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Synthesis and biological evaluation of novel semi-conservative mono-carbonyl analogs of curcumin as anti-inflammatory agents

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Curcumin, a natural product, has been shown to possess notable anti-inflammatory activities. However, the clinical application of curcumin was restricted by its poor stability and bioavailability. We have reported a series of mono-carbonyl analogs of curcumin (MACs) previously. In the present study, we synthesized 32 semi-conservative MACs and evaluated their anti-inflammatory effects in RAW 264.7 macrophages. Most of them showed enhanced abilities to inhibit the lipopolysaccharide (LPS)-induced expression of tumor necrosis factor alpha (TNF-α). The preliminary structure-activity relationship was discussed and the preliminary anti-inflammatory mechanism was also explored. The most potent analog compound **WZ35** exhibits significant protection against LPS-induced death in septic mice. These findings suggest that this kind of curcumin derivative may be used to develop promising anti-inflammatory agents to treat inflammatory diseases.

1. Introduction

Numerous studies have proved that the inflammatory response plays an important role in the pathological processes of various diseases, including sepsis,[1] cancer,[2] diabetes,[3] and inflammatory bowel[4]. In particular, sepsis, an excessive and irregular systemic inflammatory response syndrome against serious infection, is believed to be related to an exacerbated release of pro-inflammatory cytokines, such as TNF-α.[5, 6] TNF-α, a representative and multifunctional cytokine, is produced primarily by activated monocytes/macrophages and is crucial in the initiation and continuation process of inflammation and immunity.[7, 8] The expression of TNF-α is regulated by nuclear factor (NF-κB), which is a key transcription factor of lymphocytes and macrophages.[9] Thus, an effective NF-κB inhibitor is considered to be a potential anti-inflammatory drug candidate for the treatment of inflammation-related diseases.[10, 11]

Traditional non-steroidal anti-inflammatory drugs (NSAIDs), acting as inhibitors of the enzyme cyclooxygenase, are widely used for the treatment of acute or chronic conditions where pain and inflammation are present.[12, 13] However, long term use of NSAIDs is limited by their serious gastrointestinal side effects.[14, 15] The risk of gastric ulceration increases dramatically with long therapy duration and high doses. As a result, it's urgent to develop novel and potential anti-inflammatory agents. Curcumin (1,7-bis(*p*-hydroxy-*m*-methoxyphenyl)-1,6-heptdiene-3,5-dione), a yellow powder, is the major component in *Curcuma longa*. It has been demonstrated to possess multiple pharmacological activities and medicinal applications, such as anti-inflammatory,[16-18] anti-cancer,[19, 20] and anti-oxidation [21, 22]. In addition, clinical trials manifest that curcumin does not lead to discernible toxicities even under a high-dose.[23, 24] However, its poor metabolic stability and low bioavailability have dramatically restricted its therapeutic application. Several studies have found that the β-diketone moiety in the structure of curcumin contributes to its instability *in vivo* and leads its rapid degradation.[25, 26] Previously, our research group has synthesized several different Mono-carbonyl Analogs of Curcumin (MACs), most of which possess enhanced anti-inflammatory properties and stability compared to curcumin.[27-30]

More recently, some studies suggested that asymmetric MACs are able to improve survival from lethal sepsis in septic mouse model.[31, 32] However, there are big structural differences between the reported asymmetric MACs and curcumin. For the purpose of studying more structurally conserved curcumin analogues, in our ongoing research for anti-inflammatory MACs, we focused on a new mono-carbonyl analog of curcumin containing the same 4-hydroxy-3-methoxyphenyl on curcumin, named as semi-conservative MAC. This moiety, 4-hydroxy-3-methoxyphenyl, is common structural unit of many biologically active nature products, such as curcumin, vanillin, ferulic acid, guaiacol, eugenol and so on. Our pervious results and some other papers showed the presence of 4-hydroxy-3-methoxyphenyl is essential to obtain high biological activity.[29, 30, 33] Here, a series of semi-conservative MACs were synthesized and their corresponding anti-inflammatory activities were tested in mouse RAW 264.7 macrophages. Most

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of them showed enhanced abilities to inhibit the LPS-induced expression of TNF-α. Especially, the compound WZ35, a potent NF-κB inhibitor, exhibited a significant therapeutic effect in the LPS-induced septic mouse model.

2. Results and Discussion

2.1.Chemistry

The synthetic routes and structure of semi-conservative curcumin derivatives are shown in **Fig. 1**. Intermediate **3** was prepared from vanillin by protection of the alcohol group using 3,4-dihydro-2H-pyran in the presence of pyridinium *p*-toluenesulfonate (PPTS) in dichloromethane and subsequent aldol condensation with acetone in basic condition. Conversion of intermediate **3** to semi-conservative MACs except **WZ21** and **WZ26** were achieved by aldol condensation with different substituted aromatic aldehydes in basic condition, followed by hydrolysis reaction. **WZ21** and **WZ26** were prepared in acidic condition directly.

Insert Fig. 1

Fig. 1 Structures and synthesis of MACs compounds. Reagents and conditions: (i) 3,4-dihydro-2H-pyran, PPTS, CH_2Cl_2, rt; (ii) acetone, NaOH, EtOH, rt; (iii) NaOEt, EtOH, rt; (iii') HCl(g), EtOH, rt; (iv) HCl, THF, rt.

2.2.Anti-inflammatory and cytotoxic evaluation of synthetic semi-conservative MACs

The stimulation of macrophages with the bacterial endotoxin LPS induces a pro-inflammatory process through the activation of cytokine expression and release.[34] Curcumin and 32 semi-conservative MACs were evaluated for their ability to inhibit TNF-α release in mouse RAW 264.7 macrophages. Macrophages were incubated with the compounds at a concentration of 10 μM for 2 hours and then stimulated with LPS for 22 hours. The quantities of TNF-α in the media were detected by Enzyme-Linked Immunosorbant Assay (ELISA) and the protein concentrations of cells harvested from the corresponding cultural plates were used to normalize the cytokines level.

The results of the anti-inflammation assay are illustrated in **Fig. 2A**. The initial screening indicates that the majority of semi-conservative MACs showed more intense anti-inflammatory activities than curcumin at the concentration of 10 μM. Among the 32 compounds, 31 showed better inhibitory effects than curcumin on LPS-induced TNF-α expression. Particularly, compounds **WZ4**, **WZ7**, **WZ12**, and **WZ35** almost completely inhibited the release of TNF-α.

Insert Fig. 2

Fig 2 Curcumin and its analogs inhibited LPS-induced TNF-α (**A**) secretion in RAW 264.7 macrophages and the cytotoxic evaluation (**B**) in HL-7702 cells. Macrophages were plated at a density of 4.0 x 10^5/plate at 37 °C and 5% CO_2 overnight. Cells were pre-treated with curcumin or its analogs (10 μM) for 2 hours, and then treated with LPS (0.5 μg/mL) for 22 hours. TNF-α levels in the culture media were measured by ELISA and were normalized by the total protein. The results were expressed as the percent of LPS control. Each bar represents mean ± standard error of the mean (SEM) of 3-5 independent experiments. Statistical significance relative to LPS group was indicated, *p< 0.05, **p< 0.01.

Methyl thiazolyl tetrazolium (MTT) was applied to measure the cytotoxicity and safety of analogues in the human normal hepatic cell line, HL-7702, after 24 hours treatment of the cells with compounds at a concentration of 10 μM. As shown in **Fig 2B**, no compounds displayed apparent toxicity in hepatic cells, suggesting that they are relatively safe.

2.3.Preliminary structure activity relationship (SAR)

As shown in **Fig. 1**, all synthetic semi-conservative MACs are composed of two parts including ring A and ring B. Obviously, all the structural differences of synthetic compounds are presented on ring B while the ring A remains all the same. They suppressed TNF-α with inhibition ratios ranging from 6% to 96%, and most of them showed better inhibitory activity than curcumin. To investigate the SAR on ring B, the influence of group size, electronic properties, and position were tested by modifying the substituents of analogs. Obviously, the heterocycle and long chain containing analogs, such as compounds **WZ28**, **WZ33**, **WZ29**, **WZ31** and **WZ36** exhibited relatively weaker activities which indicate that too large substituents at ring B are unfavorable for the anti-inflammatory activities. On the other hand, most of mono-substituted analogs (compounds **WZ01**, **WZ03**, **WZ17** and **WZ21**) also displayed less activity than di- or tri-substituted analogs, which suggested that groups with little steric hindrance at ring B are also unfavorable for the activities. In summary, the size of substituents may play a crucial role in the anti-inflammatory activity of the asymmetrical monocarbonyl curcumin analogs.

2.4.Active compounds inhibit the LPS-induced cytokine release in a dose-dependent manner

To further confirm the anti-inflammatory properties of the active compounds, compounds **WZ08**, **WZ12**, **WZ19**, **WZ22**, **WZ35**, and **WZ37** were selected to investigate their inhibitory activity against LPS-stimulated TNF-α and interleukin-6 (IL-6) release in a dose-dependent manner. Both RAW 264.7 and primary mouse peritoneal macrophages were pretreated at different concentrations (1.1, 3.3 and 10.0 μM) of the six active analogs for 2 hours and then were incubated with LPS (0.5 μg/mL) for 22 hours. As illustrated in **Fig. 3**, all of six compounds exhibited a dose-dependent inhibition activity against LPS-induced TNF-α and IL-6 release in the two different types of macrophages. The result further suggested the potential of these semi-conservative MACs as anti-inflammatory agents.

Insert Fig. 3

Fig. 3 Six active MACs inhibited LPS-induced TNF-α and IL-6 release in a dose-dependent manner. Macrophages were plated at a density of 4× 10^5/plate overnight in 37 °C and 5% CO_2. Cells were pretreated with active compounds in a series concentration of 1.1 μM, 3.3 μM, and 10 μM for 2 hours and subsequently incubated with or without LPS (0.5 μg/mL) for 22 hours. TNF-α and IL-6 levels in the culture medium of RAW 264.7 macrophages (**A** and **B**) and primary macrophages (**C** and **D**) were measured by ELISA and were normalized by the total protein. The results were presented as the percent of LPS control. Each bar represents the mean ±SEM of three independent experiments. Statistical significance relative to the LPS group was indicated, *p< 0.05, **p< 0.01.

2.5. Active compounds inhibited LPS-induced inflammatory gene expression

Apart from TNF-α and IL-6, interleukin-1 beta (IL-1β) and cyclooxygenase-2 (COX-2) are two other important inflammatory cytokines.[35] The mRNAs levels of the pro-inflammatory cytokines, such as TNF-α, IL-6, IL-1β, and COX-2, are important and sensitive indicators of inflammation. During the inflammatory response, the expression of mRNAs for these inflammatory cytokines will be markedly up-regulated.[36] We tested the inhibitory effects of these six compounds on inflammatory gene transcription in LPS-stimulated macrophages. As shown in **Fig. 4A**, the LPS-induced up-regulated mRNA level of TNF-α, IL-6, IL-1β, and COX-2 could be significantly reduced through pretreatment with these six analogs, respectively. Furthermore, the results in the **Fig. 4B** indicated that the best compound **WZ35** possessed the inhibitory activities against the up-regulated mRNA level in a dose-dependent manner. The chemical stability of representative compounds, **WZ19** and **WZ35** were detected and measured by UV-visible absorption in phosphate buffer. The results indicate that MACs are more stable than curcumin in the buffer at physiological pH 7.4 (**Fig. S1)**.

Insert Fig. 4

Fig. 4 Six active MACs inhibited LPS-induced mRNA of inflammatory mediators release in RAW 264.7 macrophages. Macrophages were pre-treated with (A) compounds **WZ08**, **WZ12**, **WZ32**, **WZ35**, and **WZ37** at 10 μM, (B) **WZ35** at 2.5 μM, 5 μM, and 10 μM or vehicle control for 2 hours and then stimulated with LPS (0.5 μg/mL) for 6 hours. Macrophages were plated at a density of 4.0×10^5/plate for overnight in 37 °C and 5% CO_2. The mRNA levels of inflammatory mediators IL-6, TNF-α, IL-1β and COX-2 were quantified by RT-qPCR. The mRNA values for each gene was normalized to internal control β-actin mRNA and were expressed as a ratio to dimethyl sulfoxide (DMSO). Each bar represents mean ± SEM of 3-5 independent experiments. Statistical significance relative to LPS group was indicated, *p< 0.05, **p< 0.01.

2.6.Active compounds inhibited the LPS-induced activation of NF-κB

In addition, to clarify the mechanism of anti-inflammatory effects of the most active compound **WZ35**, we try to identify the possible signaling pathways responsible for the production of pro-inflammatory cytokines that are inhibited by our compounds. We tested the effect of **WZ35** on NF-κB known to be activated by LPS. NF-κB is a nuclear transcriptional factor and plays a crucial role in regulating immune response and inflammation.[37] While in an inactivated state, NF-κB is located in the cytosol and complex with the inhibitory protein IκB-α. In response to LPS, IκB kinase β phosphorylates cytoplasmic IκB-α, which results in ubiquitination, dissociation of IκB-α from NF-κB. The activated NF-κB is then translocated into the nucleus to promote the transcription of inflammatory genes.[38, 39] Since the activation of NF-κB is regulated by the degradation of IκB-α, we evaluated the effects of the analog on IκB-α degradation in LPS-stimulated RAW 264.7 cells. As shown in **Fig. 5A-B**, **WZ35** dose-dependently reduced the LPS-induced IκB-α degradation. The amount of P65 decreased in nucleus (P65N) but increased in cytoplasm (P65C) by increasing concentration of the compound. Furthermore, we also detected the binding ability of NF-κB with DNA in nucleus of LPS-stimulated RAW264.7 cells, which pretreated with a different concentration of **WZ35** by EMSA assay. As shown in **Fig. 5C**, **WZ35** significantly inhibited the NF-κB binding to DNA in a dose-dependent manner. These results demonstrate that compound **WZ35** inhibit the LPS-induced activation of NF-κB which may be involved in the anti-inflammatory actions.

Insert Fig. 5

Fig. 5 Compound **WZ35** inhibited LPS-induced NF-κB activation in RAW 264.7 macrophages. (A) Macrophages were pretreated with **WZ35** at 2.5 μM, 5 μM, and 10 μM or vehicle control for 2 hours and then treated with LPS (0.5 μg/mL) for 1 hour. The level of IκB-α and P65 were examined using a specific antibody with GAPDH or LaminB as the loading control. (B) The column figures show the normalized optical density as times of the LPS group. Bars represent the mean ± SE M of the three independent experiments (*p< 0.05, **p< 0.01 versus the LPS group). (C) Macrophages were pretreated with **WZ35** (1 μM, 2.5 μM, 5 μM, and 10 μM) or vehicle (DMSO) for 2 hours and then stimulated with LPS (0.5 μg/mL) for 1 hours, and then detected the bind ability between NF-κB and DNA in nucleus by EMSA kit.

2.7.Active compound WZ35 inhibited LPS-induced septic death in mice

It is reported that LPS, a representative endotoxin, has been implicated as a main cause of sepsis.[40, 41] Lastly, we further determined whether compound **WZ35** is able to attenuate LPS-induced septic death *in vivo*. C57BL/6 mice were injected intravenously with the compounds **WZ35** at a dosage of 10 mg/kg, 15 minutes later 20 mg/kg of LPS was injected intraperitoneally and then the survival rates were recorded for 7 days. As shown in **Fig. 6**, 100% of the animals treated with LPS alone died within 50 hours because of septic shock. The survival rates were increased from 0% to 55% by pre-treatment

with **WZ35**. These data suggest that **WZ35** exhibits anti-inflammatory activity *in vivo*.

Insert Fig. 6

Fig.6 Compound **WZ35** improve survival of mice subjected to a lethal dose of LPS. Mice were intravenously injected with 10 mg/kg **WZ35** 15 minutes before intraperitoneal injection of LPS 20 mg/kg. Survival rates were recorded for 7 days after the LPS injection at the interval of 1 day. n=9 animals in each group. Statistical significance relative to LPS group was indicated, ** p<0.01.

3. Experimental Section

3.1.Chemistry

In general, reagents, solvents, and other chemicals were used as purchased without further purification. All reagents for synthesis were obtained from Sigma Aldrich and Fluka. Thin-layer chromatography (TLC) was performed on Kieselgel 60 F_{254} plates and flash column chromatography (medium pressure liquid chromatography) purifications were carried out using Merck silica gel 60 (230-400 mesh ASTM) (Merck KGaA, Darmstadt, Germany). Melting points were determined on a Fisher-Johns melting apparatus and were uncorrected. ^{1}H NMR spectra were recorded on a Bruker 600 MHz instruments. The chemical shifts were presented in terms of parts per million with TMS as the internal reference. Electron-spray ionization mass spectra in positive mode (ESI-MS) data were recorded on a Bruker Esquire 3000t spectrometer. All general chemicals were the highest available grade.

3.1.1.Synthesis of WZ01-WZ20, WZ25, and WZ28-WZ36

A solution of intermediate compound **3** (0.002 mol) and various aromatic aldehydes (0.002 mol) in EtOH (30 mL) was added NaOEt (1 mL) and then stirred at room temperature for 3 – 4 hours. The resulting mixture was diluted with H_2O (15 mL) and extracted with EtOAc. The combined organic layers were washed with brine (15 mL) and dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was further purified by chromatography on silica gel to afford target semi-conservative MACs.

3.1.2.Synthesis of WZ01-WZ20, WZ25, and WZ28-WZ36

A solution of intermediate compound **3** (0.002 mol) and various aromatic aldehydes (0.002 mol) in EtOH (30 mL) was stirred at room temperature. Subsequently, HCl (gas) was bubbled into the mixture for 30 minutes, and the resulting reaction solution was stirred for 4 hours at room temperature. The mixture was post-processed the same as 3.1.1.

*(1E,4E)-1-(2-bromophenyl)-5-(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (**WZ01**)*
Yellow powder, 53% yield, mp 95.5-98.4 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.06(d, J = 16.2 Hz, 1H, Ar_2-CH=C), 7.68(d, J = 10.2 Hz, 1H, Ar_2-H^3), 7.64(d, J = 8.4 Hz, 1H, Ar_2-H^6), 7.37-7.34(m, 2H, Ar_2-$H^{4,5}$), 7.24(d, J = 7.8 Hz, 1H, Ar_1-CH=C), 7.20(d, J = 7.8 Hz, 1H, Ar_1-C=CH), 7.12(s, 1H, Ar_1-H^2), 7.00(d, J = 16.2 Hz, 1H, Ar_2-C=CH), 6.97(d, J = 3.6 Hz, 1H, Ar_1-H^5), 6.95(d, J = 3.6 Hz, 1H, Ar_1-H^6), 5.92(s, 1H, Ar_1-OH), 3.96(s, 3H, Ar_1-OCH_3). ESI-MS m/z: 359.3$(M+1)^+$, calcd for $C_{18}H_{15}BrO_3$: 358.02.

*(1E,4E)-1-(3-bromophenyl)-5-(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (**WZ02**)*
Yellow powder, 65% yield, mp 97.2-99.8 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.70(s, 1H, Ar_2-H^2), 7.69(d, J = 15.6 Hz, 1H, Ar_2-CH=C), 7.64(d, J = 16.2 Hz, 1H, Ar_1-CH=C), 7.30-7.27(m, 3H, Ar_2-$H^{4,5,6}$), 7.19(d, J = 7.8 Hz, 1H, Ar_1-H^5), 7.12(s, 1H, Ar_1-H^2), 7.09(d, J = 16.2 Hz, 1H, Ar_1-C=CH), 6.96(d, J = 7.8 Hz, 1H, Ar_1-H^6), 6.90(d, J = 15.6 Hz, 1H, Ar_2-C=CH), 5.92(s, 1H, Ar_1-OH), 3.97(s, 3H, Ar_1-OCH_3). ESI-MS m/z: 359.4 $(M+1)^+$, calcd for $C_{18}H_{15}BrO_3$: 358.02.

*(1E,4E)-1-(4-bromophenyl)-5-(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (**WZ03**)*
Yellow powder, 56% yield, mp 101.2-104.8 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.68(d, J = 16.2 Hz, 1H, Ar_2-CH=C), 7.66(d, J = 16.2 Hz, 1H, Ar_1-CH=C), 7.55(d, J = 8.4 Hz, 2H, Ar_2-$H^{2,6}$), 7.48(d, J = 8.4 Hz, 2H, Ar_2-$H^{3,5}$), 7.19(d, J = 8.4 Hz, 1H, Ar_1-H^5), 7.12(s, 1H, Ar_1-H^2), 7.08(d, J = 16.2 Hz, 1H, Ar_1-C=CH), 6.96(d, J = 8.4 Hz, 1H, Ar_1-H^6), 6.90(d, J = 16.2 Hz, 1H, Ar_2-C=CH), 5.91(s, 1H, Ar_1-OH), 3.96(s, 3H, Ar_1-OCH_3). ESI-MS m/z: 359.3 $(M+1)^+$, calcd for $C_{18}H_{15}BrO_3$: 358.02.

*(1E,4E)-1-(2-bromo-5-fluorophenyl)-5-(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (**WZ04**)*
Yellow powder, 61% yield, mp 175.1-177.4 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.98(d, J = 16.2 Hz, 1H, Ar_2-CH=C), 7.70(d, J = 15.6 Hz, 1H, Ar_1-CH=C), 7.60(d, J = 9.0 Hz, 1H, Ar_2-H^3), 7.40(d, J = 9.0 Hz, 1H, Ar_2-H^4), 7.19(d, J = 8.4 Hz, 1H, Ar_1-H^5), 7.12(s, 1H, Ar_1-H^2), 7.00(s, 1H, Ar_2-H^6), 6.94(d, J = 15.6 Hz, 1H, Ar_1-C=CH), 6.93(d, J = 16.2 Hz, 1H, Ar_2-C=CH), 6.89(d, J = 8.4 Hz, 1H, Ar_1-H^6), 5.91(s, 1H, Ar_1-OH), 3.96(s, 3H, Ar_1-OCH_3). ESI-MS m/z: 377.0 $(M+1)^+$, calcd for $C_{18}H_{14}BrFO_3$: 376.01.

*(1E,4E)-1-(2-bromo-6-fluorophenyl)-5-(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (**WZ05**)*
Yellow powder, 66% yield, mp 151.5-157.9 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.86(d, J = 16.2 Hz, 1H, Ar_2-CH=C), 7.68(d, J = 16.2 Hz, 1H, Ar_1-CH=C), 7.47(d, J = 7.8 Hz, 1H, Ar_2-H^3), 7.32(d, J = 16.2 Hz, 1H, Ar_2-H^5), 7.20-.7.17(m, 2H, Ar_2-H^4, Ar_1-H^5), 7.13-7.10(m, 2H, Ar_1-H^2, Ar_1-C=CH), 6.95(d, J = 8.4 Hz, 1H, Ar_1-H^6), 6.90(d, J = 16.2 Hz, 1H, Ar_2-C=CH), 5.91(s, 1H, Ar_1-OH), 3.97(s, 3H, Ar_1-OCH_3). ESI-MS m/z: 377.0 $(M+1)^+$, calcd for $C_{18}H_{14}BrFO_3$: 376.01.

*(1E,4E)-1-(4-bromo-3-fluorophenyl)-5-(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (**WZ06**)*
Yellow powder, 48% yield, mp 93.2-95.7 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.69(d, J = 15.6, 1H, Ar_2-CH=C), 7.61(d, J

=15.6, 1H, Ar_1-CH=C), 7.60(d, *J* = 7.2, 1H, Ar_2-H^5), 7.36(d, *J* = 7.2, 1H, Ar_2-H^6), 7.18(d, *J* = 8.4, 1H, Ar_1-H^5), 7.12(s, 1H, Ar_1-H^2), 7.08(d, *J* =15.6, 1H, Ar_1-C=CH), 6.96(d, *J* = 8.4, 1H, Ar_1-H^6), 6.89(d, *J* =15.6, 1H, Ar_2-C=CH), 6.86(s, 1H, Ar_2-H^2), 5.91(s, 1H, Ar_1-OH), 3.97(s, 3H, Ar_1-OCH_3). ESI-MS m/z: 376.8 $(M+1)^+$, calcd for $C_{18}H_{14}BrFO_3$: 376.01.

*(1E,4E)-1-(2,5-dibromophenyl)-5-(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (**WZ07**)*
Yellow powder, 71% yield, mp 95.2-98.0 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.96(d, *J* = 16.2 Hz, 1H, Ar_2-CH=C), 7.82(s, 1H, Ar_2-H^6), 7.70(d, *J* = 15.6 Hz, 1H, Ar_1-CH=C), 7.50(d, *J* = 8.4 Hz, 1H, Ar_1-H^5), 7.36(d, *J* = 8.4 Hz, 1H, Ar_2-H^4), 7.20(d, *J* = 8.4 Hz, 1H, Ar_2-H^3), 7.12(s, 1H, Ar_1-H^2), 7.01(d, *J* = 15.6 Hz, 1H, Ar_1-C=CH), 6.96(d, *J* = 8.4 Hz, 1H, Ar_1-H^6), 6.92(d, *J* = 16.2 Hz, 1H, Ar_2-C=CH), 5.92(s, 1H, Ar_1-OH), 3.97(s, 3H, Ar_1-OCH_3). ESI-MS m/z: 435.7, calcd for $C_{18}H_{14}Br_2O_3$: 435.93.

*(1E,4E)-1-(3-bromo-4-methoxyphenyl)-5-(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (**WZ08**)*
Yellow powder, 76% yield, mp 133.2-135.4 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.86(s, 1H, Ar_2-H^2), 7.68(d, *J* = 15.6 Hz, 1H, Ar_2-CH=C), 7.62(d, *J* = 15.6 Hz, 1H, Ar_1-CH=C), 7.51(d, *J* = 8.4 Hz, 1H, Ar_1-H^5), 7.18(d, *J* = 7.8 Hz, 1H, Ar_2-H^6), 7.12(s, 1H, Ar_1-H^2), 6.98(d, *J* = 15.6 Hz, 1H, Ar_1-C=CH), 6.95(d, *J* = 7.8 Hz, 1H, Ar_2-H^5), 6.92(d, *J* = 8.4 Hz, 1H, Ar_1-H^6), 6.90(d, *J* = 15.6 Hz, 1H, Ar_2-C=CH), 5.90(s, 1H, Ar_1-OH), 3.96(s, 3H, Ar_2-OCH_3), 3.95(s, 3H, Ar_1-OCH_3). ESI-MS m/z: 388.1, calcd for $C_{19}H_{17}BrO_4$: 388.03.

*(1E,4E)-1-(2-bromo-5-methoxyphenyl)-5-(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (**WZ09**)*
Yellow oil, 78% yield. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.00(d, *J* = 15.6 Hz, 1H, Ar_2-CH=C), 7.69(d, *J* = 15.6 Hz, 1H, Ar_1-CH=C), 7.52(d, *J* = 9.0 Hz, 1H, Ar_2-H^3), 7.12(s, 1H, Ar_1-H^2), 7.07(d, *J* =15.6 Hz, 1H, Ar_1-C=CH), 6.97(d, *J* = 8.6 Hz, 1H, Ar_1-H^5), 6.95(s, 1H, Ar_2-H^6), 6.93(d, *J* = 8.6 Hz, 1H, Ar_1-H^6), 6.90(d, *J* = 15.6 Hz, 1H, Ar_2-C=CH), 6.83(d, *J* = 9.0 Hz, 1H, Ar_2-H^4), 5.91(s, 1H, Ar_1-OH), 3.96(s, 3H, Ar_2-OCH_3), 3.85(s, 3H, Ar_1-OCH_3). ESI-MS m/z: 389.3 $(M+1)^+$, calcd for $C_{19}H_{17}BrO_4$: 388.03.

*(1E,4E)-1-(4-hydroxy-3-methoxyphenyl)-5-(2-(trifluoromethyl)phenyl)penta-1,4-dien-3-one (**WZ10**)*
Yellow powder, 81% yield, 90.4-93.2 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.04(d, *J* = 16.2 Hz, 1H, Ar_2-CH=C), 7.70(d, *J* = 16.2 Hz, 1H, Ar_1-CH=C), 7.64(d, *J* = 8.4 Hz, 1H, Ar_2-H^6), 7.74-7.36(m, 3H, Ar_2-$H^{3,4,5}$), 7.24(d, *J* = 7.8 Hz, 1H, Ar_1-H^5), 7.20(d, *J* = 7.8 Hz, 1H, Ar_1-H^6), 7.12(s, 1H, Ar_1-H^2), 7.00(d, *J* = 16.2 Hz, 1H, Ar_1-C=CH), 6.96(d, *J* = 16.2 Hz, 1H, Ar_2-C=CH), 5.92(s, 1H, Ar_1-OH), 3.97(s, 3H, Ar_1-OCH_3). ESI-MS m/z: 349.8 $(M+1)^+$, calcd for $C_{19}H_{15}F_3O_3$: 348.10.

*(1E,4E)-1-(4-hydroxy-3-methoxyphenyl)-5-(4-(trifluoromethyl)phenyl)penta-1,4-dien-3-one (**WZ11**)*
Yellow powder, 84% yield, mp 116.1-119.5 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.73(d, *J* = 16.2 Hz, 1H, Ar_2-CH=C), 7.71(d, *J* = 16.4 Hz, 1H, Ar_1-CH=C), 7.70-7.66(m, 4H, Ar_2-$H^{2,3,5,6}$), 7.20(d, *J* =16.4 Hz, 1H, Ar_1-C=CH), 7.16(d, *J* = 8.4 Hz, 1H, Ar_1-H^5), 7.12(s, 1H, Ar_1-H^2), 6.96(d, *J* = 16.4 Hz, 1H, Ar_2-C=CH), 6.92(d, *J* = 8.4 Hz, 1H, Ar_1-H^6), 5.93(s, 1H, Ar_1-OH), 3.97(s, 3H, Ar_1-OCH_3). ESI-MS m/z: 349.5 $(M+1)^+$, calcd for $C_{19}H_{15}F_3O_3$: 348.10.

*(1E,4E)-1-(2-fluoro-4-methoxyphenyl)-5-(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (**WZ12**)*
Yellow powder, 72% yield, mp 92.8-95.0 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.01(d, *J* = 16.2 Hz, 1H, Ar_2-CH=C), 7.69(d, *J* =16.2 Hz, 1H, Ar_1-CH=C), 7.52(d, *J* =8.4 Hz, 1H, Ar_2-H^6), 7.21(d, *J* = 8.4 Hz, 1H, Ar_1-H^5), 7.20(s, 1H, Ar_2-H^3), 7.12(s, 1H, Ar_1-H^2), 6.96(d, *J* =8.4 Hz, 1H, Ar_1-H^6), 6.95(d, *J* = 16.2 Hz, 2H, Ar_1-C=CH, Ar_2-C=CH), 6.82(d, *J* = 8.4 Hz, 1H, Ar_2-H^5), 5.90(s, 1H, Ar_1-OH), 3.97(s, 3H, Ar_1-OCH_3), 3.85(s, 3H, Ar_2-OCH_3). ESI-MS m/z: 329.4 $(M+1)^+$, calcd for $C_{19}H_{17}FO_4$: 328.11.

*(1E,4E)-1-(2-fluoro-5-methoxyphenyl)-5-(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (**WZ13**)*
Yellow powder, 70% yield, mp 106.2-108.5 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.79(d, *J* = 16.2 Hz, 1H, Ar_2-CH=C), 7.69(d, *J* = 15.6 Hz, 1H, Ar_1-CH=C), 7.19(d, *J* = 8.4 Hz, 1H, Ar_1-H^5), 7.15(d, *J* = 15.6 Hz, 1H, Ar_1-C=CH), 7.13(s, 1H, Ar_1-H^2), 7.09(d, *J* = 5.4 Hz, 1H, Ar_2-H^3), 7.05(d, *J* = 8.4 Hz, 1H, Ar_1-H^6), 6.94(d, *J* = 16.2 Hz, 1H, Ar_2-C=CH), 6.93(s, 1H, Ar_2-H^6), 6.90(d, *J* =5.4 Hz, 1H, Ar_2-H^4), 5.91(s, 1H, Ar_1-OH), 3.97(s, 3H, Ar_1-OCH_3), 3.83(s, 3H, Ar_2-OCH_3). ESI-MS m/z: 329.3 $(M+1)^+$, calcd for $C_{19}H_{17}FO_4$: 328.11.

*(1E,4E)-1-(2-fluoro-3-(trifluoromethyl)phenyl)-5-(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (**WZ14**)*
Brown powder, 68% yield, mp 74.6-76.1 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.81(d, *J* = 16.2 Hz, 1H, Ar_2-CH=C), 7.69(d, *J* = 16.2 Hz, 1H, Ar_1-CH=C), 7.64(t, *J* = 7.8 HZ, 1H, Ar_2-H^4), 7.30(t, *J* = 7.8 HZ, 1H, Ar_2-H^6), 7.24(d, *J* =16.2 Hz, 1H, Ar_1-C=CH), 7.19(d, *J* = 8.4 Hz, 1H, Ar_1-H^5), 7.12(s, 1H, Ar_1-H^2), 7.06-7.03(m, 1H, Ar_2-H^5), 6.96(d, *J* = 8.4 Hz, 1H, Ar_1-H^6), 6.92(d, *J* = 16.2 Hz, 1H, Ar_2-C=CH), 5.94(s, 1H, Ar_1-OH), 3.97(s, 3H, Ar_1-OCH_3). ESI-MS m/z: 367.0 $(M+1)^+$, calcd for $C_{19}H_{14}F_4O_3$: 366.09.

*(1E,4E)-1-(2,5-difluorophenyl)-5-(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (**WZ15**)*
Brown powder, 72% yield, mp 104.3-107.4 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.77(d, *J* = 16.2 Hz, 1H, Ar_2-CH=C), 7.69(d, *J* = 16.2 Hz, 1H, Ar_1-CH=C), 7.19(d, *J* =8.4 Hz, 1H, Ar_1-H^5), 7.15(d, *J* = 16.2 Hz, 1H, Ar_1-C=CH), 7.12(s, 1H, Ar_1-H^2), 7.10-7.06(m, 3H, Ar_2-$H^{3,4,6}$), 6.96(d, *J* = 8.4 Hz, 1H, Ar_1-H^6), 6.92(d, *J* = 16.2 Hz, 1H, Ar_2-C=CH), 5.92(s, 1H, Ar_1-OH), 3.97(s, 3H, Ar_1-OCH_3). ESI-MS m/z: 317.1 $(M+1)^+$, calcd for $C_{18}H_{14}F_2O_3$: 316.09.

*(1E,4E)-1-(2-fluoro-5-nitrophenyl)-5-(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (**WZ16**)*

Yellow powder, 42% yield, mp 113.1-116.8 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.27(d, *J* = 9.6 Hz, 1H, Ar_2-H^4), 7.83(d, *J* = 16.2 Hz, 1H, Ar_2-CH=C), 7.73(d, *J* = 16.2 Hz, 1H, Ar_1-CH=C), 7.69(s, 1H, Ar_2-H^6), 7.45(d, *J* = 9.6, 1H, Ar_2-H^3), 7.21(d, *J* =8.4 Hz, 1H, Ar_1-H^5), 7.13(s, 1H, Ar_1-H^2), 7.07(d, *J* = 16.2 Hz, 1H, Ar_1-C=CH), 6.93(d, *J* = 8.4 Hz, 1H, Ar_1-H^6), 6.59(d, *J* = 16.2 Hz, 1H, Ar_2-C=CH), 5.89(s, 1H, Ar_1-OH), 3.94(s, 3H, Ar_1-OCH_3). ESI-MS m/z: 344.3 $(M+1)^+$, calcd for $C_{18}H_{14}FNO_5$: 343.09.

*(1E,4E)-1-(2-chlorophenyl)-5-(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (**WZ17**)*

Yellow oil, 67% yield. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.11(d, *J* = 16.2 Hz, 1H, Ar_2-CH=C), 7.69(d, *J* = 15.6 Hz, 1H, Ar_1-CH=C), 7.45(d, *J* = 9.0 Hz, 1H, Ar_2-H^3), 7.35-7.30(m, 3H, Ar_2-$H^{4,5,6}$), 7.19(d, *J* =7.8 Hz, 1H, Ar_1-H^5), 7.12(s, 1H, Ar_1-H^2), 7.05(d, *J* =7.8 Hz, 1H, Ar_1-H^6), 6.97-6.94(m, 2H, Ar_1-C=CH, Ar_2-C=CH), 5.90(s, 1H, Ar_1-OH), 3.97(s, 3H, Ar_1-OCH_3). ESI-MS m/z: 315.1 $(M+1)^+$, calcd for $C_{18}H_{15}ClO_3$: 31407.

*(1E,4E)-1-(2,3-dichlorophenyl)-5-(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (**WZ18**)*

Yellow powder, 66% yield, mp 107.2-109.5 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.09(d, *J* = 15.6 Hz, 1H, Ar_2-CH=C), 7.70(d, *J* = 15.6 Hz, 1H, Ar_1-CH=C), 7.61(d, *J* = 7.8 Hz, 1H, Ar_2-H^4), 7.51-7.43(m, 2H, Ar_2-$H^{5,6}$), 7.19(d, *J* = 7.8 Hz, 1H, Ar_1-H^5), 7.12(s, 1H, Ar_1-H^2), 7.03(d, *J* = 15.6 Hz, 1H, Ar_1-C=CH), 6.96(d, *J* = 7.8 Hz, 1H, Ar_1-H^6), 6.94(d, *J* = 15.6 Hz, 1H, Ar_2-C=CH), 5.91(s, 1H, Ar_1-OH), 3.97(s, 3H, Ar_1-OCH_3). ESI-MS m/z: 349.0 $(M+1)^+$, calcd for $C_{18}H_{14}Cl_2O_3$: 348.03.

*(1E,4E)-1-(4-hydroxy-3-methoxyphenyl)-5-(2,4,5-trimethoxyphenyl)penta-1,4-dien-3-one (**WZ19**)*

Yellow powder, 87% yield, mp 165.7-167.4 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.04(d, *J* = 15.6 Hz, 1H, Ar_2-CH=C), 7.66(d, *J* = 15.6 Hz, 1H, Ar_1-CH=C), 7.19(d, *J* =8.4 Hz, 1H, Ar_1-H^5), 7.11(s, 2H, Ar_1-H^2, Ar_2-H^6), 6.98(d, *J* =15.6 Hz, 2H, Ar_1-C=CH, Ar_2-C=CH), 6.94(d, *J* = 8.4 Hz, 1H, Ar_1-H^6), 6.52(s, 1H, Ar_2-H^3), 5.91(s, 1H, Ar_1-OH), 3.96(s, 3H, Ar_1-OCH_3), 3.94(s, 3H, Ar_2-OCH_3), 3.91(s, 3H, Ar_2-OCH_3), 3.89(s, 3H, Ar_2-OCH_3). ESI-MS m/z: 371.1 $(M+1)^+$, calcd for $C_{21}H_{22}O_6$: 370.14.

*(1E,4E)-1-(4-hydroxy-3-methoxyphenyl)-5-(2,4,6-trimethoxyphenyl)penta-1,4-dien-3-one (**WZ20**)*

Yellow powder, 90% yield, mp 103.5-105.7 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.16(d, *J* = 16.2 Hz, 1H, Ar_2-CH=C), 7.63(d, *J* = 15.6 Hz, 1H, Ar_1-CH=C), 7.17(d, *J* = 8.4 Hz, 1H, Ar_1-H^5), 7.12(s, 1H, Ar_1-H^2), 6.95(d, *J* =15.6 Hz, 1H, Ar_1-C=CH), 6.90(d, *J* =16.2 Hz, 1H, Ar_2-C=CH), 6.84(d, *J* = 8.4 Hz, 1H, Ar_1-H^6), 6.14(s, 2H, Ar_2-$H^{3,5}$), 5.86(s, 1H, Ar_1-OH), 3.91(s, 3H, Ar_1-OCH_3), 3.86(s, 3H, Ar_2-OCH_3), 3.83(s, 3H, Ar_2-OCH_3), 3.79(s, 3H, Ar_2-OCH_3). ESI-MS m/z: 371.1 $(M+1)^+$, calcd for $C_{21}H_{22}O_6$: 370.14.

*(1E,4E)-1-(4-hydroxy-3-methoxyphenyl)-5-(3-hydroxyphenyl)penta-1,4-dien-3-one (**WZ21**)*

Yellow powder, 43% yield, mp 78.4-80.1 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.68(d, *J* = 16.2 Hz, 1H, Ar_2-CH=C), 7.66(d, *J* = 16.2 Hz, 1H, Ar_1-CH=C), 7.46-7.41(m, 1H, Ar_2-H^5), 7.19-7.16(m, 2H, Ar_1-H^5, Ar_1-H^6), 7.12(s, 1H, Ar_1-H^2), 7.06(d, *J* =16.2 Hz, 1H, Ar_1-C=CH), 6.95(d, *J* =7.8 Hz, 1H, Ar_2-H^6), 6.92(d, *J* =16.2 Hz, 1H, Ar_2-C=CH), 6.90-6.87(m, 1H, Ar_2-H^4), 5.84(s, 2H, Ar_1-OH, Ar_2-OH), 3.96(s, 3H, Ar_1-OCH_3). ESI-MS m/z: 297.3 $(M+1)^+$, calcd for $C_{18}H_{16}O_4$: 296.10.

*(1E,4E)-1-(2,4-bis(trifluoromethyl)phenyl)-5-(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (**WZ25**)*

Yellow powder, 78% yield, mp 147.9-150.3 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.66(d, *J* = 15.6 Hz, 1H, Ar_2-CH=C), 7.65(d, *J* = 15.6 Hz, 1H, Ar_1-CH=C), 7.25(s, 1H, Ar_2-H^3), 7.18(d, *J* = 7.8 Hz, 1H, Ar_1-H^5), 7.12(s, 1H, Ar_1-H^2), 7.11(d, *J* = 8.4 Hz, 1H, Ar_2-H^5), 6.96(d, *J* = 15.6 Hz, 1H, Ar_1-C=CH), 6.95(d, *J* = 7.8 Hz, 1H, Ar_1-H^6), 6.90(d, *J* = 15.6 Hz, 1H, Ar_2-C=CH), 6.87(d, *J* = 8.4 Hz, 1H, Ar_2-H^6), 5.94(s, 1H, Ar_1-OH), 3.96(s, 3H, Ar_1-OCH_3). ESI-MS m/z: 417.3 $(M+1)^+$, calcd for $C_{20}H_{14}F_6O_3$: 416.08.

*(1E,4E)-1-(2-bromo-4-hydroxyphenyl)-5-(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (**WZ26**)*

Yellow powder, 47% yield, mp 137.9-142.5 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.02(d, *J* = 15.6 Hz, 1H, Ar_2-CH=C), 7.98(s, 1H, Ar_2-H^3), 7.91(d, *J* = 8.4 Hz, 1H, Ar_2-H^6), 7.86(d, *J* = 8.4 Hz, 1H, Ar_2-H^5), 7.70(d, *J* = 15.6 Hz, 1H, Ar_1-CH=C), 7.19(d, *J* = 8.4 Hz, 1H, Ar_1-H^5), 7.12(s, 1H, Ar_1-H^2), 7.06(d, *J* = 15.6 Hz, 1H, Ar_1-C=CH), 6.96(d, *J* = 8.4 Hz, 1H, Ar_1-H^6), 6.91(d, *J* = 15.6 Hz, 1H, Ar_2-C=CH), 5.96(s, 1H, Ar_1-OH), 3.96(s, 3H, Ar_1-OCH_3). ESI-MS m/z: 375.4 $(M+1)^+$, calcd for $C_{18}H_{15}BrO_4$: 374.02.

*(1E,4E)-1-(4-hydroxy-3-methoxyphenyl)-5-(indolin-5-yl)penta-1,4-dien-3-one (**WZ28**)*

Yellow powder, 68% yield, mp 110.5-113.9 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.71(d, *J* = 15.6 Hz, 1H, Ar_2-CH=C), 7.66(d, *J* = 15.6 Hz, 1H, Ar_1-CH=C), 7.51(d, *J* = 8.4 Hz, 1H, Ar_2-H^6), 7.17(d, *J* = 8.4 Hz, 1H, Ar_1-H^5), 7.12(s, 1H, Ar_1-H^2), 6.94(d, *J* = 15.6 Hz, 1H, Ar_1-C=CH), 6.93(s, 1H, Ar_2-H^1), 6.87(d, *J* = 15.6 Hz, 1H, Ar_2-C=CH), 6.55(d, *J* = 8.4 Hz, 1H, Ar_2-H^5), 5.88(s, 1H, Ar_1-OH), 3.97(s, 1H, Ar_2-NH), 3.96(s, 3H, Ar_1-OCH_3), 3.36(m, 2H, Ar_2-N-CH_2), 2.04(m, 2H, Ar_2-CH_2). ESI-MS m/z: 322.4 $(M+1)^+$, calcd for $C_{20}H_{19}NO_3$: 321.14.

*4-((1E,4E)-5-(4-hydroxy-3-methoxyphenyl)-3-oxopenta-1,4-dien-1-yl)phenyl acetate (**WZ29**)*

Yellow oil, 52% yield. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.90(d, *J* = 15.6 Hz, 1H, Ar_2-CH=C), 7.69(d, *J* = 15.6 Hz, 1H, Ar_1-CH=C), 7.45(d, *J* = 7.8 Hz, 2H, Ar_2-H^2, Ar_2-H^6), 7.19(d, *J* = 8.4 Hz, 1H, Ar_1-H^5), 7.14(s, 1H, Ar_1-H^2), 7.09(d, *J* = 7.8 Hz, 2H, Ar_2-H^3, Ar_2-H^5), 7.07(d, *J* = 15.6 Hz, 1H, Ar_1-C=CH), 6.94(d, *J* = 8.4 Hz, 1H, Ar_1-H^6), 6.59(d, *J* = 15.6 Hz, 1H, Ar_2-C=CH), 5.90(s, 1H, Ar_1-OH), 3.94(s, 3H, Ar_1-OCH_3), 2.37(s, 3H, Ar-OOC-CH_3). ESI-MS m/z: 339.5 $(M+1)^+$, calcd for $C_{20}H_{18}O_5$: 338.12.

*(1E,4E)-1-(4-hydroxy-3-methoxyphenyl)-5-(4-isopropylphenyl)penta-1,4-dien-3-one (**WZ31**)*

Yellow powder, 76% yield, mp 133.8-136.1 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.70(d, *J* = 15.6 Hz, 1H, Ar_2-CH=C), 7.66(d, *J* = 15.6 Hz, 1H, Ar_1-CH=C), 7.56(d, *J* = 9.0 Hz, 2H, Ar_2-H^2, Ar_2-H^6), 7.18(d, *J* = 7.8 Hz, 1H, Ar_1-H^5), 7.12(s, 1H, Ar_1-H^2), 7.09(d, *J* = 9.0 Hz, 2H, Ar_2-H^3, Ar_2-H^5), 6.97(d, *J* = 15.6 Hz, 1H, Ar_1-C=CH), 6.95(d, *J* = 7.8 Hz, 1H, Ar_1-H^6), 6.59(d, *J* = 15.6 Hz, 1H, Ar_2-C=CH), 5.91(s, 1H, Ar_1-OH), 3.96(s, 3H, Ar_1-OCH_3), 3.74-3.70(m, 1H, Ar_2-CH), 1.24(d, *J* = 7.2 Hz, 6H, Ar_2-$C(CH_3)_2$). ESI-MS m/z: 323.5 $(M+1)^+$, calcd for $C_{21}H_{22}O_3$: 322.16.

*(1E,4E)-1-(6-bromobenzo[d][1,3]dioxol-5-yl)-5-(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (**WZ32**)*

Yellow oil, 69% yield. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.72(d, *J* = 16.2 Hz, 1H, Ar_2-CH=C), 7.68(d, *J* = 15.6 Hz, 1H, Ar_1-CH=C), 7.56(s, 1H, Ar_2-H^3), 7.54(s, 1H, Ar_2-H^6), 7.28(d, *J* = 8.4 Hz, 1H, Ar_1-H^5), 7.18(d, *J* = 8.4 Hz, 1H, Ar_1-H^6), 7.12(s, 1H, Ar_1-H^2), 7.05(d, *J* = 15.6 Hz, 1H, Ar_1-CH=C), 6.93(d, *J* = 16.2 Hz, 1H, Ar_2-CH=C), 6.91(s, 2H, Ar_2-O-CH_2-), 5.93(s, 1H, Ar_1-OH), 3.96(s, 3H, Ar_1-OCH_3). ESI-MS m/z: 403.5 $(M+1)^+$, calcd for $C_{19}H_{15}BrO_5$: 402.01.

*(1E,4E)-1-(4-hydroxy-3-methoxyphenyl)-5-(naphthalen-2-yl)penta-1,4-dien-3-one (**WZ33**)*

Yellow powder, 73% yield, mp 195.7-198.0 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.91-7.84(m, 7H, Ar_2-H^{2-8}), 7.77(d, *J* = 7.2 Hz, 1H, Ar_2-CH=C), 7.72(d, *J* = 16.2 Hz, 1H, Ar_1-CH=C), 7.22(d, *J* = 7.2 Hz, 1H, Ar_2-C=CH), 7.20(d, *J* = 8.4 Hz, 1H, Ar_1-H^5), 7.14(s, 1H, Ar_1-H^2), 6.98(d, *J* = 8.4 Hz, 1H, Ar_1-H^6), 6.97(d, *J* = 16.2 Hz, 1H, Ar_1-C=CH), 5.91(s, 1H, Ar_1-OH), 3.97(s, 3H, Ar_1-OCH_3). ESI-MS m/z: 331.2 $(M+1)^+$, calcd for $C_{22}H_{18}O_3$: 330.13.

*(1E,4E)-1-(2,4-dinitrophenyl)-5-(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (**WZ34**)*

Brown powder, 49% yield, mp 122.7-126.5 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.11(s, 1H, Ar_2-H^3), 8.08(d, *J* = 9.6 Hz, 1H, Ar_2-H^5), 7.72(d, *J* = 16.2 Hz, 1H, Ar_2-CH=C), 7.70(d, *J* = 9.6 Hz, 1H, Ar_2-H^6), 7.56(d, *J* = 14.4 Hz, 1H, Ar_1-CH=C), 7.20(d, *J* = 8.4 Hz, 1H, Ar_1-H^5), 7.12(s, 1H, Ar_1-H^2), 6.96(d, *J* = 14.4 Hz, 1H, Ar_1-C=CH), 6.95(d, *J* = 8.4 Hz, 1H, Ar_1-H^6), 6.91(d, *J* = 16.2 Hz, 1H, Ar_2-C=CH), 5.93(s, 1H, Ar_1-OH), 3.96(s, 3H, Ar_1-OCH_3). ESI-MS m/z: 371.1 $(M+1)^+$, calcd for $C_{18}H_{14}N_2O_7$: 370.08.

*(1E,4E)-1-(4-hydroxy-3-methoxyphenyl)-5-(2-nitrophenyl)penta-1,4-dien-3-one (**WZ35**)*

Yellow powder, 61% yield, mp 99.0-111.4 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.10(d, *J* = 16.2 Hz, 1H, Ar_2-CH=C), 8.00(d, *J* = 9.6 Hz, 1H, Ar_2-H^3), 7.72(d, *J* = 15.6 Hz, 1H, Ar_1-CH=C), 7.71-7.67(m, 3H, Ar_2-$H^{4,5,6}$), 7.56(d, *J* = 15.6 Hz, 1H, Ar_1-C=CH), 7.20(d, *J* = 8.4 Hz, 1H, Ar_1-H^5), 7.12(s, 1H, Ar_1-H^2), 6.96(d, *J* = 8.4 Hz, 1H, Ar_1-H^6), 6.91(d, *J* = 16.2 Hz, 1H, Ar_2-C=CH), 5.93(s, 1H, Ar_1-OH), 3.96(s, 3H, Ar_1-OCH_3). ESI-MS m/z: 326.7 $(M+1)^+$, calcd for $C_{18}H_{15}NO_5$: 325.10.

*(1E,4E)-1-(4-(allyloxy)phenyl)-5-(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one (**WZ36**)*

Yellow powder, 83% yield, mp 99.8-102.5 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.57(d, *J* = 9.0 Hz, 1H, Ar_2-CH=C), 7.45(d, *J* = 16.2 Hz, 1H, Ar_1-CH=C), 7.06(s, 1H, Ar_1-H^2), 6.97(d, *J* = 16.2 Hz, 1H, Ar_1-H^5), 6.95(d, *J* = 9.0 Hz, 1H, Ar_1-C=CH), 6.91(d, *J* = 16.2 Hz, 1H, Ar_2-C=CH), 6.98-6.90(m, 4H, Ar_2-$H^{2,3,5,6}$), 6.59(d, *J* = 16.2 Hz, Ar_1-H^6), 6.08-6.02(m, 1H, Ar_2-O-C-CH=C), 5.93(s, 1H, Ar_1-OH), 5.45-5.31(m, 2H, Ar_2-O-C-C=CH), 4.95(d, *J* = 5.4 Hz, 2H, Ar_2-O-CH_2-C=C), 3.93(s, 3H, Ar_1-OCH_3). ESI-MS m/z: 337.4 $(M+1)^+$, calcd for $C_{21}H_{20}O_4$: 336.14.

*(1E,4E)-1-(4-hydroxy-3-methoxyphenyl)-5-mesitylpenta-1,4-dien-3-one (**WZ37**)*

Yellow powder, 78% yield, mp 123.2-125.5 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.00(d, *J* = 16.2 Hz, 1H, Ar_2-CH=C), 7.65(d, *J* = 15.6 Hz, 1H, Ar_1-CH=C), 7.44(d, *J* = 15.6 Hz, 1H, Ar_1-C=CH), 7.17(d, *J* = 8.4 Hz, 1H, Ar_1-H^5), 7.10(s, 1H, Ar_1-H^2), 6.92(s, 2H, Ar_2-$H^{3,5}$), 6.88(d, *J* = 8.4 Hz, 1H, Ar_1-H^6), 6.71(d, *J* = 16.2 Hz, 1H, Ar_2-C=CH), 5.892(s, 1H, Ar_1-OH), 3.96(s, 3H, Ar_1-OCH_3), 2.29(s, 6H, Ar_2-$CH_3^{2,6}$), 2.21(s, 3H, Ar_2-CH_3^5). ESI-MS m/z: 323.6 $(M+1)^+$, calcd for $C_{21}H_{22}O_3$: 322.16.

3.2.Animals

Male C57BL/6 mice weighing 18–22 g were obtained from the Animal Center of Wenzhou Medical University (Wenzhou, China). The animals were housed under a constant room temperature with a 12:12 hours light-dark cycle and fed with a standard rodent diet and water. The animals were acclimatized to the laboratory for at least 7 days before being used in the experiments. Protocols involving the use of animals were approved by the Wenzhou Medical University Animal Policy and Welfare Committee (Approval documents: 2012/APWC/0102).

3.3.Cells and reagents

RAW 264.7 macrophages were incubated in DMEM medium (Gibco, Eggenstein, Germany) supplemented with 10% FBS (Hyclone, Logan, UT), 100 U/mL penicillin, and 100 mg/mL streptomycin at 37 °C with 5% CO_2. LPS purchased from Sigma was dissolved in PBS. Curcumin and its analogues were dissolved in DMSO before use. For peritoneal macrophage preparation, ICR mice were stimulated by intraperitoneal (i.p.) injection of 3 ml thioglycollate solution (0.3 g beef extract, 1 g tryptone, 0.5g sodium chloride, and 6 g soluble starch were dissolved and boiled in 100mL water. Before used, the solution was filtrated with 0.22 μm filter.) per mouse and kept in pathogen-free conditions for 3 days before peritoneal macrophage isolation. Total peritoneal macrophages were harvested by washing the peritoneal cavity with PBS 8 mL per mouse), centrifuged, then the pellet was re-suspended in RPMI 1640 medium (Gibco, Eggenstein, Germany) with 10% FBS (Hyclone, Logan, UT), 100 U/mL penicillin, and 100 mg/mL streptomycin. Nonadherent cells were removed by washing with medium at 3 hours after seeding. Experiments were

undertaken after the cells adhered firmly to the culture plates. Before used, peritoneal macrophages were cultured in RPMI-1640 medium on 35 mm plates at a density of 4.0×10^5/plate and maintained at 37 °C in a 5% CO_2-humidified air.

3.4.Determination of TNF- α and IL-6

RAW 264.7 macrophages were treated with LPS (0.5 μg/mL) in the presence or absence of compounds for 22 hours. The culture media were collected and centrifuged (1,000 rpm) at 4 °C for 5 minutes, and the supernatant was collected. TNF-α and IL-6 levels in the medium were determined with an ELISA kit (eBioScience, Inc.) according to the manufacturer's instructions. The total amount of the inflammatory factor in the medium was normalized to the total protein quantity of the viable cell pellets.

3.5.MTT assay

HL-7702 cells were seeded into 96-well plates at a density of 5000 cells per well in 1640 medium, supplemented with 5% heat-inactivated serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. All experiments were carried out 24 hours after cells were seeded. Tested compounds were dissolved in DMSO and diluted with 1640 medium to the final concentrations of 10 μM. The cells were incubated with test compounds for 24 hours before the MTT assay. A fresh solution of MTT (5 mg/mL) prepared in NaCl solution (0.9%) was added to each single well of the 96-well plate. The plates were then incubated in a CO_2 incubator for 4 hours, cells dissolved with 150 μL DMSO, and then analyzed in a multi-well-plate reader at 490 nM.

3.6.Real-time quantitative PCR

Cells were homogenized in TRIZOL kit (Invitrogen, Carlsbad, CA) for extraction of RNA according to manufacturer's protocol. Both reverse transcription and quantitative PCR were carried out using a two-step M-MLV Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen, Carlsbad, CA). Eppendorf Mastercycler ep realplex detection system (Eppendorf, Hamburg, Germany) was used for q-PCR analysis. The primers of genes including TNF-α, IL-6, IL-1β, and β-actin were synthesized by Invitrogen. The primer sequences of mouse genes used are shown as follows:

Mouse TNF-α sense: 5'-TGATCCGCGACGTGGAA-3'
Mouse TNF-α antisense: 5'-ACCGCCTGGAGTTCTGGAA-3'
Mouse IL-6 sense: 5'-CCAAGAGGTGAGTGCTTCCC-3'
Mouse IL-6 antisense: 5'-CTGTTGTTCAGACTCTCTCCCT-3'
Mouse IL-1β sense: 5'-ACTCCTTAGTCCTCGGCCA-3'
Mouse IL-1β antisense: 5'-CCATCAGAGGCAAGGAGGAA-3'
Mouse COX-2 sense 5'-TGGTGCCTGGTCTGATGATG-3'
Mouse COX-2 antisense: 5'-GTGGTAACCGCTCAGGTGTTG-3'
Mouse β-actin sense: 5'-CCGTGAAAAGATGACCCAGA-3'
Mouse β-actin antisense: 5'-TACGACCAGAGGCATACAG-3'

The amount of each gene was determined and normalized by the amount of β-actin.

3.7.Western blot

After treated, protein samples at 50 μg/lane were resolved by SDS-PAGE and the separated proteins were transferred onto nitrocellulose membranes by the wet transfer method using a Bio-Rad electrotransfer apparatus. Following transfer the blots were blocked with 5% nonfat milk in Tris buffer saline containing 0.1% Tween 20 and then incubated with primary antibodies followed by secondary antibodies. Proteins were visualized by using the enhanced chemiluminescence system. Western blot data presented are representative of those obtained in at least three separate experiments.

3.8.Preparation of Nuclear Extracts and Electrophoretic mobility shift assay (EMSA)

Nuclear protein was extracted by using nuclear and cytoplasmic extraction reagents (10 μg) and then was incubated with 1 μg poly (deoxyinosinic-deoxycytidylic acid) in binding buffer for 30 minutes at 4 °C. DNA binding activity was confirmed with a biotin-labeled oligonucleotide bio-NF-κB probe using an EMSA kit.

3.9.LPS-induced inflammatory mortality in C57BL/6 mice

The compounds **WZ35** were first dissolved with macrogol-15 hydroxystearate (a nonionic solubilizer for injection from BASF [Ludwigshafen, Germany]) with or without medium-chain triglycerides (MCT) from BASF in a water bath at 37 °C. The concentration of the compounds was 2 mg/mL. The concentration of the solubilizer ranged from 5%–10% and that of MCT ranged from 0.5%–2% in the final solution. For the vehicle, the mixture of solubilizer and MCT was prepared at 10% and 2%, respectively. Male C57BL/6 mice weighing 18–22 g were pretreated with compound **WZ35** (10 mg/kg) in a water solution by intravenous injection 15 minutes before the intraperitoneal injection of LPS (20 mg/kg). The control animals received a similar volume (200 μL) of the vehicle. The mortalities were recorded for 7 days.

4. Conclusions

In summary, we synthesized 32 semi-conservative MACs and evaluated their anti-inflammatory activities in LPS-stimulated RAW 264.7 macrophages. The majority of the compounds presented enhanced anti-inflammatory activities compared to curcumin. The SAR analysis suggests that the large substituents are unfavorable for anti-inflammatory activity. The six active compounds **WZ08**, **WZ12**, **WZ19**, **WZ32**, **WZ35**, and **WZ37** not only exhibited dose-dependent inhibition on TNF-α and IL-6 production, but also showed significant inhibition on inflammatory gene expression. Furthermore, we have confirmed that the NF-κB pathway was to be involved in the anti-inflammatory activity of the most active compounds **WZ35** and pretreatment of **WZ35** could improve the survival rate in LPS-induced acute inflammatory mouse model. Overall, these results indicate that semi-conservative MACs may be treated as potential agents for the treatment of acute inflammatory diseases.

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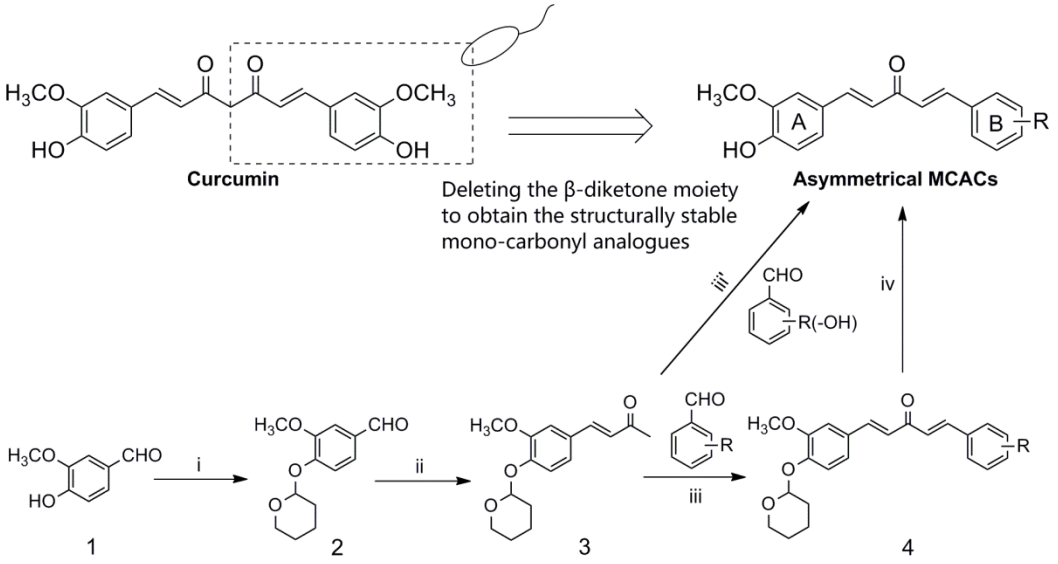
Fig. 1



Comp.	R	Comp.	R	Comp.	R
WZ01	2-Br	WZ12	2-F, 4-OCH_3	WZ26	2-Br, 4-OH
WZ02	3-Br	WZ13	2-F, 5-OCH_3	WZ28	
WZ03	4-Br	WZ14	2-F, 3-CF_3	WZ29	4-$OOCCH_3$
WZ04	2-Br, 5-F	WZ15	2,5-F	WZ31	4-$CH(CH_3)_2$
WZ05	2-Br, 6-F	WZ16	2-F, 5-NO_2	WZ32	
WZ06	4-Br, 5-F	WZ17	2-Cl	WZ33	
WZ07	2,5-Br	WZ18	2,3-Cl		
WZ08	3-Br, 4-OCH_3	WZ19	2,4,5-OCH_3	WZ34	2,4-NO_2
WZ09	2-Br, 5-OCH_3	WZ20	2,4,6-OCH_3	WZ35	2-NO_2
WZ10	2-CF_3	WZ21	3-OH	WZ36	4-$OCH_2CH{=}CH_2$
WZ11	4-CF_3	WZ25	2,4-CF_3	WZ37	2,4,6-CH_3

Fig. 1 Structures and synthesis of MACs compounds. Reagents and conditions: (i) 3,4-dihydro-2H-pyran, PPTS, CH_2Cl_2, rt; (ii) acetone, NaOH, EtOH, rt; (iii) NaOEt, EtOH, rt; (iii') HCl(g), EtOH, rt; (iv) HCl, THF, rt.

Fig. 2

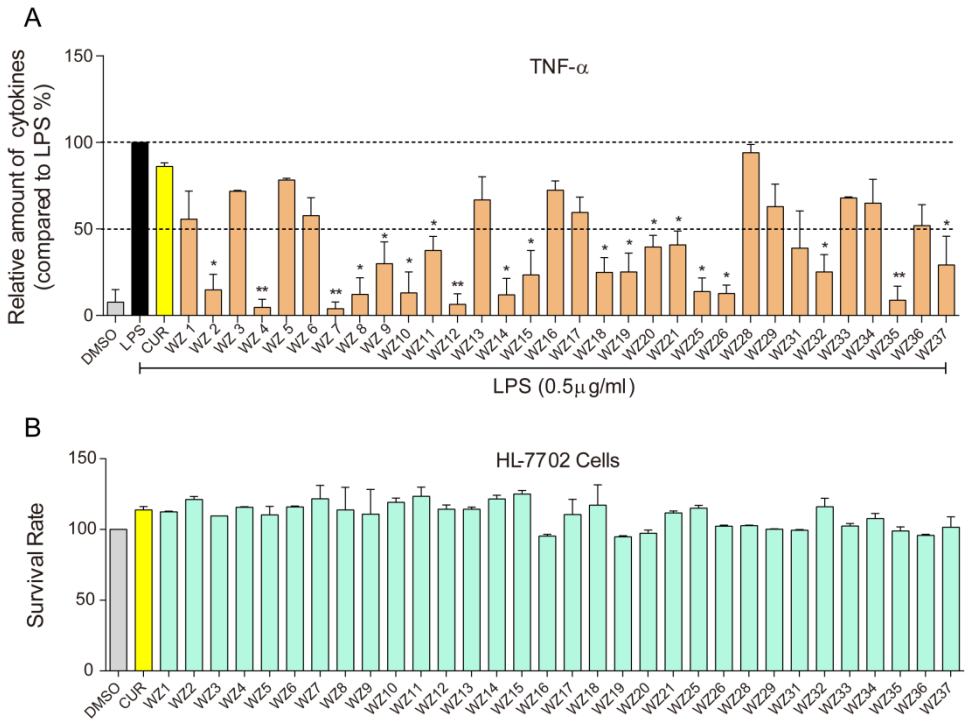


Fig. 2 Curcumin and its analogs inhibited LPS-induced TNF-α (A) secretion in RAW 264.7 macrophages and the cytotoxic evaluation (B) in HL-7702 cells. Macrophages were plated at a density of 4.0 x 10^5/plate at 37 °C and 5% CO_2 overnight. Cells were pre-treated with curcumin or its analogs (10 μM) for 2 hours, and then treated with LPS (0.5 μg/mL) for 22 hours. TNF-α levels in the culture media were measured by ELISA and were normalized by the total protein. The results were expressed as the percent of LPS control. Each bar represents mean ± standard error of the mean (SEM) of 3-5 independent experiments. Statistical significance relative to LPS group was indicated, $*p < 0.05$, $**p < 0.01$.

Fig. 3

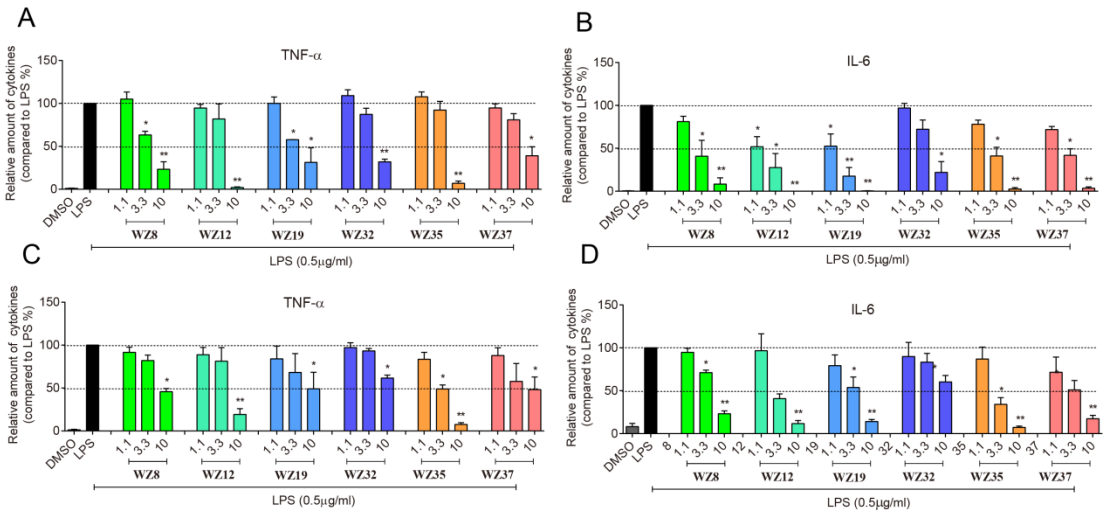


Fig. 3 Six active MACs inhibited LPS-induced TNF-α and IL-6 release in a dose-dependent manner. Macrophages were plated at a density of 4×10^5/plate overnight in 37 °C and 5% CO_2. Cells were pretreated with active compounds in a series concentration of 1.1 μM, 3.3 μM, and 10 μM for 2 hours and subsequently incubated with or without LPS (0.5 μg/mL) for 22 hours. TNF-α and IL-6 levels in the culture medium of RAW 264.7 macrophages (**A** and **B**) and primary macrophages (**C** and **D**) were measured by ELISA and were normalized by the total protein. The results were presented as the percent of LPS control. Each bar represents the mean ± SEM of three independent experiments. Statistical significance relative to the LPS group was indicated, $*p < 0.05$, $**p < 0.01$.

Fig. 4

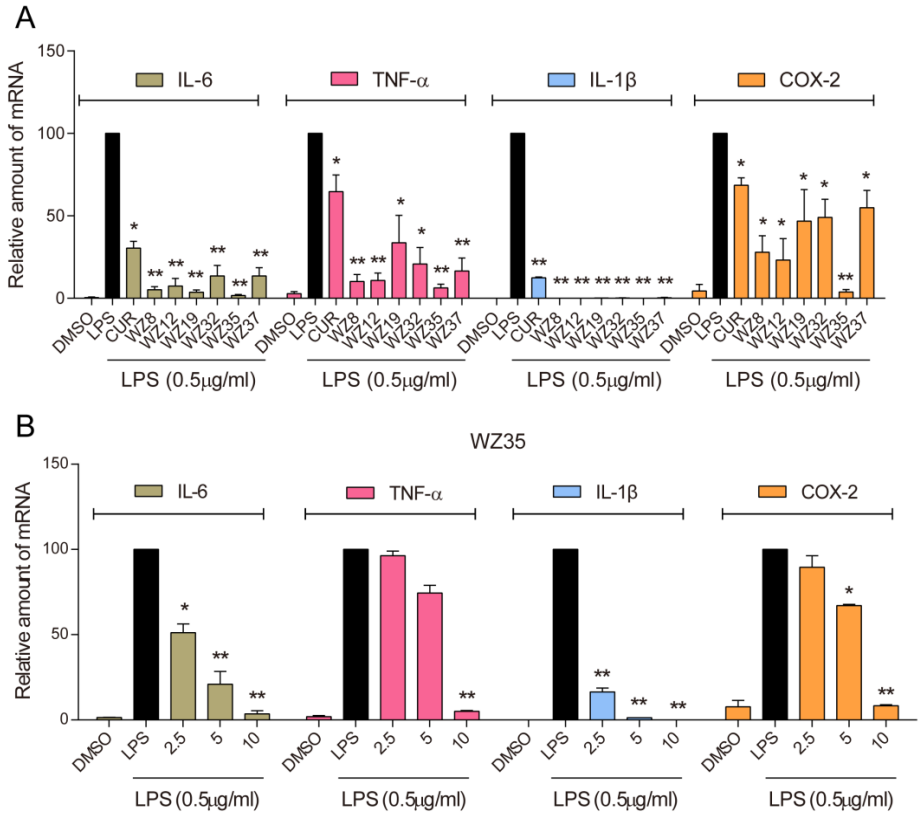


Figure 4. Six active MACs inhibited LPS-induced mRNA of inflammatory mediators release in RAW 264.7 macrophages. Macrophages were pre-treated with (A) compounds **WZ08**, **WZ12**, **WZ32**, **WZ35**, and **WZ37** at 10 μM, (B) **WZ35** at 2.5 μM, 5 μM, and 10 μM or vehicle control for 2 hours and then stimulated with LPS (0.5 μg/mL) for 6 hours. Macrophages were plated at a density of 4.0×10^5/plate for overnight in 37 °C and 5% CO_2. The mRNA levels of inflammatory mediators IL-6, TNF-α, IL-1β and COX-2 were quantified by RT-qPCR. The mRNA values for each gene was normalized to internal control β-actin mRNA and were expressed as a ratio to dimethyl sulfoxide (DMSO). Each bar represents mean ± SEM of 3-5 independent experiments. Statistical significance relative to LPS group was indicated, $*p < 0.05$, $**p < 0.01$.

Fig. 5

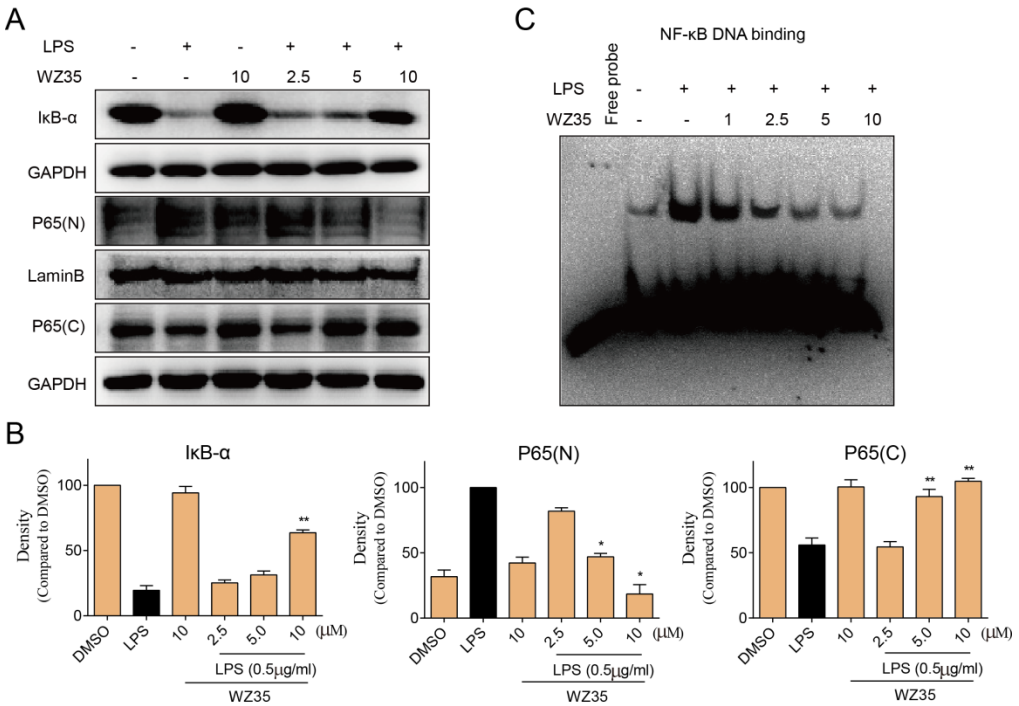


Fig. 5 Compound **WZ35** inhibited LPS-induced NF-κB activation in RAW 264.7 macrophages. (A) Macrophages were pretreated with **WZ35** at 2.5 μM, 5 μM, and 10 μM or vehicle control for 2 hours and then treated with LPS (0.5 μg/mL) for 1 hour. The level of IκB-α and P65 were examined using a specific antibody with GAPDH or LaminB as the loading control. (B) The column figures show the normalized optical density as times of the LPS group. Bars represent the mean ± SE M of the three independent experiments (*$p < 0.05$, **$p < 0.01$ versus the LPS group). (C) Macrophages were pretreated with **WZ35** (1 μM, 2.5 μM, 5 μM, and 10 μM) or vehicle (DMSO) for 2 hours and then stimulated with LPS (0.5 μg/mL) for 1 hours, and then detected the bind ability between NF-κB and DNA in nucleus by EMSA kit.

Fig. 6

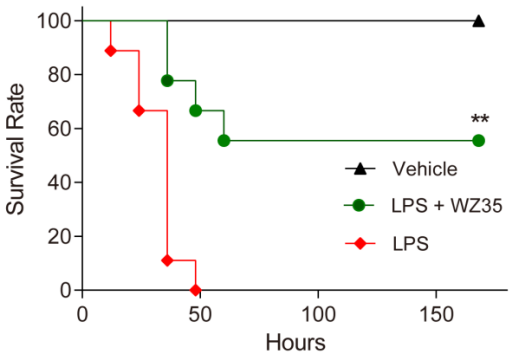


Fig. 6 Compound **WZ35** improve survival of mice subjected to a lethal dose of LPS. Mice were intravenously injected with 10 mg/kg **WZ35** 15 minutes before intraperitoneal injection of LPS 20 mg/kg. Survival rates were recorded for 7 days after the LPS injection at the interval of 1 day. n=9 animals in each group. Statistical significance relative to LPS group was indicated, ** $p<0.01$.