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Design, synthesis and anti-inflammatory effects of novel 9-*O*-substituted-berberine derivatives†‡

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Berberine, an isoquinoline alkaloid in many medicinal herbs, has been found to possess broad pharmacological activities. A series of novel C-9-*O*-substituted-berberine derivatives have been synthesized and their anti-inflammatory effects evaluated both *in vitro* and *in vivo*. Compared to berberine, the new synthetic berberine derivatives **3i** and **5e** exhibit significantly improved inhibitory activities against the release of NO, TNF-α and IL-6. Furthermore, derivatives **3i** and **5e** were found to inhibit more effectively the migration of neutrophils and primitive macrophage in transgenic zebrafish larvae with injury-provoked inflammation. Pre-treatment with derivatives **3i** or **5e** could lead to a concentration-dependent decrease in nuclear factor-κB (NF-κB) p65 and NF-κB inhibitor α (IκBα) phosphorylation and inducible nitric oxide synthase (iNOS) expression in lipopolysaccharide (LPS)-induced RAW264.7 cells, which suggested that the anti-inflammatory activities of berberine derivatives **3i** and **5e** are related to their suppression of the NF-κB signal pathway.

Introduction

Inflammation is part of the complex biological response of body tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. It is often accompanied by a rapid activation of macrophages and secretion of inflammatory mediators including nitric oxide (NO), tumor necrosis factor-α (TNF-α), cyclooxygenase-2 (COX-2), interleukin-1β (IL-1β), and interleukin-6 (IL-6). Although inflammation is usually a protection response and a beneficial process for the host,[1] excess and sustained release of pro-inflammatory cytokines can also cause damage to the host, even resulting in a systemic inflammatory reaction. In recent years, it has been widely accepted that many diseases including atherosclerosis,[2] Alzheimer's disease,[3] diabetes[4,5] and cancer[6,7] have inflammatory etiologies.

Berberine (**BBR**, Fig. 1), an isoquinoline alkaloid found in many medicinal herbs, has a long history of medicinal use in China for the treatment of gastroenteritis and bacterial diarrhea. Besides its significant antibacterial effect, berberine has also been associated with many other pharmacological activities,[8] including antiviral,[9] anti-fungal,[10] anti-diabetic,[11] anti-cancer[12,13] and anti-inflammatory.[14–16] Accumulation studies have revealed an underlying NF-κB-dependent pathway in the inhibitory effect of berberine on the expression of inflammation mediators and the production of an inflammation-related cytokine. For example, berberine can ameliorate the production of COX-2, IL-1β, IL-6, and TNF-α in the 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice through the inhibition of NF-κB activation.[15,16] The suppression effects of berberine on the production of IL-1β and TNF-α and the phosphorylation and degradation of IκB-α have also been observed in some human lung inflammation cells.[14] Moreover, berberine was found to exert a protective effect against diseases associated with inflammation such as Alzheimer's disease,[17] diabetes[18] and cancer.[19] All of these indicate the potential of berberine as a candidate anti-inflammatory drug.

Although berberine has pronounced anti-inflammatory activity, the side effects caused by long-term and especially extensive use that results from the relatively low anti-inflammatory activity of berberine severely limit its use.[20] Meanwhile, the development of **BBR** is also restricted greatly by its poor water solubility.[21,22] Structural modification of berberine at the 9-*O*-position may improve its activity and

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‡ Electronic supplementary information (ESI) available: Supplementary data contains MS and NMR spectra for all of the synthetic new compounds. See DOI: 10.1039/c5md00577a

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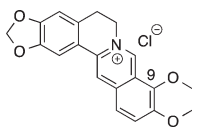


Fig. 1 Chemical structure of berberine (**BBR**).

bioavailability. Pharmacokinetic studies have revealed that the main metabolite of berberine in urine is berberrubine-9-*O*-β-glucuronide, and that *O*-demethylation might be the first step of berberine metabolism,[23–26] which suggests that the C-9-*O* skeleton might be the active framework of berberine.[27] This paper reports an effort to discover novel berberine derivatives with enhanced anti-inflammatory activity as well as improved solubility. Amino groups, especially the N-heterocycles, are found in a variety of biologically active compounds and form water-soluble salts easily with organic or inorganic acids.[28] Therefore, a series of novel 9-*O*-substituted-berberine derivatives, in which the methoxyl group was replaced by different carbamates and amines with side chains of various lengths, were designed and synthesized. The anti-inflammatory activity of these derivatives in LPS-stimulated RAW264.7 cells was examined with regard to their effects on the release of the inflammatory mediator NO. The anti-inflammatory effects of the two most active derivatives (**3i** and **5e**) were further confirmed by assessing their effects on the release of the inflammatory mediators TNF-α and IL-6 by enzyme-linked immunosorbent assays (ELISA) *in vitro* and by investigating their inhibitory activities on the migration of neutrophils and primitive macrophage in transgenic zebrafish larvae with injury-provoked inflammation *in vivo*. In addition, western blot experiments were performed in an attempt to illustrate the anti-inflammation mechanism of these two compounds.

Chemistry

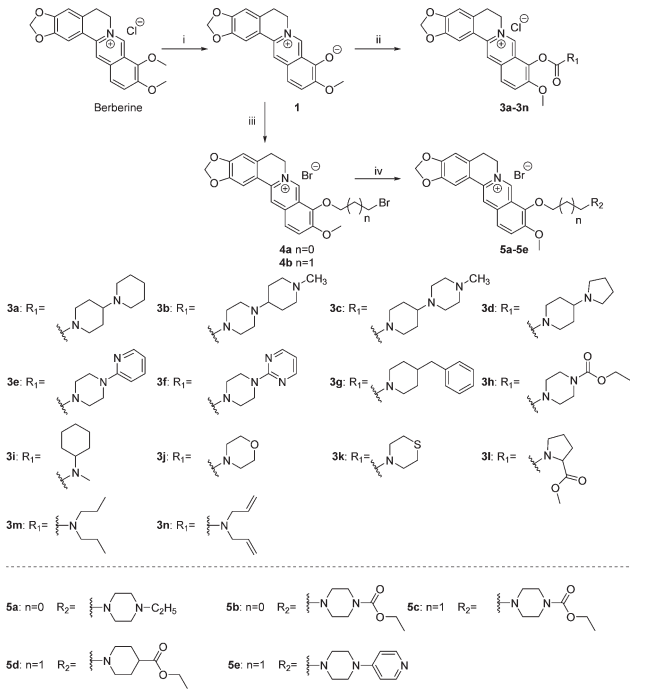
The strong hydrophilicity of berberine is likely a major cause of its poor bioavailability and low activity. Introduction of hydrophobic groups into berberine could improve its bioavailability and membrane absorption.[27] The 9-*O* position of berberine has been demonstrated to be the central point of its metabolism. Accordingly, in order to improve the anti-inflammatory activity and liposolubility of berberine, we replaced the methoxyl group in the 9-*O* position with different carbamates or introduced a side chain with terminal amino groups. The synthetic routes are shown in Scheme 1. Selective demethylation of berberine was achieved at 190 °C under vacuum, providing berberrubine as a dark red solid. A variety of secondary amines were treated with triphosgene to obtain the corresponding carbamoyl chlorides **2a–2n** which were allowed to react with berberrubine to give the berberine carbamoyl derivatives **3a–3n**.

Another series of derivatives, **5a–5e**, were synthesized by a two-step reaction, employing α,ω-dibromoalkanes and secondary amines as reagents to introduce a side chain at the C-9 position.

Results and discussion

Cell cytotoxicity assay

The cytotoxicity of the derivatives was evaluated in RAW264.7 cells using the MTT colorimetric assay. The cell viabilities of



Scheme 1 General synthetic route to the formation of berberine derivatives **3a–3q** and **5a–5i**. Reagents and conditions: (i) 20–30 mm Hg, 190 °C; (ii) R_1COCl **2a–2n**, pyridine, r.t.; (iii) alkyl bromide, K_2CO_3, MeCN, 60 °C; (iv) amine, K_2CO_3, MeCN, 80 °C.

BBR, indomethacin (an anti-inflammatory drug control) and the synthesized derivatives were determined at concentrations of 10 μM and 5 μM. As shown in Table 1, some

Table 1 Cytotoxicity of berberine and berberine derivatives to RAW264.7 cells

Compound	Viability[b] (%)	
	10 μM	5 μM
Control[a]	100.3 ± 0.04	101.5 ± 2.18
BBR[c]	97.2 ± 2.61	99.4 ± 6.97
Indo[d]	99.6 ± 5.24	99.8 ± 6.67
3a	100.1 ± 4.73	98.1 ± 3.73
3b	102.1 ± 5.76	107.1 ± 2.98
3c	103.9 ± 7.62	100.8 ± 11.62
3e	109.3 ± 8.36	103.3 ± 11.36
3f	97.6 ± 5.31	101.9 ± 3.26
3g	87.86 ± 5.24[e]	97.6 ± 6.46
3h	99.6 ± 3.43	97.9 ± 6.98
3i	83.2 ± 5.35[e]	99.3 ± 6.66
3j	96.0 ± 6.64	105.0 ± 7.76
3k	109.1 ± 8.62	96.0 ± 6.67
3m	93.9 ± 5.63[e]	101.0 ± 3.16
3n	92.2 ± 4.47[e]	100.4 ± 3.52
5c	99.1 ± 5.78	103.0 ± 4.81
5d	99.5 ± 5.33	98.5 ± 6.38
5e	100.5 ± 4.28	103.4 ± 7.06

[a] Average of 570 nm absorbance value of cells cultured normally only with fresh medium. [b] *Versus* to 100% control. [c] **BBR**: berberine. [d] Indo: indomethacin. [e] Significantly different *vs.* control group ($p < 0.05$). The given values are means ± standard error of three independent assays.

derivatives (**3g** and **3i**) showed some toxicity to RAW264.7 cells at the concentration of 10 μM, while all the derivatives had no obvious cytotoxicity to RAW264.7 cells at the concentration of 5 μM of **BBR** and indomethacin; the relative cell viabilities of the treated cells were >95%. This non-toxic concentration (5 μM) was further used in subsequent experiment processes.

Effects on NO release in RAW264.7 cells

Nitric oxide (NO) is a very active radical species that can greatly promote the process of inflammation and it is generally accepted that the presence of NO can be used as evidence of inflammation. Accordingly, the synthetic derivatives were first tested for their inhibition of NO production in LPS-induced RAW264.7 cells. It has been determined that stimulation of macrophages by LPS can significantly induce an increase in NO release. Berberine and indomethacin were also chosen as positive controls and as shown in Table 2, while most of the 9-*O*-substituted-berberine derivatives had little or no effect on the inhibition of NO release, two derivatives, **3i** and **5e**, displayed significant and improved inhibitory effects on the release of NO when compared to berberine. This was particularly true for **3i**, whose NO inhibitory rates were more than 70% at a concentration of 5 μM, much greater than that of berberine (21.1% ± 3.56%). Additionally, concentration gradient experiments further demonstrated the inhibitory effects of **3i** and **5e** on NO release, and the inhibitory effects are concentration dependent (see Fig. 2).

Effects on TNF-α and IL-6 production in RAW264.7 cells

TNF-α and IL-6 are two crucial pro-inflammatory cytokines of paramount importance in inflammatory diseases. To confirm the anti-inflammatory effects of derivatives **3i** and **5e**, we used enzyme-linked immunosorbent assay (ELISA) to measure their effects on the production of TNF-α and IL-6 in the medium. **BBR** and indomethacin were used as positive reference compounds, and the experiments were performed at a fixed concentration of 5 μM. As shown in Fig. 3A and B, derivatives **3i** and **5e** reduce not only the production of TNF-α but also the production of IL-6. Notably, treatment with **3i** and **5e**, especially with **5e**, results in a dramatic decrease in TNF-α production. Their inhibitory rates were more than 70% and 90% respectively, which were significantly better than that of berberine.

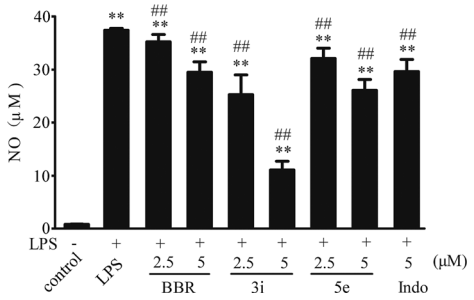


Fig. 2 Effects of berberine and its derivatives **3i** and **5e** on NO release in LPS-induced RAW264.7 cells. The experiments were performed using 2.5 μM and 5 μM of berberine and derivatives **3i** and **5e**. All values are the mean ± S.D. (n = 3–5). *Significantly different *vs.* control group ($p < 0.05$). #Significantly different *vs.* LPS group ($p < 0.05$).

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

Neutrophils and macrophages are two important immune cells in the living body. When inflammation occurs *in vivo*, they are produced in higher quantities and move towards the sites of inflammation. Consequently, transgenic zebrafish in which both macrophages and neutrophils were marked by a fluorescent protein is a useful model for the investigation of anti-inflammatory effects *in vivo*. In the present study, a double-transgenic line (*Coronilla-eGFP/Lyc-dsRed*) was used, in which neutrophils were doubly labeled with eGFP and dsRed, and primitive macrophages were labeled with eGFP only; thus, neutrophils and primitive macrophages could be distinguished by yellow (neutrophils) and green (primitive macrophages) fluorescence. The injury inflammation was induced by tail cutting, and berberine was used as a control. As shown in Fig. 4, few neutrophils (yellow) and primitive macrophages (green) were observed, mainly in the head and posterior blood posterior blood island (PBI) of the blank group for 3 h, 6 h, or 9 h. For the control group, after tail

Table 2 Effects of berberine and berberine derivatives on NO release in LPS-induced RAW264.7 cells

Compound	NO inhibition[a] (%) 5 μM	Compound	NO inhibition[a] (%) 5 μM
BBR[c]	21.1 ± 3.56**		
Indo[d]	20.8 ± 2.16**	**3i**	70.3 ± 1.55**
3a	nd[b]	**3j**	nd[b]
3b	nd[b]	**3k**	nd[b]
3c	nd[b]	**3m**	nd[b]
3e	12.9 ± 2.31**	**3n**	nd[b]
3f	nd[b]	**5c**	nd[b]
3g	nd[b]	**5d**	nd[b]
3h	9.7 ± 2.53*	**5e**	30.2 ± 1.92**

[a] The decrease of NO release *versus* the average of the amount of NO that released by cells treated by LPS only. The given values are means ± standard error of three independent assays. [b] "nd" means "not determined". [c] **BBR**: berberine. [d] Indo: indomethacin. *Significantly different *vs.* LPS group (*$p < 0.05$, **$p < 0.01$). Indo and **BBR** were as positive controls.

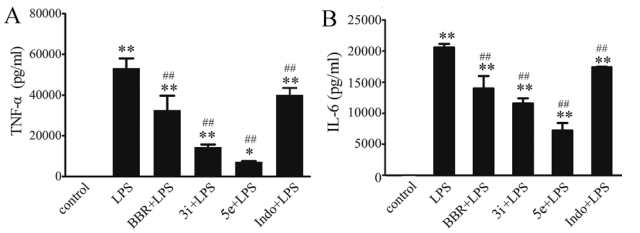


Fig. 3 Effects of berberine and derivatives **3i** and **5e** on the release of inflammatory cytokines TNF-α and IL-6 in LPS-induced RAW264.7 cells. The experiments were performed using a fixed concentration of 5 μM. All values are the mean ± S.D. (n = 3–5). *Significantly different *vs.* control group ($p < 0.05$). # Significantly different *vs.* LPS group ($p < 0.05$).

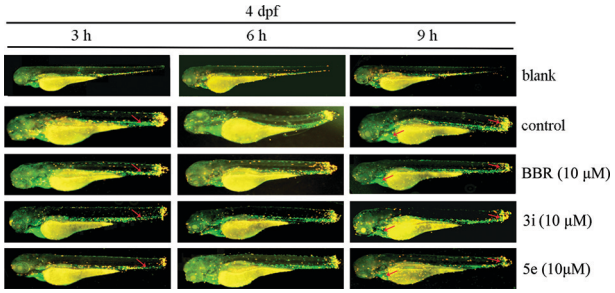


Fig. 4 Effects of berberine and derivatives **3i** and **5e** on the migration of neutrophils and macrophage in injured transgenic zebrafish larvae. 30 min after tail cutting, 4 dpf transgenic zebrafish larvae were treated with 10 μM of berberine and derivatives **3i** and **5e**. The image of neutrophil (yellow) and macrophage (green) in 3 h, 6 h, and 9 h were recorded by using a fluorescence microscope. The blank was the healthy larvae. The control was the cut-tail larvae without compound treatment. The fluoresence of 6–8 larvae in each group was recorded.

cutting, neutrophils evidently increased, greatly accumulating in the wound site, while primitive macrophages increased in the head and PBI and migrated towards the vicinity of the wound, which is consistent with the behavior described in other zebrafish inflammatory experiments.[29,30] Compared to the control group, the level of neutrophils was evidently reduced in the wounds of injured zebrafish larvae by treatment with 10 μM of derivatives **3i** and **5e** for 3 h, 6 h or 9 h. On the other hand, while primitive macrophage levels were also increased in the head, PBI and wound sites after treatment with **3i** and **5e** for 3 h and 6 h, but they migrated towards the wound more slowly than in the control group, and after treatment for 9 h, primitive macrophage levels were evidently reduced in groups treated by **3i** or **5e**. It is striking that **3i** and **5e** are more effective than berberine in inhibiting the expression and migration of primitive macrophage and neutrophils.

All these results suggest that **3i** and **5e** are excellent anti-inflammatory agents. Next, an attempt was made to explore the possible anti-inflammatory mechanism driven by **3i** and **5e**.

Western blotting for interpretation of possible mechanisms

It has been reported that the NF-κB signaling pathway is significantly involved in the regulation of inflammation in LPS-activated macrophages.[31,32] Generally, LPS can induce phosphorylation and the subsequent degradation of NF-κB inhibitor (IκB),[33] which can then lead to the activation of NF-κB and the expression of inflammatory genes.[34] The inducible nitric oxide synthase (iNOS) protein is one of the most important proteins, which is induced by various stimuli such as bacterial LPS, under the regulation of NF-κB.[35,36] Moreover, recent studies have reported that berberine can inhibit the LPS-induced phosphorylation of IκBα, activation of NF-κB p65, and expression of iNOS.[37] Therefore, in the present study, western blot analysis was employed to investigate whether the anti-inflammatory effects of derivatives **3i** and **5e** are associated with the inhibition of the NF-κB pathway, and subsequently, the repression of the expression of iNOS in LPS-stimulated macrophages. The proteins we detected included two NF-κB pathway proteins: NF-κB p65 and inhibitor kappa B alpha (IκBα) kinase as well as iNOS.

As shown in Fig. 5, when cells are pretreated with berberine or **3i** and **5e** for 2 h before LPS stimulation, the expression levels of phosphorylated NF-κB p65, IκBα and iNOS protein decreased in a concentration-dependent manner. These results indicated that, similar to berberine, the anti-inflammatory mechanisms driven by **3i** and **5e** were also at least partly associated with the suppression of the NF-κB pathway, resulting in a decrease in iNOS protein expression and NO, TNF-α and IL-6 release.

Conclusions

A series of novel 9-*O*-substituted-berberine derivatives have been synthesized and their anti-inflammatory activities were evaluated. Among these compounds, two promising anti-inflammatory agents (**3i** and **5e**) were identified, with improved anti-inflammatory activity over berberine, measured by both a decrease in the levels of NO, TNF-α and IL-6 *in vitro*, and the inhibition of migration of neutrophils and primitive macrophage in injured transgenic zebrafish larvae. Mechanism studies revealed that, like berberine, the anti-inflammatory activity of **3i** and **5e** was also due at least in part, to their suppression of the activation of the NF-κB pathway, thereby suppressing the expression of the iNOS protein. These results also illustrated that the introduction of hydrophobic groups to the C-9-*O* position of berberine is a feasible

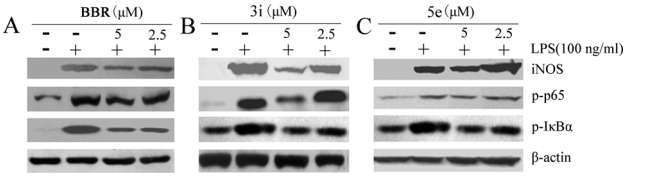


Fig. 5 Berberine and derivatives **3i** and **5e** inhibited the protein expression of iNOS and the phosphorylation of NF-κB p65 and IκBα in LPS-induced RAW264.7 cells. Cells were pretreated with berberine or derivatives **3i** and **5e** for 2 h, and then stimulated with 100 ng ml^{-1} LPS for 6 h. Protein expression of iNOS and phosphorylation of NF-κB p65 and IκBα were detected using western blot analysis.

way to obtain more effective anti-inflammatory agents from berberine. Additionally, we noticed that the improved anti-inflammatory activity of derivatives **3i** and **5e** was not consistent with their cytotoxicity, which suggests that hydrophobicity may be more important than hydrophilicity in driving anti-inflammatory activity.

Experimental

Chemistry

^{1}H and ^{13}C NMR spectra were recorded using TMS as the internal standard on a Bruker-AV spectrometer at 300 MHz and 75 MHz, respectively; ESI mass spectra were recorded on a LCQ advantage MAX or API 2000 instruments. Silica gel (200–300 mesh) used in a flash column was purchased from Qingdao Haiyang Chemical Co. Ltd.

Berberine chloride (purity of 98%) was obtained from Shanghai Medical Technology Co. Ltd., China. All other reagents were obtained commercially. CH_2Cl_2, pyridine and Et_3N were purified by distillation before use. The purity (≥98%) of the samples was determined by high-performance liquid chromatography (HPLC), conducted on a DIONEX Ultimate-3000 series system, a COMSMOSIL-C18 column (4.6 mm × 250 mm, 5 μm), eluted with a 15 : 85 or 25 : 75 H_2O/MeOH mixture containing 0.2% diethylamine, at a flow rate of 0.5 mL min^{-1}. The syntheses of the compounds were performed as follows.

Berberrubine (1)

Berberine was heated to 190 °C in vacuum to produce berberrubine which was obtained as a dark red solid; yield: 35.8%. MS(ESI): *m*/*z* 322.0 [M–Cl]$^+$, ^{1}H-NMR (CD_3OD, 300 MHz) *δ*: 3.13 (t, *J* = 6.3 Hz, 2H), 3.91 (s, 2H), 4.57 (t, *J* = 6.3 Hz, 2H), 6.05 (s, 2H), 6.79 (s, 1H), 7.34 (s, 1H), 7.50 (m, 2H), 7.88 (s, 1H), 9.24 (s, 1H). The data of ^{1}H NMR was in accordance with the literature.[38]

General procedure for the preparation of carbamoyl chlorides 2a–2n

Triphosgene (1.5 mmol) in dried CH_2Cl_2 (5 ml) and dried Et_3N (1 drop) as catalyst was added over 50 min at −10 °C to the corresponding amine (1.5 mmol) in CH_2Cl_2 (5 ml). After being stirred for 12 h at room temperature, the solvent was evaporated under vacuum to give the corresponding carbamoyl chloride **2a–2n**, which was used in the next reaction without further purification.

General procedure for the preparation of berberine derivatives 3a–3n

Berberrubine (0.3 mmol) was added in one portion to the carbamoyl chloride (1.5 mmol) obtained from the last step, and dissolved in dry pyridine (25 ml). The mixture was stirred at 25–45 °C for 1–2 hours. When the reaction was complete, the pyridine was evaporated and the residue was purified by flash column chromatography (SiO_2, $CHCl_3$: MeOH = 10 : 1) to give **3a–3n**.

9-*O*-((1,4′-Bipiperidine)-1′-carbonyl)berberrubine (3a). Yellow solid; yield: 85.7%. MS(ESI): *m*/*z* 516.6 [M–Cl]$^+$, ^{1}H-NMR (CD_3OD, 300 MHz) *δ*: 1.78–2.38 (m, 10H), 3.11 (t, *J* = 6.3 Hz, 2H), 3.25 (m, 2H), 3.54–3.68 (m, 3H), 4.09 (s, 3H), 4.35–4.7 (m, 2H), 5.01 (t, *J* = 6.3 Hz, 2H), 6.11 (s, 2H), 7.66 (s, 1H), 8.17 (m, 2H), 8.77 (s. 1H), 9.81 (s, 1H); ^{13}C-NMR (CD_3OD, 75 MHz) *δ*: 14.0, 21.6, 23.1, 25.9, 26.3, 26.7, 42.9, 43.4, 50.0, 55.9, 56.5, 63.3, 65.5, 102.3, 105.2, 108.0, 120.3, 120.5, 122.2, 125.5, 126.4, 130.7, 133.5, 134.8, 138.7, 144.1, 148.5, 150.9, 151.3, 152.2.

9-*O*-(4-(1-Methylpiperidin-4-yl)piperazine-1-carbonyl)-berberrubine (3b). Yellow solid; yield: 63.5%. MS(ESI): *m*/*z* 531.6 [M–Cl]$^+$, ^{1}H- NMR (D_2O, 300 MHz) *δ*: 2.20 (m, 2H), 2.61 (m, 2H), 3.32 (s, 3H), 3.51–3.41 (m, 3H), 3.90–3.68 (m, 8H), 4.28 (s, 3H), 4.60 (d, *J* = 8.4 Hz, 1H), 4.92 (d, *J* = 6.9 Hz, 1H), 6.31 (s, 2H), 7.14 (s, 1H), 7.40 (s, 1H), 8.19 (d, *J* = 12.3 Hz, 2H), 8.40 (s, 1H), 9.75 (s, 1H); ^{13}C-NMR (D_2O, 75 MHz) *δ*: 26.7, 43.1, 46.3, 52.0, 56.3, 57.2, 62.2, 102.6, 105.0, 108.6, 109.8, 119.5, 120.0, 121.4, 125.6, 126.7, 130.3, 133.0, 138.1, 143.4, 147.9, 150.4, 150.8, 153.4.

9-*O*-(4-(4-Methylpiperazin-1-yl)piperidine-1-carbonyl)-berberrubine (3c). Yellow solid; yield: 70.9%. MS(ESI): *m*/*z* 531.5 [M–Cl]$^+$, ^{1}H-NMR (D_2O, 300 MHz) *δ*: 2.28–2.39 (m, 2H), 2.67–2.80 (m, 2H), 3.17 (s, 3H), 3.23–3.54 (m, 5H), 3.67–4.02 (m, 10H), 4.19 (s, 3H), 4.45 (m, 2H), 6.20 (s, 2H), 7.04 (s, 1H), 7.34 (s, 1H), 8.09 (m, 2H,), 8.36 (s, 1H), 9.75 (s, 1H); ^{13}C-NMR (D_2O, 75 MHz) *δ*: 24.8, 26.7, 41.9, 43.2, 49.0, 52.9, 56.3, 57.3, 60.0, 102.6, 105.1, 108.6, 119.7, 120.1, 121.4, 125.7, 130.4, 133.2, 138.3, 143.5, 147.9, 150.4, 150.8, 153.0.

9-*O*-(4-(Pyrrolidin-1-yl)piperidine-1-carbonyl)berberrubine (3d). Yellow solid; yield: 63.9%. MS(ESI): *m*/*z* 502.5 [M–Cl]$^+$, ^{1}H-NMR ($CDCl_3$–CD_3OD, 300 MHz) *δ*: 1.80–2.29 (m, 8H), 3.19–3.25 (m, 4H), 3.47–3.71 (m, 4H), 4.11 (s, 3H), 4.36 (m, 2H), 5.07 (m, 2H), 6.12 (s, 2H), 6.92 (s, 1H), 7.61 (s, 1H), 8.20 (d, *J* = 8.7 Hz, 1H), 8.09 (d, *J* = 8.7 Hz, 1H), 8.72 (s, 1H), 9.81 (s, 1H); ^{13}C-NMR ($CDCl_3$–CD_3OD, 75 MHz) *δ*: 7.3, 22.7, 27.0, 42.9, 43.3, 51.6, 56.1, 56.9, 58.5, 61.8, 102.4, 105.4, 108.3, 120.8, 122.4, 125.6, 126.6, 130.4, 133.5, 134.7, 138.7, 143.8, 148.7, 151.1, 151.5, 152.1.

9-*O*-(4-(Pyridin-2-yl)piperazine-1-carbonyl)berberrubine (3e). Yellow solid; yield: 77.3%. MS(ESI): *m*/*z* 511.5 [M–Cl]$^+$, ^{1}H-NMR ($CDCl_3$–CD_3OD, 300 MHz) *δ*: 3.26 (t, *J* = 6.2 Hz, 2H), 3.66–3.37 (m, 6H), 3.39–4.06 (m, 2H), 4.10 (s, 3H), 4.99 (t, *J* = 6.2 Hz, 2H), 6.12 (s, 2H), 7.51 (s, 1H), 7.62 (m, 1H), 8.19 (d, *J* = 9.0 Hz, 1H), 8.07 (d, *J* = 9.0 Hz, 1H), 8.14–8.17 (m, 1H), 8.67 (s, 1H), 9.77 (s, 1H); ^{13}C-NMR ($CDCl_3$–CD_3OD, 75 MHz) *δ*: 44.0, 44.8, 45.1, 45.2, 56.1, 56.9, 77.7, 102.4, 105.4, 108.3, 108.4, 114.2, 120.0, 122.4, 125.8, 126.6, 130.2, 133.6, 135.0, 138.4, 138.7, 148.8, 151.1, 151.4, 152.5, 159.0.

9-*O*-(4-(Pyrimidin-2-yl)piperazine-1-carbonyl)berberrubine (3f). Yellow solid; yield: 66.2%. MS(ESI): *m*/*z* 512.1 [M–Cl]$^+$, ^{1}H-NMR ($CDCl_3$–CD_3OD, 300 MHz) *δ*: 3.24 (m, 2H), 3.71–3.72 (m, 2H), 3.96–3.98 (m, 4H), 4.10 (s, 3H), 4.96 (t, *J* = 6.3 Hz, 2H), 6.11 (s, 2H), 6.66 (m, 2H), 6.91 (s, 1H), 7.60 (s, 1H), 8.20

(d, J = 9.0 Hz, 1H), 8.09 (d, J = 9.0 Hz, 1H), 8.35 (s, 1H), 8.72 (s, 1H), 9.75 (s, 1H); ^{13}C-NMR ($CDCl_3$–CD_3OD, 75 MHz) δ: 27.9, 41.3, 44.0, 47.5, 57.0, 57.8, 103.3, 106.3, 109.2, 111.5, 112.4, 121.0, 121.8, 123.3, 126.6, 127.5, 131.2, 134.5, 135.9, 139.6, 144.8, 149.7, 152.1, 152.3, 153.5, 158.8, 158.8, 162.2.

9-*O*-(4-Benzylpiperidine-1-carbonyl)berberrubine (3g). Yellow solid; yield: 56.3%. MS(ESI): m/z 524.5 $[M–Cl]^+$, ^{1}H-NMR ($CDCl_3$–CD_3OD, 300 MHz) δ: 2.62 (br, 2H), 2.84–2.93 (m, 2H), 3.24 (t, J = 6.3 Hz, 2H), 3.34–3.38 (m, 2H), 3.72 (br, 2H), 3.84–4.03 (m, 2H), 4.06 (s, 3H), 5.04 (t, J = 6.3 Hz, 2H), 6.11 (s, 2H), 6.88 (s, 1H), 7.27–7.40 (m, 5H), 7.53 (s, 1H), 8.15 (d, J = 9.3 Hz, 1H), 8.02 (d, J = 9.3 Hz, 1H), 8.60 (s, 1H), 9.88 (s, 1H); ^{13}C-NMR ($CDCl_3$–CD_3OD, 75 MHz) δ: 27.2, 51.7, 52.1, 56.0, 57.0, 102.4, 105.4, 108.5, 120.0, 120.7, 122.5, 125.7, 126.5, 128.2, 128.5, 128.6, 129.9, 129.9, 130.0, 130.1, 130.2, 133.5, 135.0, 137.9, 138.6, 144.1, 148.8, 151.2, 151.4.

9-*O*-(4-(Ethoxycarbonyl)piperazine-1-carbonyl)berberrubine (3h). Yellow solid; yield: 51.6%. MS(ESI): m/z 506.1 $[M–Cl]^+$, ^{1}H-NMR ($CDCl_3$–CD_3OD, 300 MHz) δ: 1.31 (t, J = 3.3 Hz, 3H), 3.24 (t, J = 6.3 Hz, 2H), 3.63 (s, 4H), 3.25 (dt, J = 13.5 Hz, 4H), 4.07 (s, 3H), 4.19 (q, J = 7.2 Hz, 2H), 4.99 (t, J = 6.3 Hz, 3H), 6.09 (s, 2H), 6.89 (s, 1H), 7.57 (s, 1H), 8.08 (m, 2H), 8.68 (s, 1H), 9.80 (s, 1H); ^{13}C-NMR ($CDCl_3$–CD_3OD, 75 MHz) δ: 14.5, 27.5, 43.6, 44.5, 45.3, 56.5, 57.4, 62.4, 102.8, 105.9, 108.8, 120.5, 121.3, 122.8, 126.1, 127.1, 130.7, 134.0, 135.3, 139.1, 144.4, 149.2, 151.6, 151.8, 152.9, 156.2.

9-*O*-(Cyclohexyl(methyl)carbamoyl)berberrubine (3i). Yellow solid; yield: 66.1%. MS(ESI): m/z 461.2 $[M–Cl]^+$, HRMS (ESI) calcd for $C_{27}H_{29}N_2O_5$ $[M–Cl]^+$ 461.2071, found m/z 461.2100. ^{1}H-NMR ($CDCl_3$–CD_3OD, 300 MHz) δ: 1.22 (m, 2H), 1.43 (m, 2H), 1.65 (m, 3H), 1.89 (m, 3H), 2.02 (s, 1H), 2.99, 3.18,(s, 3H), 3.26 (t, J = 6.3 Hz, 2H), 3.88–4.04, 4.20–4.34 (m, 1H), 4.09 (s, 3H), 4.96 (t, J = 6.3 Hz, 2H), 6.12 (s, 1H), 7.63 (s, 1H), 8.10 (m, 2H), 8.75 (s, 1H), 9.61, 9.70 (s, 1H); ^{13}C-NMR ($CDCl_3$–CD_3OD, 75 MHz) δ: 25.1, 25.5, 25.5, 27.0, 28.7, 29.0, 29.6, 30.2, 56.0, 56.1, 56.5, 56.7, 56.8, 102.3, 105.4, 108.2, 120.1, 120.8, 122.4, 122.5, 125.6, 125.8, 126.2, 126.4, 130.2, 133.6, 135.5, 138.6, 143.8, 148.7, 151.1, 151.4, 153.6.

9-*O*-(Morpholine-4-carbonyl)berberrubine (3j). Yellow solid; yield: 56.7%. MS(ESI): m/z 435.6 $[M–Cl]^+$, ^{1}H-NMR ($CDCl_3$–CD_3OD, 300 MHz) δ: 3.26 (t, J = 6.2 Hz, 2H), 3.59–3.68 (m, 2H), 3.82–3.85 (m, 2H), 3.86–3.95 (m, 4H), 4.11 (s, 1H), 4.99 (t, J = 6.2 Hz, 2H), 6.12 (s, 2H), 6.90 (s, 1H), 7.57 (s, 1H), 8.18 (d, J = 9.0 Hz, 1H), 8.07 (d, J = 9.0 Hz, 1H), 8.66 (s, 1H), 9.78 (s, 1H); ^{13}C-NMR ($CDCl_3$–CD_3OD, 75 MHz) δ: 27.1, 44.6, 45.5, 56.1, 56.9, 66.3, 77.7, 102.4, 105.4, 108.4, 120.0, 120.8, 122.4, 125.7, 126.6, 130.2, 133.6, 134.9, 138.7, 143.9, 148.8, 151.1, 151.4, 152.5.

9-*O*-(Thiomorpholine-4-carbonyl)berberrubine (3k). Yellow solid; yield: 64.6%. MS(ESI): m/z 451.3 $[M–Cl]^+$, ^{1}H-NMR ($CDCl_3$–CD_3OD, 300 MHz) δ: 2.76 (m, 2H), 2.85 (m, 2H), 3.24 (t, J = 6.2 Hz, 2H), 3.88 (m, 2H), 4.09 (s, 3H), 4.15 (m, 2H), 4.95 (t, J = 6.2 Hz, 2H), 6.10 (s, 1H), 6.91 (s, 1H), 7.63 (s, 1H), 8.18 (d, J = 9.0 Hz, 1H), 8.09 (d, J = 9.0 Hz, 1H), 8.71 (s, 1H), 9.69 (s, 1H); ^{13}C-NMR ($CDCl_3$–CD_3OD, 75 MHz) δ: 27.7, 27.9, 28.0, 57.0, 57.8, 103.3, 106.3, 109.2, 121.0, 121.8, 123.3, 126.6, 127.5, 131.2, 134.5, 136.0, 144.8, 149.7, 152.1, 152.2, 153.4.

9-*O*-(2-(Methoxycarbonyl)pyrrolidine-1-carbonyl)-berberrubine (3l). Yellow solid; yield: 44.6%. MS (ESI): m/z 477.2 $[M–Cl]^+$, ^{1}H-NMR ($CDCl_3$–CD_3OD, 300 MHz) δ: 2.06–2.16 (m, 4H), 2.30–2.50 (m, 1H), 3.23–3.27 (m, 2H), 3.62–3.64 (m, 2H), 3.67, 3.68 (s, 3H), 3.95, 3.97 (s, 3H), 4.87–4.97 (m, 2H), 6.07 (s, 1H), 6.93 (s, 1H), 7.57 (s, 1H), 8.03–8.10 (m, 2H), 8.67 (s, 1H), 9.58, 9.69 (s, 1H); ^{13}C-NMR ($CDCl_3$–CD_3OD, 75 MHz) δ: 24.5, 25.5, 28.1, 31.1, 32.1, 53.2, 53.5, 57.5, 57.7, 57.9, 58.0, 61.0, 61.3, 103.8, 106.7, 109.5, 121.6, 121.9, 123.4, 123.6, 127.0, 127.1, 128.1, 132.0, 134.9, 135.4, 135.9, 140.0, 145.4, 149.9, 152.3, 152.8, 152.9, 153.2.

9-*O*-(Dipropylcarbamoyl)berberrubine (3m). Yellow solid; yield: 92.8%. MS(ESI): m/z 449.2 $[M–Cl]^+$, ^{1}H-NMR ($CDCl_3$–CD_3OD, 300 MHz) δ: 1.69–1.74 (m, 6H), 1.74–1.90 (m, 2H), 2.91 (t, J = 8.1 Hz, 2H), 3.25 (t, J = 6.2 Hz, 3H), 3.56(t, J = 7.8 Hz, 2H), 4.06 (s, 3H), 4.95 (t, J = 6.2 Hz, 2H), 6.09 (s, 1H), 6.91 (s, 1H), 7.60 (s, 1H), 8.18 (d, J = 9.0 Hz, 1H), 8.08 (d, J = 9.0 Hz, 1H), 8.73 (s, 1H), 9.60 (s, 1H); ^{13}C-NMR ($CDCl_3$-CD_3OD, 75 MHz) δ: 11.3, 11.5, 20.3, 21.9, 22.7, 28.0, 50.9, 51.1, 57.7, 103.3, 106.4, 109.3, 121.1, 121.9, 123.4, 126.7, 127.3, 131.3, 134.6, 136.3, 139.6, 144.7, 149.7, 152.0, 152.4, 154.7.

9-*O*-(Diallylcarbamoyl)berberrubine (3n). Yellow solid; yield: 74.9%. MS(ESI): m/z 445.3 $[M–Cl]^+$, ^{1}H-NMR (DMSO-d_6, 300 MHz) δ: 3.22 (m 2H), 3.95 (d, J = 5.2 Hz, 2H), 4.03 (s, 3H), 4.17 (d, J = 4.5 Hz, 2H), 4.96 (t, J = 6.1 Hz, 2H), 5.28–5.39 (m, 4H), 5.88 (ddd, J = 22.3, 10.3, 5.3 Hz, 1H), 6.07 (ddd, J = 15.9, 10.5, 5.4 Hz, 1H), 6.18 (s, 2H), 7.10 (s, 2H), 7.81 (s, 1H), 8.26 (d, J = 9.3 Hz, 1H), 8.18 (d, J = 9.3 Hz, 1H), 9.03 (s, 1H), 9.78 (s, 1H); ^{13}C-NMR (DMSO-d_6, 75 MHz) δ: 26.2, 49.5, 49.7, 55.5, 57.3, 102.1, 105.6, 108.5, 117.2, 117.3, 120.4, 120.6, 121.6, 126.1, 126.2, 130.9, 132.8, 132.9, 133.6, 134.7, 144.4, 147.7, 150.0, 150.8, 152.3.

General procedure for the synthesis of 9-*O*-(ω-bromoalkyl)berberrubine 4a–4b

Alkyl bromides (1,2-dibromoethane or 1,3-dibromopropane; 15 mmol) and K_2CO_3 (15 mmol) were added to a stirred solution of berberrubine (3.1 mmol) in MeCN (30 ml) at 60 °C. When the reaction (monitored by TLC) was complete, the suspension was filtered. The residue obtained by evaporation from the filtrate was then purified by flash column chromatography (SiO_2, $CHCl_3$: MeOH = 12 : 1) to give corresponding bromide **4a–4b**.

General procedure for the synthesis of berberine derivatives 5a–5e

K_2CO_3 (3 mmol) and corresponding amines (1 mmol) were added to 9-*O*-(ω-bromoalkyl)berberrubine (**4a–4b**) (1 mmol) in MeCN (20 ml) at 80 °C. After stirring for 12–24 h, the solution was filtered and the filtrate was concentrated under reduced pressure. The residue was purified by flash column chromatography (SiO_2, $CHCl_3$: MeOH = 12 : 1) to give **5a–5e**.

9-*O*-(2-(4-Ethylpiperazin-1-yl)ethyl)berberrubine (5a). Yellow solid; yield: 33.5%. MS(ESI): *m*/*z* 462.5 $[M-Br]^+$, ^{1}H-NMR ($CDCl_3$–CD_3OD, 300 MHz) δ: 1.38 (t, J = 7.3 Hz, 3H), 2.74–2.82 (m, 2H), 3.01–3.17 (m, 10H), 3.23 (m, 2H), 4.12 (s, 3H), 4.57 (t, J = 5.2 Hz, 2H), 5.06 (t, J = 6.2 Hz, 2H), 6.11 (s, 2H), 6.93 (s, 1H), 7.62 (s, 1H), 8.05 (q, J = 9.2 Hz, 2H), 8.67 (s, 1H), 9.89 (s, 1H); ^{13}C-NMR ($CDCl_3$–CD_3OD, 75 MHz) δ: 8.9, 27.1, 43.3, 50.1, 51.3, 51.8, 56.1, 56.5, 56.8, 70.7, 102.3, 105.3, 108.2, 120.3, 122.1, 123.7, 126.2, 130.2, 133.7, 138.3, 143.0, 145.0, 148.6, 150.6, 150.8.

9-*O*-(2-(4-(Ethoxycarbonyl)piperazin-1-yl)ethyl)berberrubine (5b). Yellow solid; yield: 39.7%. MS(ESI): *m*/*z* 506.4 $[M-Br]^+$, ^{1}H-NMR ($CDCl_3$–CD_3OD, 300 MHz) δ: 1.26 (t, J = 7.1 Hz, 3H), 2.61–2.65 (m, 4H), 2.98 (t, 2H), 3.27 (t, J = 6.2 Hz, 2H), 3.50 (m, 4H), 4.10 (s, 3H), 4.13 (q, J = 6.9 Hz, 2H), 4.56 (t, J = 5.3 Hz, 2H), 4.99 (t, J = 6.2 Hz, 2H), 6.10 (s, 2H), 6.89 (s, 1H), 7.54 (s, 1H), 8.00 (m, 2H), 8.56 (s, 1H), 9.93 (s, 1H); ^{13}C-NMR ($CDCl_3$–CD_3OD, 75 MHz) δ: 14.2, 27.3, 43.3, 52.8, 56.1, 56.8, 57.9, 61.7, 70.6, 102.3, 105.3, 108.4, 120.1, 120.4, 122.3, 123.6, 126.4, 130.0, 133.6, 138.2, 143.3, 145.1, 148.7, 150.6, 151.0, 155.8.

9-*O*-(3-(4-(Ethoxycarbonyl)piperazin-1-yl)propyl)-berberrubine (5c). Yellow solid; yield: 63.3%. MS(ESI): *m*/*z* 520.5 $[M-Br]^+$, ^{1}H-NMR ($CDCl_3$–CD_3OD, 300 MHz) δ: 1.25 (t, J = 7.2 Hz, 3H), 2.19 (m, 2H), 2.56 (m, 4H), 2.72 (t, J = 7.8 Hz, 2H), 3.26 (t, J = 6.2 Hz, 2H), 3.54 (m, 4H), 4.06 (s, 3H), 4.12 (q, J = 7.2 Hz, 2H), 4.46 (t, J = 6.4 Hz, 2H), 5.04 (t, J = 6.2 Hz, 2H), 6.08 (s, 2H), 6.85 (s, 1H), 7.47 (s, 1H), 7.94 (m, 2H), 8.47 (s, 1H), 9.85 (s, 1H); ^{13}C-NMR ($CDCl_3$–CD_3OD, 75 MHz) δ: 14.4, 27.2, 27.4, 43.2, 44.5, 52.7, 54.8, 56.2, 56.9, 61.7. 73.0, 102.3, 105.3, 108.5, 120.1, 120.3, 122.2, 123.2, 126.5, 130.1, 133.6, 138.1, 143.7, 145.0, 148.6, 150.5, 150.9, 155.7.

9-*O*-(3-(4-(Ethoxycarbonyl)piperidin-1-yl)propyl)-berberrubine (5d). Yellow solid; yield: 58.3%. MS(ESI): *m*/*z* 520.5 $[M-Br]^+$, ^{1}H-NMR ($CDCl_3$–CD_3OD, 300 MHz) δ: 1.26 (t, J = 7.1 Hz, 3H), 2.06–2.11 (m, 2H), 2.14–2.24 (m, 2H),2.40–2.49 (m, 2H), 2.65 (m, 1H), 3.09 (br, 2H), 3.24 (t, J = 6.0 Hz, 2H), 3.35–3.47 (m, 4H), 4.07 (s, 3H), 4.16 (q, J = 7.1 Hz, 2H), 4.53 (t, J = 5.8 Hz, 2H), 5.16 (t, J = 6.2 Hz, 2H), 6.09 (s, 2H), 6.85 (s, 1H), 7.46 (s, 1H), 7.92 (m, 2H), 8.42 (s, 1H), 10.15 (s, 1H); ^{13}C-NMR ($CDCl_3$–CD_3OD, 75 MHz) δ: 13.9, 25.7, 25.8, 25.9, 27.3, 51.6, 54.3, 55.9, 57.0, 61.1, 71.6, 77.4, 102.3, 105.2, 108.5, 120.1, 121.8, 123.0, 126.5, 130.2, 133.5, 138.2, 143.5, 145.3, 148.6, 150.0, 150.9, 173.5.

9-*O*-(3-(4-(Pyridin-4-yl)piperazin-1-yl)propyl)berberrubine (5e). Yellow solid; yield: 44.9%. MS(ESI): *m*/*z* 526.5 $[M-Br]^+$, HRMS (ESI) calcd for $C_{31}H_{33}N_4O_4$ $[M-Br]^+$ 526.2575, found *m*/*z* 526.2570. ^{1}H-NMR ($CDCl_3$–CD_3OD, 300 MHz) δ: 2.34–2.16 (m, 2H) 2.78 (m, 5.0 Hz, 6H), 3.27 (t, J = 6.2 Hz, 2H), 3.73–3.58 (m, 4H), 4.10 (s, 3H), 4.50 (t, J = 6.5 Hz, 2H), 5.07 (t, J = 6.3 Hz, 2H), 6.09 (s, 2H), 6.87 (s, 1H), 6.98 (d, J = 7.4 Hz, 2H), 7.47 (s, 1H), 7.97 (s, 2H), 8.10 (d, J = 7.3 Hz, 2H), 8.50 (s, 1H),9.87 (s, 1H); ^{13}C-NMR ($CDCl_3$–CD_3OD, 75 MHz) δ: 27.2, 27.3, 45.9, 52.3, 54.5, 56.2, 56.9, 72.9, 77.6, 102.3, 105.2, 107.9, 108.5, 120.1, 120.3, 122.1, 123.2, 126.6, 130.2, 133.6, 138.2, 142.5, 143.7, 144.9, 148.6, 150.5, 150.9, 156.4.

Biological assays and experimental procedures

Cell culture and reagents

RAW264.7 murine macrophages were cultured in DMEM (Gibco) containing 10% FBS (Gibco), penicillin/streptomycin (Invitrogen) at 37 °C in a 5% CO_2 humidified atmosphere. All compounds, including indomethacin, berberine and derivatives were dissolved in DMSO to a concentration of 50 mM and the amount of DMSO was 0.1% (v/v) when the cells were treated. LPS (*Escherichia coli* 0127:B8) and Griess reagent (modified) (G4410) were purchased from Sigma. LPS was dissolved in sterile PBS at a concentration of 10 mg ml^{-1}. For inflammation experiments, cells were pretreated with compounds for 2 h prior to stimulation by LPS (100 ng ml^{-1}). TNF-α enzyme immunoassay kits (EK2822) and IL-6 enzyme immune assay kits (EK2062) were purchased from Multi Sciences Biotechnology. IP cell lysis buffer (P0013) and PMSF (ST506) were purchased from Beyotime Biotechnology. Anti-beta actin monoclonal antibody (E021020-01) was purchased from (EarthOx, LLC, CA). Primary anti-rabbit inducible NO synthase (iNOS) (#2982) and NF-κB pathway sampler kit (#9936S) including anti-phosphorylation of nuclear factor-κB (NF-κB) p65, anti-phosphorylation of IκBα (Ser32/36), anti-rabbit IgG HRP-linked antibody and anti-mouse IgG HRP-linked antibody were purchased from Cell Signaling Technology. BCA protein assay kit (KGPBCA) and enhanced ECL Detection Kit (KGP1124) were purchased from KeyGEN Biotechnology.

Cell cytotoxicity assay

Following incubation with compounds for 48 h, the cytotoxicity effects of the compounds were measured using an MTT assay. Briefly, 20 μL of 5 mg mL^{-1} MTT was added to each well and the cells were incubated further for 4 h. Then the cell medium in each well was removed and 150 μL of DMSO was added to each well to dissolve the formazan crystals in the well, and the optical density of the formazan solution was measured on a microplate reader (Synergy™ HT, BIOTEK, America) at 570 nm (OD_{570}). Control cells were cultured with fresh medium only. The optical density of formazan generated by control cells was taken as 100% survival. Cell viability (%) = compound (OD_{570})/control (OD_{570}) × 100%.

Amount of NO assay

When cells were stimulated by LPS for 24 h, cell supernatant was collected to determine the amount of NO with the Griess reagent according to the manufacturer's instructions. Briefly, 100 μl of Griess reagent was mixed with an equal volume of supernatant medium in a 96-well plate and the plate was incubated for 10 min at room temperature, and then measured on a microplate reader at 540 nm using serial dilutions of $NaNO_2$ as a standard. Indomethacin was used as a positive drug in this experiment. NO inhibition rate = [control (OD_{540}) − compound (OD_{540})]/[control (OD_{540}) − blank (OD_{540})] ×

100%. The control was treated with LPS only. The blank was cultured with fresh medium only.

Cytokine enzyme-linked immunosorbent assay (ELISA)

When cells were stimulated by LPS for 24 h, TNF-α and IL-6 levels in cell culture supernatant were measured by using commercially available mouse TNF-α and IL-6 enzyme immune assay kits according to the manufacturer's instructions. Indomethacin was as a positive drug in this experiment.

Zebrafish larvae live image analysis

Neutrophil/macrophage double transgenic zebrafish (*Coronilla-eGFP/Lyc-dsRed*), in which macrophage express is linked with eGFP and neutrophils express is double linked with eGFP and dsRed, was obtained from Zebrafish Research Centre of Southern Medical University in China. The adult zebrafish was raised separately in a water-purification system (manufactured by the Shanghai Aquarium Devices Company) at 28.5 °C with 14 h light and 10 h dark every day. When mating, female and male were transferred to the same mating pool in a ratio of 1 : 2. Embryos were collected to a dish and maintained by egg water (culture water containing 5–10% methylene blue, Sigma) and 0.003% phenylthiourea (PTU, Sigma) was added to egg water within 12–24 h to inhibit zebrafish melanin formation. Egg water was replaced every day. At 4 days post fertilization (dpf), healthy larvae were anesthetized by 0.1 mg ml^{-1} tricaine for 5 min; and the tails were cut by a sterile sharp blade, then washed by egg water for three times to remove the remaining tricaine and then recovered in egg water. 30 min later, the injured larvae were plated to a 24-well microplate and treated with compounds diluted in egg water. Cut-tail larvae maintained in egg water containing DMSO (0.2%) were used as a control and non-cut-tail larvae maintained in egg water were used as a blank. The fluorescence photos of larvae treated after 3 h, 6 h, and 9 h were recorded by using a fluorescence microscope (Olympus 1 × 71). The fluorescence of 6–8 larvae in each group was recorded. All experiments were performed in compliance with the relevant laws and institutional guidelines, and the Zebrafish Research Centre of Southern Medical University in China has approved the experiments.

Western blot analysis

After LPS-stimulation for 6 h, the cells were washed twice with ice-cold PBS and lysed with IP cell lysis buffer supplemented with 1 mM PMSF for 30 min at 4 °C. The cell lysates were centrifuged at 12 000 × *g* for 10 min at 4 °C, the supernatant was collected and the total protein concentration was determined using a BCA protein assay kit. An equal amount of protein was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked using 5% skim milk and sequentially incubated with primary antibodies at 4 °C overnight, and subsequently with secondary antibodies at room temperature for 2 h, followed by ECL detection.

Statistical analysis

Data are presented as means ± standard error. Student's *t*-test was used for comparisons between two experiments. A value of $p < 0.05$ was considered statistically significant.

Conflicts of interest

The authors declare no competing interest.

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