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ARTICLE

Strigolactones: A plant phytohormone as novel anti-inflammatory agents

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Strigolactones (SLs) are a novel class of plant hormones with enormous potential in the prevention and treatment of inflammation. To further investigate the anti-inflammatory activities of SLs, a representative SL, GR24 and its reductive products of D-ring were synthesized and their anti-inflammatory activities were fully evaluated both in vitro and in vivo models. Among these compounds, two most active optical isomers (2a and 6a) demonstrated strong inhibitory activity on the release of inflammatory cytokines including nitric oxide (NO), tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) by blocking the nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways, as well as greatly inhibited the migration of neutrophils and macrophages in fluorescent protein labeled zebrafish larvae model. These results identified the promising anti-inflammatory effects of SLs, and suggested that both the absolute configuration of SL and the α, β-unsaturated D-ring structure are essential for anti-inflammatory activity.

Introduction

Multifaceted biological functions in mammals were recognized in massive phytohormones, abscisic acids (ABA)[1], methyl jasmonates (MJ)[2], and cytokinins (CTK)[3, 4], for instance, which indicates highly potential in the treatment of various clinical diseases. It seems those phytohormones are likely to be an excellent source for the discovery of potential drugs from food and plants. The strigolactones (SLs) are a novel family of plant hormones produced in potential host plants such as wheat, rice and corn, which control shoot branching architecture by inhibiting growth and self renewal of axillary meristem cells[5]. As far, most initial studies were focus on the bioactivity of SLs of their action as a germination stimulant of parasitic seed weeds[6-9]. Interestingly, Yarden et al. recently reported that SL and its analogues are potent inhibitors of mammosphere formation and cancer stem-like cell survival, which opens up the possibility to discover promising anticancer agents from SLs[10-12]. As we known, inflammation is an immune response initiated by pathogen invasion or tissue injury, and increasing studies have realized that it is always accompanied by various diseases, such as cancer[13, 14], atherosclerosis[15, 16], diabetes[17, 18], etc. However, no study related to SLs' promising anti-inflammatory effects have been reported.

To date, more than 15 natural SLs have been isolated from plant root extracts or exudates[19]. Based on the structure-activity relationships, a number of synthetic SL analogues, so-called GR compounds, were designed and synthesized. Among them, GR24, a synthetic analogue of SLs with simpler structure, is the most active and widely applicable as a reference compound for research into parasitic weeds, such as Striga and Orobanche in bioassays[20-23]. Since its inhibitory effects toward breast cancer cell lines growth and survival[10], GR24 become an interesting leading compound and research hotspot in pharmaceutical studies.

Therefore, in the present work, the (±)-GR24 and their reductive products of D-ring were synthesized and the anti-inflammatory effects were evaluated on both in vitro and in vivo models, followed by our in-depth study on their mechanism by western-blot analysis. Toxicity on cells were also evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The results showed the promising anti-inflammatory activity of GR24, especially two most active optical isomers (**2a** and **6a**), which greatly inhibited the release of TNF-α and NO in LPS-activated murine macrophages by affecting MAPK and NF-κB pathways. These findings provided supports for the potential future pharmaceutical application of SLs as a novel anti-inflammatory leading compound.

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Results and discussion

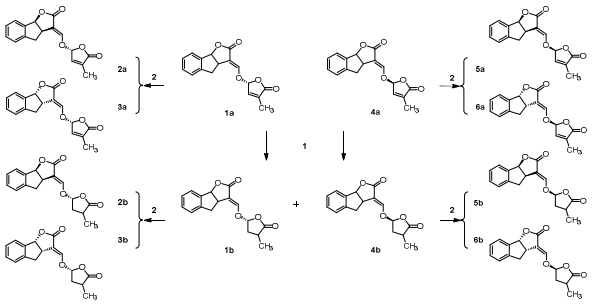
Preparation of optically active synthetic GR24 derivatives

The synthetic routes of GR24 followed previously reported methods, with some improvements to increase the yield (shown in Scheme S1). The synthetic of GR24 enantiomers (**2a**, **3a**, **5a** and **6a**) are shown in Scheme 1, in order to investigate the effect of double bond in D-ring, reduction product GR24 derivatives (**1b** and **4b**) were obtained by a chemical reduction method from **1a** and **4a**, respectively. The optically active reduction GR24 derivatives (**2b**, **3b**, **5b** and **6b**) were separated by chiral column chromatography. All the GR24 enantiomers were confirmed by ^{1}H NMR, ^{13}C NMR and MS (show in supplementary information). The structures of **2a**, **6a** and positive control dexamethasone were shown in Figure 1.

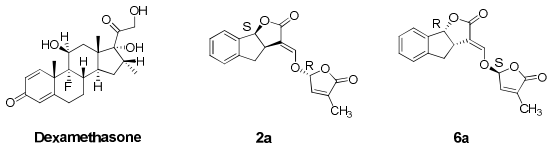
Inhibition of NO production in (LPS)-stimulated *RAW264.7* cells

Nitric oxide (NO) acts as a significant pro-inflammatory mediator for acute or chronic inflammation. It is generally reported that NO inhibitors may offer potential opportunities to develop new therapeutic methods for inflammatory diseases[24, 25]. In this study, the inhibitory activity against NO release of synthesized strigolactones compounds were firstly investigated in lipopolysaccharide (LPS)-stimulated *RAW264.7* cells. As depicted in Table 1, it was found that a novel class of GR24 derivatives (**1a-6a**) displayed improving NO inhibitory activities compared to dexamethasone. In particular, series **a** (**1a-6a**) exhibited better activities than series **b** (**1b-6b**). Among which, compounds **2a**, **2b**, **6a** and **6b** showed better activities than corresponding compounds **3a**, **3b**, **5a** and **5b**.

This work confirmed that the unreduced D-ring of strigolactones plays an essential role in anti-inflammation bioactivity. Compounds **2a**, **2b** and **3a**, **3b** have the (8bS, 2'R) and (8bR, 2'R)-configuration, respectively. In contrast, compounds **6a**, **6b** and **5a**, **5b** have the configuration of (8bR, 2'S) and (8bS, 2'S), respectively. So we can speculate that the α,β-unsaturated furanones, including strigolactones with their



Scheme 1. Synthesis of GR24 derivatives: (1) H_2/Pd, 1 h; (2) Optically active column.



Dexamethasone 2a 6a

Figure 1. Chemical structures of dexamethasone, **2a** and **6a**.

Table 1. The inhibitory effects of the synthesized compounds on NO production in LPS-stimulated *RAW264.7* cells.

Compounds	IC_{50} (μM)[a]	Compounds	IC_{50} (μM)[a]
Dex[b]	2.66±0.50	1b	42.05±5.77
1a	4.06±0.47	2b	32.27±4.23
2a	3.14±0.24	3b	60.45±6.54
3a	6.34±0.76	4b	44.48±4.72
4a	4.32±0.39	5b	64.59±4.32
5a	6.62±0.51	6b	35.44±2.83
6a	3.51±0.22		

[a] Results were showed as means ± SD (n=4) of at least three independent experiments.

[b] Dex: dexamethasone.

D-ring, are important chemical pharmacophore. Besides, compounds **3a**, **3b**, **5a** and **5b** showed weak bioactivity in vitro because of the steric-hindrance.

Cytotoxicity in *RAW264.7* cells

To determine whether the NO inhibitory activities of **2a** and **6a** were related to cell viabilities, a further investigation was demonstrated on the effect of compounds upon *RAW264.7* cells growth, by adopting the method of MTT assay[26]. As can be seen from Table 2, the relative cell viabilities of *RAW264.7* were all above 99% which indicated no obvious cytotoxicity of the tested agents (dexamethasone, **2a**, **6a** and LPS) were shown at the detected concentrations. A further indication of this study also showed that NO inhibitory effects of **2a** and **6a** were likely to be attributed to the structure of these two compounds. These non-toxic concentrations were also used in the following experiments.

Inhibition of TNF-α and IL-6 production in *RAW264.7* cells

TNF-α and IL-6 play an important role in many biological processes and has been proposed as the main contributing factors to induce inflammatory diseases[27, 28]. The inhibitory effect of **2a** and **6a** on the production of pro-inflammatory cytokines is one of the major and effective methods for treatment of inflammation. So the expression of TNF-α and IL-6 cytokines in *RAW264.7* cells was investigated and measured by ELISA[29]. Dexamethasone was also used as a positive reference drug. The effects of these three compounds (**2a**, **6a** and dexamethasone) were evaluated using the concentrations of 10 μM, 5 μM, 2.5 μM and 1.25 μM in a time-dependent manner.

Table 2. Effects of compounds on the viability of *RAW264.7* cells

Compounds	Concentrations	Cell viability (%)[a]
Blank[b]		100.0±3.5
Dex[c]	10 μM	100.2±2.1
2a	10 μM	100.2±1.6
6a	10 μM	99.2±2.1
LPS	500 ng/mL	99.8±2.0
LPS	100 ng/mL	101.5±4.2

[a] Results were expressed as means ± SD (n=5) of three independent experiments.

[b] Blank: the cells cultured with fresh medium only.

[c] Dex: dexamethasone.

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Table 3. The inhibitory effects of dexamethasone, 2a and 6a on LPS-induced TNF-α production in *RAW264.7* cells.

		TNF-α (pg/mL)[a]		
Compounds		6 h	12 h	24 h
Blank		75.9±6.5	107.2±10.4	204.1±23.8
LPS		4377.1±318.6$^{\#\#}$	5313.7±420.9$^{\#\#}$	9635.7±377.3$^{\#\#}$
LPS+Dex[b]	10 μM	2655.0±106.2**	3393.5±280.9**	6458.3±362.9**
	5 μM	3219.4±208.8**	4063.6±326.8**	7533.7±260.2**
	2.5 μM	3671.8±97.6**	4455.7±446.9*	8187.9±184.9**
	1.25 μM	4056.3±231.3	4810.1±462.8	8748.1±370.5**
LPS+2a	10 μM	3862.3±300.1*	4685.5±348.6*	8660.7±363.2**
	5 μM	4140.7±427.4	5036.1±413.2	9085.5±327.1*
	2.5 μM	4276.9±415.4	5186.9±386.2	9397.7±483.9
	1.25 μM	4388.3±425.2	5303.7±405.9	9565.4±450.4
LPS+6a	10 μM	3927.4±222.8*	4721.3±286.7*	8796.7±342.8**
	5 μM	4209.3±316.3	5079.1±376.0	9318.5±348.4
	2.5 μM	4307.9±271.9	5212.4±404.2	9444.5±260.6
	1.25 μM	4367.3±264.7	5319.6±354.3	9582.9±362.0

$^{\#\#}P < 0.01$, $^{\#}P < 0.05$ versus the blank (cultured with fresh medium only) group; $^{**}P < 0.01$, $^{*}P < 0.05$ versus the LPS (treated with LPS only) group.

[a] Results were showed as means ± SD (n=3) of three independent experiments.

[b] Dex: dexamethasone.

Table 4. The inhibitory effects of dexamethasone, **2a** and **6a** on LPS-induced IL-6 production in *RAW264.7* cells.

		IL-6 (pg/mL)[a]		
Compounds		6 h	12 h	24 h
Blank		2.3±0.5	6.8±0.7	12.4±1.9
LPS		472.3±25.8$^{\#\#}$	1394.0±62.6$^{\#\#}$	1966.6±102.3$^{\#\#}$
LPS+Dex[b]	10 μM	269.5±38.9**	791.0±82.9**	924.1±84.1**
	5 μM	332.8±32.8**	927.7±57.4**	1336.5±117.4**
	2.5 μM	393.4±30.9**	1120.3±60.7**	1594.9±95.4**
	1.25 μM	422.7±23.0*	1240.5±42.8**	1770.4±107.2*
LPS+2a	10 μM	302.3±21.8**	869.0±47.2**	1002.2±87.5**
	5 μM	387.1±15.0**	1090.7±30.2**	1499.2±43.3**
	2.5 μM	433.6±14.2*	1267.7±40.5**	1729.3±11.3**
	1.25 μM	461.2±27.3	1351.9±57.7	1892.3±47.2
LPS+6a	10 μM	340.6±37.8**	998.2±84.1**	1061.3±82.1**
	5 μM	410.3±26.2**	1177.0±21.7**	1561.1±162.1**
	2.5 μM	447.9±19.7	1296.0±42.8*	1787.6±65.3*
	1.25 μM	470.1±25.7	1374.3±56.2	1936.5±137.1

$^{\#\#}P < 0.01$, $^{\#}P < 0.05$ versus the blank (cultured with fresh medium only) group; $^{**}P < 0.01$, $^{*}P < 0.05$ versus the LPS (treated with LPS only) group.

[a] Results were showed as means ± SD (n=3) of three independent experiments.

[b] Dex: dexamethasone.

According to the results (Table 3, Table 4, Figure 2 and Figure 3), we found that the levels of TNF-α and IL-6 in LPS-stimulated *RAW264.7* cells were significantly decreased by **2a** and **6a** treatment in concentration- and time-dependent manners as compared with that in *RAW264.7* control cells, and their inhibitory effects were slightly weaker than that of the positive control drug treatment on both TNF-α and IL-6 production at all three (6 h, 12 h and 24 h) detection time. For example, compounds **2a**, **6a**, and dexamethasone at 10 μM could significantly reduce the production of LPS-induced inflammation cytokine IL-6 with the inhibition index of 1002.2±87.5 pg/mL, 1061.3±82.1 pg/mL, and 924.1±84.1 pg/mL, respectively. So the above results found in our laboratory suggested that **6a**, especially **2a**, were excellent agents which showed obvious anti-inflammation effect. Next, in order to further examine the regulatory effect of **2a** and **6a** on inflammatory response, numerous efforts were performed to explore the possible anti-inflammatory mechanism of **2a** and **6a** .

Effects of GR24 on migration of neutrophils and primitive macrophages in the injuries of transgenic zebrafish larvae

In order to further evaluate anti-inflammatory activity of GR24 derivatives, the injury transgenic zebrafish larvae was used as in vivo model for the next step. To our best knowledge, neutrophils and macrophages are two most prominent types of inflammatory cells in living body. Once inflammation occurs in vivo, higher quantities of them were produced and were

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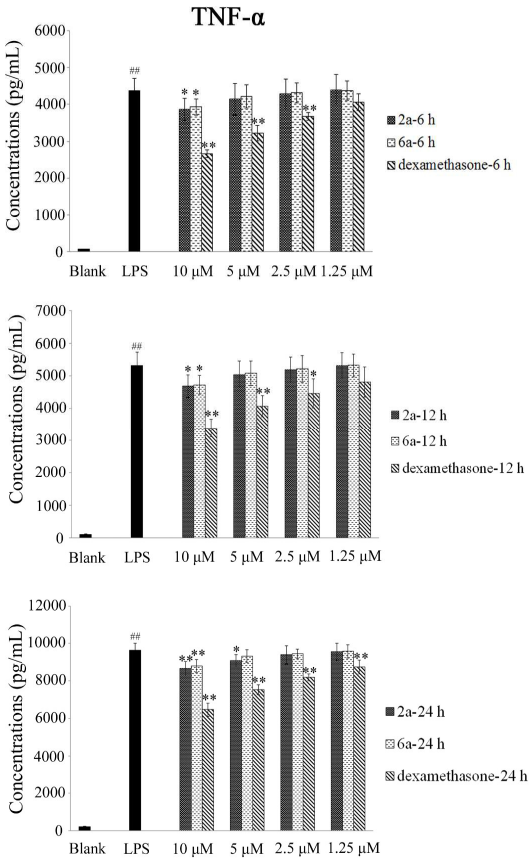

Figure 2. Effects of dexamethasone, 2a and 6a on the LPS-induced production of TNF-α in *RAW264.7* cells. *RAW264.7* cells were treated with dexamethasone, 2a and 6a (at the concentrations of 10 μM, 5 μM, 2.5 μM and 1.25 μM) and LPS (100ng/mL) for 6 h, 12 h and 24 h. Data were presented as means ± SD (n=3). $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ versus the blank (cultured with fresh medium only) group; $^{**}P < 0.01$, $^{*}P < 0.05$ versus the LPS (treated with LPS only) group.

being transported to the inflammation sites. In this study, the anti-inflammatory effects of **2a** and **6a** were investigated using a double-transgenic line (Coronilla-eGFP/Lyc-dsRed), in which neutrophils were double labeled by eGFP and dsRed, presenting yellow color; while primitive macrophages were labeled by eGFP only, presenting green color.

The generation of injury inflammation was induced by tail-cutting; dexamethasone was used as the control drug. As shown in Figure 4, the blank group without tail cutting, which was the healthy larvae, always generated a clear image. But after tail cutting for 3 h and 6 h, the control group generated a fluorescence image, in which visualize these inflammatory cells neutrophils and primitive macrophages obviously accumulated around the wound. Compared to the control group, the level of neutrophils and primitive macrophages around the wound

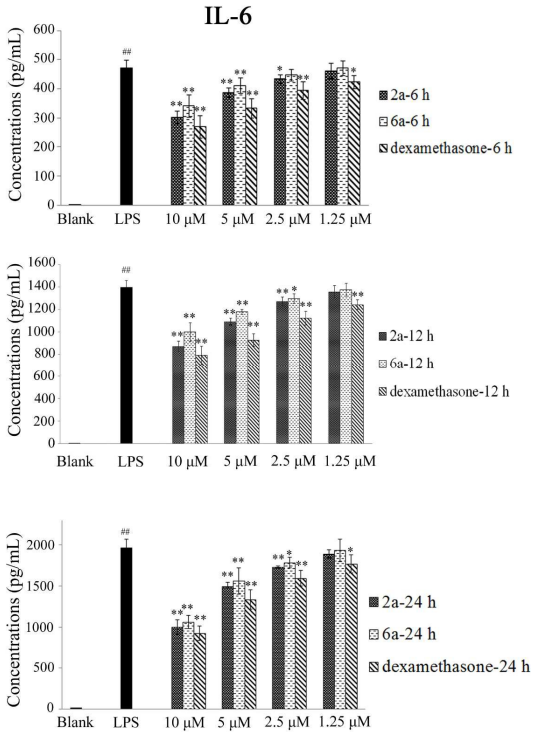

Figure 3. Effects of dexamethasone, 2a and 6a on the LPS-induced production of IL-6 in *RAW264.7* cells. *RAW264.7* cells were treated with dexamethasone, 2a and 6a (at the concentrations of 10 μM, 5 μM, 2.5 μM and 1.25 μM) and LPS (100ng/mL) for 6 h, 12 h and 24 h. Data were presented as means ± SD (n=3). $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ versus the blank (cultured with fresh medium only) group; $^{**}P < 0.01$, $^{*}P < 0.05$ versus the LPS (treated with LPS only) group.

slightly reduced after treatment with 5 μM of **2a** and **6a**, but obviously reduced after treatment with 10 and 20 μM dosages during the evaluation time, which is similar to positive control group. This is also consistent with the behavior described in our early reports[30]. In summary, all these results illustrate the in vivo anti-inflammation activity of GR24 derivatives **2a** and **6a**. Therefore, further efforts were essential to explore their possible anti-inflammatory mechanism.

Western Blot for interpreting possible mechanism

It has been reported that NF-κB and MAPK pathways were quite paramount and significant in inflammation by interplaying with various inflammatory diseases because of their crucial roles in the regulation of the intracellular and extracellular production of inflammatory cytokines in activated macrophages, such as NO, TNF-α, IL-6, and other inflammatory mediators[31-33]. Accumulating literatures indicated that the inflammatory mediators induced by LPS could activate NF-κB and MAPK signaling pathways[34, 35].

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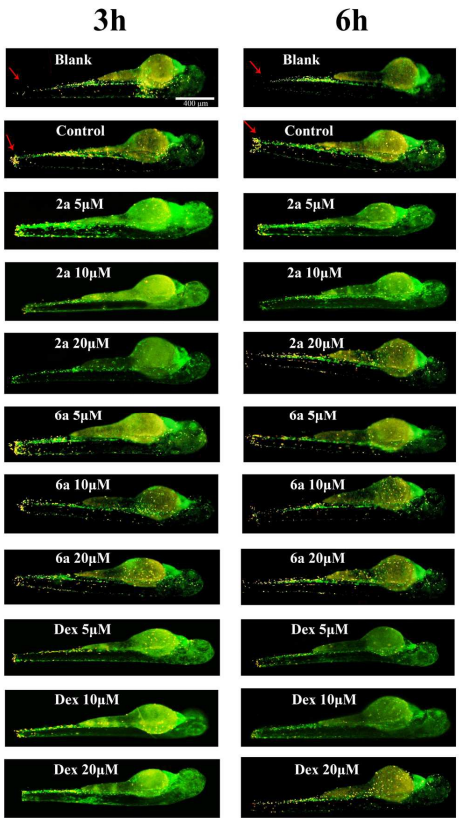

Figure 4. Effects of **2a** and **6a** on migration of neutrophils and macrophage in injury transgenic zebrafish larvae. After tail cutting for half hour, 4 dpf transgenic zebrafish larvae were treated by **2a** and **6a** (5, 10 and 20 μM). Dexamethasone (Dex) was used as control drug. The image of neutrophil (yellow) and macrophage (green) in 3 h and 6 h were recorded by fluorescent microscope. The blank were healthy larvae. The control were cut-tail larvae but without compounds treatment. 6-8 larvae in each group were recorded. Scale bar = 400 μm.

Therefore, in order to investigate whether the anti-inflammatory effects of **2a** and **6a** were associated with the activation of NF-κB and MAPK, western blot analysis were employed in this study. Herein, the proteins we detected including two NF-κB pathway proteins: NF-κB p65 and inhibitor kappa B alpha (IκBα) kinase[36-38], as well as two MAPK cascades: extracellular regulated protein kinases (ERK1/2, or p42/44) and p38 MAPK[39-41]. As shown in Figure 5, the levels of phosphorylated NF-κB p65, IκBα, ERK1/2 and p38 MAPK was significantly increased by treatment of LPS, and decreased in varying degrees in *RAW264.7* cells by **2a** and **6a** treatment. Furthermore, we observed that the phosphorylations of NF-κBp65, IκBα, ERK1/2 and p38 MAPK were obviously inhibited after treatment with **2a** and **6a** in a concentration dependent manner when compared with LPS-induced macrophages, while they had slightly antagonized effect on phosphorylation of IκBα. Taken together, it can be concluded that anti-inflammation effects of **2a** and **6a** were due in part, to their ability to suppress the activation of NF-κB and MAPK cascades, resulting in decreased NO, TNF-α and IL-6 levels.

To further investigate the anti-inflammatory mechanism of GR24, reverse docking of **2a** in the NF-κB signaling pathway

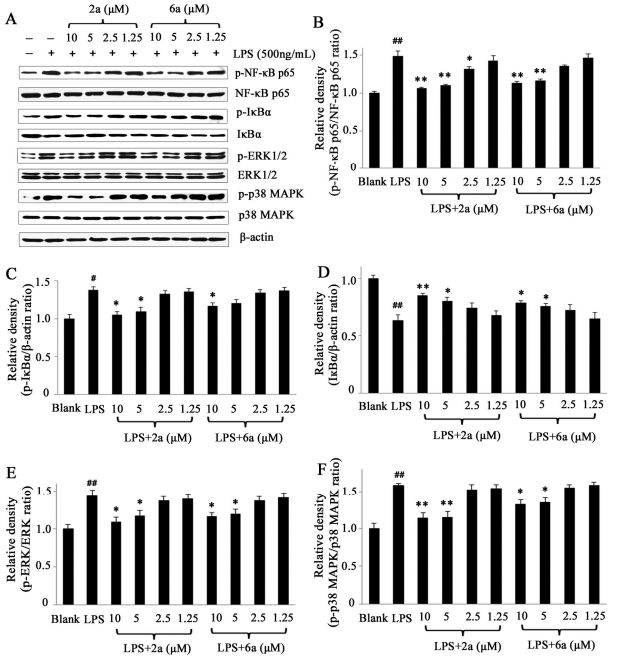

Figure 5. Effects of compounds **2a** and **6a** on the LPS-induced phosphorylation of NF-κB p65, IκBα, ERK1/2 and p38 MAPK in *RAW264.7* cells. *RAW264.7* cells were treated with **2a** (10 μM, 5 μM, 2.5 μM, 1.25 μM), **6a** (10 μM, 5 μM, 2.5 μM, 1.25 μM), and LPS (500 ng/mL) for 4 h. The levels of NF-κB p65, IκBα, ERK1/2, and p38 MAPK proteins, and their phosphorylated forms were analyzed using western blotting. Data were presented as means ± SD (n=3). $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ versus the blank (cultured with fresh medium only) group; $^{**}P < 0.01$, $^{*}P < 0.05$ versus the LPS (treated with LPS only) group.

was performed. 62 proteins of NF-κB were obtained from PDB website, and its protein target structure database was built (Table S1). The molecular docking software AutoDock Vina was used to dock **2a** with the protein targets. According to the docking scores and the docking conformational interaction, the potential targets were selected. 3 target proteins including PARP1, CK2, and AKT were found to interact with GR24 with high affinity. By comparison of their docking conformational interaction (Figures S1~S3), PARP1 is superior to CK2 and AKT. Anti-inflammation of GR24 relates to its function of competitive inhibition of the 3 target proteins, which provides an important reference for the study of the anti-inflammatory mechanism of strigolactone and its analogues. However, it also needs to be confirmed further.

Conclusions

In conclusion, a novel class of synthetic GR24 derivatives was synthesized and their bioactivities were evaluated. To our knowledge, the anti-inflammatory activity of SLs and derivatives was studied for the first time. Among these SLs, two promising compounds (**2a** and **6a**) for the treatment of inflammatory diseases were identified. In respect of the in vitro anti-inflammatory experiment, a similar or even better inhibitory effect was demonstrated on the release of inflammatory mediator and cytokines (NO, TNF-α, and IL-6) comparing to dexamethasone. Moreover, the migration of

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neutrophils and primitive macrophage were also measured in a in vivo zebrafish model, the results also revealed a strongly inhibition on **2a** and **6a**. It was demonstrated that anti-inflammation activity of **2a** and **6a** was due at least in part, to their ability to suppress the activation of NF-κB and MAPK cascades, resulting in decreased NO, TNF-α and IL-6 levels. These results further confirm that compounds with the unreduced D-ring generally exhibit improved anti-inflammation activity, which suggests that reserved D-ring would be an effective strategy to develop preferable anti-inflammatory agents. Further work will be undertaken in our group in order to evaluate the possible anti-inflammatory action target of these SLs.

Experimental

Apparatus

^{1}H and ^{13}C NMR spectra were taken in $CDCl_3$ with a Bruker 300 spectrometer (Karlsruhe, Germany), using tetramethylsilane (TMS) as an internal standard. Chemical shifts are shown in δ (ppm). Mass spectra were recorded with a Thermo Finnigan LCQ Advantage MAX mass spectrometer (Applied Biosystems, 4000 Q TRAP). Optical rotation was obtained on a SEPA-300 (Horiba, Tokyo, Japan). Melting points were determined with a Reichert Thermopan microscope and are uncorrected. Kieselgel 60 F_{254} (Merck, Germany) was used for column chromatography, and pre-coated silica gel 60 F_{254} plates were used to analyze thin layer chromatography (TLC). Spots were visualized using a UV lamp. The fluorescence photos of larvae were recorded by using a fluorescence microscope (Olympus, Japan).

Materials and reagents

RAW264.7 murine macrophages was purchased from American Type Culture Collection (ATCC) and cultured in DMEM containing 10% FBS. Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco (Invitrogen, California, USA). The lipopolysaccharide (LPS, Escherichia coli 0127:B8) and MTT were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Dexamethasone was purchased from national institutes for food and drug control (Beijing, China). N-(1-naphthyl) ethylenediamine dihydrochloride and sulfanilamide were purchased from Sinopharm chemical reagent Co. Ltd (Shanghai, China). TNF-α enzyme immunoassay kits (EK2822) and IL-6 enzyme immune assay kits (EK2062) were purchased from Multi Sciences Biotechnology (Hangzhou, China). Phorbol-12-myristate-13-acetate (PMA, S1819), IP cell lysis buffer (P0013) and phenylmethanesulfonyl fluoride (PMSF, ST506) were purchased from Beyotime Biotechnology (Shanghai, China). Anti-beta actin monoclonal antibody (E021020-01) was purchased from EarthOx, LLC, CA. Primary anti-rabbit inducible NO synthase (iNOS, #2982), NF-κB pathway sampler Kit (#9936S), p-MAPK family antibody sampler kit (#9910S) and MAPK family antibody sampler kit (#9926) were purchased from Cell Signaling Technology. BCA protein assay kit (KGPBCA) was purchased from KeyGEN Biotechnology (Nanjing, China). The enhanced chemiluminescent (ECL) Detection Kit (WBKLS0050) was purchased from Merck Millipore Corporation (Merck KGaA, Darmstadt, Germany).

Chemicals

(±)-GR24 and its derivatives were synthesized from commercially available material indan-1-one using previously reported methods[42-44] as an epimer mixtures of **1a** and **4a** (ca. 1:1) at C-2', which were then separated by chromatography on silica gel with hexane/EtOAc (v/v, 7:3) as the elution. Then, a solution of **1a**/**4a** (50 mg) in ethyl acetate (5mL) was added Pd/C (5mg). The mixture was stirred under hydrogen atmosphere for 0.5 h at room temperature before being filtered, and the filtrate was concentrated in vacuo to provide the crude product which was purified by flash chromatography on a silica gel column to provide the diastereomer **1b**/**4b**. The optical isomers of **2a** and **3a** were achieved by an optically active column from compound **1a**, as reported by Ueno[45]. Similarly, the isomers of **5a** and **6a** were also prepared from compound **4a**. Additionally, a series of optical isomers of **2b, 3b, 5b** and **6b** were obtained in the same way, respectively (Scheme 1). All products in stock including dexamethasone were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 50 mM and the amount of DMSO was fixed at 0.1% (v/v) when added to cells.

Cell culture

RAW264.7 murine macrophages cells were routinely cultured in DMEM with 10% fatal calf serum (FCS), 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 5% CO_2 humidified atmosphere.

Assay for NO production

RAW264.7 cells, a murine macrophage cell line, were inoculated at 5×10^4 cells per well in 96-well plate and left to culture for 18 h before any treatment. The concentrations of each compound including the positive drug (dexamethasone) were designed to 100 μM, 50 μM, 20 μM, 10 μM, 5 μM, 2 μM and 1 μM. The cells were then pre-treated with different concentrations of compounds for 2 h before stimulation with LPS (100 ng/mL), while the cells in control group were treated with LPS only. After stimulated for 24 h by LPS, the NO produced in the culture medium was quantified by Greiss reagent, in brief, transferred 100 μL cell culture supernatant to another 96-well plate, added 100 μL of Griess reagent and incubated for 10 min at room temperature. Then the absorbance of samples was measured at 540 nm (OD_{540}) in a microplate reader (Bio-Rad Laboratories, CA, USA). Dexamethasone was used as the positive control. IC_{50}, the half maximal inhibitory concentration, represented the concentration of a compound that is required for inhibition of 50% NO production in *RAW264.7* cells and was determined constructing a dose-response curve in vitro.

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Cell cytotoxicity

Cell cytotoxicity of *RAW264.7* cells was measured by MTT assay. Briefly, cells were seeded at 4×10^3 cells per well in a 96-well plate. After cultured overnight, the cells were treated with different compounds for 48 h. Then to each well, 20 μL of 0.5 mg/mL MTT solution was added. Cells were incubated for 4 h at 37 °C. After four hours, cell culture supernatant was removed and then followed by solubilization with 150 μL dimethyl sulfoxide (DMSO) solutions. The optical density was measured at 570 nm (OD_{570}) using a Bio-Radmicroplate reader. Each experiment was repeated thrice, and the data was calculated from three measurement results. The density of formazan formed in blank group was set as 100% of viability. Cell viability (%) = [Compound (OD_{570}) - Blank (OD_{570})] / [Control (OD_{570}) - Blank (OD_{570})] × 100%

Blank: cultured with fresh medium only

Control: the cells cultured with fresh medium only

Compound: the cells treated with different compounds

Measurement of TNF-α and IL-6

The levels of TNF-α and IL-6 were measured with the mouse ELISA kit (TNF-α: MultiSciences, EK2822; IL-6: MultiSciences, EK2062) which is operated according to the manufacturer's instructions. All samples were obtained as follows: *RAW264.7* cells (5×10^5 cells/well) were cultured in 24-well plate and pretreated with 10 μM, 5 μM, 2.5 μM, 1.25 μM of compounds for 2 h, and then LPS (100 ng/mL) was added. The production of TNF-α and IL-6 was incubated for 6 h, 12 h and 24 h.

Zebrafish larvae live image analysis

The neutrophil/macrophage double transgenic zebrafish (Coroninla-eGFP / Lyc-dsRed) of **2a** and **6a** was performed according to our previously study[30, 46-48]. The preparing of four days post fertilization zebrafish larvae, tail-cutting surgery, compound treatment and image analysis were all followed this reported methods with a minor modification. Dexamethasone was used as the control drug. All experiments were performed in compliance with the relevant laws and institutional guidelines, and the Zebrafish Research Centre of Southern Medical University in China has approved the experiments.

Western Blot analysis.

RAW264.7 cells were seeded into a 6-well culture plate at a density of 2×10^6 cells/well. Then, the culture medium was replaced by fresh medium containing different concentrations of compounds (10 μM, 5 μM, 2.5 μM, and 1.25 μM), and LPS (500 ng/mL) was added. After cultured for another 4 h, the cells were harvested and lysed with cell lysates which contained IP buffer (Beyotime, P0013) and 1 mM phenylmethanesulfonyl fluoride (PMSF: Beyotime, ST506) for 30 min at 4 °C. The cell supernatant was collected by centrifugation at 14,000×g for 10 min at 4 °C. Protein concentration was determined using the BCA protein assay kit (Thermo Scientific, 23227). Furthermore, each protein sample was mixed with a quarter volume of 5X SDS-PAGE sample loading buffer and the mixture was boiled for 10 min. Equal amounts of total cellular protein were separated in a 12.5% SDS-PAGE gel and then transferred to polyvinylidenedifluoride membranes (Bio-Rad). After wet transfer, the membranes were blocked for 4 h at room temperature in 5% non-fat dry milk in TBS containing 0.1% Tween 20 (TBST), washed 3 times in TBST, incubated with the primary antibody (anti-phosphorylation of ERK1/2 (Thr202/Tyr204), anti-ERK1/2, anti-phosphorylation of p38 (Thr180/Tyr182), anti-p38, anti-phosphorylation of IκBα (Ser32/36), anti-IκBα, anti-phosphorylation of NF-κB p65, and anti-NF-κB p65) at 4 °C overnight (all the primary antibodies were purchased from Cell Signaling Technology and diluted in TBST at the ratio of 1:1000), washed 3 times in TBST, incubated with anti-rabbit or anti-mouse secondary antibody (1:1000 in TBST, Cell Signaling Technology) for 1.5 h at room temperature, washed in TBST and exposed to ECL reagents.

Statistical analysis

Results are presented as mean ± standard error (SD). Statistical analysis were performed using Student's t test (two-tailed, paired) versus vehicle controls and regarded as being significant when $p < 0.05$.

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Conflict of Interest

The authors declare no competing interests.

References

1 P. Sakthivel, N. Sharma, P. Klahn, M. Gereke and D. Bruder, *Curr. Med. Chem.*, 2016, **23**, 1549-1570.
2 S. Cohen and E. Flescher, *Phytochemistry*, 2009, **70**, 1600-1609.
3 Y. Ishii, S. Sakai and Y. Honma, *Biochim. Biophys. Acta, Mol. Cell Res.*, 2003, **1643**, 11-24.
4 P. Mlejnek, *J. Cell. Biochem.*, 2001, **83**, 678-689.
5 P. B. Brewer, H. Koltai and C. A. Beveridge, *Mol. Plant*, 2013, **6**, 18-28.
6 Y. Kapulnik, P.M. Delaux, N. Resnick, E. Mayzlish-Gati, S. Wininger, C. Bhattacharya, N. Séjalon-Delmas, J.P. Combier, G. Bécard and E. Belausov, *Planta*, 2011, **233**, 209-216.

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7 C. Rameau, *C. R. Biol.*, 2010, **333**, 344-349.
8 V. Gomez-Roldan, S. Fermas, P. B. Brewer, V. Puech-Pagès, E. A. Dun, J.P. Pillot, F. Letisse, R. Matusova, S. Danoun and J.-C. Portais, *Nature*, 2008, **455**, 189-U22.
9 M. Umehara, A. Hanada, S. Yoshida, K. Akiyama, T. Arite, N. Takeda-Kamiya, H. Magome, Y. Kamiya, K. Shirasu and K. Yoneyama, *Nature*, 2008, **455**, 195-U29.
10 C. Pollock, H. Koltai, Y. Kapulnik, C. Prandi and R. Yarden, *Breast Cancer Res. Treat.*, 2012, **134**, 1041-1055.
11 C. B. Pollock, S. McDonough, V. S. Wang, H. Lee, L. Ringer, X. Li, C. Prandi, R. J. Lee, A. S. Feldman and H. Koltai, *Oncotarget*, 2014, **5**, 1683-1698.
12 E. Mayzlish-Gati, D. Laufer, C. F. Grivas, J. Shaknof, A. Sananes, A. Bier, S. Ben-Harosh, E. Belausov, M. D. Johnson and E. Artuso, *Cancer Biol. Ther.*, 2015, **16**, 1682-1688.
13 J. K. Kundu and Y.J. Surh, *Mutat. Res., Rev. Mutat. Res.*, 2008, **659**, 15-30.
14 S. I. Grivennikov, F. R. Greten and M. Karin, *Cell*, 2010, **140**, 883-899.
15 P. Libby, P. M. Ridker and A. Maseri, *Circulation*, 2002, **105**, 1135-1143.
16 P. Libby, *Arterioscler., Thromb., Vasc. Biol.*, 2012, **32**, 2045-2051.
17 A. S. Major and D. G. Harrison, *Circulation*, 2011, **124**, 2809-2811.
18 W. Xie and L. Du, *Diabetes, Obes. Metab.*, 2011, **13**, 289-301.
19 X. Xie, K. Yoneyama and K. Yoneyama, *Annu. Rev. Phytopathol.*, 2010, **48**, 93-117.
20 E. Dor, D. M. Joel, Y. Kapulnik, H. Koltai and J. Hershenhorn, *Planta*, 2011, **234**, 419-427.
21 C. Bhattacharya, P. Bonfante, A. Deagostino, Y. Kapulnik, P. Larini, E. G. Occhiato, C. Prandi and P. Venturello, *Org. Biomol. Chem.*, 2009, **7**, 3413-3420.
22 A. S. Mwakaboko and B. Zwanenburg, *Bioorg. Med. Chem.*, 2011, **19**, 5006-5011.
23 G. H. Nefkens, J. W. J. Thuring, M. F. Beenakkers and B. Zwanenburg, *J. Agric. Food Chem.*, 1997, **45**, 2273-2277.
24 L. S. Tedesco, J. Fuseler, M. Grisham, R. Wolf and S. C. Roerig, *Pain*, 2002, **95**, 215-223.
25 H.Y. Tseng, S.H. Wu, W.-H. Huang, S.F. Wang, Y.N. Yang, N. Mahindroo, T. Hsu, W.T. Jiaang and S.J. Lee, *Bioorg. Med. Chem. Lett.*, 2005, **15**, 2027-2032.
26 M. Taira, K. Sasaki, S. Saitoh, T. Nezu, M. Sasaki, S. Kimura, K. Terasaki, K. Sera, T. Narushima and Y. Araki, *Dent. Mater. J.*, 2006, **25**, 726-732.
27 R. Sharma, S. Agrawal, A. Saxena and R. Sharma, *J. Interferon Cytokine Res.*, 2013, **33**, 384-391.
28 K. Popko, E. Gorska, A. Stelmaszczyk-Emmel, R. Plywaczewski, A. Stoklosa, D. Gorecka, B. Pyrzak and U. Demkow, *Eur. J. Med. Res.*, 2010, **15**, 120-122.
29 J. Bienvenu, L. Coulon, C. Doche, M. Gutowski and G. Grau, *Eur. Cytokine Network*, 1993, **4**, 447-451.
30 M.Y. Huang, J. Lin, Z.J. Huang, H.G. Xu, J. Hong, P.H. Sun, J.L. Guo and W.M. Chen, *MedChemComm*, 2016, **7**, 658-666.
31 W. Li, H. Huang, Y. Zhang, T. Fan, X. Liu, W. Xing and X. Niu, *Eur. J. Pharmacol.*, 2013, **715**, 62-71.
32 C. Xie, J. Kang, M. E. Ferguson, S. Nagarajan, T. M. Badger and X. Wu, *Mol. Nutr. Food Res.*, 2011, **55**, 1587-1591.
33 C. Xie, J. Kang, Z. Li, A. G. Schauss, T. M. Badger, S. Nagarajan, T. Wu and X. Wu, *J. Nutr. Biochem.*, 2012, **23**, 1184-1191.
34 S. Chen, J. Xiong, Y. Zhan, W. Liu and X. Wang, *Cell. Mol. Neurobiol.*, 2015, **35**, 523-531.
35 D. B. Reddy and P. Reddanna, *Biochem. Biophys. Res. Commun.*, 2009, **381**, 112-117.
36 T. D. Gilmore, *Cancer biology*, 1997, **8**, 61-62.
37 Y. Yamamoto and R. B. Gaynor, *J. Clin. Invest.*, 2001, **107**, 135-142.
38 K. Ashikawa, S. Majumdar, S. Banerjee, A. C. Bharti, S. Shishodia and B. B. Aggarwal, *J. Immunol.*, 2002, **169**, 6490-6497.
39 S. M. Wurgler-Murphy and H. Saito, *Trends Biochem. Sci.*, 1997, **22**, 172-176.
40 J. M. Kyriakis and J. Avruch, *Physiol. Rev.*, 2012, **92**, 689-737.
41 E. Herlaar and Z. Brown, *Mol. Med. Today*, 1999, **5**, 439-447.
42 S. Wigchert, E. Kuiper, G. Boelhouwer, G. Nefkens, J. Verkleij and B. Zwanenburg, *J. Agric. Food Chem.*, 1999, **47**, 1705-1710.
43 J. W. J. Thuring, G. H. Nefkens and B. Zwanenburg, *J. Agric. Food Chem.*, 1997, **45**, 2278-2283.
44 E. M. Mangnus, F. J. Dommerholt, R. L. De Jong and B. Zwanenburg, *J. Agric. Food Chem.*, 1992, **40**, 1230-1235.
45 K. Ueno, M. Fujiwara, S. Nomura, M. Mizutani, M. Sasaki, H. Takikawa and Y. Sugimoto, *J. Agric. Food Chem.*, 2011, **59**, 9226-9231.
46. M.Y. Huang, J. Lin, K. Lu, H.G. Xu, Z.Z. Geng, P.H. Sun and W.M. Chen, *J. Agric. Food Chem.*, 2016, **64**, 2893-2900.
47. H.B. Zhou, D.T. Yang, N. E. Lvleva, N. E. Mircescu, R. Niessner and C. Haisch, *Anal. Chem.*, 2014, **86**, 1525-1533.
48. H.B. Zhou, D.T. Yang, N. P. Lvleva, N. E. Mircescu, S. Schubert, R. Niessner, A. Wieser and C. Haisch, *Anal. Chem.*, 2015, **87**, 6553-6561.

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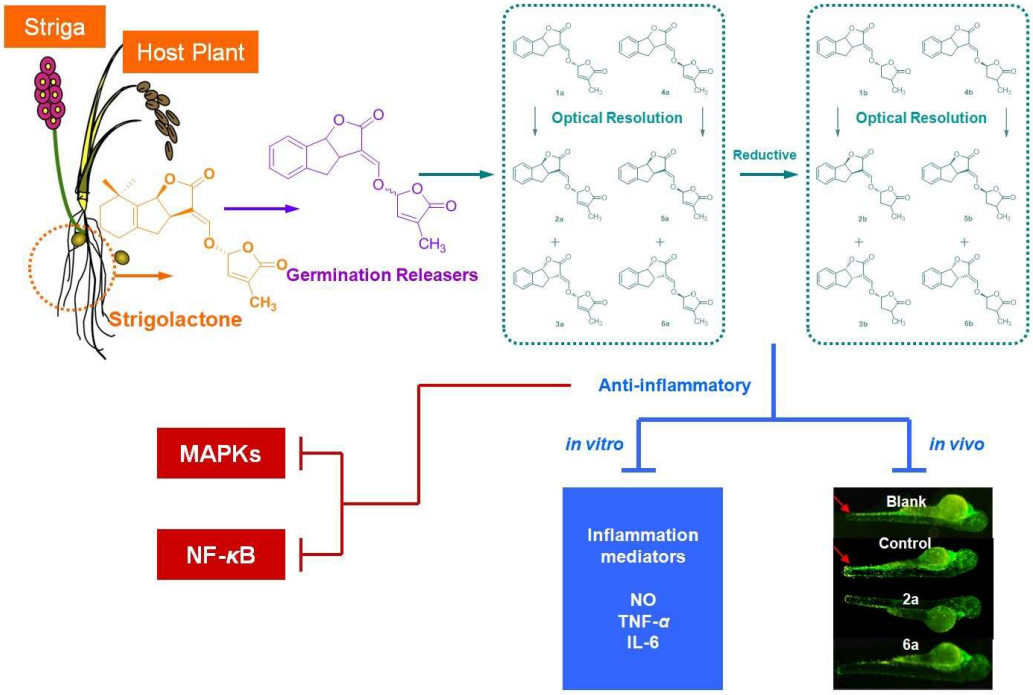
Table of Contents Graphic



Strigolactones (SLs)，A representative SL, GR24 and its reductive products of D-ring were synthesized and their anti-inflammatory activities were fully evaluated on both in vitro and in vivo models.