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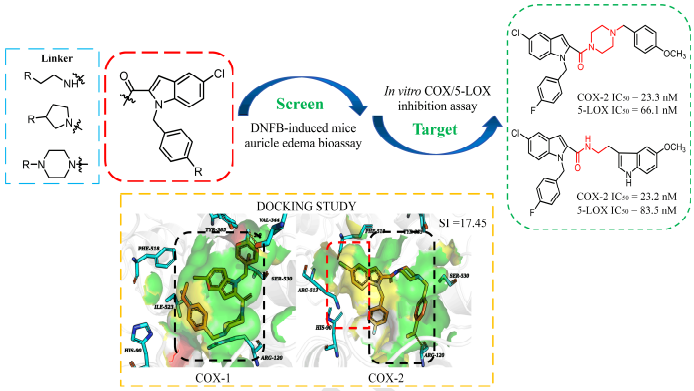

Design, Synthesis, biological evaluation and docking study of novel Indole-2-amide as anti-inflammatory agents with dual inhibition of COX and 5-LOX

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Abstract

In this work, a series of novel indole-2-amide compounds were designed, synthesized, characterized and the anti-inflammatory activity *in vivo* were evaluated. Compounds **8a**, **10b**, **12h**, and **12l** exhibited marked anti-inflammatory activity in 2,4-Dinitrofluorobenzenethe (DNFB) - induced mice auricle edema model. Further, compounds **8a 10b** and **12h** exhibited potential *in vitro* COX-2 inhibitory activity (IC_{50} = 21.86, 23.3 and 23.21 nM, respectively), while the reference drug celecoxib was 11.20 nM. The most promising compound **10b** was exhibited the highest selectivity for COX-2 (selectivity index (COX-1/COX-2) = 17.45) and moderate 5-LOX inhibitory activity (IC_{50} = 66 nM), which comparable to positive controlled zileuton (IC_{50} = 38.91 nM). In addition, the test results showed compounds **10b** and **12h** no significant cytotoxic activity on normal cells (RAW264.7). Further, at the active sites of the COX-1, COX-2 co-crystals, **3b** and **4l** showed higher binding forces in the molecular docking study, which consistent with the results of *in vitro* experiments. These results demonstrated that these compounds had dual inhibitory activity of COX/5-LOX, providing clues for further searching for safer and more effective anti-inflammatory drugs.

Keywords: Indole; Anti-inflammatory; COXs; 5-LOX; Docking

1. Introduction

As one of the most frequently used drugs in the world, traditional non-steroidal anti-inflammatory drugs (NSAIDs) achieve antipyretic, analgesic and anti-inflammatory effects by inhibiting COX enzyme-associated inflammatory cytokines synthesis [1]. COX is one of the key enzymes in the arachidonic acid (AA) metabolic pathway. It include two isoenzymes (COX-1 and COX-2), which mediates the production of prostaglandins and thromboxanes from arachidonic acid [2]. According to current research, COX-1 is an intrinsic enzyme, and mainly distributed in the stomach, kidney and platelets, which can maintain the gastric and renal homeostasis; COX-2 is an inducible enzyme, and its expression is enhanced in the case of tissue damage, inflammation, *etc* [3]. Therefore, long-term use of traditional NSAIDs such as aspirin may cause serious gastrointestinal side effects, which are very unfavorable for the treatment of some chronic inflammation [4]. Compared with traditional NSAIDs, selective COX-2 inhibitors significantly reduce gastrointestinal side effects, but at the same time increase the likelihood of cardiovascular disease [5,6].

5-lipoxygenase (5-LOX) is one of the three major isozymes of liquid oxygen (LOX), involved in the metabolic process of AA, and there is a compensatory mechanism with COX [7]. The Leukotrienes (LTs) ultimately produced by the LOX pathway are considered as important mediators of inflammatory and allergic diseases, and are believed to be closely related to cancer and cardiovascular disease [7,8]. Therefore, it is possible to decrease the potential cardiovascular disease risk of NSAIDs by inhibiting 5-LOX and reducing the synthesis of LTs. At the same time, studies have shown that LTs are involved in the gastrointestinal reactions caused by NSAIDs [9]. It is worth noting that although 5-LOX plays an important role in various physiological processes such as inflammation, the 5-LOX inhibitor - Zileuton don’t show an obvious anti-inflammatory effect in related experiments [10].

Therefore, it is possible to cut down side effects such as gastrointestinal tract and cardiovascular while maintaining the basic activity of NSAIDs through strategy of COX/5-LOX co-inhibition, thereby obtaining safer and more effective

anti-inflammatory drugs.

As a dual inhibitor of COX/5-LOX, licofelone and darbufelone were first designed and entered the clinic as an analgesic and anti-inflammatory drug, but failed to be marketed due to efficacy or toxicity. Therefore, for the time being, the design and research of dual inhibitors is still urgently needed in the market [11]. And in recent years, researchers have also discovered some excellent compounds with dual inhibitory potential. among which, Mostafa *etc* designed and synthesized a compound as COX/5-LOX double inhibition by using quinoline as a backbone and a 2-amide structure as a linker, Its IC_{50} values of COX-1 = 8.6 μM, COX-2 = 1.13 μM and 5-LOX = 7.61 μM, both comparable to positive drugs [12]. In addition, indole ring is an important fragment with anti-inflammatory effects, and indomethacin has been used as a non-steroidal anti-inflammatory drug [13,14]. Palwinder and co-workers combined three heterocyclic structures of indole-chrysin-barbiturate to synthesize compound **2** and exhibited extremely high double inhibitory activity with IC_{50} values of COX-2 = 1 nM and 5-LOX = 1.5 nM, respectively, better than celecoxib and zileuton [15]. And Reddy synthesized a series of compounds by modifying the structure of *N*-benzyl indole, in which compound **3** showed good COX/LOX dual inhibitory activity with IC_{50} values of COX-2 = 3.9 μM and 5-LOX = 94 μM [16]. (**Fig. 1)**

Encouraged by these findings, 5-chloroindole-2-carboxylic acid was used as the fundamental structure to construct amide bond by ethylamine, pyrrolidine and piperazine, and simultaneously combined with the tryptamine and ligustrazine fragments which have a wide range of biological activities, in order to obtain compounds which have good anti-inflammatory activity (**Fig. 2**). The first six compounds were synthesized and evaluated for their *in vivo* anti-inflammatory activity, and the most active compound **8a** was selected for further modification to confer different electronic and lipophilic properties to the molecules. Similarly, the newly synthesized fourteen compounds were also evaluated for their *in vivo* anti-inflammatory activity. Further, the most active compounds **8a**, **10b**, **12h**, **12l**

were investigated for their *in vitro* COX/5-LOX inhibition. In addition, we also conducted molecular docking studies on compounds **10b** and **12h** to explore feasible combinations of compounds with the two enzymes.

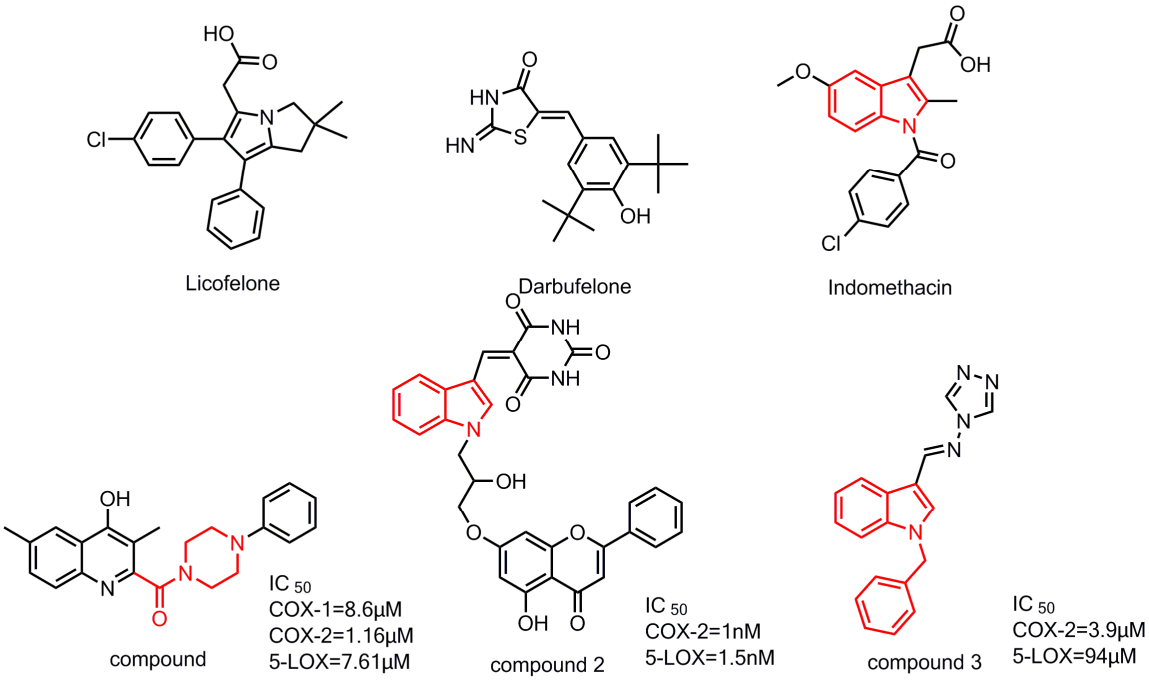


Fig. 1 Chemical structure of indole-based COX or LOX inhibitor compound and dual COX/LOX copound of contain amide

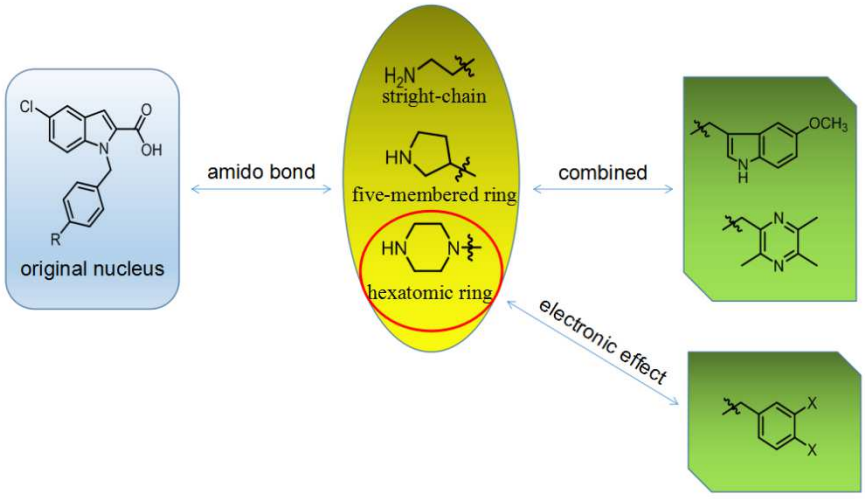


Fig. 2 Idea of newly designed indole-2-carboxamide

2. Result and discussion

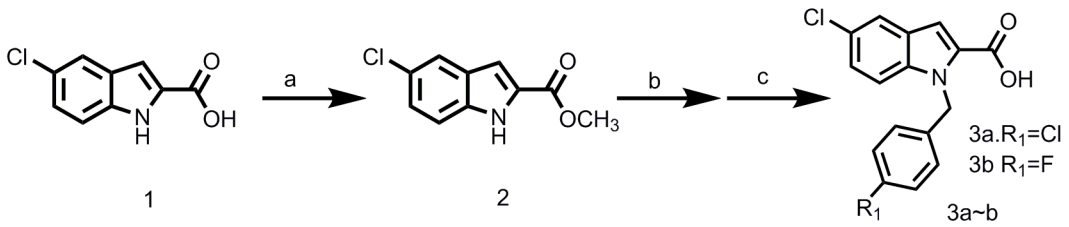
2.1. Chemical synthesis

Schemes 1 - 4 describes the synthetic route for intermediates and final compounds.

The preparation of the carboxylic acid fragments **3a-b** are described in **Scheme 1**. Since there were two active sites in the 5-Chloroindole-2-carboxylic acid, the carboxylic acid group was first protected by methyl esterification. The obtained methyl ester was reacted with 4-chlorobenzyl chloride or 4-fluorobenzyl chloride, and NaH was added to increase the reactivity to obtain **3a-b** [17]. The two steps had good yields of 91.5% and 86.4%, respectively. The structures of the compounds **3a-b** were confirmed by ^{1}H NMR and ^{13}C NMR spectroscopic analyses as well as MS spectrometry, and the absence of indole N**H** peak of the compound **2** and presence of a peak of two protons integration due to benzyl group at 5.87 ppm in ^{1}H NMR spectrum of **3a-b** confirmed the reaction. Finally, they were hydrolyzed in a hot alkaline solution to obtain **3a-b**.

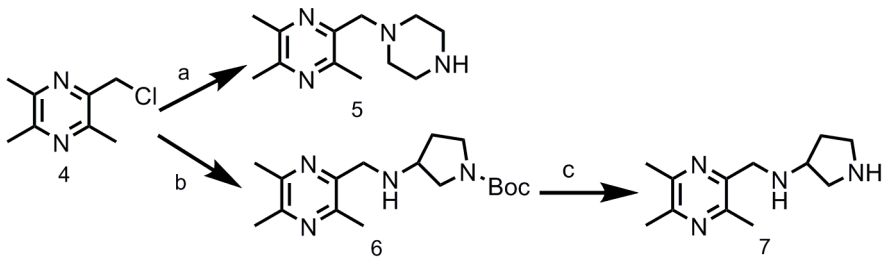
The preparation of amino fragments **5** and **7** were described in **Scheme 2**. Dichloromethane solution of **4** with an excess of anhydrous piperazine afforded amine **5** in 77% yield. On the other hand, compound **4** was subjected to substitution using 1 - Boc - 3 - aminopyrrolidine in dichloromethane to yield intermediate **6**. And compound **6** was hydrolyzed to the amine **7** using trifluoroacetic acid (TFA) under an alkaline environment [18].

The formation of compounds' amide bond are described in **Schemes 3** and **4**. The HOBT and EDCI were first combined with the carboxylic acid fragment to form an active lipid, which increaseed the reactivity, and then added the above-mentioned amine fragment to form an amide under an alkaline environment [19]. All the structures of indole-2-carboxamides were confirmed by ^{1}H NMR and ^{13}C NMR spectroscopic analyses as well as MS spectrometry. Taking compound **8a** as an example, ^{1}H NMR spectra showed the presence of a peak assigned to ArC**H$_2$**N at 5.5 ppm and a peak assigned to Piperazine-C**H$_2$** at 3.5 ppm. At the same time, the mass spectrometry data further confirmed the structure of **8a**, which gave a molecular ion m/z 521.17 $(M+H)^{+}$.



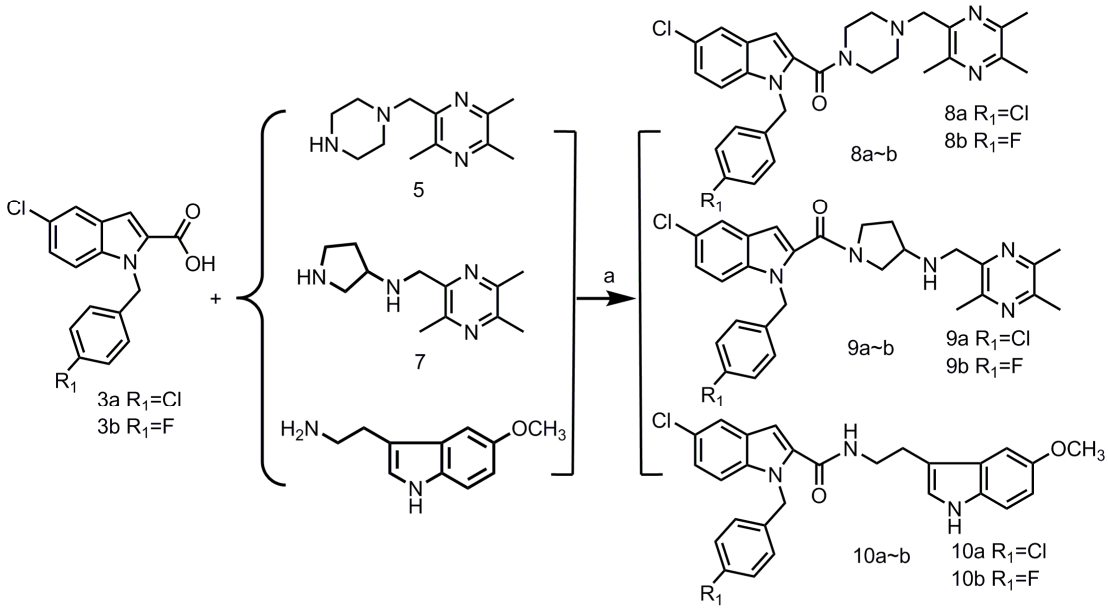
Reagents and conditions: (a) $SOCl_2$, Methanol, 75 □; (b) Appropriate benzyl, NaH, DMF, rt; (c) NaOH, alcohol, 75 □.

Scheme 1 Synthetic pathways for compounds **1-3**



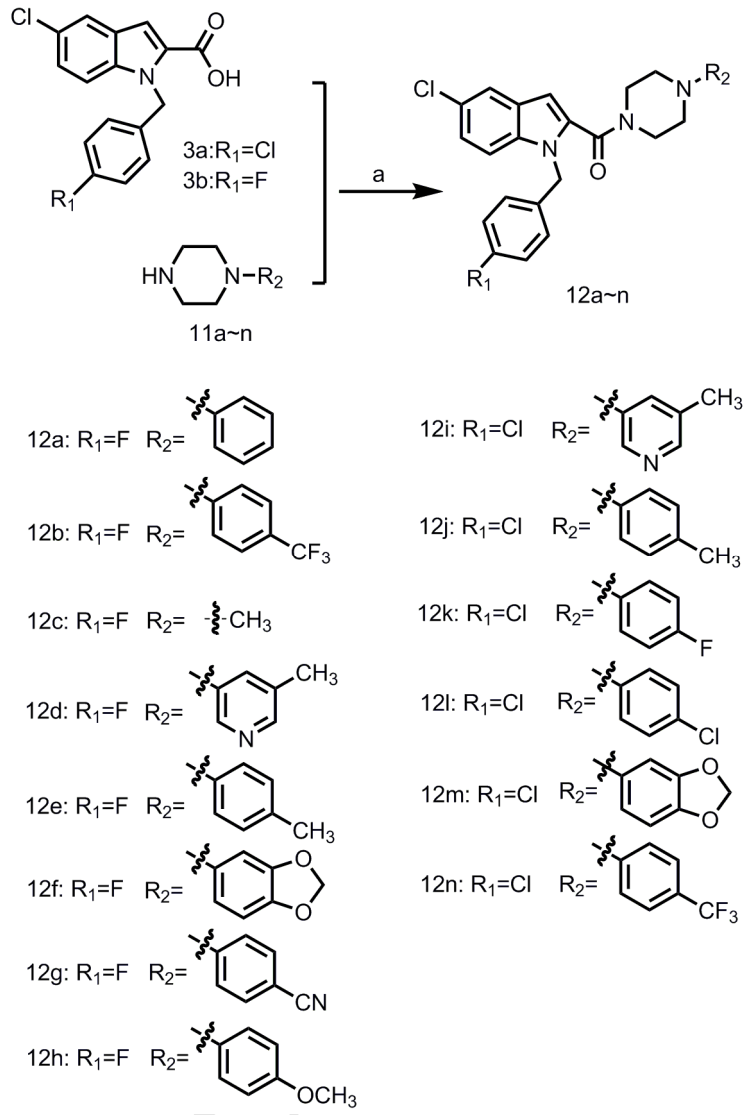
Reagents and conditions: (a) piperazine, CH_2Cl_2, rt; (b) 1-Boc-3-aminopyrrolidine, THF, reflux; (c) TFA, CH_2Cl_2, rt

Scheme 2 Synthetic pathways for compounds **5-7**



Reagents and conditions: (a) EDCI, HOBT, TEA, DMF, rt

Scheme 3 Synthetic pathway for compounds **8a-b**, **9a-b**, **10a-b**



Reagents and conditions: (a) EDCI, HOBT, TEA, DMF, rt

Scheme 4 Synthetic pathway for compounds **12a-n**

2.2. Biological evaluation

2.2.1. Anti-inflammatory activity

The anti-inflammatory activity of the synthesized compounds was evaluated by utilizing the mice auricle edema model using dexamethasone as a positive drug [20]. The experimental results were shown in **Table 1** and **Table 2** and expressed as means ± standard deviation (SD). And one-way analysis of variance was performed on the control group and the test group using SPSS 23.0 statistical software. The difference was significant at $p < 0.05$.

In the inflammatory model, the compounds were divided into two batches to evaluate the activity of inhibition of mouse ear swelling. Each test compound was

administered by intragastric administration (positive group was given dexamethasone 0.5 mg/kg, and compound group was given 5 mg/kg, respectively). After 6 days of adaptive feeding, DNFB was used to stimulate inflammation, and the mice were sacrificed 24 hours later. The left ear and the right ear were weighed and the swelling degree and inhibition rate were calculated [20].

Among the first activity results, **8a** (64.9%) and **9b** (52.5%) had significant inhibitory activity compared with the control group ($p < 0.01$). There was no significant difference between **8a** and the positive drug dexamethasone. **12h** (43.1%) and **12l** (40.5%) had significant inhibitory activity compared with the model group ($p < 0.01$), which **12h** was equivalent to the positive drug.

Table 1

The percentages of edema inhibition of compounds **8a-b, 9a-b, 10a-b** and dexamethasone

Compound	Dose levels (mg/kg)	left ear (mg)	Right ear (mg)	swelling degree (mg)	inhibition (%)
Blank	-	14.3±1.0	13.9±1.2	-	-
Control	-	13.5±1.2	30.9±3.5	17.4±3.6	-
Dexamethasone	0.5	13.8±1.0	20.1±4.8	6.3±4.7^{aa}	63.8
8a	5	12.9±0.8	19.0±3.2	6.1±2.5^{aa}	64.9
8b	5	12.5±1.1	21.5±3.7	9.0±4.3^{a}	48.3
9a	5	12.5±0.9	22.5±3.7	10.0±3.1	42.5
9b	5	12.9±0.6	21.7±3.4	8.8±4.1^{a}	49.4
10a	5	13.8±0.9	23.5±3.2	9.7±4.1^{a}	44.3
10b	5	13.8±1.5	22.5±4.6	8.7±4.7^{aa}	50.0

$^{A}p < 0.05$, $^{aa}p < 0.01$ vs control

swelling degree (mg) = W_R - W_L

inhibition (%) = ((W_R - W_L) control - (W_R - W_L) treated) / (W_R - W_L) control * 100%

W_R = Average weight of the right ear; W_L = Average weight of the left ear

Table 2

The percentages of edema inhibition of compounds **12a-n** and dexamethasone

Compound	Dose levels (mg/kg)	left ear (mg)	Right ear (mg)	swelling degree (mg)	inhibition (%)
Blank	-	11.8±0.7	12.2±0.7	-	-
Control	-	12.5±1.1	25.7±2.2	13.2±2.3	-
Dexamethasone	0.5	12.3±0.8	19.3±1.2	7±1.9^{aa}	46.8
12a	5	11.3±1.0	21.3±3.2	10±3.7	24.0
12b	5	11.5±1.0	21±2.8	9.5±3.8	27.8
12c	5	12.3±1.0	20.7±3.4	8.3±3.2^{a}	36.7
12d	5	13.8±1.0	23.7±3.6	9.9±3.4	25.4
12e	5	12.1±0.7	21±2.7	8.8±2.3^{a}	32.9
12f	5	12±0.9	20.7±3.8	8.7±3.9^{a}	34.2
12g	5	12.5±1.1	21±2.4	8.5±2.9^{a}	35.4
12h	5	11.7±0.8	19.2±1.9	7.5±2.1^{aa}	43.1
12i	5	13.9±1.2	22.9±3.1	9.0±2.9	29.8
12j	5	12.5±1.4	20.5±3.0	8.0±2.8^{a}	39.2
12k	5	12.2±0.7	21.5±3.7	9.3±3.6	29.1
12l	5	12±0.9	19.8±2.1	7.8±2.9^{aa}	40.5
12m	5	12.2±0.7	21±2.9	8.8±3.4^{a}	32.9
12n	5	12±0.9	21.2±3.2	9.2±2.4^{a}	30.4

$^{A}p < 0.05$, $^{aa}p < 0.01$ vs control

swelling degree (mg) = W_R - W_L

inhibition (%) = ((W_R - W_L) control - (W_R - W_L) treated) / (W_R - W_L) control * 100%

W_R = Average weight of the right ear; W_L = Average weight of the left ear

2.2.2. *In vitro* COX-1/COX-2 inhibition assay

Further, compounds with high anti-inflammatory activity such as **8a**, **10b**, **12h**, and **12l** were evaluated for their COX inhibitory activity *in vitro* by immunofluorescence analysis, and indomethacin and celecoxib were used as positive controls [21]. The mean fluorescence values were calculated at an emission wavelength of 587 nm and the potency of the testing compounds was determined as

the concentration causing 50% enzyme inhibition (IC_{50}). In addition, the selectivity index (IS) of COX-2 was calculated.

As shown in **Table 3**, all compounds exhibited good inhibitory ability to COX-2 and selectivity to COX-2 (IC_{50} = 31.91-21.86 nm) was higher than that of COX-1 (IC_{50} = 406.6-195.0 nm). The SI ranges from 8.4 to 17.45. All the tested compounds had higher COX inhibition than indomethacin but slightly less inhibition of COX-2 than celecoxib. These results indicated that compounds with good anti-inflammatory activity exhibited high inhibitory activity against COX, and inhibition COX may be one of the pathways by which these compounds exert anti-inflammatory effects.

Table 3

In vitro COX-1, COX-2 and 5-LOX enzymatic inhibition assay of the compounds **8a**,**10b**,**12h** and **12l**.

Compound	IC_{50}[a] (nM)			
	COX-1	COX-2	COX-1/COX-2 selectivity index(SI)[b]	5-LOX
8a	312.4±28.75	21.86±1.37	14.29	339.0±30.62
10b	406.6±35.65	23.3±1.39	17.45	66.14±6.26
12h	195.0±10.34	23.21±1.51	8.4	83.54±6.28
12l	361.4±21.35	31.91±1.78	11.33	209.5±9.57
Indomethacin	489.9±18.73	1466±86.59	0.33	ND
Celecoxib	>500	11.20±0.97	>40	ND
Zileuton	ND	ND	ND	38.91±2.16

a IC_{50}: Represents the concentration of the test compound that is required for 50% inhibition *in vitro*.

b SI: IC_{50} (COX-1) / IC_{50} (COX-2)

ND: not determined

2.2.3 *In vitro* 5-LOX inhibition assay

Similarly, the evaluation of 5-LOX inhibitory activity was performed on **8a**, **10b**, **12h** and **12l**, and the obtained data is listed in **Table 3** [22]. Among them,compounds

10b and **12h** had moderate inhibitory activities (IC_{50} = 66.14 and 83.54 nM, respectively) while the positive control zileuton was 38.91 nM. However, **8a** and **12l** showed no significant inhibitory activity against 5-LOX. By analyzing the structure of the compounds, we had found that the electron-donating group of methoxy group may increase the inhibitory activity of the compound for 5-LOX compared to the electron withdrawing group.

2.2.4 *In vitro* cytotoxic activity

Further, compounds **10b** and **12h** were evaluated for their cytotoxicity on normal macrophage cell line (RAW264.7). The results showed that compounds 10b and 12h had no significant effect on the survival of RAW264.7 cells below 50 μM (including 50 μM). The cell survival rate of each concentration group was not significant difference from that of the blank group (**Fig. 3**).

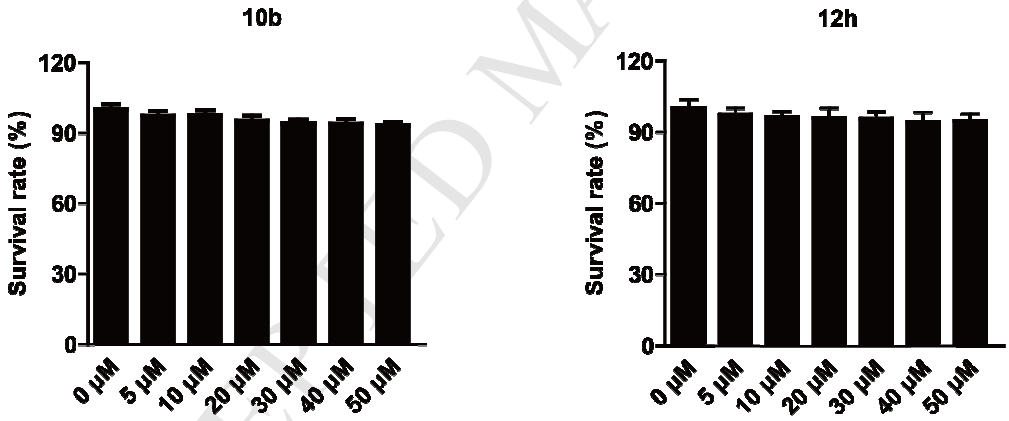


Fig. 3 Cell viability experiment was performed at 5-50 μM concentration and there was no significantly cytotoxic of compounds **10b** and **12h** at the concentration up to 50 μM.

2.3 Molecular docking study

In order to investigate the possible binding conformations of compounds **10b** and **12h** with either COX-1 (PDB code 1PGF) [23] or COX-2 (PDB code 1CX2) [24] receptors, molecular docking study was carried out using AUTODOCK 4.2.6 modeling software [25]. The docking results were processed with PYMOL to obtain the molecular chimeric maps, and the positions at which the compounds formed

hydrogen bonds and binding pockets were analyzed [26].

To ensure the feasibility of the docking results, we extracted the eutectic ligands IMM and SC-558 from the 3D protein structure of COX-1 and COX-2. **In Fig. 4**, the docking method of IMM (**A**) and SC-558 (**B**) was compared with the eutectic ligand to verify the accuracy of the docking study. The same docking parameters were used in subsequent docking of **10b** and **12h**.

Fig. 5 (A) showed the combination of **12h** and COX-1. **12h** enter the active pockets of HIS90, ARG120, SER530 and TYR385 [27], and combined HIS90 with hydrogen bonds. In **Fig. 5 (B)**, the combination of **12h** and IMM was similar, and the docking scores for **12h** and IMM were -11.74 and -10.62, respectively. Be different from Ile523 in COX-1, COX-2 binding site was present an additional side pocket because of the amino acid residue Val523, which increases the active area of COX-2 by 20% [28]. The results of the docking simulation of **12h** into COX-1 active site showed that this compound was forced to adopt a longitudinal binding pattern due to presence of Ile523, which closed the side pocket and pushed the compound to the bottom of the active site. On the contrary, in **Fig. 6** (**B** and **D**), the orientation of the 5-chloroindole moieties in **10b** and **12h**, respectively within the additional side pocket of COX-2 was similar to fitting of sulfonamide moiety of SC-558 into the same side pocket. Due to the different chemical nature of the sulfonamides in the chlorine atom, **figure 5** illustrated that the compounds **10b** and **12h** have certain COX-2 selectivity, and may also explain the difference in activity between the compounds and celecoxib [29]. Simultaneously, **12h** forms a hydrogen bond with the important residue ARG120, which is the same as AA (**Fig 6** (**B**)), while **10b** forms a hydrogen bond with the active pocket apical residue TYR385. These two hits exhibited similar binding pattern and interactions to that of the cocrystallized SC-558 ligand (**Fig 6** (**D**)) [30].

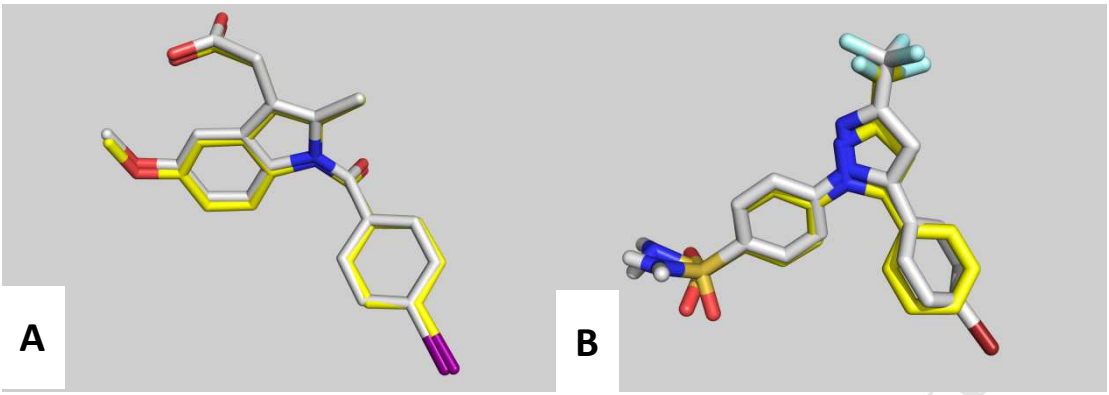


Fig 4 (A) Comparison between the docked pose of IMM (yellow) as produced by docking experiment and the co-crystallized ligand of this inhibitor within COX-1 (gray, PDB code: 1PGF). (B) Comparison between the docked pose of SC-558 (yellow) as produced by docking experiment and the co-crystallized ligand of this inhibitor within COX-2 (gray, PDB code: 1CX2).

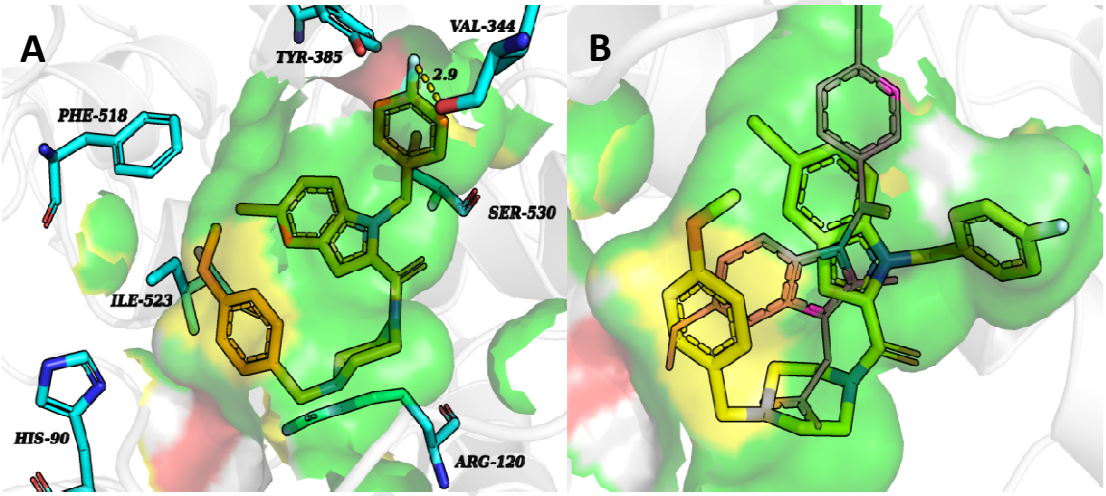
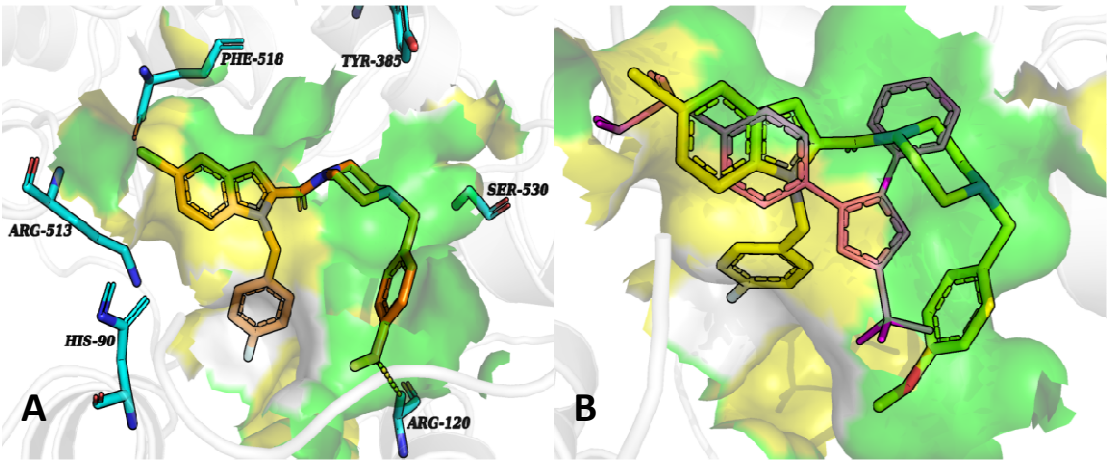


Fig 5 (A) Docking and binding pattern of compound **12h** (orange) into COX-1 active site (PDB code: 1PGF); (B) The superimposition of the docked pose **12h** (yellow) and the co-crystallized IMM (magenta) within active site of COX-1. Dashed lines represent hydrogen bonds. The hydrophobic area of active site was represented by green; and the hydrophilic area was indicated by yellow.



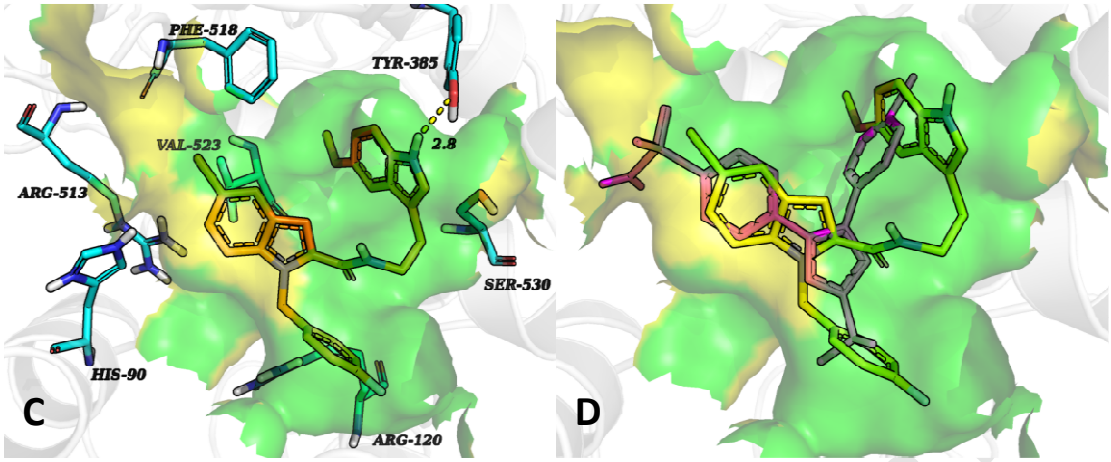


Fig 6 (A) Docking and binding pattern of compound **12h** (orange) into COX-2 active site (PDB code: 1CX2). (B) The superimposition of the docked pose **12h** (yellow) and the co-crystallized SC-558 (magenta) within active site of COX-2. (C) Docking and binding pattern of compound **10b** (orange) into COX-2 active site (PDB code: 1CX2). (D) The superimposition of the docked pose **10b** (yellow) and the co-crystallized SC-558 (magenta) within active site of COX-2. Dashed lines represent hydrogen bonds. The hydrophobic area of active site was represented by green; and the hydrophilic area was indicated by yellow.

3 Conclusion

In the present study, a series of 2-indole-amides derivative was synthesized as anti-inflammatory agents. Compounds **8a**, **10b**, **12h** and **12l** exhibited good anti-inflammatory activity in the mice auricle edema model. There was no significant cytotoxic activity on RAW264.7. Further, the experiments conducted in-depth studies on the anti-inflammatory mechanisms of there compounds. In the enzyme immunosuppression experiment, the compounds were found to have a good inhibitory activity against the COX/5-LOX enzyme. Among them, **13i** had the best inhibitory activity against COX-1 (IC_{50} = 195 nM); **9a**, **11b**, **13i** had potent inhibitory activities against COX-2, which were 21.86 nM, 23.3 nM and 23.21 nM, respectively; while **11b** and **13i** exhibited 5-LOX inhibitory activity (IC_{50} = 66.14 nM, 83.54 nM, respectively). Therefore, were it was confirmed that the compounds exert its anti-inflammatory activity by inhibiting the COX/5-LOX pathway. According to Guo Zongru's strategy of moderate inhibition[31], due to the mutual restriction of COX-1 and COX-2 in the body, excessive inhibition of COX-2 will break this kind of balance, which will lead to some side effects that are not expected [31]. Compounds **10b** and **12h** exhibited moderate inhibition of the COX/5-LOX and temperate selectivity for

COX-2, which may be more safer than celecoxib. In addition, docking studies have explained and supported the results of *in vitro* enzyme activity experiments. By analyzing the binding pattern of the compounds to the active site of the enzyme, it was find that the 5-chloroindole structure in the compounds **10b** and **12h** entered the COX-2 side pocket, which was similar to the sulfonamide group in SC-558. In conclusion, combined with the results of the mice auricle edema and the enzyme immunosuppression experiments, this study led to discovery of two promising compounds with high potency against COX and 5-LOX, providing clues for further discovery of safe and effective anti-inflammatory drugs.

4. Experimental

4.1. Chemistry

The reagents and solvents for reaction were purchased from common commercial suppliers. If necessary, purification processes were carried out prior to their use. Melting points are determined on melting point apparatus (RDCSY-I) and are uncorrected. 1H NMR and 13C NMR spectra were recorded on 400 MHz and 100 MHz instruments (Bruker, Fallanden, Switzerland), respectively, with tetramethylsilane (TMS) as internal standard. MS spectra were measured with a Hewlett-Packard 1100 LC/MSD spectrometer (Agilent, Waldbronn, Germany).

4.1.1. methyl 5-chloro-1*H*-indole-2-carboxylate (**2**)

To a solution of 5-Chloroindole-2-carboxylic acid (5g, 25.6 mmol, 1 eq) in 30 mL of MeOH was added $SOCl_2$ (3.7g, 30.8mmol, 1.2 eq) dropwise within 30 min at 0 □. Subsequently, the reaction mixture was stirred at 35 □ for 2 h. Then, the solvent was evaporated under reduced pressure, and the residue was purified by column chromatography using ethylacetate/petroleum, giving intermediate **2** as a yellow solid, yield 93%. m.p. 212.2-214.7 □. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.95 (s, 1H, N**H**), 7.67 (s, 1H, Ar**H**), 7.35 (d, *J* = 8.7 Hz, 1H, Ar**H**), 7.31-7.25 (m, 2H, ArH, ArC**H**), 3.95 (s, 3H, OC**H$_3$**); ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.1, 135.1, 128.4, 126.6, 126.0, 121.8, 112.98, 112.93, 108.1, 52.2.

4.1.2. 1-benzyl-5-chloro-1*H*-indole-2-carboxylic acid (**3a-b**)

To a stirred suspension of NaH (60 percent dispersion inmineral oil; 0.7 g, 17.3 mmol, 1.2 eq) in DMF (10 mL) was added dropwise solution of **1** (3g, 14.4 mmol, 1 eq) in DMF (30 mL) at 0 □, and the mixture was stirred at room temperature for 30 min. To the mixture was added dropwise 4-Chlorobenzyl chloride or 4-Fluorobenzyl chloride (14.4 mmol, 1 eq), and the resulting mixturewas stirred at room temperature for 30 min. The reaction was quenched with water and acidified with acetic acid. The formed precipitate was filtered, washed with water, and dried under vacuum to obtain the gray solid.

To a solution of the above compounds (7.8 mmol, 1 eq) in ethanol (30 ml), 10 % NaOH solution (15 ml) was added with stirring for 15 min at 75 □. The residue after removal of ethanol under reduced pressure was then taken into water and acidified with concentrated hydrochloric acid. The formed precipitate was filtered, washed with water, and dried under vacuum to give **3a-b** as white solid.

4.1.2.1. 5-chloro-1-(4-chlorobenzyl)-1*H*-indole-2-carboxylic acid (**3a**)

Compound **3a** was synthesized according to the synthetic procedure given above as white solid in yield of 85%, ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.80 (d, *J* = 2.0 Hz, 1H, Ar**H**), 7.61 (d, *J* = 8.9 Hz, 1H, Ar**H**), 7.37 – 7.29 (m, 4H, Ar**H**, ArC**H=**), 7.05 – 7.01 (m, 2H, Ar**H**), 5.87 (s, 2H, NC**H$_2$**Ar), COOH (none); ESI-Mass for $C_{16}H_{11}C_{l2}NO_2$: m/z $(M+H)^+$ 317.7

4.1.2.1. 5-chloro-1-(4-fluorobenzyl)-1*H*-indole-2-carboxylic acid (**3b**)

Compound **3b** was synthesized according to the synthetic procedure given above in yield of 86%, m.p. 202.8-205.2; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 7.79 (d, *J* = 2.1 Hz, 1H, Ar**H**), 7.62 (d, *J* = 8.7 Hz, 1H, Ar**H**), 7.30 (s, 1H, ArC**H=**), 7.29 (dd, *J* = 2.0, 8.7 Hz, 1H, Ar**H**), 7.01 - 7.09 (m, 4H, Ar**H**), 5.87 (s, 2H, NC**H$_2$**Ar), COOH (none); ESI-Mass for $C_{16}H_{11}ClFNO_2$: m/z $(M+H)^+$ 301.8

4.1.3. 2,3,5-trimethyl-6-(piperazin-1-ylmethyl)pyrazine (**5**)

Anhydrous piperazine (3.0 g, 34.9 mmol, 6 eq) was dissolved in dichloromethane (60 mL). 2-(chloromethyl)-3,5,6-trimethylpyrazine (0.99g, 5.8 mmol, 1 eq) in dichloromethane (30 mL) was added dropwise to the solution at room temperature . The reaction mixture was stirred for

about 4 h. Then, the mixture was washed with saturated K_2CO_3 solution (30 mL × 3) and water (30 mL × 3). The organic layer was dried over anhydrous Na_2SO_4. The filtrate was evaporated under reduced pressure and desired products **5**. ESI-Mass for $C_{16}H_{11}ClFNO_2$: m/z $(M+H)^+$ 221.11.

4.1.4. tert-butyl3-(((3,5,6-trimethylpyrazin-2-yl)methyl)amino)pyrrolidine-1-carboxylate (**6**)

1-Boc-3-aminopyrrolidine (1.08 g, 5.8 mmol, 1 eq) was dissolved in THF (60 mL). 2-(chloromethyl)-3,5,6-trimethylpyrazine (1g, 5.8 mmol, 1 eq) in THF (30 mL) was added dropwise to the solution at room temperature . The reaction mixture was stirred at reflux for 4 hours. Then, the mixture was evaporated under reduced pressure after filtered. Obtaining oil **6** for the next reaction

4.1.5. N-((3,5,6-trimethylpyrazin-2-yl)methyl)pyrrolidin-3-amine (**7**)

To a stirred solution of 6 (1g, 3.1 mmol, 1 eq) in dichloromethane (20 mL) was added dropwise trifluoroacetic acid (3.5 g, 31 mmol, 10 eq), and the mixture was stirred at room temperature for 12 h. The progress of the reaction was monitored by TLC. After concentration at reduced pressure, the residue was purified by flash column chromatography (methanol/dichloromethane = 1:20 - 1:10) afforded the title compound 7.

4.1.6. (1-benzyl-5-chloro-1*H*-indol-2-yl)(4-((3,5,6-trimethylpyrazin-2-yl)methyl)piperazin-1-yl) methanone (**8a-b**)

To a stirred solution of **3a-b** (1.65 mmol,1 eq.) in DMF (25 ml), HOBT (3.3 mmol,2 eq.) and EDCI (4.12 mmol 2.5 eq.) was added at 25 □. After 15 min, **6** (0.36 g 1.65 mmol,1 eq.) dissolved in DMF (10 mL) was added. The reaction mixture was continued to keep at 25 □ with stirring for 4 h. A saturated solution of citric acid was slowly added with stirring for an additional 30 min followed by addition of H_2O and EtOAc. The organic layer was washed with brine, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was purified by flash column chromatography (PE/EA = 5:1 - 2:1) afforded the title compounds (**8a-b**).

Compounds 8a-b were synthesized according to the synthetic procedure given above (See details in Supplemental Data).

4.1.7. (1-benzyl-5-chloro-1*H*-indol-2-yl)(3-(((3,5,6-trimethylpyrazin-2-yl)methyl)ami-no) pyrrolidin-1-yl)methanone (**9a-b**)

To a stirred solution of **3a-b** (1.65 mmol,1 eq.) in DMF (25 ml), HOBT (3.3 mmol,2 eq.) and EDCI (4.12 mmol, 2.5 eq.) was added at 25 □. After 15 min, **8** (0.36 g, 1.65 mmol, 1 eq) dissolved in DMF (10 mL) was added. The reaction mixture was continued to keep at 25 □ with stirring for 4 h. A saturated solution of citric acid was slowly added with stirring for an additional 30 min followed by addition of H_2O and EtOAc. The organic layer was washed with brine, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was purified by flash column chromatography (PE/EA= 5:1 - 2:1) afforded the title compounds (**9a-b**).

Compounds 9a-b were synthesized according to the synthetic procedure given above (See details in Supplemental Data).

4.1.8. 1-benzyl-5-chloro-N-(2-(5-methoxy-1*H*-indol-3-yl)ethyl)-1H-indole-2-carboxa-mide (**10a-b**)

To a stirred solution of **3a-b** (1.65 mmol,1 eq) in DMF (25 ml), HOBT (3.3 mmol,2 eq) and EDCI (4.12 mmol, 2.5 eq) was added at 25 □. After 15 min, 5-Methoxytryptamine (0.31 g, 1.65 mmol, 1 eq) dissolved in DMF (10 mL) was added. The reaction mixture was continued to keep at 25 □ with stirring for 4 h. A saturated solution of citric acid was slowly added with stirring for an additional 30 min followed by addition of H_2O and EtOAc. The organic layer was washed with brine, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was purified by flash column chromatography (PE/EA = 5:1-2:1) afforded the title compound (**10a-b**).

Compounds 10a-b were synthesized according to the synthetic procedure given above (See details in Supplemental Data).

4.1.9. (1-benzyl-5-chloro-1*H*-indol-2-yl)(4-benzylpiperazin-1-yl)methanone (**12a-n**)

To a stirred solution of **3a-b** (1.65mmol,1 eq) in DMF (25 ml), HOBT (3.3 mmol,2 eq) and EDCI (4.12 mmol 2.5 eq) was added at 25 □. After 15 min, monosubstituted benzyl piperazine (**11a-n**) (1.65 mmol,1 eq) dissolved in DMF (10 mL) was added. The reaction mixture was continued to keep at 25 □ with stirring for 4 h. A saturated solution of citric acid was slowly added with stirring for an additional 30 min followed by addition of H_2O and EtOAc. The organic

layer was washed with brine, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was purified by flash column chromatography (PE/EA = 5:1-2:1) afforded the title compound (**12a-n**).

Compounds 12a-n were synthesized according to the synthetic procedure given above (See details in Supplemental Data).

4.2. Biological activity

4.2.1. DNFB-induced mice auricle edema bioassay

Materials: male ICR mice weighing (18 - 22 g); 1% DNFB solution (composed by 0.1 mL DNFB solution, 8 mL acetone and 2 mL olive oil); Dexamethasone; sodium carboxymethylcellulose solution (CMC-NA) were provided by Hefei Yigong Medicine Co., Ltd.

Experimentation: animals were randomly divided into 9 groups according to weights, 6 in each group. They were were normal group, model group, positive group and other 6 compound groups. The normal group and the model group were given the same dose of the normal saline. The positive group was given dexamethasone 0.5 mg/kg, and the compound groups were given compound 5 mg/kg, respectively. All drugs were adiministered by intragastric administration. The drug was administered once a day for 6 days. After 7 days of adaptive feeding, the normal group did not do any treatment. In the other groups, the right ear of the mice were uniformly coated with 10 uL of 1 % DNFB solution, and the left ear was coated with the same amount of solution of acetone and olive oil for comparison. After 24 hours, the animals were sacrificed, and round ear slices were cut off from the same sites of left and right ears by 8 mm hole puncher, and weighed to calculate the swelling degree and inhibition rate. Calculation was carried out according to the following equations.

swelling degree (mg) = VR - VL

Inhibitory rate of ear swelling (%) = ((VR - VL) control - (VR - VL) treated) / (VR - VL) control * 100%

VR = Average weight of the right ear; VL = Average weight of the left ear

4.2.2. *In vitro* COX-1/COX-2 inhibition assay

The inhibitory COX activity of the tested compounds and the reference was assayed using

COX-1 Inhibitor Screening Kit (Fluorometric) (BioVision, Inc., Mountain View, CA, USA) ; COX-2 Inhibitor Screening Kit (Beyotime Biotech Co., Ltd, China) according to the manufacturer’s instructions. COX-1/COX-2 Assay Buffer (398 μL), COX-1 Cofactor (2 μL) and Arachidonic acid solution were placed in a 96-well plate. The test compounds were added to above solution. The plate incubated for 5 min at 37 ℃ and fluorescence was measured. The excitation wavelength was 535 nm and the emission wavelength was 587 nm.

The recording groups were divided into RFU (Relative Fluorescence Unit) blank control, RFU 100% enzyme activity control, RFU positive drug control, and RFU test compound. Calculation of the inhibition rate was carried out according to the following equations

Inhibition rate (%) = (RFU (enzyme) - RFU (test compound / positive)) / (RFU (enzyme) - RFU (blank) * 100%

RFU (enzyme) = RFU 100% enzyme activity control; RFU (test compound) = RFU test compound; RFU (blank) = RFU blank control; RFU (positive) = RFU positive drug control.

The assays were performed in triplicate and the IC_{50} values were calculated from the concentration curves.

4.2.3. *In vitro* 5-LOX inhibition assay

The inhibitory activity of the test compounds against soya bean 5-LOX was evaluated by using Abnova lipoxygenase assay kit (catalog No. KA1329, Cayman Chemicals). IC_{50} values of the tested compounds were carried out according to procedures and instructions given with the assay kit and in accordance to previously reported methods

4.2.4. *In vitro* cytotoxicity

RAW264.7 cell line was purchased from the American Tissue Culture Collection (ATCC, Manassas, VA, USA).

Suspensions of tested cell lines (ca. 1.0 × 105 cells/ml) were placed in 96-well microtiter plates and incubated with serum-free medium for 2 h at 37℃ and 5% CO2. Subsequently, the supernatant was discarded and incubate with RPMI 1640 medium containing different concentrations of compounds (concentration: 0 μM, 5 μM, 10 μM, 20 μM, 30 μM, 40 μM, 50 μM) for 24 h. Four hours before the end of incubation, add 20 μL of MTT solution (5 mg/mL) to each

well. After the incubation, the 96-well plate was centrifuged at 1500 rpm for 3 minutes, the supernatant of each well was discarded, 150 μL of DMSO was added to each well, and the cell shaker was shaken for 10 min. After the crystals were sufficiently dissolved, the OD570 was measured by a microplate reader.

4.2.5. Docking studies

The molecular docking simulation was performed using **ANTODOCK 4.2.6** imbedded into **AutoDockTools - 1.5.6**. The active sites were generated from the co-crystallized ligands (IMM and SC-558)[23, 24] within COX-1 and COX-2 protein structures (PDB codes: 1PGF and 1CX2), respectively. Polar hydrogens were added to all ligands and proteins with the **AutoDock Tools (ADT)** program prior to docking with **Autodock 4.2.6** program[25]. A grid box 40 Å in size was centered on the active site of protein. This docking approach was validated by successful pose-retrieval of the co-crystallized ligands of COX-2 crystal structures. All graphical representations in **Figs. 4 - 6** were rendered using **PyMOL**[26].

5. Conflict of interest

There are no conflicts of **interest**.

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References

[1] Fabio Marsico, Stefania Paolillo, Pasquale P. Filardi, et al. NSAIDs and cardiovascular risk. Journal of Cardiovascular Medicine. 18 (2017) e40–e43.

[2] Jacqueline K. Innes, Philip C. Calder. Omega-6 fatty acids and inflammation. Prostaglandins, Leukotrienes and Essential Fatty Acids. 132 (2018) 41-48.

[3] Giovanni de Gaetano, Maria Benedetta Donati, Chiara Cerletti. Prevention of thrombosis and vascular inflammation: benefits and limitations of selective or combined COX-1, COX-2 and 5-LOX inhibitors. Trends in Pharmacological Sciences. 24 (2003) 245-252.

[4] P. Jaismy Jacob, S.L. Manju, K.R. Ethiraj, et al. Safer anti-inflammatory therapy through dual COX-2/5-LOX inhibitors: A structure-based approach. European Journal of Pharmaceutical Sciences. 121 (2018) 356-381.

[5] Timothy D Warner, Jane A Mitchell. COX-2 selectivity alone does not define the cardiovascular risks associated with non-steroidal anti-inflammatory drugs. The Lancet. 371 (2008) 270-273.

[6] Jane A. Mitchell, Timothy D. Warner. COX isoforms in the cardiovascular system: understanding the activities of non-steroidal anti-inflammatory drugs. Nature reviews Drug discovery. 5 (2006) 75-86.

[7] Gaganpreet Kaur, Om Silakari. Multiple target-centric strategy to tame inflammation. Future Medicinal Chemistry. 9 (2017) 1361-1376

[8] Hyde, C. A; Missailidis, S. Inhibition of arachidonic acid metabolism and its implication on cell proliferation and tumour-angiogenesis. International Immunopharmacology. 9 (2009) 701-15.

[9] J Martel-Pelletier; D Lajeunesse; P Reboul, et al. Therapeutic role of dual inhibitors of 5-LOX and COX, selective and non-selective non-steroidal anti-inflammatory drugs. Annals of the Rheumatic Diseases. 62 (2003) 501-509.

[10] CJ Hawkey, LM Dube, LV Rountree, et al. A Trial of Zileuton Versus Mesalazine or Placebo in the Maintenance of Remission of Ulcerative Colitis. Gastroenterology. 112 (1997) 718-724.

[11] S. Tries, W. Neupert, S. Laufer. The mechanism of action of the new antiinflammatory

compound ML3000: inhibition of 5-LOX and COX-1/2. Inflammation Research. 51 (2002) 135-143.

[12] Abdelrahman, M. H, Youssif, B. G. M, Abdelgawad, M. A, et al. Synthesis, biological evaluation, docking study and ulcerogenicity profiling of some novel quinoline-2-carboxamides as dual COXs/LOX inhibitors endowed with anti-inflammatory activity. European Journal of Medicinal Chemistry. 127 (2017) 972-985.

[13] Sravanthi, T. V, Manju, S. L. Indoles - A promising scaffold for drug development. European Journal of Pharmaceutical Sciences. 91 (2016) 1-10.

[14] Lamie, P. F, Ali, W. A. M, Bazgier, V, et al. Novel N-substituted indole Schiff bases as dual inhibitors of cyclooxygenase-2 and 5-lipoxygenase enzymes: Synthesis, biological activities *in vitro* and docking study. European Journal of Medicinal Chemistry. 123 (2016) 803-813.

[15] Palwinder Singh, Jagroop Kaur, Gurjit Singh. Tri-block Conjugates: Identification of a Highly Potent Anti-inflammatory Agent. Journal of Medicinal Chemistry. 58 (2015) 5989-6001.

[16] Reddy, M. V, Billa, V. K, Pallela, V. R, et al. Design, synthesis, and biological evaluation of 1-(4-sulfamylphenyl)-3-trifluoromethyl-5-indolyl pyrazolines as cyclooxygenase-2 (COX-2) and lipoxygenase (LOX) inhibitors. Bioorganic & Medicinal Chemistry. 16 (2008) 3907-16.

[17] Joshua S Alford, Huw M. L. Davies. Mild Aminoacylation of Indoles and Pyrroles through a Threecomponent Reaction with Ynol Ethers and Sulfonyl Azides. Journal Of The American Chemical Society. 136 (2014) 10266-9.

[18] Clelia Dallanoce, Paola Bazza, Giovanni Grazioso, et al. Synthesis of Epibatidine-Related Δ2-Isoxazoline Derivatives and Evaluation of Their Binding Affinity at Neuronal Nicotinic Acetylcholine Receptors. European Journal of Organic Chemistry. 16 (2006) 3746-3754.

[19] Bowers A A, West N, Newkirk T L, et al. Synthesis and histone deacetylase inhibitory activity of largazole analogs: alteration of the zinc-binding domain and macrocyclic scaffold. Organic Letters, 11 (2009) 1301-4.

[20] Younghee Yun, Kyuseok Kim, Inhwa Choi, et al. Topical Herbal Application in the Management of Atopic Dermatitis: A Review of Animal Studies. Mediators of Inflammation. 2014 (2014) 1-8.

[21] P J Loll, D Picot, O Ekabo. Synthesis and Use of Iodinated Nonsteroidal

Antiinflammatory Drug Analogs as Crystallographic Probes of the Prostaglandin H2 Synthase Cyclooxygenase Active Site. 35 (1996) 7330-40.

[22] Fa-Qian Shen, Zhong-Chang Wang, Song-Yu Wu, et al. Synthesis of novel hybrids of pyrazole and coumarin as dual inhibitors of COX-2 and 5-LOX. Bioorganic & Medicinal Chemistry Letters. 27 (2017) 3653-3660.

[23] Kulmacz RJ, Lands WE. Requirements for hydroperoxide by the cyclooxygenase and peroxidase activities of prostaglandin H synthase. Prostaglandins. 25 (1983) 531-40.

[24] Kurumbail RG, Stevens AM, Gierse JK, et al. Structural basis for selective inhibition of cyclooxygenase-2 by anti-inflammatory agents. Nature. 384 (1996) 644-8.

[25] Garrett M. Morris, Ruth Huey, Arthur J. Olson. Using AutoDock for ligand-receptor docking. Curr Protoc Bioinformatics. 8 (2008) 8-14.

[26] Lill MA, Danielson ML. Computer-aided drug design platform using PyMOL. Journal Of Computer-Aided Molecular Design. 25 (2011) 13-9.

[27] Gino Cingolani, Andrea Panella, Maria Grazia Perrone, et al. Structural basis for selective inhibition of Cyclooxygenase-1 (COX-1) by diarylisoxazoles mofezolac and 3-(5-chlorofuran-2-yl) -5-methyl-4-phenylisoxazole (P6). European Journal of Medicinal Chemistry. 138 (2017) 661-668.

[28] Scott W. Rowlinson, James R. Kiefer, Jeffery J. Prusakiewicz, et al. A novel mechanism of cyclooxygenase-2 inhibition involving interactions with Ser-530 and Tyr-385. Journal of Biological Chemistry. 278 (2003) 45763-9.

[29] Mary E. Konkle, Anna L. Blobaum, Christopher W. Moth, et al. Conservative Secondary Shell Substitution In Cyclooxygenase-2 Reduces Inhibition by Indomethacin Amides and Esters via Altered Enzyme Dynamics. Biochemistry. 55 (2016) 348-59.

[30] Mosaad S. Mohamed, Yara E. Mansour, Hatem K. Amin, et al. Molecular modelling insights into a physiologically favourable approach to eicosanoid biosynthesis inhibition through novel thieno [2, 3-b] pyridine derivatives. Journal Of Enzyme Inhibition And Medicinal Chemistry. 33 (2018) 755-767.

[31] GUO Zong-Ru, Innovation of anti-inflammatory drugs - strategy of moderate inhibition of cyclooxygenases. Acta Pharmaceutica Sinica. 40 (2005) 967 - 969.

- A series of novel indole-2-amide compounds were designed, synthesized.
- Construct amide bond by ethylamine, pyrrolidine and piperazine.
- **8a**, **10b**, **12h**, and **12l** showed most potent in vivo anti-inflammatory activity.
- 10b and 12h showed significant suppressive activity for COX/5-LOX.
- Conducted molecular docking studies on compounds **10b** and **12h**.