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Synthesis of mono-carbonyl analogues of curcumin and their effects on inhibition of cytokine release in LPS-stimulated RAW 264.7 macrophages

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ABSTRACT

Curcumin has been reported to possess multifunctional bioactivities, especially the ability to inhibit pro-inflammatory induction. We previously demonstrated that the mono-carbonyl analogues of curcumin possessed improved pharmacokinetic profiles both in vitro and in vivo. In this study, we synthesized and examined a series of 5-carbon linker-containing mono-carbonyl analogues of curcumin with potent inhibitory activities against TNF-α and IL-6 release in LPS-stimulated RAW 264.7 macrophages. Discussion and conclusions are given regarding structure-activity relationships (SAR). The two most potent analogues among the tested compounds, **B75** and **C12**, exhibited anti-inflammatory abilities in a dose-dependent manner in macrophages. This raises the possibility that mono-carbonyl analogues of curcumin might serve as potential agents for the treatment of various inflammatory diseases.

1. Introduction

Proinflammation is a widespread phenomenon that is associated with various diseases including cardiovascular diseases and cancer.[1–3] Numerous cytokines are present with proinflammation. For example, TNF-α and IL-6 are two inflammatory cytokines involved in the pathogenesis of cardiovascular diseases, cancer and diabetes.[4–6] Anti-inflammatory candidates targeting proinflammatory cytokines or inhibiting the over-expressions of cytokines are a major focus of current drug development.[7–9]

Curcumin, 1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, is a key active component in the traditional herb *Curcuma Longa* and has been extensively investigated for its potential biological benefits, including anti-tumor, anti-inflammation, cardio-protection and anti-virus.[10–13] Presently, curcumin has been evaluated in clinic trials for the treatment of many diseases such as liver disease, rheumatoid arthritis, infectious diseases and cancers.[14] The therapeutic effects of curcumin are attributed to its activity on a wide range of molecular targets, particularly a series of inflammatory factors and cytokines.[15] Curcumin is able to inhibit the production of proinflammatory cytokines, both at the mRNA and protein levels. The decrease in the expression of cytokines by curcumin is thought to be mediated by the inhibition of the activation of NF-κB and AP-1.[16–18]

In spite of the favorable biological properties of curcumin, there are drawbacks that limit the development of curcumin as a potential therapeutic agent, including low bioavailability and instability at neutral to basic conditions.[19,20] For example, with oral administration of 450–3600 mg/day in a phase I trial, the curcumin blood concentration in plasma and target tissues is under the detection limit.[21] Thus, the weakness of a pharmacokinetic profile in vivo of curcumin significantly inhibits its clinical application.

It is believed that the presence of an active methylene group and β-diketone moiety contributes to the instability of curcumin under physiological conditions, and induces rapid degradation and metabolism of curcumin.[22,23] In our previous study, we designed and synthesized a series of mono-carbonyl analogues of curcumin by deleting the β-diketone moiety.[24] We have also evaluated a total of 87 analogues for anti-inflammatory properties using lipopolysaccharide (LPS)-stimulated mouse macrophages and discuss the primary structure–activity relationship (SAR).[25,26] The evidence from a degradation degree assay in pH 7.4 buffer in vitro and a pharmacokinetic study in vivo demonstrated that mono-carbonyl analogues exhibit enhanced stability and improved pharmacokinetic profiles.[24] In this study, we further present 23 newly designed mono-carbonyl analogues of curcumin and their SAR results for their anti-inflammatory activities in mouse RAW 264.7 macrophages. Following initial examination, we further

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show that compounds **B75** and **C12** prevented LPS-induced inflammation in a dose-dependent manner.

2. Result and discussion

2.1. Chemistry

Three series of mono-carbonyl analogues of curcumin, 1,5-diaryl-1,4-pentadiene-3-ones (**B**), together with cyclopentanone (**A**) and cyclohexanone (**C**) analogues, were designed by displacing beta-diketone moiety with a single carbonyl group based on explanations we reported previously.[24] Different substituents with opposing electronic properties in the benzene rings were designed to investigate and discuss the structure–activity relationship. As shown in Figure 1, compounds **12** and **67–75** were synthesized by coupling the appropriate aromatic aldehyde with cyclohexanone, cyclopentanone or acetone in an alkaline medium, respectively. The general process for synthesis of these compounds was previously reported.[27] The synthetic yields, melting points, ^{1}H NMR and ESI-MS analysis of unpublished compounds are described in the Section 4. The diaryl structure is confirmed by the absence of methyl protons adjacent to the carbonyl group in the ^{1}H NMR spectra of **A**-class compounds and the absence of two methylene protons near to the central carbonyl in the spectra of **B**- and **C**-class compounds.

2.2. Inhibitory effects on LPS-induced TNF-α and IL-6 release by curcumin analogues

Lipopolysaccharide (LPS) is an important structural component of the outer membrane of gram-negative bacteria, and it is a well-studied immunostimulator that induces a systemic inflammation response,[28] and especially, the expression of proinflammatory cytokines, such as TNF-α and IL-6. Here, curcumin and its 23 synthetic analogues in the 5-carbon linker series were evaluated for their inhibitory abilities against LPS-induced TNF-α and IL-6 release in mouse RAW 267 macrophages. The macrophages were pre-treated with 10 μM compounds for 2 h and then incubated with 0.5 μg/ml LPS for 22 h. The amount of TNF-α and IL-6 in media were detected thru enzyme-linked immunosorbant assays (ELISA) and normalized by protein concentration of cells harvested in homologous culture plates.

The results of the anti-inflammatory assay of three analog classes are shown in Fig. 2A and B, respectively. Among these 23 compounds, the majority inhibited LPS-induced TNF-α and IL-6 expression to various degrees and compounds **B68**, **A69**, **B69**, **B72**, **C72**, **B75** and **C12** exhibited the highest inhibitory abilities against LPS-induced TNF-α expression (Fig. 2A), and compounds **A68**, **B68**, **B69**, **B72**, **C72**, **A73**, **B75** and **C12** showed inhibitory effects within 60% against IL-6 release compared to the LPS-control. Compounds **B75** and **C12**, especially, were more potent than the leading curcumin at the same concentration in inhibiting LPS-induced TNF-α and IL-6 expression. **C12**, a 3-(dimethylamino) propoxyl-substituted compound, showed the strongest inhibitory effect on LPS-induced TNF-α and IL-6 release among the tested analogues and its inhibitory rates reached 96.9% and 97.1%, respectively, compared to the LPS-control.

2.3. Structure–activity relationship analysis of curcumin analogues

As an excellent leading compound, curcumin has been investigated in depth in the field of medicinal chemistry. A number of analogues of curcumin have also been designed and synthesized for the development of new anti-inflammatory and anti-cancer drugs.[29–31] Our previous publications demonstrated that the structural replacement of C_7 'ene-[1,3-dioxo]-ene' linker in curcumin by C_5 'ene-oxoene' linker forms mono-carbonyl analogues and leads to the enhancement of stability in vitro and improvement of pharmacokinetic profiles in vivo.[24,31] In our previous studies,[26] a series of mono-carbonyl derivatives of curcumin containing 1,4-pentadiene-3-one linker and their cyclopenta- and cyclohexa-analogues,

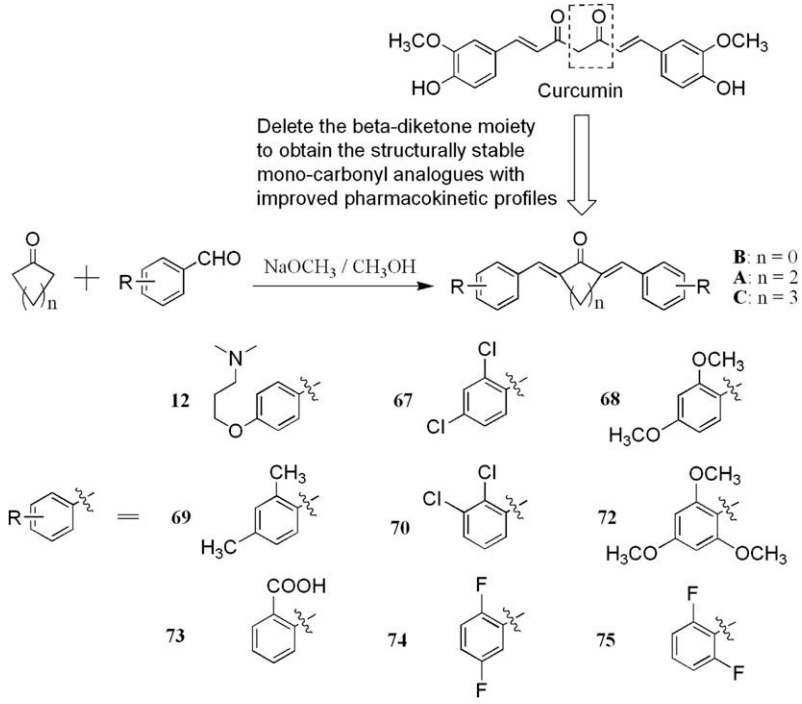

Figure 1. Chemical structure of curcumin and the design of its mono-carbonyl analogues as well as general synthesis and chemical structures of mono-carbonyl analogues of curcumin.

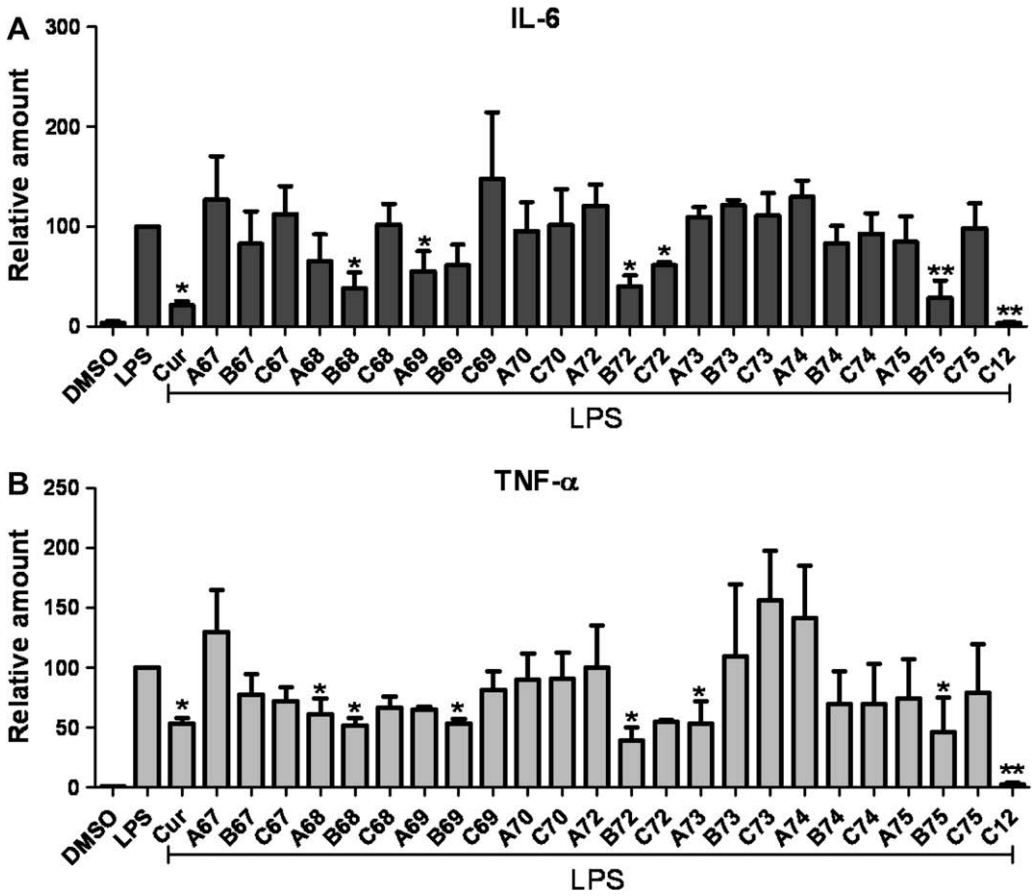

Figure 2. Curcumin and its analogues inhibited LPS-induced TNF-α and IL-6 secretion in RAW 264.7 macrophages. Macrophages were plated at a density of 4.0×10^5/plate for overnight in 37 °C and 5% CO_2. Cells were pre-treated with curcumin or its analogues (10 μM) for 2 h, then treated with LPS (0.5 μg/ml) for 22 h. TNF-α and IL-6 levels in the culture media were measured by ELISA and were normalized to the total protein. The results are expressed as percent of LPS control. Each bar represents mean ± SE of 3–5 independent experiments. Statistical significance relative to LPS is indicated, $^*p$ <0.05.

exhibited an ability to inhibit LPS-induced TNF-α and IL-6 expression in mouse macrophages. Further, our studies found that five active compounds exhibited inhibitory effects on LPS-induced TNF-α, IL-1β, IL-6, MCP-1, COX-2, PGES, iNOS and p65 NF-κB mRNA production.[25] The previous results indicated that these mono-carbonyl analogues may possess anti-inflammatory activities comparable to curcumin despite the absence of the β-diketone.

Concurrently, 23 mono-carbonyl analogues were synthesized and their inhibitory effects on TNF-α and IL-6 release were evaluated (Fig. 1). Among 16 compounds which exhibited high inhibitory abilities against LPS-induced TNF-α and/or IL-6 expression, the percentage of **B**-class compounds was 53.3%. As reported previously, it is observed here that acetone-derived **B**-class analogues are more effective than cyclopentanone-derived **A**-class and cyclohexanone-derived **C**-class analogues, indicating that the structure of a 5-canbonyl linker may have a role on such activities.

Previous reports have found that the electric property of a substituent in the 4′-position plays an important role in anti-inflammatory activities.[25] In this study, compounds **70**, **73** and **74** without substituents at the para position of the phenyl rings showed little inhibitory activity; and an electron-withdrawing chloro substituent at the 4′-position removes the anti-inflammatory activities of compound **67**. In comparison, methoxyl-containing **68** and **72** and methyl-containing **69** showed significant inhibitory activities against LPS-induced TNF-α and IL-6. The *N*,*N*-dimethyl-propyl-alkalized **C12** exhibited the strongest bioactivity among the tested compounds.

These results indicate that the bioactivity of analogues against inflammation induced by LPS is associated with electronegativity of the 4′-substituent: the electron-donating ability of the 4′-substituent may increase the anti-inflammatory abilities of the mono-carbonyl analogues whereas electric neutrality and electron-withdrawing moiety may reduce or remove such bioactivity.

Among all 23 compounds, **C12** showed the highest potential as an anti-inflammatory agent. In our previous publication[25], we reported that a structurally similar compound, **A12**, 2,6-bis(4-(3-(dimethylamino)propoxy)benzylidene)cyclohexanone, possessed intensive inhibitory effects against LPS-induced expression of inflammatory factors. Here, our data demonstrated again that *N*,*N*-dimethyl-propoxy containing long-chain analogues may be considered as promising compounds for developing anti-inflammatory candidates. However, further studies including analysis of the SAR of N-containing long-chain substituents and examination of the underlying molecular mechanisms of these kinds of compounds at the transcriptional or posttranscriptional level are necessary to further establish this investigational pathway.

2.4. B75 and C12 inhibit TNF-α and IL-6 release in a dose-dependent manner

Among the active analogues above, two compounds, **B75** and **C12**, demonstrated the highest activities and low cytotoxicity (data not shown) in macrophages. They were chosen for further evaluation of their dose-dependent inhibitory effects against LPS-induced

TNF-α and IL-6 release. RAW 264.7 macrophages were pre-treated with **B75** or **C12** in a series of concentrations (1, 2.5, 5.0 and 10 μM) for 2 h and were subsequently incubated with LPS (0.5 μg/ml) for 22 h. As shown in Figure 3, our data indicated a dose-dependent inhibition of LPS-induced TNF-α and IL-6 release by **B75** and **C12**. The inhibition of TNF-α and IL-6 release by **B75** and **C12** in a dose-dependent manner demonstrated their potential for development as anti-inflammatory agents.

Combined with our previous studies[24] regarding to the stability and pharmacokinetics of these mono-carbonyl analogues, we considered that these mono-carbonyl analogues without β-diketone may lend themselves favorably to the development of curcumin-based anti-inflammatory drugs from both pharmacokinetic and pharmacological standpoints.

3. Conclusions

In conclusion, we examined a series of 5-carbon linker-containing mono-carbonyl analogues of curcumin with potent inhibitory activities against TNF-α and IL-6 release in LPS-stimulated RAW 264.7 macrophages. Discussion and conclusions were made regarding structure-activity relationships. Compounds **B75** and **C12** were the most potent analogues and exhibited anti-inflammatory abilities in a dose-dependent manner in macrophages. This presents the possibility that mono-carbonyl analogues of curcumin might serve as potential agents for the treatment of various inflammatory diseases.

4. Experimental section

4.1. Chemical synthesis

Melting points were determined on a Fisher-Johns melting apparatus and were uncorrected.[1] H NMR spectra were recorded on a Varian INOVA-400 spectrometer. 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 3000$^+$ spectrometer. Column chromatography purifications were carried out on Silica Gel 60 (E. Merck, 70–230 mesh). The general procedure for synthesis of these compounds was the same as reported in our previous papers.[27] Briefly, an amount of 7.5 mmol acetone (**B**-class), cyclopentanone (**A** class), or cyclohexanone (**C**-class) was added to a solution of 15 mmol arylaldehyde in MeOH (10 ml). The solution was stirred at room temperature for 20 min, followed by dropwise addition of $NaOCH_3/CH_3OH$ (1.5 ml, 7.5 mmol). The mixture was stirred at room temperature and monitored with TLC. When the reaction was finished, the residue was poured into saturated NH_4Cl solution and filtered. The precipitate was washed with water and cold ethanol, and dried in vacuum. The solid was

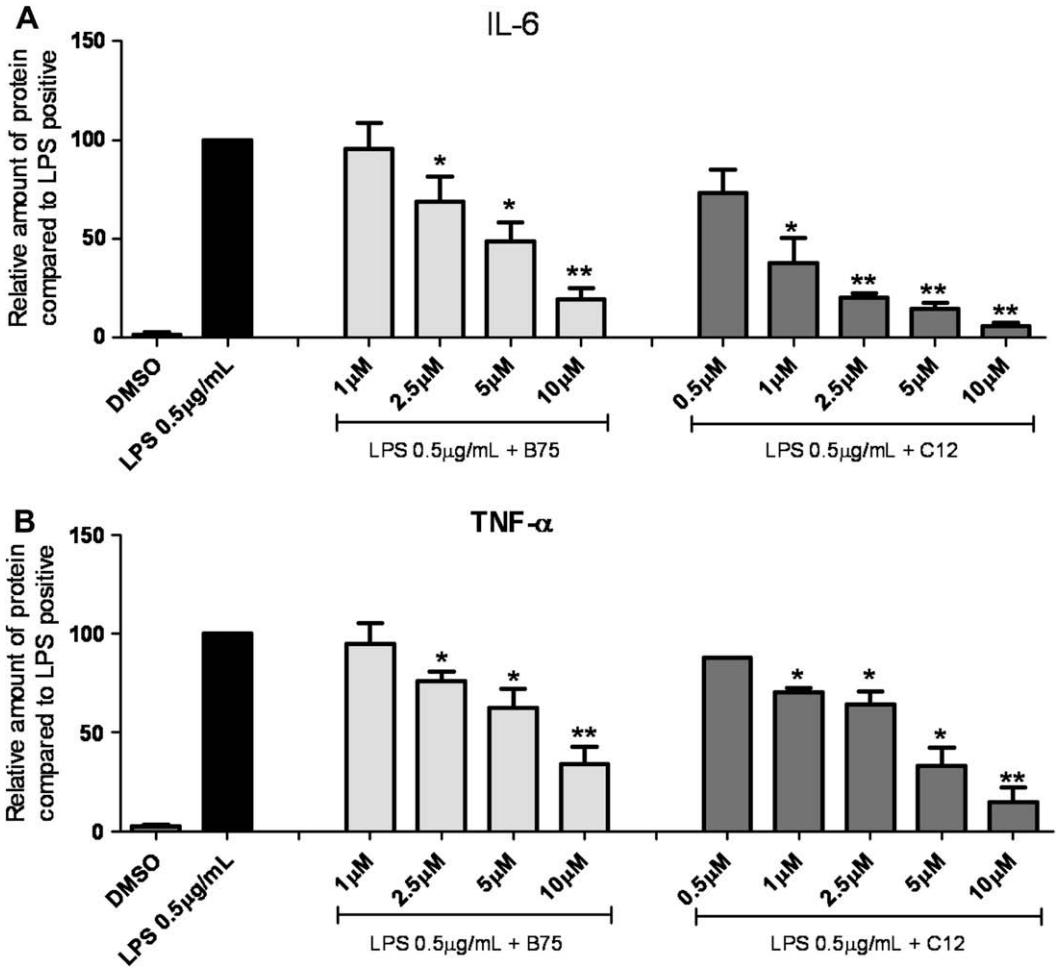

Figure 3. **B75** and **C12** inhibited LPS-induced TNF-α and IL-6 release in a dose-dependent manner in RAW 264.7 macrophages. Macrophages were plated at a density of 4.0×10^6/plate overnight in 37 °C and 5% CO_2. Cells were pre-treated with specific compounds as indicated for 2 h, followed by LPS (0.5 μg/ml) treatment for 22 h. TNF-α and IL-6 levels in the culture media were measured by ELISA and were normalized to the total protein amount. The results are expressed as percent of LPS control. Each bar represents mean ± SE of 3–5 independent experiments. Statistical significance relative to LPS is indicated, * p <0.05; ** p <0.01.

purified by chromatography over silica gel using CH_2Cl_2/CH_3OH as the eluent to yield compounds.

4.1.1. (2*E*,5*E*)-2,5-Bis(2,4-dichlorobenzylidene)cyclopentanone (A67)

Yellow powder, 82.7% yield, mp 209.4–212 °C [206–208 °C, lit.[32]]. ESI-MS *m/z*: 399.16 $(M+1)^+$, calcd for $C_{19}H_{12}Cl_4O$: 398.11.

4.1.2. (1*E*,4*E*)-1,5-Bis(2,4-dichlorophenyl)penta-1,4-dien-3-one(B67)

Yellow powder, 67.7% yield, mp 160.4–163.8 °C [168–169 °C, lit.[33]]. ESI-MS *m/z*: 373.32 $(M+1)^+$, calcd for $C_{17}H_{10}Cl_4O$: 372.07.

4.1.3. (2*E*,6*E*)-2,6-Bis(2,4-dichlorobenzylidene)cyclohexanone (C67)

Yellow powder, 73.9% yield, mp 160.1–163 °C [163–164 °C, lit.[32]].

4.1.4. (2*E*,5*E*)-2,5-Bis(2,4-dimethoxybenzylidene) cyclopentanone(A68)

Yellow powder, 34.1% yield, mp 176.1–180.3 °C. ^1H NMR ($CDCl_3$) δ: 2.98 (4H, s, CH_2–CH_2), 3.84 (6H, s, Ar^4-O–$CH_3 \times 2$), 3.86 (6H, s, Ar^2-O–$CH_3 \times 2$), 6.46 (2H, s, Ar-$H^3 \times 2$), 6.52 (2H, d, $J = 8.0$ Hz, Ar-$H^5 \times 2$), 7.49 (2H, d, $J = 8.0$ Hz, Ar-$H^6 \times 2$), 7.94 (2H, s, Ar-CH=C $\times 2$). ESI-MS *m/z*: 381.3 $(M+1)^+$, calcd for $C_{23}H_{24}O_5$: 380.43.

4.1.5. (1*E*,4*E*)-1,5-Bis(2,4-dimethoxyphenyl)penta-1,4-dien-3-one(B68)

Yellow powder, 35.7% yield, mp 132.7–136.5 °C [138–139 °C, lit.[34]].

4.1.6. (2*E*,6*E*)-2,6-Bis(2,4-dimethoxybenzylidene)cyclohexanone (C68)

Yellow powder, 36.2% yield, mp 155.8–160.7 °C. ^1H NMR ($CDCl_3$) δ: 1.74 (2H, t, $J = 6.0$ Hz, $>CH_2$), 2.82 (4H, t, $J = 6.0$ Hz, CH_2–C–CH_2), 3.82 (12H, s, –O–$CH_3 \times 4$), 6.46 (2H, s, Ar-$H^3 \times 2$), 6.49 (2H, d, $J = 6.0$ Hz, Ar-$H^5 \times 2$), 7.26 (2H, d, $J = 6.0$ Hz, Ar-$H^6 \times 2$), 7.95 (2H, s, Ar-CH=C $\times 2$). ESI-MS *m/z*: 395.5 $(M+1)^+$, calcd for $C_{24}H_{26}O_5$: 394.46.

4.1.7. (2*E*,5*E*)-2,5-Bis(2,4-dimethylbenzylidene)cyclopentanone (A69)

Yellow powder, 72.6% yield, mp 128.8–131.8 °C. ^1H NMR ($CDCl_3$) δ:2.35 (6H, s, Ar^4-$CH_3 \times 2$), 2.43 (6H, s, Ar^2-$CH_3 \times 2$), 2.99 (4H, s, CH_2-CH_2), 7.04 (2H, s, Ar-$H^5 \times 2$), 7.07 (2H, s, Ar-$H^3 \times 2$), 7.40 (2H, d, $J = 8.0$ Hz, Ar-$H^6 \times 2$), 7.79 (2H, s, Ar-CH=C $\times 2$). ESI-MS *m/z*: 317.4 $(M+1)^+$, 339.3 (M+Na), calcd for $C_{23}H_{24}O$: 316.44.

4.1.8. (1*E*,4*E*)-1,5-Bis(2,4-dimethylphenyl)penta-1,4-dien-3-one(B69)

Yellow powder, 38.2% yield, mp 95.5–98 °C. ^1H-NMR ($CDCl_3$) δ: 2.34 (6H, s, Ar^4-$CH_3 \times 2$), 2.44 (6H, s, Ar^2-$CH_3 \times 2$), 6.96 (2H, d, $J = 16.0$ Hz, =CH–C=O $\times 2$), 7.03 (2H, d, $J = 8.0$ Hz, Ar-$H^5 \times 2$), 7.05 (2H, s, Ar-$H^3 \times 2$), 7.56 (2H, d, $J = 8.0$ Hz, Ar-$H^6 \times 2$), 8.00 (2H, d, $J = 16.0$ Hz, Ar-CH=C $\times 2$). ESI-MS *m/z*: 292.4 $(M+1)^+$, 603.9 $(2\ M+Na)^+$, calcd for $C_{21}H_{22}O$: 290.4.

4.1.9. (2*E*,6*E*)-2,6-Bis(2,4-dimethylbenzylidene)cyclohexanone (C69)

Yellow powder, 36.8% yield, mp 118.6–121.5 °C. ^1H NMR ($CDCl_3$) δ: 1.70 (2H, m, $>CH_2$), 2.32 (6H, s, Ar^4-$CH_3 \times 2$), 2.34 (6H, s, Ar^2-$CH_3 \times 2$), 2.77 (4H, t, $J = 4.0$ Hz, CH_2–C–CH_2), 7.01 (2H, d, $J = 8.0$ Hz, Ar-$H^5 \times 2$), 7.05 (2H, s, Ar-$H^3 \times 2$), 7.16 (2H, d, $J = 8.0$ Hz, Ar-$H^6 \times 2$), 7.88 (2H, s, Ar-CH=C $\times 2$). ESI-MS *m/z*: 331.6 $(M+1)^+$, calcd for $C_{24}H_{26}O$: 330.46.

4.1.10. (2*E*,5*E*)-2,5-Bis(2,3-dichlorobenzylidene)cyclopentanone (A70)

Yellow powder, 82.6% yield, mp 204.6–206.8 °C [215–216 °C, lit.[35]]. ^1H NMR ($CDCl_3$) δ: 2.95 (4H, s, CH_2–CH_2), 7.25 (2H, d, $J = 8.0$ Hz, Ar-$H^6 \times 2$), 7.41 (2H, d, $J = 8.0$ Hz, Ar-$H^5 \times 2$), 7.47 (2H, d, $J = 8.0$ Hz, Ar-$H^4 \times 2$), 7.88 (2H, s, Ar-CH=C $\times 2$). ESI-MS *m/z*: 399.89 $(M+1)^+$, calcd for $C_{19}H_{12}Cl_4O$: 398.11.

4.1.11. (2*E*,6*E*)-2,6-Bis(2,3-dichlorobenzylidene)cyclohexanone (C70)

Yellow powder, 78.8% yield, mp 176.4–179.6 °C. ^1H NMR ($CDCl_3$) δ: 1.75 (2H, m, $>CH_2$), 2.72 (4H, t, $J = 4.0$ Hz, CH_2–C–CH_2), 7.21 (4H, d, $J = 8.0$ Hz, Ar-$H^{5,6} \times 2$), 7.44 (2H, t, $J = 8.0$ Hz, Ar-$H^4 \times 2$), 7.85 (2H, s, Ar-CH=C $\times 2$). ESI-MS *m/z*: 413.2 $(M+1)^+$, calcd for $C_{20}H_{14}Cl_4O$: 412.14.

4.1.12. (2*E*,5*E*)-2,5-Bis(2,4,6-trimethoxybenzylidene) cyclopentanone(A72)

Yellow powder, 24.0% yield, mp 195–197.8 °C. ^1H NMR ($CDCl_3$) δ: 2.51 (4H, s, CH_2–CH_2), 3.78 (12H, s, $Ar^{2,6}$-O–$CH_3 \times 2$), 3.82 (6H, s, Ar^4-O–$CH_3 \times 2$), 6.12 (4H, s, Ar-$H^{3,5} \times 2$), 7.55 (2H, s, Ar-CH=C $\times 2$). ESI-MS *m/z*: 441.6 $(M+1)^+$, calcd for $C_{25}H_{28}O_7$: 440.49.

4.1.13. (1*E*,4*E*)-1,5-Bis(2,4,6-trimethoxyphenyl)penta-1,4-dien-3-one(B72)[30]

Yellow powder, 17.0% yield, mp 210.4–215.4 °C. ^1H-NMR ($CDCl_3$) δ: 3.84 (6H, s, Ar^4-O–$CH_3 \times 2$), 3.88 (12H, s, $Ar^{2,6}$-O–$CH_3 \times 2$), 6.12 (4H, s, Ar-$H^{3,5} \times 2$), 7.44 (2H, d, $J = 18.0$ Hz, –CH–C=O $\times 2$), 8.11 (2H, d, $J = 18.0$ Hz, Ar-CH=C $\times 2$). ESI-MS *m/z*: 415.3 $(M+1)^+$, calcd for $C_{23}H_{26}O_7$: 414.45.

4.1.14. (2*E*,6*E*)-2,6-Bis(2,4,6-trimethoxybenzylidene)cyclohexanone(C72)[29]

Yellow powder, 39.7% yield, mp 201.8–203.4 °C. ^1H NMR ($CDCl_3$) δ: 1.62 (2H, t, $J = 6.0$ Hz, CH_2), 2.43 (4H, t, $J = 6.0$ Hz, CH_2–C–CH_2), 3.79 (12H, s, $Ar^{2,6}$-O–$CH_3 \times 2$), 3.82 (6H, s, Ar^4-O–$CH_3 \times 2$), 6.13 (4H, s, Ar-$H^{3,5} \times 2$), 7.61 (2H, s, Ar-CH=C $\times 2$). ESI-MS *m/z*: 456.1 $(M+1)^+$, calcd for $C_{26}H_{30}O_7$: 454.51.

4.1.15. Bis(2-carboxylbenzylidene)cyclopentanone(A73)[36]

Green powder, 87.9% yield, mp >260 °C. ^1H-NMR (HDO) δ: 2.73 (4H, s, CH_2–CH_2), 7.32–7.44 (4H, m, Ar-$H^{4,6} \times 2$), 7.52–7.63 (4H, m, Ar-$H^5 \times 2$, Ar-CH=C $\times 2$), 7.80 (2H, s, Ar-$H^3 \times 2$). ESI-MS *m/z*: 347.3 $(M-1)^-$, calcd for $C_{21}H_{26}O_5$: 348.35.

4.1.16. Bis(2-carboxylbenzylidene)acetone(B73)

Green powder, 52.2% yield, mp >260 °C. ^1H-NMR (HDO) δ: 7.11 (2H, d, $J = 18.0$ Hz, =CH–C=O $\times 2$), 7.29–7.34 (4H, m, Ar-$H^{4,6} \times 2$), 7.51 (4H, m, Ar-$H^5 \times 2$, Ar-CH=C $\times 2$), 7.61 (2H, d, $J = 18.0$ Hz, Ar-CH=C $\times 2$), 7.87 (2H, s, Ar-$H^3 \times 2$). ESI-MS *m/z*: 321.2 $(M-1)^-$, calcd for $C_{19}H_{14}O_5$: 322.31.

4.1.17. Bis(2-carboxylbenzylidene)cyclohexanone(C73)

Green powder, 77.7% yield, mp >260 °C. ^1H NMR (HDO) δ: 1.65 (2H, m, $>CH_2$), 2.66 (4H, t, $J = 8.0$ Hz, CH_2–C–CH_2), 7.36 (2H, d, $J = 8.0$ Hz, Ar-$H^4 \times 2$), 7.41 (2H, t, $J = 8.0$ Hz, Ar-$H^6 \times 2$), 7.44 (2H, s, Ar-CH=C $\times 2$), 7.51 (2H, d, $J = 8.0$ Hz, Ar-$H^5 \times 2$,), 7.84 (2H, s, Ar-$H^3 \times 2$). ESI-MS *m/z*: 361.3 $(M-1)^-$, calcd for $C_{22}H_{18}O_5$: 362.38.

4.1.18. (2*E*,5*E*)-2,5-Bis(2,5-difluorobenzylidene)cyclopentanone (A74)

Yellow powder, 29.4% yield, mp 208.1–209.8 °C. ^1H NMR ($CDCl_3$) δ: 3.05 (4H, s, CH_2–CH_2), 7.06–7.10 (6H, m, Ar-H), 7.73 (2H, s, Ar-CH=C $\times 2$). ESI-MS *m/z*: 334.5 $(M+1)^+$, 687.3 $(2\ M+Na)^+$, calcd for $C_{19}H_{12}F_4O$: 332.29.

4.1.19. (1*E*,4*E*)-1,5-bis(2,5-difluorophenyl)penta-1,4-dien-3-one(B74)

Yellow powder, 22.0% yield, mp 84.4–90.5 °C. ^{1}H NMR ($CDCl_3$) δ: 7.08 (2H, d, J = 18.0 Hz, =CH–C=O × 2), 7.09–7.13 (2H, m, Ar-H^6 × 2), 7.29–7.32 (4H, m, Ar-$H^{3,4}$ × 2), 7.79 (2H, d, J = 18.0 Hz, Ar-CH=C × 2). ESI-MS m/z: 306.2 $(M+1)^+$, calcd for $C_{17}H_{10}F_4O$: 306.25.

4.1.20. (2*E*,6*E*)-2,6-Bis(2,5-difluorobenzylidene)cyclohexanone (C74)

Yellow powder, 45.3% yield, mp 132–135.4 °C. ^{1}H NMR ($CDCl_3$) δ: 1.80 (2H, m, $>CH_2$), 2.80 (4H, t, J = 6.0 Hz, CH_2–C–CH_2), 6.99–7.08 (6H, m, Ar-H), 7.74 (2H, s, Ar-CH=C × 2). ESI-MS m/z: 348.1 $(M+1)^+$, 715.4 (2M+Na), calcd for $C_{20}H_{14}F_4O$: 346.32.

4.1.21. (2*E*,5*E*)-2,5-Bis(2,6-difluorobenzylidene)cyclopentanone (A75)

Yellow powder, 79.2% yield, mp 146.8–149.6 °C. ^{1}H NMR ($CDCl_3$) δ: 2.73 (4H, s, CH_2–CH_2), 6.92–6.95 (4H, m, Ar-$H^{3,5}$ × 2), 7.29–7.34 (2H, m, Ar-H^4 × 2), 7.50 (2H, s, Ar-CH=C × 2). ESI-MS m/z: 334.4 $(M+1)^+$, 687.4 (M+Na), calcd for $C_{19}H_{12}F_4O$: 332.29.

4.1.22. (1*E*,4*E*)-1,5-Bis(2,6-difluorophenyl)penta-1,4-dien-3-one(B75)

Yellow powder, 49.3% yield, mp 135–138.5 °C. ^{1}H NMR ($CDCl_3$) δ: 6.95 (4H, t, Ar-$H^{3,5}$ × 2), 7.29 (2H, d, J = 18.0 Hz, =CH–C=O × 2), 7.31–7.36 (2H, m, Ar-H^4 × 2), 7.81 (2H, d, J = 18.0 Hz, Ar-CH=C × 2). ESI-MS m/z: 307.7 $(M+1)^+$ 329.6 (M+Na) 635.3 (2 M+Na), calcd for $C_{17}H_{10}F_4O$: 306.25.

4.1.23. (2*E*,6*E*)-2,6-Bis(2,6-difluorobenzylidene)cyclohexanone (C75)

Yellow powder, 42.5% yield, mp 127.4–130.8 °C. ^{1}H NMR ($CDCl_3$) δ: 1.73 (2H, m, $>CH_2$), 2.59 (4H, s, CH_2–C–CH_2), 6.91–6.94 (4H, m, Ar-$H^{3,5}$ × 2), 7.27–7.32 (2H, m, Ar-H^4 × 2), 7.55 (2H, s, Ar-CH=C × 2). ESI-MS m/z: 348.7 $(M+1)^+$, 715.2 (2M+Na), calcd for $C_{20}H_{14}F_4O$: 346.32.

4.1.24. (2*E*,6*E*)-2,6-Bis(4-(3-(dimethylamino)propoxy) benzylidene)cyclohexanone (C12)

Green powder, 64.7% yield, mp 89.7–91.5 °C. ^{1}H NMR ($CDCl_3$) δ: 1.85 (2H, m, $>CH_2$), 1.97 (4H, m, $-CH_2-$ × 2), 2.25 (12H, s, N–CH_3 × 4), 2.45 (4H, t, J = 6.9 Hz, N–CH_2- × 2), 2.91 (4H, t, J = 4.8 Hz, CH_2–CH_2), 4.02 (4H, m, O–CH_2 × 2), 6.92 (4H, d, J = 8.4 Hz, Ar-$H^{3,5}$ × 2), 7.26 (2H, s, Ar-CH=C × 2), 7.43 (4H, d, J = 8.4 Hz, Ar-$H^{2,6}$ × 2). ESI-MS m/z: 477.3 $(M+1)^+$, calcd for $C_{30}H_{40}N_2O_3$: 476.65.

4.2. Cell line and reagents

Mouse RAW 264.7 macrophages were obtained from the American Type Culture Collection (ATCC, USA). Cell culture reagents were obtained from Gibco. Fetal bovine serum was from HyClone and was heat-inactivated for 30 min at 65 °C. LPS purchased from Sigma was dissolved in PBS. Curcumin and its analogues were dissolved in DMSO.

4.3. Cell treatment and ELISA assay

Mouse RAW 264.7 macrophages were incubated in DMEM media (Gibco) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C with 5% CO_2. Cells were pre-treated with 10 μM of curcumin, analogues or vehicle control for 2 h, then treated with LPS (0.5 μg/ml) for 22 h. After treatment, the culture media and cells were collected separately. The culture media collected were centrifuged at 1000 rpm for 10 min. The levels of TNF-α and IL-6 in the media were determined by ELISA using mouse TNF-α and mouse IL-6 ELISA Kits (BOSTER, USA). After centrifugation, the supernatant was separated and stored at −70 °C until use. Cells were washed with PBS and harvested with cell lysis buffer (Tris–HCl 20 mM, NP40 1%, NaCl 150 mM, EDTA 2 mM, Na3VO4 200 mM, SDS 0.1%, NaF 20 mM). The mixed liquor was shaken vigorously for 10 min in lysis buffer at 0 °C. After being centrifuged at 12,000 rpm for 30 min at 4 °C, the total protein was collected and the concentrations were determined using Bio-Rad protein assay reagents. The total amount of the inflammatory factor in the media was normalized to the total protein amount of the viable cell pellets.

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