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Discovery and evaluation of piperid-4-one-containing mono-carbonyl analogs of curcumin as anti-inflammatory agents

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ABSTRACT

We previously reported the design and discovery of three series of 5-carbon linker-containing mono-carbonyl analogs of curcumin (MCACs) as excellent anti-inflammatory agents. In continuation of our ongoing research, we designed and synthesized the fourth series of MCACs, whose central linker is a piperid-4-one. Their inhibitory effects against IL-6 production were evaluated in lipopolysaccharide (LPS)-stimulated macrophages. Among them, compounds **F8**, **F29**, **F33**, **F35**, and **F36** exhibited the IC_{50} values under 5 μM. The structure–activity relationship was discussed. Mechanistically, **F35** and **F36** dose-dependently prevented LPS-induced NF-κB and ERK activation. Finally, pretreatment with **F35** and **F36** significantly protected the C57B/L6 mice from LPS-induced septic death. Together, these data present a series of new analogs of curcumin as promising anti-inflammatory agents.

1. Introduction

Inflammatory response is a common pathological process of a variety of diseases. Besides immunological diseases and inflammation, many important diseases in the preliminary stage such as cardiovascular disease, atherosclerosis, cancer, and type II diabetes can be initiated and aggravated by over-expression and activation of pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α).[1–4] As a result, pharmacological intervention of new small molecules, which are capable of reducing the expression of inflammatory cytokines, has been recently proposed as a possible method for the prevention and treatment of these diseases.[1,3]

Curcumin (Fig. 1) is a yellow natural product isolated from turmeric.[5] Research shows curcumin is a highly pleiotropic molecule capable of interacting with numerous molecular targets involved in inflammation.[5] Based on early cell culture and animal research, clinical trials indicate curcumin may have potential as a therapeutic agent in diseases such as inflammatory bowel disease, pancreatitis, arthritis, and chronic anterior uveitis, as well as certain types of cancer.[6] Curcumin modulates the inflammatory response by inhibiting the production of the inflammatory cytokines such as TNF-α and IL-1, -2, -6, and -12.[5,6]

Although this molecule has already been the subject of several clinical trials for development as a drug, the rapid plasma clearance and low bioavailability significantly limit its therapeutic usefulness.[7] For the purpose of finding novel derivatives with increased systemic bioavailability and enhanced pharmacological activity, chemical modifications as well as synthesis of curcumin analogues have been attempted by many research groups to find a better treatment for various diseases linked to inflammation.[7–13] The β-diketone moiety in curcumin's structure has been considered to be responsible for its instability, fast metabolism and poor bioavailability.[7] In our previous study, 3 series of 5-carbon linker-containing mono-carbonyl analogues of curcumin (MCACs), 1,5-diaryl-1,4-pentadiene-3-ones, together with cyclopentanone and cyclohexanone analogues, were designed by displacing β-diketone moiety with a single carbonyl group.[8–13] They were evaluated the anti-inflammatory activity, and some exhibit promising pharmacological effects and are undergoing preclinical study. Our lab works for the discovery of new anti-inflammatory agents from MCACs. In continuation of our ongoing research, we present here the fourth series of MCACs in our lab, whose central linker is a piperid-4-one structure (Fig. 1). We evaluated here the inhibitory effects of 34 piperidone-containing analogs against IL-6 production in lipopolysaccharide (LPS)-stimulated macrophages. Further, active compounds were selected for the study of anti-inflammatory mechanism and in vivo therapeutic effects against LPS-induced septic death in mouse models. These results suggest their potential to serve as new anti-inflammatory agents.

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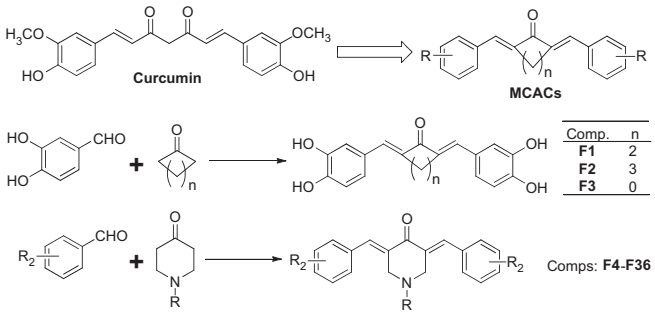

Comp.	R_1	R_2	Comp.	R_1	R_2
F4	$CH_2CH_2CH_3$	3-OCH_3, 4-OH	F21	CH_2CH_3	2-COOH
F5	$CH_2CH_2CH_3$	2-Cl	F22	CH_2CH_3	2,4,6-OCH_3
F6	$CH_2CH_2CH_3$	3,4-OH	F23	CH_2CH_3	2,5-OCH_3
F8	$CH_2CH_2CH_3$	3,4-OCH_3	F24	CH_2CH_3	2-Br
F9	CH_3	2,4-Cl	F25	CH_2CH_3	3,4-F
F10	CH_3	4-$N(CH_3)_2$	F26	CH_2CH_3	3,4-OCH_3
F11	CH_3	3-OCH_3, 4-OH	F27	H	4-OCH_3
F12	CH_3	2-Cl	F28	H	2-F
F13	CH_3	2-F	F29	H	4-OH
F14	CH_3	4-OH	F30	H	2,4,6-OCH_3
F15	CH_3	4-F	F31	H	2,4-OCH_3
F16	CH_3	2-OCH_3	F32	H	3,4-OH
F17	CH_3	2,3-OCH_3	F33	H	3-OH
F18	CH_3	3,4-OCH_3	F35	H	3,4,5-OCH_3
F19	CH_2CH_3	2,4-Cl	F36	H	3,4-OCH_3
F20	CH_2CH_3	4-$N(CH_3)_2$			

Figure 1. Structures of synthetic compounds. Reagents and conditions: (I) CH_3CH_2OH, HCl, rt or reflux; (II) CH_3CH_2OH/H_2O, NaOH or HCl, rt or reflux.

2. Result and discussion

2.1. Chemistry

In continuation of our ongoing research on potential anti-inflammatory agents from MCACs, we designed 4 series of 5-carbon-linker mono-carbonyl analogues as follows: 1,5-diaryl-1,4-pentadiene-3-ones, 2,6-(diarylidene) cyclohexanone, 2,5-(diarylidene) cyclopentanone, and 3,5-(diarylidene) piperid-4-one. Herein, we present the analogues containing the structure of 3,5-(diarylidene) piperid-4-one. In this skeleton, various substitutes on the benzene rings were used for the structure–activity relationship analysis. The synthesis and structures of compounds **F1**–**F36** are shown in Figure 1. Compounds were synthesized by direct aldol condensation of substituted benzaldehyde with the ketones in acidic or alkaline media. Compounds with hydroxy groups in the benzene rings (**F1**–**F4**, **F6**, **F11**, **F14**, **F29**, **F32** and **F33**) were synthesized using HCl as a catalyst; among these compounds, **F3**, **F4**, **F6** and **F11** were prepared in a condition of reflux, while **F1**, **F2**, **F14**, **F29**, **F32** and **F33** were obtained at room temperature. Other compounds were synthesized using NaOH as catalyst at 4–8 °C. Compound **F8**, **F17**, **F18**, **F22** and **F26** were purified by recrystallization, and others were purified by silica gel column chromatography. The yields of pure products were in the range of 12–96%. Details of yields, melting points, and spectral analysis in ESI-MS and ^{1}H NMR of these compounds are described in Section 4.

2.2. Inhibitory effects of these compounds on LPS-induced IL-6 release

IL-6 is a well-characterized cytokine that plays an important role in many inflammatory diseases caused by endotoxins.[14] 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, especially the expression of proinflammatory cytokines such as IL-6.[14] Thus, the anti-inflammatory activity of these synthetic compounds was characterized by their inhibition on LPS-induced IL-6 secretion in mouse RAW264.7 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 IL-6 in media was detected through enzyme-linked immunosorbant assays (ELISA) and normalized by protein concentration of cells harvested in homologous culture plates.

The result of anti-inflammatory evaluation is shown in Figure 2A. A majority of piperid-4-one-containing MCACs exhibited high inhibition against LPS-induced IL-6 expression. Among 34 tested compounds, 26 compounds showed stronger activity than curcumin at the same dosage, and 23 compounds showed inhibitory rates higher than 50%. Compound **F8**, **F18**, **F29**, **F35** and **F36** showed the strongest inhibitory effect on LPS-induced IL-6 release and their inhibitory rates reached 90%, 90%, 89%, 96%, and 98%, respectively, compared to the LPS control.

Compounds **F1**–**F3** only showed moderate inhibition of IL-6 expression. Combined with the anti-inflammatory screening data on MCACs that we previously published,[8–13] it is generally observed that piperid-4-one-containing MCACs are more effective against LPS-induced IL-6 expression than previous three series of 5-carbon linker-containing mono-carbonyl analogues of curcumin (MCACs). In addition, electron-withdrawing halogen substituents in the benzene rings appear to increase the anti-inflammatory activity. Compounds (**F5**, **F13**, **F15**, **F24** and **F28**) with halogen in the 2-position of the benzene rings showed good activity. The effects of electron-donating substituents in the benzene rings on the anti-inflammatory activity of analogs varied from the type of groups. The methoxyl group is always favorable to the anti-inflammatory effects, while the hydroxyl group and *N*,*N*-dimethyl amino group show uncertain roles in bioactivity. For piperid-4-one derivatives, the N-substitution with different groups should play an important role in the IL-6-inhibitory activity. We observed that *N*-methyl and *N*-propyl substitution seem to slightly increase the activity; however, the SAR profile of the substitution with *N*-methyl, *N*-ethyl, *N*-propyl and hydrogen on the anti-inflammatory actions of piperid-4-one-containg MCACs is still confused and require further investigation according to our results.

2.3. Active compounds inhibit LPS-induced IL-6 release in a dose dependent manner

Among active compounds above, five of the most active compounds **F8**, **F29**, **F33**, **F35**, and **F36** were selected for further dose-dependent evaluation and they exhibited no obvious cytotoxicity in macrophages (data not shown). Cells were pretreated with active compounds at 1, 5, and 10 μM for 2 h, followed by incubation with LPS (0.5 μg/mL) for 22 h. The results are shown in Figure 2B. All of five compounds exhibit good dose-dependent inhibition of LPS-induced IL-6 release, with the IC_{50} values under 5 μM. Compound **F35** exhibits the best activity and its IC_{50} is under 1 μM. This result further suggests the potential of the piperid-4-one-containg MCACs as anti-inflammatory agents.

2.4. Active compounds show different mechanism in NF-κB and MAPK pathways

LPS is recognized by toll-like receptor 4 (TLR4) and activate MyD88-dependent pathways, one of which culminates in activation of the transcription factor nuclear factor (NF)-κB and the other leads to activation of mitogen-activated protein kinase

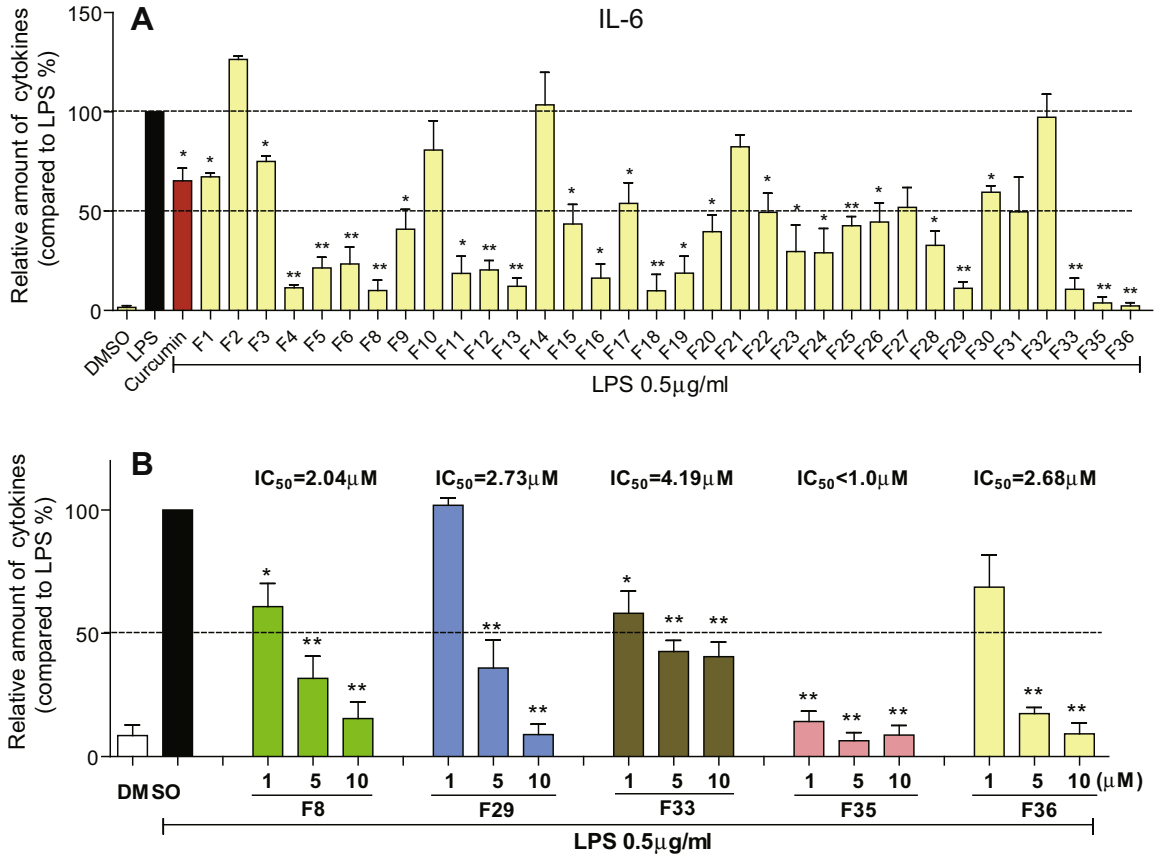

Figure 2. Compounds inhibited LPS-induced IL-6 release in Mouse RAW 264.7 macrophages. Mouse RAW 264.7 macrophages were plated with a density of 1.2×10^6/plate for overnight. (A) An inhibitory screening of compounds at 10 μM. (B) A dose-dependent evaluation of active compounds at 1, 5, and 10 μM. Cells were treated with vehicle (DMSO) or curcumin or compounds for 2 h, then incubated with LPS (0.5 μg/mL) for 22 h. The culture media and the cells were collected, respectively. The IL-6 levels in culture media were determined with an ELISA kit and were normalized by the total protein concentration of cells. The results were showed in the percent of LPS control. Each bar represents mean ± SE of 3–6 independent experiments. Statistical significance relative to LPS was expressed, $^*p$ <0.05, $^{**}p$ < 0.01.

(MAPKs).[14,15] Both of these two could regulate the expression of inflammatory factors including IL-6 at the transcriptional level in response to various inflammatory stimuli containing LPS. In NF-κB signaling, IκB degradation frees NF-κB p65 subunit and allows it to translocate to the nucleus, followed by turning on transcription of inflammatory genes.[14,15] MAPKs include three kinases: the extracellular-signal-regulated kinase (ERK), the JUN N-terminal kinase (JNK) and the p38 kinase.[14,15] Among them, p38 has also been identified to regulate the cytokine expression at the post-transcriptional level.

To gain insight into involved signaling, we determined whether MAPKs and NF-κB might be involved in anti-inflammatory actions of active curcumin analogues. In order to closely imitate the pathological practice, the primary mouse peritoneal macrophages (MPMs) were employed here instead of RAW264.7 cell line. MPMs were treated with compounds before LPS stimulation. LPS induced the IκB degradation and the phosphorylation of ERK, p38, and JNK as shown in Figure 3. A pretreatment with active **F35** and **F36** significantly reversed LPS-induced degradation of IκB, while **F8** and **F29** had no effect on it (Fig. 3A). **F35** and **F36** further showed dose-dependent inhibition against LPS-induced IκB degradation (Fig. 3B). We also tested the effects of four compounds on LPS-induced MAPK phosphorylation. Figure 3C revealed that **F36** could significantly block the phosphorylation of ERK and JNK, **F35** only suppressed ERK activation, while **F8** and F**29** showed no effect on all three MAP kinases. Subsequently, the inhibitory activity of **F35** and **F36** against LPS-induced ERK activation was confirmed by dose-dependent experiments (Fig. 3D). A similar result was also observed in **F36**'s inhibition on JNK phosphorylation in a dose-dependent manner (Fig. 3E). In addition, pretreatment with curcumin at 10 μM exhibited obvious inhibition only on JNK phosphorylation in LPS-stimulated macrophages (Fig. 3C).

compound **F35** seems to exert anti-inflammatory actions partly via inhibiting NF-κB and ERK pathways; the inhibition of **F36** on inflammatory cytokines is accompanied with inactivation of NF-κB, ERK, and JNK. However, the bioactivities of **F8** and **F29** may be NF-κB/MAPKs-independent. Curcumin has been identified as a multi-target compound and there is recent evidence that curcumin can inhibit MAPKs and NF-κB at relatively high concentrations. We have previously demonstrated that several curcumin analogues affected NF-κB and ERK/JNK pathways.[16,17] These results indicated a mechanistic difference among active anti-inflammatory curcumin analogues, although they are derived from the same structural lead. This suggests that the structural modification reduces and even changes the possible molecular targets of curcumin. It is really worthy to be further investigated in the future for the chemical and structural features influencing the biological mechanism of curcumin analogs.

2.5. F35 and F36 attenuated the LPS-induced septic death in mice

As a major endotoxin, LPS has been implicated as a major cause of sepsis.[18] A number of different approaches have been investigated to treat and/or prevent the septic shock associated with infections caused by Gram-negative bacteria, including blockage of one or more of the cytokines induced by LPS signaling.[15,18]

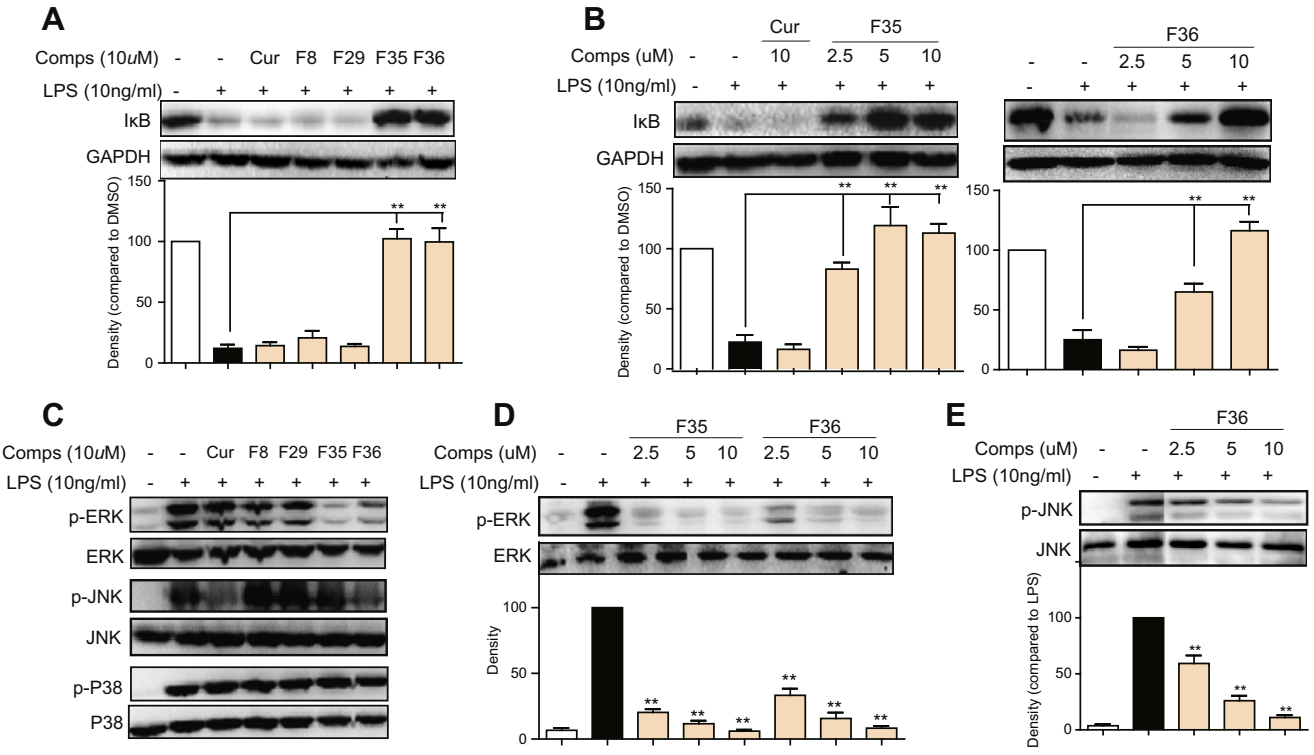

Figure 3. Active compounds **F8**, **F19**, **F35**, and **F36** affected LPS-induced NF-κB and MAPK signaling activation in MPMs. (A) Four compounds affected IκB degradation; (B) F35 and F36 dose-dependently reversed LPS-induced IκB degradation; (C) Four compounds inhibited the phosphorylation of JNK, ERK, and p38; (D) **F35** and F36 dose-dependently inhibited ERK activation; (E) **F36** dose-dependently decreased JNK phosphorylation (For clarity, lanes 3rd–5th showing the effects of **F35** at three concentrations have been cut. The full unmodified gels are not shown.). MPMs were plated with a density of 1.2×10^6/plate for overnight. Cells were pretreated with DMSO or curcumin (Cur) or indicated compounds at indicated concentrations for 2 h, then treated with LPS (10 ng/mL) for 1 h. The cells were collected and lysated. The protein level of IκB, p-JNK, p-ERK, or p-p38 was detected by western blot with GAPDH or corresponding total protein as loading control. Representative blots of 4–6 dependent experiments in each study are shown. The column figures show the normalized optical density as a percentage of control. Bars represent the mean ± SEM of 3–5 independent experiments: (**) p <0.01.

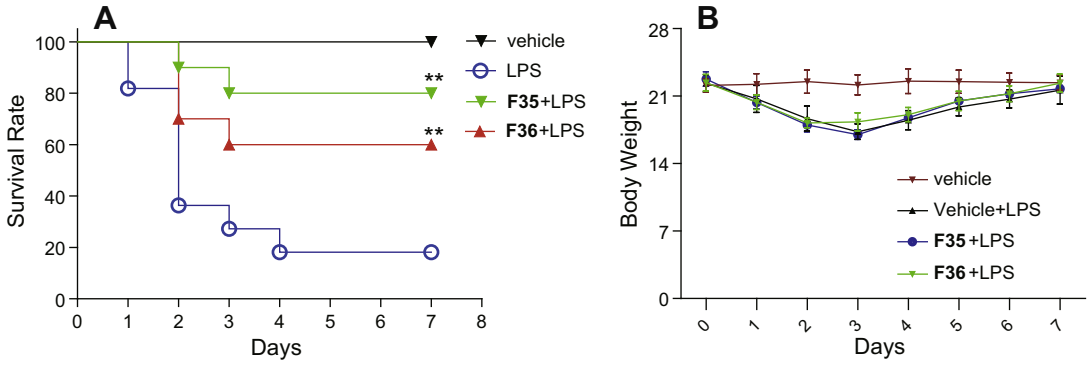

Figure 4. **F35** and **F36** attenuated LPS-induced septic shock in vivo. Male C57BL/6 mice were pretreated with **F35** or **F36** (ip, 20 mg/kg) or vehicle, followed by injection of LPS (iv, 20 mg/kg). Survival (A) and body weight (B) were recorded for 7 days at an interval of 24 h after the LPS injection. n = 10 animals in each group. ** p <0.01 versus LPS group.

Our data have demonstrated the inhibitory effects of these analogs on LPS-induced signaling activation and IL-6 release. For the potential clinical application, we further determine whether **F35** and **F36** are able to attenuate LPS-induced septic death in mice. Compounds were used in a water-soluble preparation for intraperitoneal (ip) administration. Mice were injected with LPS at the dosage of 20 mg/kg intravenously (iv) 15 min after the ip injection of **F35** or **F36**, and their survival rates were monitored for seven days, respectively. As shown in Figure 4A, 80% of animals treated with LPS alone died within 4 days as a result of the septic shock. In animals receiving **F35** or **F36** at 15 mg/kg 15 min prior to LPS injection, the survival rates were significantly increased compared to that of the control group (80% survival in **F35**-treated group and 60% survivals in **F36**-treated group, p <0.01 in both groups vs LPS group). Meanwhile, the body weight of compound-treated mice decreased during day 0–3, but regained slowly 3 days after LPS treatment (Fig. 4B). Thus, our data provide the in vivo evidence for the anti-inflammatory effects of these piperid-4-one-containing MCACs.

3. Conclusion

In summary, we synthesized and screening several piperid-4-one-containing analogues of curcumin, which exhibited strong

inhibition on LPS-induced IL-6 release in RAW264.7 macrophage cell. Generally, this kind of MCAC is more effective against LPS-induced IL-6 expression than previous 3 series of 5-carbon linker-containing MCACs. Their SAR profiles were discussed and active compounds were selected for further anti-inflammatory investigation. Compounds **F8**, **F29**, **F33**, **F35** and **F36** showed dose-dependent inhibition on LPS-induced IL-6 expression, and pretreatment of **F35** and **F36** prolonged survival in LPS-induced acute inflammatory mouse model. We also test the effects of active compounds on NF-κB and MAPK signaling pathways. The results indicate that they exert cytokine-inhibitory activity via different signaling mechanism, although they are derived from the same lead. These findings suggest that piperid-4-one-containing MCACs may be promising anti-inflammatory agents and have potential in the therapy of sepsis and other inflammatory diseases. In the next step, we may examine the introduction of the other heteroatom into the central linker of MCACs. Further studies are also necessary to investigate the underlying molecular mechanisms and different targets of these analogues at the transcriptional or post-transcriptional level.

4. Experimental section

4.1. Chemical synthesis

All chemical reagents were obtained from Sigma–Aldrich, Fluka, and Aladdin (Beijing, China). Silica gel (GF254) for thin-layer chromatography and column chromatography (100–200 and 200–300 mesh) were obtained from Aladdin. Melting points were tested on a Fisher-Johns melting apparatus. Electron-spray ionization mass spectra (ESI-MS) data were determined on a Bruker Esquire HCT spectrometer. The ^{1}H NMR spectra data was recorded on a 600 MHz spectrometer (Bruker Corporation, Switzerland).

All compounds were synthesized by aldol condensation between aryl aldehydes and ketones (acetone, cyclohexanone, cyclopentanone, piperid-4-one, 1-methylpiperid-4-one, 1-ethyl piperid-4-one, or 1-propylpiperid-4-one). The general procedure for synthesis of these compounds is briefly described as following. The piperid-4-one (2 mmol) and corresponding aryl (4 mmol) aldehydes are dissolved in the mixture solvent of ethanol and water (10:1), and other ketones (2 mmol) and corresponding aryl aldehydes (4 mmol) are dissolved in ethanol absolute. The compounds containing –OH group in the benzene ring were catalyzed by HCl. Other compounds were catalyzed by NaOH at 5–8 °C. All reactions were monitored by the silica gel TLC. At the end of the reaction, water is added into the reaction mixture to precipitate the product. Compound **F3**–**F6**, **F9**, **F11**–**F16**, **F19**–**F21**, **F25**, **F27**–**F36** were purified by column chromatography using PE/EA or $CHCl_3/CH_3OH$. The crude of other compounds were recrystallized in the ethanol or the mixture of trichloromethane and ethanol. Their structures were determined by spectral data from ESI-MS and ^{1}H NMR. The spectral data of new or unreported compounds are shown below.

4.1.1. (2*E*,5*E*)-2,5-Bis(3,4-dihydroxybenzylidene) cyclopentanone (F1)

13.7% Yield, mp 260.7 °C. ^{1}H NMR (DMSO-d_6), δ: 9.563 (br s, 2H, OH-4), 9.211 (br s, 2H, OH-3), 7.238 (s, 2H, Ar–CH=C × 2), 7.113 (d, J = 1.8 Hz, 2H, Ar-H^2 × 2), 7.006 (dd, J_1 = 1.8 Hz, J_2 = 8.4 Hz, 2H, Ar-H^6 × 2), 6.834 (d, J = 8.4 Hz, 2H, Ar-H^5 × 2), 3.001 (s, 4H, CH_2–O–CH_2). ESI-MS m/z: 323.1$(M-1)^-$, calcd for $C_{19}H_{16}O_5$: 324.33.

4.1.2. (2*E*,6*E*)-2,6-Bis(3,4-dihydroxybenzylidene)cyclohexanone (F2)

69% Yield, mp 234.4 °C. ^{1}H NMR (DMSO-d_6), δ: 9.438 (br s, 2H, OH-4), 9.131 (br s, 2H, OH-3), 7.446 (s, 2H, Ar–CH=C × 2), 6.980 (d, J = 1.8 Hz, 2H, Ar-H^2 × 2), 6.873 (dd, J_1 = 1.8 Hz, J_2 = 8.4 Hz, 2H, Ar-H^6 × 2), 6.799 (d, J = 8.4 Hz, 2H, Ar-H^5 × 2), 2.845 (t, J = 4.8 Hz, 4H, CH_2–C–CH_2), 1.725 (t, J = 4.8 Hz, 2H, C–CH_2–C). ESI-MS m/z: 339.1$(M+1)^+$, calcd for $C_{20}H_{18}O_5$: 338.35.

4.1.3. (1*E*,4*E*)-1,5-Bis(3,4-dihydroxyphenyl)penta-1,4-dien-3-one (F3)

15% Yield, mp 149–151.7 °C [150–154 °C, lit.[19]]. ^{1}H NMR (DMSO-d_6) δ: 9.697 (s, 2H, Ar–CH=C × 2), 9.524 (s, 2H, Ar–C=CH × 2), 7.265 (m, 4H, Ar-H^2 × 2, Ar-H^5 × 2), 6.904 (d, 2H, Ar-H^6 × 2). ESI-MS m/z: 296.9$(M-1)^-$, calcd for $C_{17}H_{14}O_5$: 298.29. ESI-MS m/z: 296.9$(M-1)^-$, calcd for $C_{17}H_{14}O_5$: 298.29.

4.1.4. (3*E*,5*E*)-3,5-Bis(4-hydroxy-3-methoxybenzylidene)-1-propylpiperid-4-one (F4)

36.5% Yield, mp 213.8–214.7 °C. ^{1}H NMR (DMSO-d_6) δ: 7.833 (S, 2H, Ar–CH=C × 2), 7.139 (s, 2H, Ar-H^2 × 2), 7.033 (d, J = 8.4 Hz, 2H, Ar-H^6 × 2), 6.946 (d, J = 8.4 Hz, 2H, Ar-H^5 × 2), 3.842 (s, 6H, 3-OCH_3 × 2), 3.356 (S, 4H, –CH_2–N–CH_2–), 2.500 (s, 2H, N–CH_2), 1.678-1.716 (m, 2H, –CH_2–), 0.889 (t, J = 7.2 Hz, 3H, –CH_3). ESI-MS m/z: 410.1 $(M+1)^+$, calcd for $C_{24}H_{27}NO_5$: 409.47.

4.1.5. (3*E*,5*E*)-3,5-Bis(2-chlorobenzylidene)-1-propylpiperid-4-one (F5)

76.6% Yield, mp 126.2–127.9 °C. ^{1}H NMR ($CDCl_3$), δ: 8.003 (s, 2H, Ar–CH=C × 2), 7.452–7.468 (m, 2H, Ar-H^6 × 2), 7.281–7.320 (m, 4H, Ar-H^3 × 2, Ar-H^4 × 2), 7.235–7.251 (m,2H, Ar-H^5 × 2), 3.692 (s, 4H, N–CH_2–C × 2), 2.420 (t, J = 7.8 Hz, 2H, N–CH_2), 1.343–1.384 (m, 2H, N–C–CH_2), 0.809 (t, J = 7.8 Hz, 3H, CH_3). ESI-MS m/z: 386.2, 388.1 $(M+1)^+$, calcd for $C_{22}H_{21}Cl_2NO$: 386.31.

4.1.6. (3*E*,5*E*)-3,5-Bis(3,4-dihydroxybenzylidene)-1-propylpiperid-4-one (F6)

43.15% Yield, mp 232.2–233.8 °C. ^{1}H NMR (DMSO-d_6), δ: 7.700 (s, 2H, Ar–CH=C × 2), 7.221 (d, J = 1.8 Hz, 2H, Ar-H^2 × 2), 6.900 (d, J = 7.8 Hz, 2H, Ar-H^6 × 2), 6.871 (d, J = 7.8 Hz, 2H, Ar-H^5 × 2), 4.524 (s, 4H, CH_2–N–CH_2), 3.146–3.151 (m, 2H, N–CH_2),1.655–1.694 (m, 2H, N–C–CH_2), 0.912–0.925 (m, 3H, CH_3). ESI-MS m/z: 380.1 $(M-1)^-$, calcd for $C_{22}H_{23}NO_5$: 381.42.

4.1.7. (3*E*,5*E*)-3,5-Bis(3,4-dimethoxybenzylidene)-1-propylpiperid-4-one (F8)

61.1% Yield, mp 162.7–165.2 °C. ^{1}H NMR ($CDCl_3$), δ: 7.769 (s, 2H, Ar–CH=C × 2), 7.021 (dd, J_1 = 1.8 Hz, J_2 = 8.4 Hz, 2H, Ar-H^6 × 2), 6.955 (d, J = 1.2 Hz, 2H, Ar-H^2 × 2), 6.928 (d, J = 8.4 Hz, 2H, Ar-H^5 × 2), 3.952 (s, 6H, 3-OCH_3 × 2), 3.924 (s, 6H, 4-OCH_3 × 2), 3.854 (s, 4H, CH_2–N–CH_2), 2.520 (t, 2H, N–CH_2), 1.483 (m, 2H, CH_3CH_2), 0.884 (t, J = 7.2 Hz, 3H, CH_3). ESI-MS m/z: 438.3$(M+1)^+$, calcd for $C_{26}H_{31}NO_5$: 437.53.

4.1.8. (3*E*,5*E*)-3,5-Bis(2,4-dichlorobenzylidene)-1-methylpiperid-4-one (F9)

88.6% Yield, mp 145.9–147.6 °C. ^{1}H NMR ($CDCl_3$), δ: 7.913 (s, 1H, Ar–CH=C), 7.481 (d, J = 1.8 Hz, 2H, Ar-H^3 × 2), 7.305 (dd, J_1 = 1.8 Hz, J_2 = 7.8 Hz, 2H, Ar-H^6 × 2), 7.187 (d, J = 7.8 Hz, 2H, Ar-H^5 × 2), 3.735 (s, 4H, CH_2–N–CH_2), 2.453 (s, 3H, N–CH_3). ESI-MS m/z: 427.8, 425.9, 429.8$(M+1)^+$, calcd for $C_{20}H_{15}Cl_4NO$: 427.15.

4.1.9. (3*E*,5*E*)-3,5-Bis(4-(dimethylamino)benzylidene)-1-methylpiperid-4-one (F10)

16.8% Yield, mp 225.8–227.3 °C [223–225 °C, lit.[21]]; ^{1}H NMR ($CDCl_3$), δ: 7.785 (s, 2H, Ar–CH=C × 2), 7.345 (d, J = 9.0 Hz, 4H, Ar-H^2 × 2, Ar-H^6 × 2), 6.716 (d, J = 8.4 Hz, 4H, Ar-H^3 × 2, Ar-H^5 × 2), 3.845 (s, 4H, CH_2–N–CH_2), 3.023 (s, 12H, 4-N(CH_3) × 2), 2.510 (s, 3H, N–CH_3). ESI-MS m/z: 376.1$(M+1)^+$, calcd for $C_{24}H_{29}N_3O$: 375.51.

4.1.10. (3*E*,5*E*)-3,5-Bis(4-hydroxy-3-methoxybenzylidene)-1-methylpiperid-4-one (F11)

17.1% Yield, mp 190.2–192.7 °C [195–197 °C, lit.[20]]. ^{1}H NMR (DMSO-d_6) δ: 7.821 (S, 2H, Ar–CH=C × 2), 7.418 (dd, J_1 = 1.8 Hz, J_2 = 7.8 Hz, 1H, Ar-H^6 × 2), 7.131 (d, J = 1.2 Hz, 2H, Ar-H^2×2), 6.969 (d, J = 8.4 Hz, 2H, Ar-H^6 × 2), 3.841 (s, 6H, 3-OCH$_3$ × 2), 3.345 (S, 4H, –CH$_2$–N–CH$_2$–), 2.500 (s, 3H, N–CH$_3$). ESI-MS *m*/*z*: 382.1(M+1)$^+$, calcd for $C_{22}H_{23}NO_5$: 381.42

4.1.11. (3*E*,5*E*)-3,5-Bis(2-chlorobenzylidene)-1-methylpiperid-4-one (F12)

78.5% Yield, mp 143.9–145.2 °C [150–151 °C, lit.[21]]. ^{1}H NMR ($CDCl_3$), δ: 8.010 (s, 2H, Ar–CH=C × 2), 7.458 (d, J = 9.0 Hz, 2H, Ar-H^6 × 2), 7.285–7.322 (m, 4H, Ar-H^3 × 2, Ar-H^4 × 2), 7.231–7.262 (m, 2H, Ar-H^5 × 2), 3.659 (s, 4H, CH$_2$–N–CH$_2$), 2.379 (s, 3H, N–CH$_3$). ESI-MS *m*/*z*: 358.3, 360.1, 361.1(M+1)$^+$, calcd for $C_{20}H_{17}Cl_2NO$: 358.26.

4.1.12. (3*E*,5*E*)-3,5-Bis(2-fluorobenzylidene)-1-methylpiperid-4-one (F13)

95.2% Yield, mp 138.5–141.8 °C. ^{1}H NMR ($CDCl_3$), δ: δ: 7.902 (s, 2H, Ar–CH=C × 2), 7.355–7.371 (m, 2H, Ar-H^6 × 2), 7.294 (dt, J = 1.2 Hz, 7.2 Hz, 2H, Ar-H^3 × 2), 7.187 (dt, J_1 = 0.6 Hz, J_2 = 7.2 Hz, 2H, Ar-H^4 × 2), 7.128 (dt, J_1 = 0.6 Hz, J_2 = 8.4 Hz, 2H, Ar-H^5 × 2), 3.653 (s, 4H, N–CH$_2$ × 2), 2.409 (s, 3H, N–CH$_3$). ESI-MS *m*/*z*: 326.2(M+1)$^+$, calcd for $C_{20}H_{17}F_2NO$: 325.35.

4.1.13. (3*E*,5*E*)-3,5-Bis(4-hydroxybenzylidene)-1-methylpiperid-4-one (F14)

36.7% yield, mp 116.5–117.8 °C. ^{1}H NMR (DMSO-d_6) δ: 7.758 (d, J = 8.4 Hz, 4H, Ar–CH=C × 2, Ar-H^2 × 2, Ar-H^6 × 2), 6.927 (d, J = 8.4 Hz, 4H, Ar-H^3 × 2, Ar-H^5 × 2), 3.324 (S, 4H, –CH$_2$–N–CH$_2$–), 2.498 (s, 3H, N–CH$_3$). ESI-MS *m*/*z*: 319.7(M−1)$^-$, calcd for $C_{20}H_{19}NO_3$: 321.37.

4.1.14. (3*E*,5*E*)-3,5-Bis(4-fluorobenzylidene)-1-methylpiperid-4-one (F15)

67.8% Yield, mp 173.8–175.8 °C. ^{1}H NMR (DMSO-d_6) δ: 7.559–7.595 (m, 6H, Ar–CH=C × 2, Ar-H^6 × 2, Ar-H^2 × 2), 7.311 (d, J = 8.4 Hz, 4H, Ar-H^3 × 2, Ar-H^5 × 2), 3.316 (S, 4H, –CH$_2$–N–CH$_2$–), 2.500 (s, 3H, N–CH$_3$). ESI-MS *m*/*z*: 324.36(M−1)$^-$, calcd for $C_{20}H_{17}F_2NO$: 325.35.

4.1.15. (3*E*,5*E*)-3,5-Bis(2-methoxybenzylidene)-1-methylpiperid-4-one (F16)

35.92% Yield, mp 108.2–110.9 °C [117–118 °C, lit.[21]]. ^{1}H NMR ($CDCl_3$), δ: 8.093 (s, 2H, Ar–CH=C × 2), 7.335–7.363 (m, 2H, Ar-H^6 × 2), 7.183 (dd, J_1 = 1.2 Hz, J_2 = 7.8 Hz, Ar-H^4 × 2), 6.974 (t, J = 7.8 Hz, Ar-H^5 × 2), 6.924 (d, J = 8.4 Hz, Ar-H^3 × 2), 3.857 (s, 6H, 2-OCH$_3$ × 2), 3.735 (s, 4H, CH$_2$–N–CH$_2$), 2.404 (s, 3H, N–CH$_3$). ESI-MS *m*/*z*: 350.1 (M+1)$^+$, calcd for $C_{22}H_{23}NO_3$: 349.42.

4.1.16. (3*E*,5*E*)-3,5-Bis(2,3-dimethoxybenzylidene)-1-methylpiperid-4-one (F17)

38.1% Yield, mp 126.6–128.4 °C. ^{1}H NMR ($CDCl_3$), δ: 7.173 (s, 2H, Ar–CH=C × 2), 7.052 (d, J = 7.8 Hz, 2H, Ar-H^6 × 2), 7.027 (t, J = 7.8 Hz, 2H, Ar-H^4 × 2), 7.003 (d, J = 7.8 Hz, 2H, Ar-H^5 × 2), 3.887 (s, 4H, CH$_2$–N–CH$_2$), 3.851–3.876 (m, 12H, 2-OCH$_3$ × 2, 3-OCH$_3$ × 2), 3.838 (s, 3H, N-CH$_3$). ESI-MS *m*/*z*: 410.2(M+1)$^+$, calcd for $C_{24}H_{27}NO_5$: 409.47.

4.1.17. (3*E*,5*E*)-3,5-bis(3,4-dimethoxybenzylidene)-1-methylpiperid-4-one (F18)

52.3% Yield, mp 157.3–159.4 °C. ^{1}H NMR ($CDCl_3$), δ: 7.785 (s, 2H, Ar–CH=C × 2), 6.999 (dd, J_1 = 1.2 Hz, J_2 = 8.4 Hz, 2H, Ar-H^6 × 2), 6.936 (d, J = 1.8 Hz, 2H, Ar-H^2 × 2), 6.916 (d, J = 8.4 Hz, 2H, Ar-H^5 × 2), 3.937 (s, 6H, 3-OCH$_3$ × 2), 3.915 (s, 6H, 4-OCH$_3$ × 2), 3.833 (s, 4H, CH$_2$–N–CH$_2$), 2.497 (s, 3H, N–CH$_3$). ESI-MS *m*/*z*: 410.1(M+1)$^+$, calcd for $C_{24}H_{27}NO_5$: 409.47.

4.1.18. (3*E*,5*E*)-3,5-Bis(2,4-dichlorobenzylidene)-1-ethylpiperid-4-one (F19)

92.21% Yield, mp 100.4–103.7 °C. ^{1}H NMR ($CDCl_3$), δ: 7.925 (s, 1H, Ar–CH=C), 7.487 (d, J = 1.8 Hz, 2H, Ar-H^3 × 2), 7.294 (dd, J_1 = 1.8 Hz, J_2 = 7.8 Hz, 2H, Ar-H^6 × 2), 7.175 (d, J = 7.8 Hz, 2H, Ar-H^5 × 2), 3.662 (s, 4H, N–CH$_2$ × 2), 2.546 (q, J = 7.2 Hz, 2H, N–CH$_2$), 0.996 (t, J = 7.2 Hz, 3H, CH$_3$). ESI-MS *m*/*z*: 442.1, 440.2, 444.0(M+1)$^+$, calcd for $C_{21}H_{17}C_{l4}NO$: 441.18.

4.1.19. (3*E*,5*E*)-3,5-Bis(4-(dimethylamino)benzylidene)-1-ethylpiperid-4-one (F20)

34.2% Yield, mp 188.6–191.1 °C. ^{1}H NMR ($CDCl_3$), δ: 7.797 (s, 2H, Ar–CH=C × 2), 7.355 (d, 4H, Ar-H^2 × 2, Ar-H^6 × 2), 6.720 (d, 4H, Ar-H^3 × 2, Ar-H^5 × 2), 3.909 (s, 4H, N–CH$_2$ × 2), 2.670 (q, J = 7.2 Hz, 2H, N–CH$_2$), 1.115 (t, J = 7.2 Hz, 3H, CH$_3$). ESI-MS *m*/*z*: 390.3(M+1)$^+$, calcd for $C_{25}H_{31}N_3O$: 389.53.

4.1.20. 2-{[(3*E*,5*E*)-5-[(2-Carboxyphenyl)methylidene]-1-ethyl-4-oxopiperidin-3-ylidene]methyl}benzoic acid (F21)

25.0% Yield, mp 64.8–66.9 °C. ^{1}H NMR ($CDCl_3$), δ: 7.889 (d, 2H, Ar-H^3 × 2), 7.709 (t, 2H, Ar–CH=C × 2), 7.592 (m, 4H, Ar-H^5 × 2, Ar-H^6 × 2), 3.932 (m, 4H, CH$_2$–N–CH$_2$). ESI-MS *m*/*z*: 390.1(M−1)$^-$, calcd for $C_{23}H_{21}NO_5$: 391.42.

4.1.21. (3*E*,5*E*)-3,5-Bis(2,4,6-trimethoxybenzylidene)-1-ethylpiperid-4-one (F22)

18.66% Yield, mp 193.7–196.2 °C. ^{1}H NMR ($CDCl_3$), δ: 7.782 (s, 2H, Ar–CH=C × 2), 6.118 (s, 4H, Ar-H^3 × 2, Ar-H^5 × 2), 3.876 (s, 6H, 4-OCH$_3$ × 2), 3.800 (s, 12H, 2-OCH$_3$ × 2, 6-OCH$_3$ × 2), 3.482 (s, 4H, CH$_2$–N–CH$_2$), 2.467 (m, 2H, NCH$_2$), 0.896 (t, J = 7.2 Hz, 3H, CH$_3$). ESI-MS *m*/*z*: 484.4(M+1)$^+$, calcd for $C_{27}H_{33}NO_7$: 483.55.

4.1.22. (3*E*,5*E*)-3,5-Bis(2,5-dimethoxybenzylidene)-1-ethylpiperid-4-one (F23)

69.01% Yield, mp 88.9–92.1 °C. ^{1}H NMR ($CDCl_3$), δ: 8.010 (s, 2H, Ar–CH=C × 2), 6.875 (d, J = 3.0 Hz, 2H, Ar-H^3 × 2), 6.855 (s, 2H, Ar-H^6 × 2), 6.775 (d, J = 3.0 Hz, 2H, Ar-H^4 × 2), 3.751–3.807 (m, 12H, OCH$_3$ × 4), 3.761 (s, 4H, N–CH$_2$ × 2), 2.541 (q, J = 7.2 Hz, 2H, N–CH$_2$), 0.996 (t, J = 7.2 Hz, 3H, CH$_3$). ESI-MS *m*/*z*: 424.2(M+1)$^+$, calcd for $C_{25}H_{29}NO_5$: 423.5.

4.1.23. (3*E*,5*E*)-3,5-bis(2-bromobenzylidene)-1-ethylpiperid-4-one (F24)

84.5% Yield, mp 133.4–136 °C. ^{1}H NMR ($CDCl_3$), δ: 7.946 (s, 2H, Ar–CH=C × 2), 7.655 (d, J = 7.8 Hz, 2H, Ar-H^3 × 2), 7.346 (t, J = 7.2 Hz, 2H, Ar-H^5 × 2), 7.187–7.236 (m, 4H, Ar-H^4 × 2, Ar-H^6 × 2), 3.667 (s, 4H, N–CH$_2$ × 2), 7.525 (q, J = 7.2 Hz, 2H, N–CH$_2$), 0.972 (t, J = 7.2 Hz, 3H, CH$_3$). ESI-MS *m*/*z*: 462.1, 463.9, 460.3, 462.9, 464.9(M+1)$^+$, calcd for $C_{21}H_{19}Br_2NO$: 461.19.

4.1.24. (3*E*,5*E*)-3,5-Bis(3,4-difluorobenzylidene)-1-ethylpiperid-4-one (F25)

68.69% Yield, mp 122.2–124.3 °C. ^{1}H NMR ($CDCl_3$), δ: 7.688 (s, 2H, Ar–CH=C × 2), 7.191–7.232 (m, 4H, Ar-H^2 × 2, Ar-H^6 × 2), 7.133–7.151 (m, 1H, Ar-H^5 × 2), 3.766 (s, 4H, N–CH$_2$ × 2), 2.635 (q, J = 7.2 Hz, 2H, N–CH$_2$), 1.082 (t, J = 7.2 Hz, 3H, CH$_3$). ESI-MS *m*/*z*: 376.0, calcd for $C_{21}H_{17}F_4NO$: 375.36.

4.1.25. (3*E*,5*E*)-3,5-Bis(3,4-dimethoxybenzylidene)-1-ethylpiperid-4-one (F26)

66.32% Yield, mp 188.7–190.8 °C. ^{1}H NMR ($CDCl_3$), δ: 7.783 (s, 2H, Ar–CH=C × 2), 7.017 (dd, J_1 = 1.8 Hz, J_2 = 8.4 Hz, 2H,

Ar-H^6 × 2), 6.950 (d, J = 1.8 Hz, 2H, Ar-H^2 × 2), 6.922 (d, J = 8.4 Hz, 2H, Ar-H^3 × 2), 3.920 (m, 12H, OCH_3 × 4), 3.888 (s, 4H, N–CH_2–C × 2), 1.085 (t, J = 7.2 Hz, 3H, CH_3), 2.641 (d, J = 7.2 Hz, 2H, N–CH_2). ESI-MS m/z: 424.5$(M+1)^+$, calcd for $C_{25}H_{29}NO_5$: 423.5.

4.1.26. (3*E*,5*E*)-3,5-Bis(4-methoxybenzylidene)piperid-4-one (F27)

11.1% Yield, mp 182.0–184.6 °C [180–182 °C, lit.[22]] ESI-MS m/z: 336.1$(M+1)^+$, calcd for $C_{21}H_{21}NO_3$: 335.4.

4.1.27. (3*E*,5*E*)-3,5-Bis(2-fluorobenzylidene)piperid-4-one (F28)

44.68% Yield, mp 139.6–142.5 °C. ^{1}H NMR ($CDCl_3$), δ: 7.884 (s, 2H, Ar–CH=C × 2), 7.285–7.389 (m, 4H, Ar-H^3 × 2, Ar-H^6 × 2), 7.204 (t, J = 7.2 Hz, 2H, Ar-H^4 × 2), 7.148 (t, J = 9.0 Hz, 2H, Ar-H^5 × 2), 4.049 (s, 4H, CH_2–N–CH_2). ESI-MS m/z: 312.3$(M+1)^+$, calcd for $C_{19}H_{15}F_2NO$: 311.33.

4.1.28. (3*E*,5*E*)-3,5-Bis(4-hydroxybenzylidene)piperid-4-one (F29)

7.6% Yield, mp 298.5 °C decompose [>300 °C, lit.[23]]. ^{1}H NMR (DMSO-d_6), δ: 10.270 (s, 2H, Ar–CH=C × 2), 7.392 (d, J × 8.4 Hz, 4H, Ar-H^2 × 2, Ar-H^6 × 2), 6.920 (d, J × 8.4 Hz, 4H, Ar-H^3 × 2, Ar-H^5 × 2), 4.451 (s, 4H, CH_2–N–CH_2). ESI-MS m/z: 308.1 $(M+1)^+$; 306.0 $(M-1)^-$, calcd for $C_{19}H_{17}NO_3$: 307.34.

4.1.29. (3*E*,5*E*)-3,5-Bis(2,4,6-trimethoxybenzylidene)piperid-4-one (F30)

30.61% Yield, mp 152.3–156.1 °C. ^{1}H NMR ($CDCl_3$), δ: 7.662 (s, 2H, Ar–CH=C × 2), 6.124 (s, 4H, Ar-H^3 × 2, Ar-H^5 × 2), 3.843 (s, 6H, 4-OCH_3 × 2), 3.799 (s, 12H, 2-OCH_3 × 2, 6-OCH_3 × 2), 3.686 (s, 4H, CH_2-N-CH_2). ESI-MS m/z: 456.2$(M+1)^+$, calcd for $C_{25}H_{29}NO_7$: 455.19.

4.1.30. (3*E*,5*E*)-3,5-Bis(2,4-dimethoxybenzylidene)piperid-4-one (F31)

41.61% Yield, mp 150.7–154.1 °C. ^{1}H NMR ($CDCl_3$), δ: 7.993 (s, 2H, Ar–CH=C × 2), 7.108 (d, J = 8.4 Hz, 2H, Ar-H^6 × 2), 6.500 (d, J = 7.8 Hz, 2H, Ar-H^5 × 2), 6,464 (s, 2H, Ar-H^3 × 2), 4.046 (s, 4H, N–CH_2–C × 2), 3.843 (s, 6H, 2-OCH_3 × 2), 3.834 (s, 6H, 4-OCH_3 × 2), 1.975 (br s, 1H, N–H). ESI-MS m/z: 396.1 $(M+1)^+$, calcd for $C_{23}H_{25}NO_5$: 395.45.

4.1.31. (3*E*,5*E*)-3,5-Bis(3,4-dihydroxybenzylidene)piperid-4-one (F32)

31% Yield, mp 81.6–83.5 °C. ESI-MS m/z: 337.8 $(M-1)^-$, calcd for $C_{19}H_{13}F_4NO$: 339.34.

4.1.32. (3*E*,5*E*)-3,5-Bis(3-hydroxybenzylidene)piperid-4-one (F33)

41.2% Yield, mp >300 °C. ^{1}H NMR ($CDCl_3$), δ: 8.955 (s, 2H, Ar–CH=C × 2), 7.764 (s, 2H, Ar-H^2 × 2), 7.405 (t, 2H, Ar-H^6 × 2), 6.967–6.996 (m, 4H, Ar-H^4 × 2, Ar-H^5 × 2), 3.905 (s, 4H, CH_2–N–CH_2). ESI-MS m/z: 308.7$(M+1)^+$, calcd for $C_{19}H_{16}O_4$: 307.34.

4.1.33. (3*E*,5*E*)-3,5-Bis(3,4,5-trimethoxybenzylidene)piperid-4-one (F35)

29.73% Yield, mp 193.7–194.0 °C. ^{1}H NMR ($CDCl_3$), δ: 7.734 (s, 2H, Ar–CH=C × 2), 6.615 (s, 4H, Ar-H^2 × 2, Ar-H^6 × 2), 4.210 (s, 4H, CH_2–N–CH_2), 3.930 (s, 6H, 4-OCH_3 × 2), 3.880 (s, 12H, 3-OCH_3 × 2, 5-OCH_3 × 2). ESI-MS m/z: 456.2$(M+1)^+$, calcd for $C_{25}H_{29}NO_7$: 455.5.

4.1.34. (3*E*,5*E*)-3,5-Bis(3,4-dimethoxybenzylidene)piperid-4-one (F36)

8.39% Yield, mp 162.2–165.4 °C. ^{1}H NMR ($CDCl_3$), δ: 7.758 (s, 2H, Ar–CH=C × 2), 7.008 (d, J = 8.4 Hz, 2H, Ar-H^2 × 2), 6.908–6.933 (m, 4H, Ar-H^5 × 2, Ar-H^6 × 2), 4.191 (s, 4H, N–CH_2–C × 2), 3.895–3.947 (m, 12H, OCH_3 × 4), 1.780 (br s, 1H, N–H). ESI-MS m/z: 396.2$(M+1)^+$, calcd for $C_{23}H_{25}NO_5$: 395.45.

4.2. Animals

Male C57BL/6 and ICR mice weighing 18–22 g were obtained from the Animal Center of Wenzhou Medical College (Wenzhou, China). Animals were housed at a constant room temperature with a 12:12 hour 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 used in experiments. Protocols involving the use of animals were approved by the Wenzhou Medical College Animal Policy and Welfare Committee (Approval documents: 2009/APWC/0031).

4.3. Reagents and cells

Chemical reagents and lipopolysaccharide (LPS) were purchased from Sigma (Louis, MO). Saline was prepared as 0.9% NaCl solution. Mouse RAW 264.7 macrophages were obtained from the American Type Culture Collection (ATCC, USA). 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. The mouse primary peritoneal macrophage (MPMs) were obtained from ICR mice according to the method described in our previous paper.[22]

4.4. Determination of IL-6 by ELISA method

After treatment of cells with indicated compounds and LPS, the IL-6 levels in medium were determined with an ELISA kit (eBioScience, San Diego, CA) according to the manufacturer's instructions and the method described in our previous paper.[16] The total amount of the inflammatory factor in the medium was normalized to the total protein quantity of the viable cell pellets.

4.5. Western blot analysis

The treated cells were collected and lysated, then 40–60 μg of the whole cell lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to a nitrocellulose membrane. Each membrane was preincubated for 1 h at room temperature in tris-buffered saline, pH 7.6, containing 0.05% tween 20 and 5% non-fat milk. The nitrocellulose membrane was incubated with specific antibodies against p-JNK, JNK, IκB, p-p38, p38, p-ERK, ERK, or Actin (Santa Cruz Biotech Co. LTD, CA), respectively. Immunoreactive bands were then detected by incubating with secondary antibody conjugated with horseradish peroxidase and visualized using enhanced chemiluminescence reagents (Bio-Rad, Hercules, CA).

4.6. LPS-induced inflammatory mortality in mice

Compounds were firstly dissolved with macrogol 15 hydroxystearate (a nonionic solubilizer for injection from BASF) with or without medium chain triglycerides (MCT, from BASF) in water bath at 37 °C. The concentration of compounds was 2 mg/mL. The concentration of solubilizer was ranged 5–10%, and MCT 0.5–2% in 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 compounds (20 mg/kg) in a water solution by ip injection 15 min before the iv injection of LPS (20 mg/kg). Control animals received a similar volume (200 μL) of vehicle. Body weight change and mortality were recorded for 7 days.

4.7. Statistical analysis

All experiments were repeated more than three times. The results are showed in the mean ± SE. Statistics were analysed using student's *t*-test mode in GraphPad Pro (GraphPad, San Diego, CA). p values less than 0.05 (p <0.05) were considered as significance.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.03.057. These data include MOL files and InChiKeys of the most important compounds described in this article.

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