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Design, Synthesis and Evaluation of 3-Substituted Coumarin Derivatives as Anti-Inflammatory Agents

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Coumarin moiety has garnered momentous attention especially in the design of compounds with significant biological activities. In this work, a series of 3-substituted coumarin derivatives 6a−6l were synthesized and fully characterized. Most of the compounds could obviously inhibit the activity of COX-1 at the concentration of 10 μM. Besides, 6h and 6l exhibited highest inhibitory effects against COX-2 with inhibition rates of 33.48% and 35.71%, respectively. Detailed structure-activity relationships (SARs) were also discussed. In vivo studies, 6b, 6i and 6l could remarkably repress the xylene-induced ear swelling in mice at the dose of 20 mg/kg. Especially, 6l seemed to be the most effective compound at the dose of 10 mg/kg, displaying favorable anti-inflammatory activity comparable to indomethacin. All of these findings suggested that 6l might be utilized as a candidate for the treatment of inflammatory diseases.

Key words 3-substituted coumarin; anti-inflammatory; tumor necrosis factor; cyclooxygenase; xylene-induced ear swelling;

Introduction

Inflammation is a primary defensive response of living tissue to various damage factors, such as biological pathogens, toxic chemicals, irritants and other harmful stimuli.[1,2] As a complex biological and physiological process, inflammation is characterized by five main symptoms, including swelling, redness, heat, pain and local dysfunction.[3] Inflammation is a protective immune response and is usually beneficial. However, persistent and exaggerated inflammation will promote tissue damage and lead to diseases, for instance, arthritis, sepsis, atherosclerosis, and even cancer.[4-6]

Based on the structure and therapeutic mechanism, anti-inflammatory drugs can be divided into two types, of which nonsteroidal anti-inflammatory drugs (NSAIDs) are the most widely administered drugs for the treatment of inflammation.[7] NSAIDs, such as indomethacin and ibuprofen, act their antipyretic, analgesic and anti-inflammatory effects by inhibiting cyclooxygenase-1 and cyclooxygenase-2 (COX-1 and COX-2), which are key enzymes involved in the pathway that produces prostaglandins (PGs).[8,9] However, NSAIDs may cause some unexpected side effects, such as peptic ulcer, liver damage and anaphylaxis.[10] Thus, it is still quite necessary for us to develop and explore anti-inflammatory drugs with better therapeutic effects.

The coumarin skeleton, also known as benzo-α-pyrone, has attracted voluminous attention for its ability to form non-covalent interaction with the active sites of the target protein.[11] Given its favorable pharmacological activity, benzo-α-pyrone has been used as an indispensible structural subunit for the discovery of drugs with improved pharmacological profiles.[12] In recent years, coumarins and related derivatives have displayed their diverse biological activities, such as anti-cancer,[13] antibacterial,[14] antioxidant[15] and anti-inflammatory[16]. Furthermore, some coumarins with different pharmacophores at C-3 position have been evaluated for anti-inflammatory activities.[17-20]

Sulfone and sulfoxide derivatives containing heterocyclic moieties belong to an important class of active compounds possessing various biological activities.[21,22] It has been reported that the combination of distinct pharmacophores in the same structure is very likely to obtain compounds with significant activity.[23] Thus, in order to develop novel anti-inflammatory agents, benzyl sulfone/sulfoxide moieties were introduced to the C-3 position of coumarin skeleton and the target compounds, 3-substituted coumarin derivatives were designed and synthesized (Fig. 1 and Chart 1). Anti-inflammatory activity of compounds **6a–6l** was preliminarily evaluated in mouse RAW 264.7 macrophages. Most of the compounds could markedly restrain the release of lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)-α at non-cytotoxic concentrations. Besides, all compounds were evaluated for cyclooxygenase inhibitory activity in cellular level by the enzyme-linked immuno-sorbent assay (ELISA) in vitro. In addition, **6b**, **6c**, **6d**, **6h**, **6i**, **6l** were selected for further anti-inflammatory study in vivo by the xylene-induced ear swelling method.

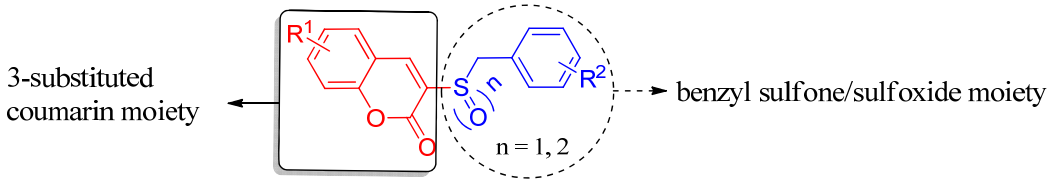


Fig. 1. The design of 3-substituted coumarin derivatives

Results and Discussion

Chemistry

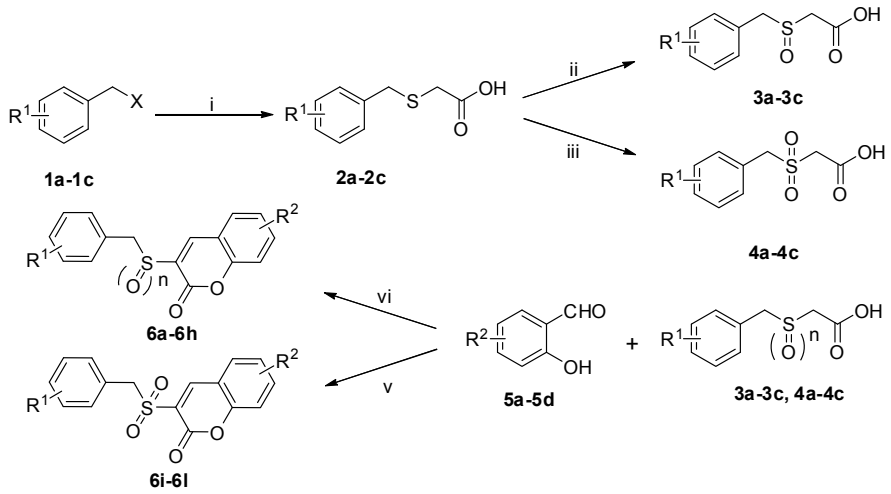
The target compounds **6a–6l** were synthesized via a three-step synthetic route from substituted benzylchloride/bromide (**1a–1j**) as outlined in Chart 1. The starting material **1a–1c** were treated with mercaptoacetic acid at the presence of sodium hydroxide to give benzylmercaptoacetic acids **2a–2c** in 63–87% yields.[24] Treatment of **2a–2c** with 30% hydrogen peroxide at room temperature gave benzylsulfinylacetic acids **3a–3c**[24] or at heating condition gave benzylsulfonylacetic acids **4a–4c** with satisfactory yields.[25] Finally, the target compounds **6a–6l** were synthesized via knoevenagel reaction[26] between **3a–3c** or **4a–4c** and substituted salicylaldehydes (**5a–5d**). The expected compounds **6a–6h** were prepared from **3a**, **3b**, **3c** or **4c** with **5a–5d** at the presence of EDCI in 26–58% yields. And **6i–6l** were obtained from **4a**, **4b** with **5a**, **5c** or **5d** in acetic anhydride with different yields ranging from 29% to 62%. All the target compounds were purified by recrystallization or flash chromatography and their structures were confirmed by ^{1}H-NMR, ^{13}C-NMR and HRMS spectra analysis.

Cell Viability Assay

The cytotoxicity of coumarin derivatives **6a–6l** (2.5 μM, 5 μM, 10 μM and 20 μM) on RAW 264.7 macrophages was evaluated by CCK-8 (Cell Counting Kit-8, WST-8) assay[27] after 24 h of treatment. As observed from the cell viability data in Fig. 2(A), at the concentration of up to 10 μM, all compounds generated no cytotoxicity to RAW264.7 with cell viability higher than 85%. At the concentration of 20 μM, the viability of **6h**-treated cells was just 81.79%. Thus, the concentration of 10 μM was selected to evaluate coumarin derivatives in the following TNF-α detection.

Evaluation of TNF-α Production Induced by LPS

Macrophages are well known to play an important role in the initiation and development of inflammation.[28] Activated macrophages induced by LPS produce cytokines such as TNF-α, interleukin, and pre-inflammatory mediators, including nitric oxide (NO) and PGs.[29] Studies have proved that over release of cytokines and pro-inflammatory mediators will lead to inflammatory diseases.[30] In order to evaluate the anti-inflammatory activity of all the target compounds **6a–6l** in vitro, ELISA was used to screen the production of TNF-α induced by LPS in RAW 264.7 macrophages.[31] As showed in Fig. 2(B), at the concentration of 10 μM, most of the tested compounds could significantly inhibit the secretion of TNF-α compared with the LPS group. Especially, **6c**, **6d**, **6h** and **6i** most strongly restrained the secretion of TNF-α.



1a: R^1 = 3-OCH_3, X = Cl

1b: R^1 = 4-OCH_3, 3-NO_2, X = Br

1c: R^1 = 4-Br, X = Br

2a, 3a, 4a: R^1 = 3-OCH_3

2b, **3b**, **4b**: R^1 = 4-OCH_3, 3-NO_2

2c, **3c**, **4c**: R^1 = 4-Br

5a: R^2 = 4,6-$(OCH_3)_2$

5b: R^2 = 5-OCH_3

5c: R^2 = 4-OCH_3

5d: R^2 = 5-Br

6a: R^1 = 3-OCH_3, R^2 = 5,7-$(OCH_3)_2$, n = 1

6b: R^1 = 4-Br, R^2 = 6-OCH_3, n = 2

6c: R^1 = 4-Br, R^2 = 6-OCH_3, n = 1

6d: R^1 = 4-Br, R^2 = 5,7-$(OCH_3)_2$, n = 2

6e: R^1 = 4-Br, R^2 = 5,7-$(OCH_3)_2$, n = 1

6f: R^1 = 4-OCH_3, 3-NO_2, R^2 = 5,7-$(OCH_3)_2$, n = 1

6g: R^1 = 4-OCH_3, 3-NO_2, R^2 = 7-OCH_3, n = 1

6h: R^1 = 3-OCH_3, R^2 = 6-Br, n = 1

6i: R^1 = 3-OCH_3, R^2 = 5,7-$(OCH_3)_2$, n = 2

6j: R^1 = 4-OCH_3, 3-NO_2, R^2 = 5,7-$(OCH_3)_2$

6k: R^1 = 4-OCH_3, 3-NO_2, R^2 = 7-OCH_3

6l: R^1 = 3-OCH_3, R^2 = 6-Br

Chart 1. Reagents and conditions: (i) $HSCH_2COOH$, NaOH, CH_3OH, rt, 1-2 h, 63%–87%; (ii) H_2O_2 , NaOH, H_2O, rt, 4 h, 61%–88%; (iii) H_2O_2, CH_3COOH, 55 °C, 5 h, 63%–77%; (iv) EDCI, DMAP, CH_3CN, rt, 1 h, 26%–58%; (v) CH_3COONa, $(CH_3CO)_2O$, 110 °C, 0.5 h, 29%–62%.

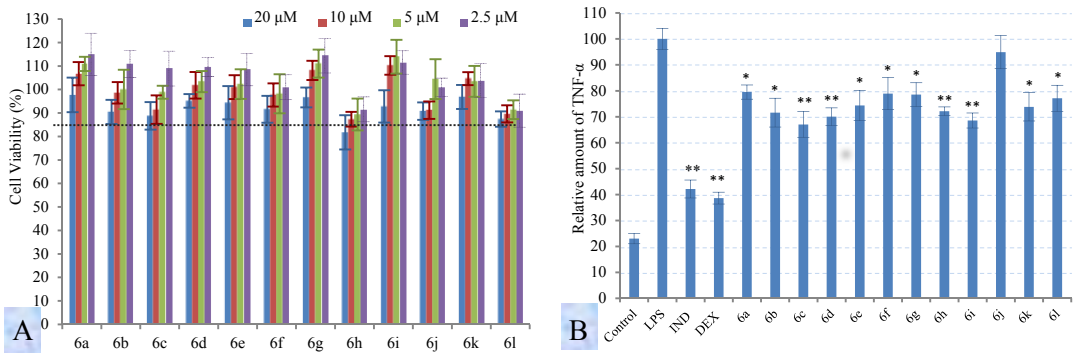


Fig. 2. (A) In vitro viability rate of RAW264.7 cells treated with compounds **6a–6l** at different concentrations of 2.5 μM–20 μM. The results were presented as the percent of LPS control; (B) Effects of coumarin derivatives on LPS-induced TNF-α production in RAW264.7 cells.

Data were expressed as the mean ± SD of three independent experiments. Compared with the LPS group, *P<0.01, **P<0.001. DEX: dexamethasone; IND: indomethacin.

In vitro Cyclooxygenase Inhibition and SAR Study

The 12 newly synthesized compounds **6a**–**6l** were first evaluated for cyclooxygenase inhibitory activity in cellular level by ELISA assay,[7] using indomethacin as a comparison. Based on the results displayed in Table 1, all compounds exhibited favorable inhibitory activity against COX-1 at the concentration of 10 μM, except for **6j**. Compounds **6a**, **6e** and **6g** exhibited excellent inhibitory potency with inhibition rates of 46.76%, 46.24% and 45.57%, respectively, which were comparable to that of indomethacin (51.11%). Compounds **6b**, **6c**, **6d**, **6h**, **6i**, **6k** and **6l** also exhibited moderate inhibitory effects against COX-2 with inhibition rates above 25% at the concentration of 10 μM. Besides, **6h** and **6l** exhibited the highest potency with inhibition rates of 33.48% and 35.71%, respectively, and could be potent COX-2 inhibitors.

Table 1. The COX-1 and COX-2 inhibitory activities of compounds **6a**–**6l**

Inhibition Rates (%) / Compound	COX-1		COX-2	
	5 μM	10 μM	5 μM	10 μM
IND	35.14 ± 5.88	51.11 ± 6.59	19.02 ± 2.17	35.26 ± 5.34
6a	21.66 ± 3.97	46.76 ± 4.56	13.01 ± 3.54	21.58 ± 4.59
6b	21.00 ± 2.97	34.74 ± 6.44	16.80 ± 5.56	25.47 ± 4.89
6c	29.99 ± 2.81	43.59 ± 2.87	16.24 ± 3.93	21.02 ± 2.37
6d	23.25 ± 5.28	41.61 ± 4.24	13.79 ± 4.79	24.36 ± 3.36
6e	28.67 ± 3.90	46.24 ± 4.64	8.90 ± 2.00	12.46 ± 3.91
6f	23.65 ± 3.60	36.20 ± 6.00	10.01 ± 2.37	13.46 ± 3.21
6g	25.63 ± 6.44	45.57 ± 1.99	10.12 ± 1.90	15.24 ± 2.41
6h	30.38 ± 2.78	31.70 ± 5.96	16.13 ± 2.59	33.48 ± 5.02
6i	19.82 ± 2.39	41.22 ± 4.38	20.58 ± 5.24	32.15 ± 3.72
6j	12.15 ± 4.59	19.02 ± 4.20	11.90 ± 3.18	19.35 ± 4.54
6k	11.62 ± 4.80	40.29 ± 7.05	9.45 ± 2.22	22.91 ± 2.52
6l	31.04 ± 5.24	38.84 ± 5.17	18.91 ± 2.73	35.71 ± 4.01

From the data of COX-1 inhibitory activities, some structure–activity relationships (SARs) can be observed: (i) the bioactivity of sulfoxides was higher than the corresponding sulfones (**6a** > **6i**, **6c** > **6b**); (ii) the type, number and position of R^1 seemed to play important roles for the activity: 7-OCH_3 > 5,7-$(OCH_3)_2$ > 6-Br (**6g** > **6f**, **6a** > **6h**). When it came to COX-2 inhibitory activity, some interesting SARs were illustrated: (i) the bioactivity of sulfones was higher than the corresponding sulfoxides (**6i** > **6a**, **6b** > **6c**); (ii) the type, number and position of R^1 also played important roles for the activity: 6-Br (**6h**)> 5,7-$(OCH_3)_2$ (**6a**), 7-OCH_3 (**6g**) > 5,7-$(OCH_3)_2$ (**6f**).

NSAIDs exert their anti-inflammatory effects mainly by inhibiting COX-2, while the inhibition of COX-1 may contribute to their unwanted side effects, such as gastric and renal damage.[32] Compounds with higher inhibitory potency on COX-2 but lower inhibitory activity against COX-1 were thought to be idea anti-inflammatory agents. Then **6b**, **6c**, **6d**, **6h**, **6i**, **6l** were selected for further study in vivo.

Anti-inflammatory Activity Evaluation in vivo

The process of inflammation is related to the increase of blood flow, capillary permeability and migration of macrophages and neutrophils from capillaries to interstitial spaces. As more fluid continues to accumulate in the interstitial space, the damaged tissue begins to swell.[33] Thus, swelling becomes one

of the main symptoms of inflammation.[34] The anti-inflammatory activities in vivo were screened in mice model of xylene-induced ear swelling, with dexamethasone and indomethacin as reference drugs. According to the results in Table 2, the target compounds (**6b**, **6c**, **6d**, **6h**, **6i** and **6l**) exhibited different degrees of anti-inflammatory activities under the experimental conditions. At the dose of 20 mg/kg, these compounds could obviously repress ear swelling with inhibition rates from 27.76% to 41.43%. At the dose of 10 mg/kg, **6b**, **6i** and **6l** suppressed the swelling with inhibition rates above 30%. Remarkably, compound **6l** exhibited the best anti-inflammatory activity, with inhibition rate of 34.56%, which was comparable to indomethacin.

Table 2. Inhibitory effects of **6a**–**6l** on xylene-induced ear swelling

Group	Swelling degree (mg)		Inhibition rate (%)	
	20 mg/kg	10 mg/kg	20 mg/kg	10 mg/kg
Control	16.01 ± 3.81		–	
DEX	N.T.	7.02 ± 1.94**	N.T.	56.14
IND	N.T.	9.92 ± 2.14**	N.T.	38.03
6b	9.43 ± 2.19**	10.96 ± 2.03**	41.08	31.58
6c	11.57 ± 2.99*	12.84 ± 2.84	27.76	19.78
6d	10.83 ± 2.40**	11.93 ± 2.99*	32.34	25.47
6h	10.40 ± 3.07**	11.40 ± 3.05*	35.04	28.80
6i	9.72 ± 1.68**	11.57 ± 2.67**	39.28	30.46
6l	9.38 ± 2.83**	10.48 ± 3.12**	41.43	34.56

Data were expressed as the mean ± SD, n = 9. Compared with the LPS group, *P < 0.05, **P < 0.01.

"NT": not test.

Conclusion

In summary, to obtain effective lead compounds that can serve as anti-inflammatory agents, we have designed and synthesized a total of twelve coumarin derivatives linked substituted benzyl sulfone/sulfoxide moieties at C-3 position. The anti-inflammatory effects of these compounds were evaluated in vitro and in vivo, including the inhibition of TNF-α production induced by LPS in RAW 264.7 macrophages, cyclooxygenase inhibition study and xylene-induced ear swelling in mice. Results of the in vitro study provided evidence that most of the compounds could repress the release of TNF-α and exhibited favorable inhibitory activity against COX-1 at the concentration of 10 μM. Moreover, **6h** and **6l** exhibited the highest inhibitory potency on COX-2. In addition, at the dose of 20 mg/kg, the active compounds **6b**, **6c**, **6d**, **6h**, **6i** and **6l** could obviously repress ear swelling in vivo. Especially, **6l** displayed satisfactory inhibitory activity similar to indomethacin at the dose of 10 mg/kg. All of these results reveal that **6l** may be a lead compound working on cyclooxygenase in inflammation therapy and is worthy of further study and optimization.

Experimental **Supplementary** General chemistry methods, synthesis procedures, spectral data, and bioassay methods are given in Supplemental information.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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