favorable and selective COX-2 inhibitor, which displayed anti-inflammatory via the COX-2 pathway and was scarcely any toxicity due to little COX-1 inhibition. The results of this study help to find a promising compound with stronger activities and low toxicity toward the development of new therapeutic agent to fight against inflammation.

4. Experimental protocols

4.1. Materials and measurements

All chemicals and reagents used in current study were analytical grade. All the ^{1}H NMR spectra were recorded on a Bruker DPX 300 or DRX 500 model Spectrometer in DMSO-d_6 and chemical shifts were reported in ppm (δ). ESI-MS spectra were recorded on a Mariner System 5304 Mass spectrometer. Elemental analyses were performed on a CHN-O-Rapid instrument. TLC was performed on the glass-backed silica gel sheets (silica gel 60 Å GF254) and visualized in UV light (254 nm). Column chromatography was performed using silica gel (200–300 mesh) eluting with ethyl acetate and petroleum ether.

4.2. Synthesis

4.2.1. General synthetic procedure of chalcones (3a–3t)

Equimolar portions of the appropriately aromatic aldehydes (3 mmol, 1 equiv) and substituted acetophenone (3 mmol, 1 equiv) were dissolved in approximately 20 mL of ethanol. The mixture was allowed to stir for several minutes at 0 °C to let dissolve. Than a 1 mL aliquot of a 40% aqueous potassium hydroxide solution was then slowly added dropwise to the reaction flask via a self-equalizing addition funnel. The reaction solution was allowed to stir at room temperature for approximately 4–6 h. Most commonly, a precipitate formed and was then collected by suction filtration.

4.2.2. General synthetic procedure of pyrazole derivatives (4a–4t)

A mixture of chalcone (2 mmol), thiosemicarbazide (3 mmol), and KOH (2 mmol) was refluxed in ethanol (30 mL) for 12 h. After cooling, the solution was poured into mass of ice-water and stirred for a few minutes. The precipitate was filtered and crystallized from ethanol.

4.2.3. General synthetic procedure of dihydro-pyrazolyl-thiazolinone derivatives (5a–5t)

A mixture of compound **4** (1 mmol), bromoacetic acid (1.2 mmol), acetic anhydride (2 mmol), and sodium acetate (2 mmol) was dissolved in acetic acid (20 mL) and kept stirring in 80 °C for 6–8 h. After cooling, the solution was poured into mass of ice-water and stirred for a few minutes. The precipitate was filtered and crystallized from methylene dichloride : ethanol = 1:1.

4.2.3.1. 2-(3-(3,4-Dimethylphenyl)-5-phenyl-4,5-dihydro-1*H*-pyrazol-1-yl)thiazol-4(5*H*)-one (5a). Yield 64%; mp 202–204 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 2.34 (s, 6H), 3.34 (d, J = 14.32 Hz, 1H), 3.92 (s, 2H), 3.98 (dd, J_1 = 6.96 Hz, J_2 = 10.89 Hz, 1H), 5.84 (d, J = 8.04 Hz, 1H), 7.16 (d, J = 8.25 Hz, 1H), 7.28 (d, J = 8.22 Hz, 3H), 7.43 (d, J = 6.84 Hz, 2H), 7.52–7.56 (m, 2H). ESI-MS: 350.45 ($C_{20}H_{20}N_3OS$, $[M+H]^+$). Anal. Calcd for $C_{20}H_{19}N_3OS$: C, 68.74; H, 5.48; N, 12.02. Found: C, 68.80; H, 5.50; N, 12.06.

4.2.3.2. 2-(3-(3,4-Dimethylphenyl)-5-(4-fluorophenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)thiazol-4(5*H*)-one (5b). Yield 84%; mp 220–222 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): 2.33 (s, 6H), 3.34 (dd, J_1 = 3.20 Hz, J_2 = 17.85 Hz, 1H), 3.94 (s, 2H), 3.96 (dd, J_1 = 5.34 Hz, J_2 = 15.84 Hz, 1H), 5.88 (d, J = 8.05 Hz, 1H),7.16 (d, J = 8.26 Hz, 2H), 7.23 (d, J = 7.90 Hz, 1H), 7.45 (d, J = 8.40 Hz, 2H), 7.50 (d, J = 7.75 Hz, 1H), 7.57 (s, 1H). ESI-MS: 368.44 ($C_{20}H_{19}FN_3OS$, $[M+H]^+$). Anal. Calcd for $C_{20}H_{18}FN_3OS$: C, 65.38; H, 4.94; N, 11.44. Found: C, 65.45; H, 4.96; N, 11.47.

4.2.3.3. 2-(5-(4-Chlorophenyl)-3-(3,4-dimethylphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)thiazol-4(5*H*)-one (5c). Yield 85%; mp 234–236 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 2.32 (s, 6H), 3.34 (d, J = 10.62 Hz, 1H), 3.86 (s, 2H), 3.92 (dd, J_1 = 6.26 Hz, J_2 = 10.89 Hz, 1H), 5.80 (d, J = 5.22 Hz, 1H), 6.99–7.02 (m, 2H), 7.21–7.26 (m, 2H), 7.51 (d, J = 4.65 Hz, 2H), 7.58 (s, 1H). ESI-MS: 384.89 ($C_{20}H_{19}ClN_3OS$, $[M+H]^+$). Anal. Calcd for $C_{20}H_{18}ClN_3OS$: C, 62.57; H, 4.73; N, 10.95. Found: C, 62.62; H, 4.71; N, 10.98.

4.2.3.4. 2-(5-(4-Bromophenyl)-3-(3,4-dimethylphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)thiazol-4(5*H*)-one (5d). Yield 85%; mp 262–264 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 2.33 (s, 6H), 3.34 (d, J = 17.19 Hz, 1H), 3.71 (s, 2H), 3.87 (dd, J_1 = 6.91 Hz, J_2 = 12.89 Hz, 1H), 5.87 (s, 1H), 7.20–7.26 (m, 3H), 7.30 (d, J = 8.04 Hz, 2H), 7.51 (d, J = 8.43 Hz, 1H), 7.59 (d, J = 8.43 Hz, 1H). ESI-MS: 429.35 ($C_{20}H_{19}BrN_3OS$, $[M+H]^+$). Anal. Calcd for $C_{20}H_{18}BrN_3OS$: C, 56.08; H, 4.24; N, 9.81. Found: C, 56.05; H, 4.24; N, 9.84.

4.2.3.5. 2-(3-(3,4-Dimethylphenyl)-5-(*p*-tolyl)-4,5-dihydro-1*H*-pyrazol-1-yl)thiazol-4(5*H*)-one (5e). Yield 82%; mp 219–221 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 2.33 (s, 9H), 3.36 (dd, J_1 = 3.48 Hz, J_2 = 17.76 Hz, 1H), 3.72 (s, 2H), 3.81 (d, J = 17.01 Hz, 1H), 5.84 (d, J = 7.89 Hz, 1H), 7.10–7.18(m, 4H), 7.21 (d, J = 7.86 Hz, 1H), 7.51 (d, J = 7.86 Hz, 1H), 7.59 (d, J = 6.78 Hz, 1H). ESI-MS: 364.48 ($C_{21}H_{22}N_3OS$, $[M+H]^+$). Anal. Calcd for $C_{21}H_{21}N_3OS$: C, 69.39; H, 5.82; N, 11.56. Found: C, 69.37; H, 5.81; N, 11.59.

4.2.3.6. 2-(3-(3,4-Dimethylphenyl)-5-(4-methoxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)thiazol-4(5*H*)-one (5f). Yield 86%; mp 223–225 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 2.33 (s, 6H), 2.38 (s, 3H), 3.35 (d, J = 15.32 Hz, 1H), 3.81 (s, 2H), 3.92 (dd, J_1 = 4.38 Hz, J_2 = 18.02 Hz, 1H), 5.86 (d, J = 7.64 Hz, 1H), 6.98 (d, J = 7.38 Hz, 2H), 7.16- 7.18 (m, 3H), 7.54 (d, J = 5.46 Hz, 2H). ESI-MS: 380.48 ($C_{21}H_{22}N_3O_2S$, $[M+H]^+$). Anal. Calcd for $C_{21}H_{21}N_3O_2S$: C, 66.47; H, 5.58; N, 11.07. Found: C, 66.53; H, 5.61; N, 11.11.

4.2.3.7. 2-(5-(3-Chlorophenyl)-3-(3,4-dimethylphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)thiazol-4(5*H*)-one (5g). Yield 78%; mp 229–231 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 2.33 (s, 6H), 3.38 (d, J = 14.84 Hz, 1H), 3.85 (s, 2H), 3.98 (d, J = 16.84 Hz, 1H), 5.82 (d, J = 8.22 Hz, 1H), 7.14–7.19 (m, 2H), 7.31–7.34 (m, 2H), 7.49 (d, J = 4.34 Hz, 1H), 7.54 (d, J = 6.34 Hz, 1H), 7.60 (s, 1H). ESI-MS: 384.89 ($C_{20}H_{19}ClN_3OS$, $[M+H]^+$). Anal. Calcd for $C_{20}H_{18}ClN_3OS$: C, 62.57; H, 4.73; N, 10.95. Found: C, 62.62; H, 4.75; N, 10.91.

4.2.3.8. 2-(3-(3,4-Dimethylphenyl)-5-(*m*-tolyl)-4,5-dihydro-1*H*-pyrazol-1-yl)thiazol-4(5*H*)-one (5h). Yield 74%; mp 215–218 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 2.34 (s, 9H), 3.37 (d, J = 16.84 Hz, 1H), 3.76 (s, 2H), 3.84 (dd, J_1 = 7.23 Hz, J_2 = 17.04 Hz, 1H), 5.82 (s, 1H), 7.08–7.13 (m, 3H), 7.18 (d, J = 8.65 Hz, 1H), 7.51 (d, J = 8.36 Hz, 2H), 7.56 (d, J = 6.42 Hz, 1H). ESI-MS: 364.48 ($C_{21}H_{22}N_3OS$, $[M+H]^+$). Anal. Calcd for $C_{21}H_{21}N_3OS$: C, 69.39; H, 5.82; N, 11.56. Found: C, 69.43; H, 5.81; N, 11.59.

4.2.3.9. 2-(5-(2-Chlorophenyl)-3-(3,4-dimethylphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)thiazol-4(5*H*)-one (5i). Yield 79%; mp 232–233 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 2.31 (s, 6H), 3.36 (d, J = 15.34 Hz, 1H), 3.91 (s, 2H), 4.02 (dd, J_1 = 4.24 Hz, J_2 = 14.38 Hz, 1H), 5.85 (d, J = 8.34 Hz, 1H), 7.16 (d, J = 5.36 Hz, 1H), 7.22–7.26 (m, 3H), 7.52 (d, J = 4.46 Hz, 2H), 7.73 (d, J = 5.46 Hz, 1H). ESI-MS: 384.89 ($C_{20}H_{19}ClN_3OS$, $[M+H]^+$). Anal. Calcd for $C_{20}H_{18}ClN_3OS$: C, 62.57; H, 4.73; N, 10.95. Found: C, 62.63; H, 4.76; N, 10.92.

4.2.3.10. 2-(3-(3,4-Dimethylphenyl)-5-(*o*-tolyl)-4,5-dihydro-1*H*-pyrazol-1-yl)thiazol-4(5*H*)-one (5j). Yield 75%; mp 217–219 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 2.34 (s, 3H), 3.37 (d, J = 18.24 Hz, 1H), 3.87 (s, 2H), 4.08 (dd, J_1 = 6.83 Hz, J_2 = 15.82 Hz, 1H), 5.82 (d, J = 10.24 Hz, 1H), 6.94 (d, J = 5.34 Hz, 1H), 7.14–7.17 (m, 2H), 7.22 (d, J = 8.62 Hz, 1H), 7.45–7.52 (m, 3H). ESI-MS: 364.48 ($C_{21}H_{22}N_3OS$, $[M+H]^+$). Anal. Calcd for $C_{21}H_{21}N_3OS$: C, 69.39; H, 5.82; N, 11.56. Found: C, 69.46; H, 5.84; N, 11.59.

4.2.3.11. 2-(3-(3,4-Dichlorophenyl)-5-phenyl-4,5-dihydro-1*H*-pyrazol-1-yl)thiazol-4(5*H*)-one (5k). Yield 66%; mp 244–246 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.36 (d, J = 16.82 Hz, 1H), 3.83 (s, 2H), 4.01 (dd, J_1 = 7.13 Hz, J_2 = 14.64 Hz, 1H), 5.86 (d, J = 10.14 Hz, 1H), 7.27–7.29 (m, 3H), 7.41 (d, J = 8.04 Hz, 2H), 7.70 (s, 1H), 7.87 (d, J = 6.85 Hz, 2H). ESI-MS: 391.29 ($C_{18}H_{14}Cl_2N_3OS$, $[M+H]^+$). Anal. Calcd for $C_{18}H_{13}Cl_2N_3OS$: C, 55.39; H, 3.36; N, 10.77. Found: C, 55.44; H, 3.35; N, 10.80.

4.2.3.12. 2-(3-(3,4-Dichlorophenyl)-5-(4-fluorophenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)thiazol-4(5*H*)-one (5l). Yield 83%; mp 252–254 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.36 (d, J = 14.23 Hz, 1H),

3.91 (s, 2H), 4.02 (dd, J_1 = 4.63 Hz, J_2 = 12.24 Hz, 1H), 5.87 (d, J = 8.84 Hz, 1H), 7.19 (d, J = 6.24 Hz, 2H), 7.28 (d, J = 4.58 Hz, 2H), 7.69 (s, 1H) 7.86 (d, J = 6.24 Hz, 2H). ESI-MS: 409.28 ($C_{18}H_{13}Cl_2FN_3OS$, $[M+H]^+$). Anal. Calcd for $C_{18}H_{12}Cl_2FN_3OS$: C, 52.95; H, 2.96; N, 10.29. Found: C, 53.02; H, 2.98; N, 10.32.

4.2.3.13. 2-(5-(4-Chlorophenyl)-3-(3,4-dichlorophenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)thiazol-4(5*H*)-one (5m). Yield 85%; mp 260–262 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.36 (d, J = 14.26 Hz, 1H), 3.88 (s, 2H), 3.98 (dd, J_1 = 4.36 Hz, J_2 = 10.45 Hz, 1H), 5.87 (d, J = 8.43 Hz, 1H), 7.44 (d, J = 6.04 Hz, 2H), 7.49 (d, J = 5.84 Hz, 2H), 7.69 (d, J = 5.34 Hz, 1H), 7.86 (d, J = 14.22 Hz, 2H). ESI-MS: 425.73 ($C_{18}H_{13}Cl_3N_3OS$, $[M+H]^+$). Anal. Calcd for $C_{18}H_{12}Cl_3N_3OS$: C, 50.90; H, 2.85; N, 9.89. Found: C, 50.95; H, 2.87; N, 9.92.

4.2.3.14. 2-(5-(4-Bromophenyl)-3-(3,4-dichlorophenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)thiazol-4(5*H*)-one (5n). Yield 86%; mp 285–287 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.33 (d, J = 16.32 Hz, 1H), 3.86 (s, 2H), 3.95 (dd, J_1 = 6.38 Hz, J_2 = 14.04 Hz, 1H), 5.85 (s, 1H), 7.19 (d, J = 7.46 Hz, 2H), 7.69 (d, J = 7.36 Hz, 1H), 7.86 (d, J = 6.82 Hz, 2H), 7.96 (d, J = 8.62 Hz, 2H). ESI-MS: 470.18 ($C_{18}H_{13}BrCl_2N_3OS$, $[M+H]^+$). Anal. Calcd for $C_{18}H_{12}BrCl_2N_3OS$: C, 46.08; H, 2.58; N, 8.96. Found: C, 46.03; H, 2.56; N, 8.91.

4.2.3.15. 2-(3-(3,4-Dichlorophenyl)-5-(*p*-tolyl)-4,5-dihydro-1*H*-pyrazol-1-yl)thiazol-4(5*H*)-one (5o). Yield 82%; mp 249–251 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 2.34 (s, 3H), 3.36 (dd, J_1 = 4.06 Hz, J_2 = 14.63 Hz, 1H), 3.87 (s, 2H), 3.98 (dd, J_1 = 5.26 Hz, $J2$ = 15.42 Hz, 1H), 5.89 (d, J = 6.36 Hz, 1H), 7.15–7.18 (m, 4H), 7.65 (d, J = 6.34 Hz, 1H), 7.85 (d, J = 5.38 Hz, 2H). ESI-MS: 405.31 ($C_{19}H_{16}Cl_2N_3OS$, $[M+H]^+$). Anal. Calcd for $C_{19}H_{15}Cl_2N_3OS$: C, 56.44; H, 3.74; N, 10.39. Found: C, 56.49; H, 3.75; N, 10.42.

4.2.3.16. 2-(3-(3,4-Dichlorophenyl)-5-(4-methoxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)thiazol-4(5*H*)-one (5p). Yield 86%; mp 253–255 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.36 (dd, J_1 = 4.24 Hz, J_2 = 17.84 Hz, 1H), 3.82 (s, 3H), 3.88 (s, 2H), 4.02 (dd, J_1 = 5.36 Hz, J_2 = 14.44 Hz, 1H), 5.86 (d, J = 7.36 Hz, 1H), 6.94 (d, J = 8.42 Hz, 2H), 7.20 (d, J = 7.62 Hz, 2H), 7.72 (d, J = 6.42 Hz, 1H), 7.82–7.86 (m, 2H). ESI-MS: 421.31 ($C_{19}H_{16}Cl_2N_3O_2S$, $[M+H]^+$). Anal. Calcd for $C_{19}H_{15}Cl_2N_3O2S$: C, 54.29; H, 3.60; N, 10.00. Found: C, 54.26; H, 3.62; N, 10.05.

4.2.3.17. 2-(5-(3-Chlorophenyl)-3-(3,4-dichlorophenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)thiazol-4(5*H*)-one (5q). Yield 78%; mp 256–258 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.34 (dd, J_1 = 3.64 Hz, J_2 = 17.54 Hz, 1H), 3.87 (s, 2H), 3.94 (dd, J_1 = 6.42 Hz, J_2 = 13.24 Hz, 1H), 5.86 (d, J = 6.42 Hz, 1H), 7.14 (d, J = 5.86 Hz, 1H), 7.32–7.36 (m, 2H), 7.49 (s, 1H), 7.68 (d, J = 5.18 Hz, 1H), 7.86 (d, J = 8.32 Hz, 2H). ESI-MS: 425.73 ($C_{18}H_{13}Cl_3N_3OS$, $[M+H]^+$). Anal. Calcd for $C_{18}H_{12}Cl_3N_3OS$: C, 50.90; H, 2.85; N, 9.89. Found: C, 50.90; H, 2.85; N, 9.89.

4.2.3.18. 2-(3-(3,4-Dichlorophenyl)-5-(*m*-tolyl)-4,5-dihydro-1*H*-pyrazol-1-yl)thiazol-4(5*H*)-one (5r). Yield 81%; mp 247–248 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 2.34 (s, 3H), 3.33 (d, J = 10.67 Hz, 1H), 3.90 (s, 2H), 3.95 (dd, J_1 = 6.62 Hz, J_2 = 12.42 Hz, 1H), 5.87 (d, J = 5.68 Hz, 1H), 7.08 (d, J = 6.04 Hz, 2H), 7.16 (d, J = 5.43 Hz,1H), 7.58–7.63 (m, 2H), 7.86 (d, J = 6.34 Hz, 2H). ESI-MS: 405.31 ($C_{19}H_{16}Cl_2N_3OS$, $[M+H]^+$). Anal. Calcd for $C_{19}H_{15}Cl_2N_3OS$: C, 56.44; H, 3.74; N, 10.39. Found: C, 56.49; H, 3.76; N, 10.43.

4.2.3.19. 2-(5-(2-Chlorophenyl)-3-(3,4-dichlorophenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)thiazol-4(5*H*)-one (5s). Yield 75%; mp 220–222 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.36 (dd, J_1 = 5.08 Hz, J_2 = 15.48 Hz, 1H), 3.86 (s, 2H), 4.03 (dd, J_1 = 5.94 Hz, J_2 = 15.38 Hz, 1H), 5.86 (d, J = 8.14 Hz, 1H), 7.22–7.28 (m, 3H), 7.67 (d, J = 5.89 Hz, 1H), 7.72 (d, J = 8.12 Hz, 1H), 7.88 (d, J = 7.24 Hz, 2H). ESI-MS: 425.73 ($C_{18}H_{13}Cl_3N_3OS$, $[M+H]^+$). Anal. Calcd for $C_{18}H_{12}Cl_3N_3OS$: C, 50.90; H, 2.85; N, 9.89. Found: C, 50.96; H, 2.87; N, 9.86.

4.2.3.20. 2-(3-(3,4-Dichlorophenyl)-5-(*o*-tolyl)-4,5-dihydro-1*H*-pyrazol-1-yl)thiazol-4(5*H*)-one (5t). Yield 76%; mp 262–264 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 2.34 (s, 3H), 3.36 (dd, J_1 = 4.46 Hz, J_2 = 16.94 Hz, 1H), 3.86 (s, 2H), 4.04 (dd, J_1 = 6.26 Hz, J_2 = 14.84 Hz, 1H), 5.82 (s, 1H), 6.95 (d, J = 5.34 Hz, 1H), 7.15–7.20 (m, 2H), 7.38–7.41 (m, 1H), 7.69 (d, J = 8.22 Hz, 1H), 7.86 (d, J = 8.24 Hz, 2H). ESI-MS: 405.31 ($C_{19}H_{16}Cl_2N_3OS$, $[M+H]^+$). Anal. Calcd for $C_{19}H_{15}Cl_2N_3OS$: C, 56.44; H, 3.74; N, 10.39. Found: C, 56.49; H, 3.76; N, 10.42.

4.3. Molecular modeling (docking) studies

Molecular docking of compound **5a** into the 3D COX-1/COX-2 complex structure (PDB code: 1PGF/1cx2) was carried out using the Discovery Stutio (version 3.1) as implemented through the graphical user interface DS-LigandFit protocal.

The three-dimensional structures of the aforementioned compounds were constructed using Chem 3D ultra 11.0 software [Chemical Structure Drawing Standard; Cambridge Soft corporation, USA (2009)], then they were energetically minimized by using MOPAC with 100 iterations and minimum RMS gradient of 0.10. The crystal structures of COX-2 complex were retrieved from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). All bound water and ligands were eliminated from the protein and the polar hydrogen was added. The whole COX-2 complex was defined as a receptor and the site sphere was selected based on the ligand binding location of SC-558, then the SC-558 molecule was removed and **5a** was placed during the molecular docking procedure. Types of interactions of the docked protein with ligand were analyzed after the end of molecular docking.

4.4. Cytotoxicity test

The cytotoxic activity in vitro was measured using the MTT assay. RAW264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS) (Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. In all cell cultures, each compound was prepared in dimethyl sulphoxide (DMSO) followed by dilution with culture medium to desired concentrations, and DMSO final concentration was 0.1%. DMSO at 0.1% was added into control (RAW264.7 cells were treated with LPS only) and blank (RAW264.7 cells only, without any treatments) groups and showed no effects on cells.

Cells were plated at a density of 5×10^4 cells in a 96-well plate, and compounds were added to each plate at the indicated concentrations. The incubation was permitted at 37 °C, 5% CO_2 atmosphere for 24 h before the cytotoxicity assessments. 20 μL MTT reagent (4 mg/mL) was added per well 4 h before the end of the incubation. Four hours later, the plate was centrifugaled at 1200 rcf for 5 min and the supernatants were removed, each well was added with 200 μL DMSO. The absorbance was measured at a wavelength of

570 nm (OD 570 nm) on an ELISA microplate reader. The results were summarized in Table 1.

4.5. In vitro cyclooxygenase (COX) inhibition assays

The ability of the test compounds **5a–5t** to inhibit COX-1 and COX-2 was determined using chemiluminescent enzyme assays kit (Cayman Chemical, Ann Arbor, MI, USA) according to previously reported method.[28]

4.6. Western blotting

After incubation, cells were harvested and washed with PBS, then lysed in lysis buffer (30 mm Tris, pH 7.5, 150 mm NaCl, 1 mm phenylmethylsulfonyl fluoride, 1 mm Na_3VO_4, 1% Nonidet P-40, 10% glycerol, and phosphatase and protease inhibitors). After centrifugation at 12,000*g* for 5 min, the supernatant was collected as total protein. The concentration of the protein was determined by a BCA™ protein assay kit (Pierce, Rockford, IL, USA). The protein samples were separated by 10% SDS–PAGE and subsequently electrotransferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% nonfat milk for 2 h at room temperature. The blocked membrane was probed with the indicated primary antibodies overnight at 4 °C, and then incubated with a horse radish peroxidase (HRP)-coupled secondary antibody. COX-2 protein expression was measured as shown in Figure 2.

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