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Screening anti-inflammatory components from Chinese traditional medicines using a peritoneal macrophage/cell membrane chromatography-offline-GC/MS method

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ABSTRACT

We report the development of an analytical method combining cell membrane chromatography (CMC) with gas chromatography/mass spectrometry (GC/MS). This was applied to the purification and identification of anti-inflammatory components from traditional Chinese medicines. The stationary phase of the CMC employed mouse peritoneal macrophage (PM) cell membranes. We investigated the performance of the PM/CMC-offline-GC/MS method using hydrocortisone (HC) and dexamethasone (DM) as standards. The method was then applied to the identification of anti-inflammatory components in extracts of Rhizoma *Atractylodes macrocephala* (RAM) and Rhizoma *Atractylodes lancea* Thunb DC (RALD). The major components from both species retained by CMC were identified as atractylenolide I (AO-I) by GC/MS. Competition experiments' results showed that AO-I and lipopolysaccharide (LPS) bound competitively to cell surface receptors while AO-I and HC had only partly overlapping binding sites on the PM membrane. In vitro experiments revealed that AO-I was able to inhibit LPS-induction of TNF-α, IL-1β and NO production in a dose-dependent manner. IC50 values were 5.3 μg/mL, 5.1 μg/mL and 7.5 μg/mL, respectively. The PM/CMC-offline-GC/MS method is an effective screening system for the rapid detection, enrichment, and identification of target components from complex samples.

1. Introduction

Peritoneal macrophages (PMs) constitute an important class of immune cells. Receptors expressed at the cell surface include the toll-like receptors (TLRs) [1], membrane-bound glucocorticoid receptors (mGCRs) [2,3], leukotriene receptors (LTRs) [4], platelet activating factor (PAF) receptor [5,6], and vascular endothelial growth factor (VEGF) receptor-1 (Flt-1) [7]. The TLRs are perhaps the most important membrane receptors in relation to inflammatory processes in PMs. Binding of gram-negative bacterial endotoxin lipopolysaccharide (LPS) to plasma membrane TLRs leads to an inflammatory response by activation of the nuclear factor κB (NF-κB) pathway and the release of pro-inflammatory cytokines including tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β). Moreover, glucocorticoids such as hydrocortisone act both at plasma membrane mGCRs and at intracellular glucocorticoid receptors, and can reduce the inflammatory response by blocking the NF-κB pathway. These receptors provide important targets for drug development.

Cell membrane chromatography (CMC) offers a powerful approach to the study of ligand–receptor interactions [8,9]. Whereas radioactive ligand assay (RLA) is the standard method for studying these interactions [10,11], a significant correlation between results obtained with CMC and RLA has been reported [11–13]. CMC has previously been applied to the screening of medicinal plants for active components targeting membrane receptors [14–16]. Gas chromatography/mass spectrometry (GC/MS) is most commonly used for separation and identification of unknown components and is particularly applicable to volatile components often encountered in Chinese medicine [17,18]. We have therefore sought to develop a combined PM/CMC-offline-GC/MS method for the efficient detection and identification of active components in complex samples. We report here the development of a combined CMC–GC/MS method based on peritoneal macrophages (Fig. 1). The method was used to analyze two medicinal plants, Rhizoma *Atractylodes macrocephala* (RAM) and Rhizomza *Atractylodes lancea* (RAL), for anti-inflammatory compounds. Hydrocortisone and dexamethasone were used as positive controls. We report the preliminary characterization of pathways mediating the

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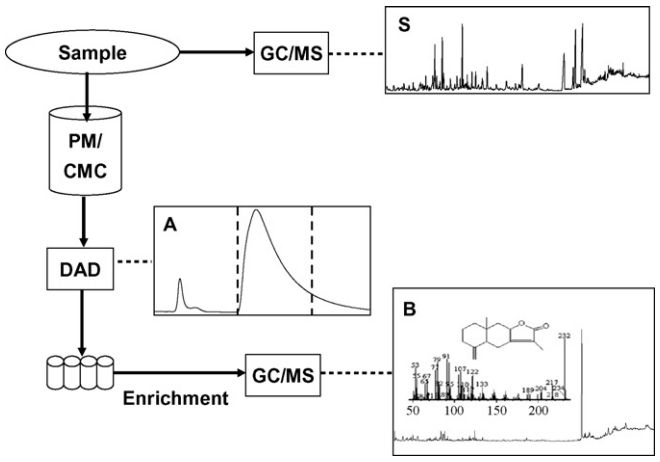

Fig. 1. Schematic outline of the PM/CMC-offline-GC/MS method. (A) CMC chromatography using PM/CMC; GC/MS, the GC/MS system. (B) Total ion current chromatograms and the mass spectra of the retention components. (S) Total ion current chromatograms of the samples analyzed. *Abbreviations*: PM/CMC, peritoneal macrophage (PM) cell membrane chromatography (CMC) column; DAD, diode array detector; GC/MS, gas chromatography with mass spectrometry.

anti-inflammatory effects of active components identified by this method.

2. Materials and methods

2.1. Materials

Silica gel (ZEX-II, 100–200 mesh) was obtained from Qingdao Meigao Chemical Company (Qingdao, PR China). RPMI-1640 medium was purchased from Gibco (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO), 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT), ethylenediamine tetra-acetic acid (EDTA), lipopolysaccharide, trypan blue dye and trypsin were purchased from Sigma (Saint Louis, MO, USA). The enzyme immunosorbent assay (ELISA) kit for mouse TNF-α and IL-1β were purchased from R&D Systems (Minneapolis, MN, USA). HPLC grade methanol and ethyl acetate were purchased from Fisher Scientific (Pittsburgh, PA, USA). Hydrocortisone (HC), dexamethasone (DM), and atractylenolide I (AO-I) were supplied by the National Institute for the Pharmaceutical and Biological Products of China. Rhizoma *A. macrocephala* and Rhizomza *A. lancea* Thunb DC (RALD) were purchased from the TCM Store (Xi'an, PR China).

2.2. Standard solutions

Standard stock solutions (1 mg/mL each) of HC, DM, AO-I were prepared in ethyl acetate. Mixed standard solution I contained 1 mg/mL of both HC and DM. Mixed standard solution II contained 1 mg/mL of both HC and AO-I.

2.3. Sample preparation

Essential oils of RAM and RALD were extracted using supercritical CO_2. Dried RAM and RALD roots were separately powdered (~60 mesh), 2 kg of powder was placed into a 5 L supercritical extraction vessel and subjected to slow heating. When the temperature of extraction vessel reached 50 °C a compressor pump was employed to maintain pressure and temperature at 20.0 MPa and 50 °C, respectively, in the extraction vessel, and at 10.0 MPa and 30 °C in the separation vessel. Cyclic extraction was performed for 3 h with a CO_2 flow rate of 40 kg/h and generated yellow oil extracts. Extraction yields were 2.5% and 2.1% for RAM and RALD, respectively.

2.4. Preparation of peritoneal macrophage (PM) cell membrane chromatography (CMC) columns

BALB/c mice (25–30 g) were from the Animal Center at Xi'an Jiaotong University (Xi'an, China). Mice were injected (ip) with 2 mL of 3% thioglycollate 4 d before sacrifice. PMs were collected by lavaging the peritoneal cavity with 5 mL of RPMI-1640. Cells were collected by centrifugation, washed, and suspended in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and maintained in a humidified incubator with 5% CO_2 at 37 °C. Cells were purified by adherence to tissue culture plates for 2 h. The viability of the macrophages was assessed by trypan blue dye exclusion. We routinely measured viabilities of greater than 90% in all preparations. PM cell membranes were prepared as previously described [19]. Cells (7×10^6) were washed 3 times with normal saline solution centrifuging each time ($650 \times g$, 5 min, 4 °C) and resuspended into suspension buffer (50 mM Tris–HCl pH 7.4). The resulting homogenate was centrifuged ($200 \times g$, 5 min), the pellet discarded, and the supernatant was centrifuged at $15{,}000 \times g$ for 20 min at 4 °C. The supernatant was discarded, the membrane pellet was washed (suspension buffer), recentrifuged as before, and the membrane pellet suspended into 5 mM phosphate-buffered solution (PBS pH 7.4). The PM cell membrane stationary phase (PM-CMSP) was prepared as described [9]. Briefly, the membrane suspension was added to 0.05 g activated (105 °C, 30 min) silica carrier under vacuum at 4 °C with gentle agitation. The homogenate obtained was packed into a column by a wet method to generate the PM/CMC column (10 mm × 3.1 mm, 5 μm).

2.5. PM/CMC assay

A HPLC system and a 32 Karat workstation (Beckman Coulter, Fullerton, CA, USA) were used in conjunction with the PM/CMC column. The mobile phase was 5 mM PBS (pH 7.4) with a flow rate of 0.2 mL/min and a column temperature of 37 °C. The detection wavelength ranged from 220 nm to 240 nm. The chromatographic system was stabilized (~1.5 h) before sample injection. 1 μL of standard solutions or RAM or RALD samples were injected. During "recognition analysis", fractions were collected into 96-well plates every 0.3 min using a Model SC-100 fraction collector (Beckman Coulter). Retention fractions from the chromatogram were combined and evaporated with a SpeedVac concentrator (5301, Eppendorf, Germany). After extraction with 100 μL ethyl acetate by vigorous agitation for 5 min, samples were analyzed by GC/MS.

2.6. Gas chromatography with mass spectrometry (GC/MS)

Standard solutions, standard mixed solutions I and II, and all samples retained by PM/CMC were analyzed by GC/MS. A capillary gas chromatography coupled mass spectrometer (GCMS-QP2010 Shimadzu, Kyoto, Japan) with a Rtx-5MS capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness, Restek, CA, USA) was used. Helium (purity 99.999%) was the carrier at a constant column flow of 2.0 mL/min. Initial temperature was 140 °C ramped at 10 °C/min to 280 °C and held for 8 min. Inlet temperature was maintained at 280 °C. For RAM, RALD essential oils and corresponding PM/CMC samples, initial temperature was 120 °C ramped at 5 °C/min to 180 °C and held for 12 min; then ramped at 20 °C/min to 300 °C and held for 5 min. Inlet temperature was maintained at 280 °C. The mass spectrometer was operated in total ion current (TIC) scanning mode, and we got TIC chromatogram. The mass range scanned was from 40 *m*/*z* to 700 *m*/*z*. Electron impact energy was set

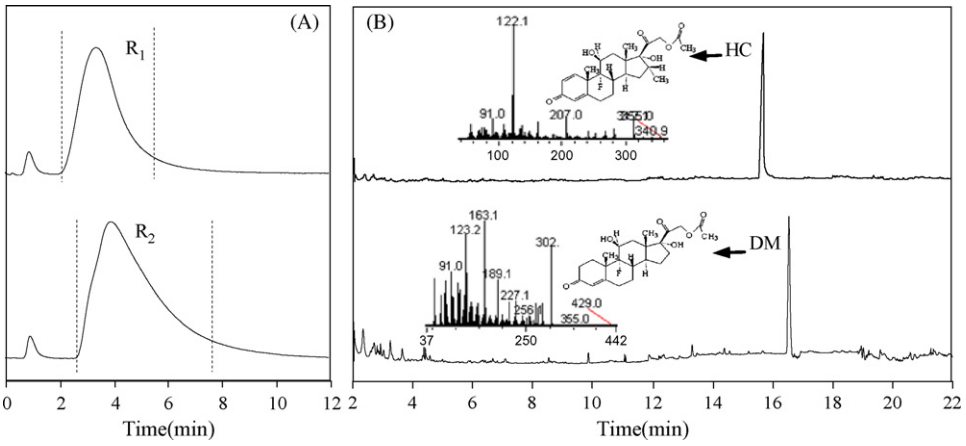

Fig. 2. Chromatograms of standard solutions using the PM/CMC-offline-GC/MS method. (A) PM/CMC chromatograms of standard solutions including HC (R_1) and DM (R_2). (B) Total ion current chromatograms of PM/CMC fractions indicated by dotted lines and identified as HC and DM. S, Total ion current chromatograms of HC and DM.

at 70 eV. Data were collected using GC/MS Analysis Station software and analyzed using a NIST library (Shimadzu, Kyoto, Japan).

2.7. Replacement experiments

The interaction between AO-I and the TLRs was determined by competition experiments. LPS was dispersed at different concentrations into 5 mM PBS (pH 7.4). The capacity factors (k') of hydrocortisone and atractylenolide I were determined using different concentrations of these molecules added to the LPS preparations once the PM/CMC system reached equilibrium.

$$k' = \frac{t_R - t_0}{t_0}$$

where t_R is the retention time of the retention components, and t_0 is the dead time of system. t_R and t_0 were calculated by using a previously described approach [9].

Then hydrocortisone or atractylenolide I was added to a 5 mM PBS with different concentrations, respectively, and the k' values of atractylenolide I and hydrocortisone were determined too.

2.8. MTT assay for cell viability

The cytotoxicity of LPS, AO-I, and HC was evaluated using the MTT (1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan) assay. In brief, 2×10^4 cells per well were plated into 96-well plates and incubated at 37 °C for 24 h. The medium was changed and cells were treated with AO-I (final concentrations 1–100 μg/mL in 0.05% DMSO), HC (final concentration 1–100 μg/mL in 0.05% DMSO), alone or in combination with LPS (final concentration 10 μg/mL). Control and LPS wells received the same amount of DMSO (the added 0.05% DMSO allows solubilization of AO-I in aqueous solution). After 24 h of incubation at 37 °C, 20 μL of MTT (5 mg/mL) was added to each well and 4 h later the cells were lysed with 150 μL DMSO. Plates were agitated gently for 10 min. After 30 min cytotoxicity was assessed by OD570 measurements as described [20].

2.9. Atractylenolide I and hydrocortisone inhibition of TNF-α, IL-1β, and nitric oxide (NO) production by LPS-stimulated peritoneal macrophages

Cells were seeded into 24-well plates (5×10^5 cells per well) in a final volume of 500 μL and incubated at 37 °C under 5% CO_2 for 3 h. Cells were then treated with LPS (final concentration 10 μg/mL) alone or in combination with different concentrations of AO-I or HC (dissolved in DMSO) and incubated for a further 24 h. Control and LPS wells received the same amount of DMSO. Supernatants were collected and assayed for TNF-α and IL-1β using an ELISA kit according to the protocol supplied by the manufacturer. NO content was determined by the Griess reaction [21] and the amount of nitrite was calculated from a $NaNO_2$ standard curve.

3. Results and discussion

3.1. Performance of the PM/CMC-offline-GC/MS method

We first addressed the ability of peritoneal macrophage cell membrane chromatography in conjunction with GC/MS to retain and identify standard compounds. Fig. 2A shows the PM/CMC chromatograms of standard hydrocortisone and dexamethasone solutions. Both HC (R_1) and DM (R_2) showed obvious retention characteristics, with retention times of 3.6 min and 4.0 min, respectively. Selected fractions were collected as indicated on the figure (dotted lines), extracted and injected into the GC/MS system for identification. Mass spectrometric data (Fig. 2B) confirmed the identities of the R_1 and R_2 peaks as HC and DM, respectively.

The PM/CMC chromatogram of mixed standard solution I is shown in Fig. 3A. Four fractions (R_0, R_1, R_2 and R_3) were collected as indicated on the figure (dotted lines). Fractions R_1 and R_2 displayed

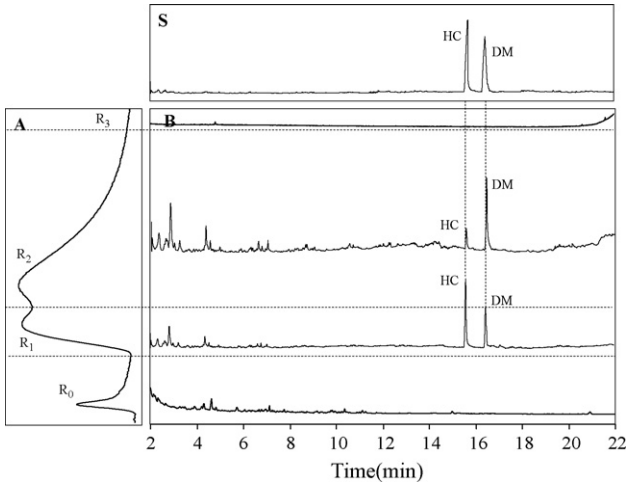

Fig. 3. Chromatograms of the mixed standard solution I using the PM/CMC-offline-GC/MS method. (A) PM/CMC chromatogram of mixed standard solutions I; 4 fractions (R_0, R_1, R_2 and R3) were collected as indicated (dotted lines in the chromatograms). (B) Total ion current chromatograms of the R_0, R_1, R_2 and R_3 fractions. (S) Total ion current chromatogram of the mixed standard solution I (HC plus DM).

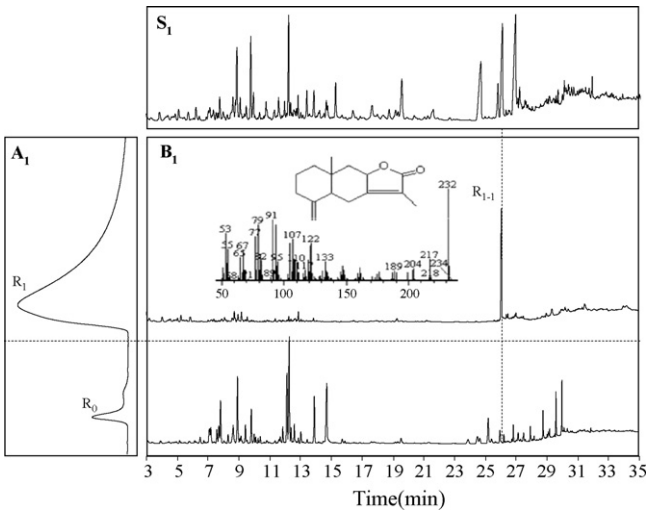

Fig. 4. Chromatograms of RAM extracts using the PM/CMC-offline-GC/MS method. (A_1) PM/CMC chromatogram of the supercritical CO_2 extract of RAM; the R_1 fraction was collected. (B_1) Total ion current chromatograms of the R_1 fraction; the major retained peak (R_{1-1}) was identified as AO-I. (S_1) Total ion current chromatograms of the supercritical CO_2 extract of RAM.

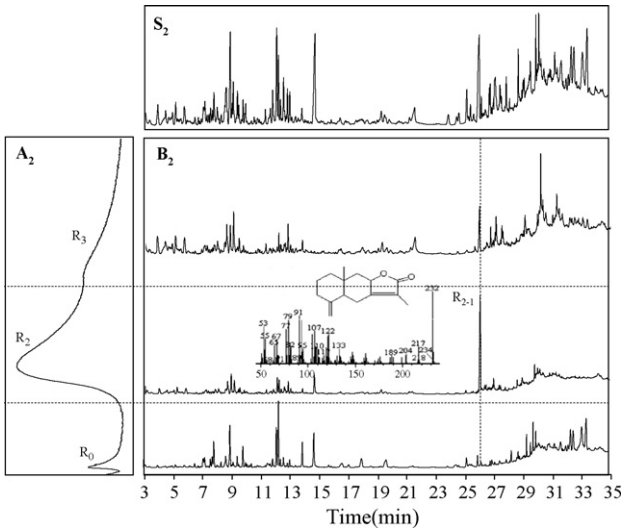

Fig. 5. Analysis of the RALD extract using PM/CMC-offline-GC/MS. (A_2) PM/CMC chromatogram of the supercritical CO_2 extract from RALD; the R_2 fraction was collected. (B_2) Total ion current chromatograms of the R_2 fraction; the major retained peak (R_{2-1}) was identified as AO-I. (S_2) Total ion current chromatograms of the supercritical CO_2 extract of RALD.

obvious retention characteristics. Mixed standard solutions I and each of the fractions were analyzed by GC/MS (Fig. 3S and B). Comparison of retention times identified HC and DM in the R_1 and R_2 fractions, these molecules were absent from R_0 and R_3. Retention of HC and DM may be attributed to interaction with mGCRs [22] abundant in the macrophage membrane. Retention time showed a positive correlation with interaction strength.

3.2. Practical application

The PM/CMC-offline-GC/MS method was then applied to the screening of active components from 2 traditional medicinal Rhizomas *A. macrocephala* and Rhizomas *A. lancea* Thunb DC. PM/CMC analysis of the RAM supercritical CO_2 extract is shown in Fig. 4. The R_1 peak (Fig. 4A_1) displayed retention characteristics similar to HC and DM controls (Figs. 2A and 3A). The R_1 fraction was collected, extracted, and analyzed by GC/MS (Fig. 4B_1). Peak R_{1-1} was the major retention component of the R_1 fraction. Mass spectrometry identified this molecule as atractylenolide I (AO-I). Comparison of Fig. 4S_1 and B_1 confirmed that AO-I was the active component. PM/CMC analysis of the supercritical CO_2 RALD extract is shown in Fig. 5. Peak R_{2-1} was the main retention component of the R_2 fraction. This was also identified as AO-I by mass spectrometry. We conclude that AO-I is the major retained component in extracts of both RAM and RALD.

These results demonstrate that the PM/CMC-offline-GC/MS method can be used to screen for active components in a complex sample. Table 1 presents the quantitation of AO-I according to the areas under the TIC chromatograms peaks (Figs. 4 and 5). The molecule represented 10.2% and 6.3% of the RAM and RALD extracts, increasing to 84.6% and 72.5% in the fractions enriched for AO-I. The enrichment ratio between the extracts and retained fractions were 8.2- and 11.5-fold, respectively.

Table 1
The enrichment ratios of AO-I after CMC with peritoneal macrophage cell membranes (peak area ratios, %).

	In the extracts	In the fractions	Enrichment ratio
RAM	10.2	84.6	8.2
RALD	6.3	72.5	11.5

3.3. Validation of screening results

To validate the PM/CMC-offline-GC/MS protocol as a screening method, the mixed standard solution II (HC and AO-I) was analyzed. The PM/CMC chromatogram of mixed standard solution II is presented in Fig. 6A_3. Three fractions were collected (R_0, R_1 and R_2). R_1 comprised the retained fraction. Mixed standard solution II and each fraction were analyzed by GC/MS (Fig. 6S_3 and B_3). Comparison of retention times revealed that HC and AO-I were both present in the R_1 fraction and absent from the R_0 and R_3 fractions (Fig. 6).

3.4. Interaction between atractylenolide I and cell membrane receptors

The interactions between AO-I, LPS, and cell membrane receptors were determined by competition experiments with *k′–C*

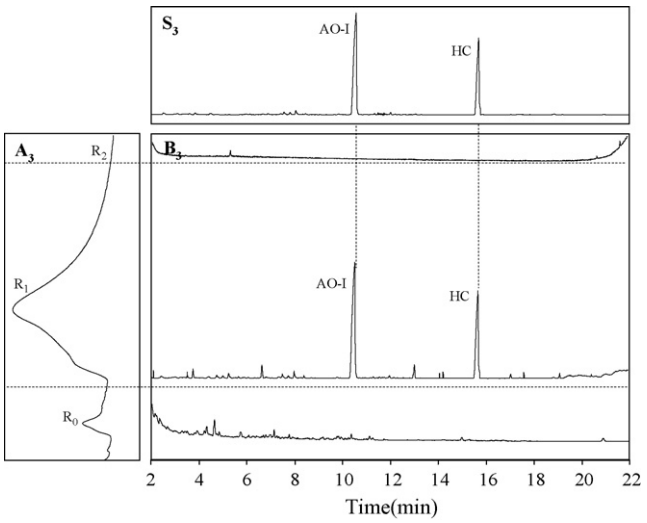

Fig. 6. Chromatogram of the mixed standard solution II using the PM/CMC-offline-GC/MS method. (A_3) PM/CMC chromatogram of the mixed standard solution II; 3 fractions (R_0, R_1, and R_2) were collected as indicated (dotted lines). (B_3) Total ion current chromatograms of the R_0, R_1, and R_2 fractions. (S_3) Total ion current chromatograms of the mixed standard solution II (HC and AO-I).

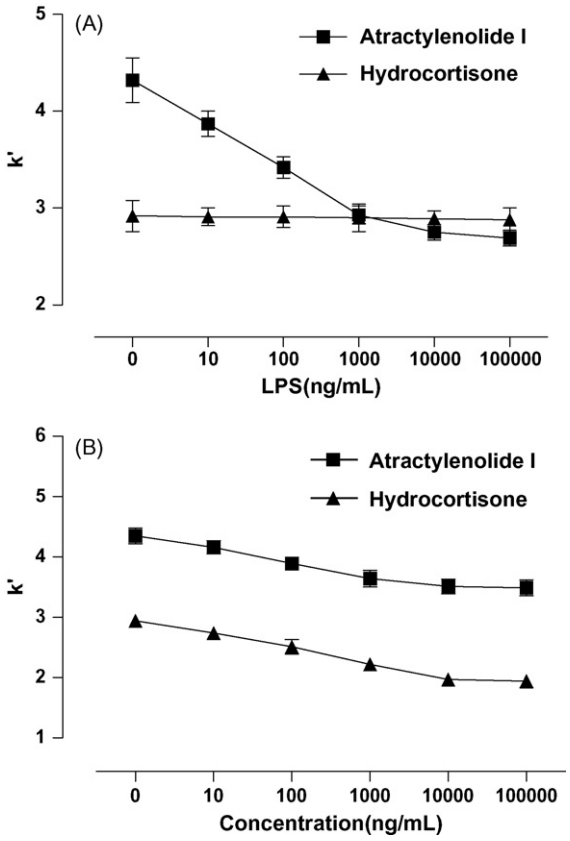

Fig. 7. Interaction between AO-I and cell membrane receptors. (A) Effect of LPS on the chromatographic retention characteristics of HC and AO-I. (B) Competition of AO-I retention by HC, and competition of HC retention using AO-I Data were obtained from 5 independent experiments and are expressed as means ± SD.

displacement curves (Fig. 7). The k' values of AO-I decreased when the concentration of LPS increased, indicating that AO-I binding to its major known receptor on PM cells, TLRs, was competed by LPS (Fig. 7A). At LPS concentrations greater than 1 μg/mL, AO-I k' values became invariable, indicating that competitive binding had achieved dynamic equilibrium. This experiment also revealed that k' values of HC were maintained even at high LPS concentration, showing that HC and LPS do not compete for binding, and indicating that HC, unlike AO-I, does not bind to TLRs.

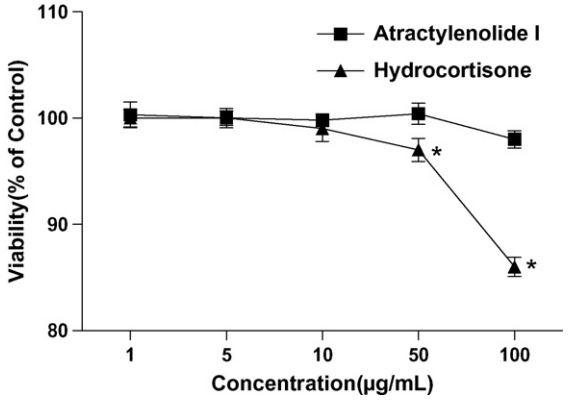

Fig. 8. Effects of HC and AO-I on peritoneal macrophage cell viability determined by the MTT assay. Data were obtained from 5 independent experiments and were expressed as means ± SEM. (*) Statistically significant ($p < 0.01$) difference from the control group.

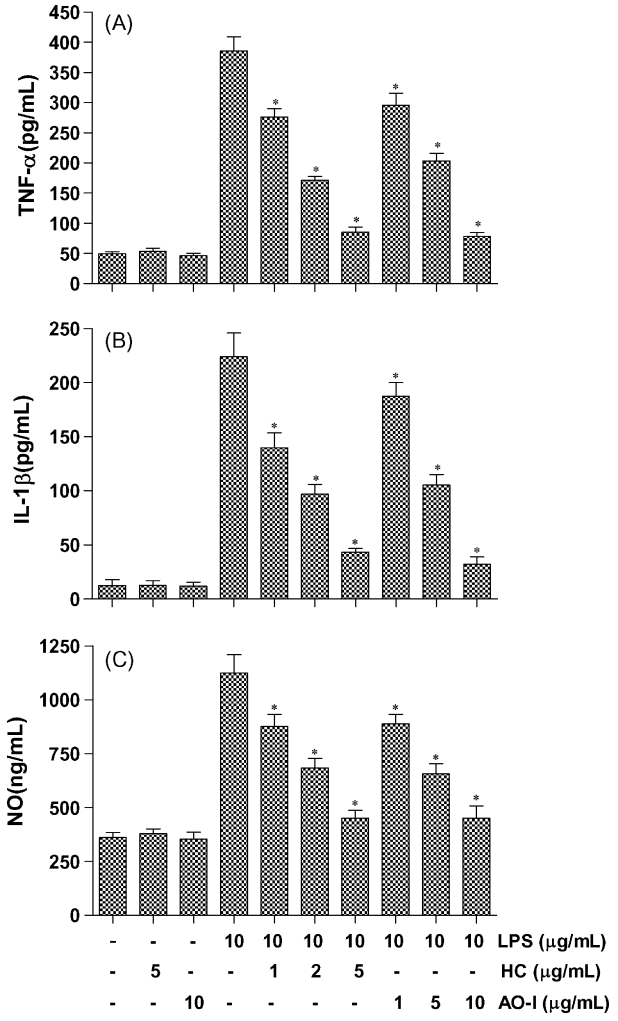

Fig. 9. Effects of AO-I and HC on TNF-α (A), IL-1β (B) and NO (C) production by LPS-stimulated peritoneal macrophages. Data were obtained from t independent experiments and were expressed as means ± SEM. (*) Statistically significant ($p < 0.05$) difference from the LPS (10 μg/mL) group.

Different concentrations of HC or AO-I in 5 mM PBS (pH 7.4) were analyzed and k'–C displacement curves were obtained (Fig. 7B). This revealed that k' values of AO-I and HC decreased as their respective concentrations increased. This indicates that these 2 molecules have partly identical PM/CMC binding sites. When the concentrations of HC or AO-I exceeded 1 μg/mL or 10 μg/mL, respectively, k' values became generally invariable, indicating that dynamic equilibrium had been achieved.

3.5. Cell viability

The cytotoxicity of these compounds was assessed using the MTT assay. As shown in Fig. 8, HC had a slight inhibitory effect while atractylenolide I showed no cytotoxicity at concentrations ranging from 1 to 50 μg/mL. However, AO-I and HC inhibited cell viability at concentrations greater than 50 μg/mL. The concentrations of AO-I and HC applied in subsequent experiments were lesser than 50 μg/mL. LPS alone or in combination with AO-I (1–10 μg/mL) or HC (1–5 μg/mL) showed no cytotoxic effects.

3.6. Effects of atractylenolide I and hydrocortisone on TNF-α and IL-1β induction by LPS

LPS interacts with TLRs on the peritoneal macrophage cell membrane. The TLR4 pathway has been shown to be involved

in the expression of TNF-α, IL-1β and iNOS after LPS challenge of macrophages and glucocorticoids can block this pathway [23]. To investigate interaction between AO-I, HC, and LPS-induced inflammation, levels of TNF-α and IL-1β were determined following LPS treatment of PMs (Fig. 9). Resting state PMs released 49.3 ± 3.7 pg/mL of TNF-α and 12.4 ± 5.6 pg/mL of IL-1β over 24 h. On LPS stimulation, TNF-α and IL-1β production was markedly increased to 385.8 ± 23.4 pg/mL and 223.8 ± 22.1 pg/mL, respectively.

No significant difference in TNF-α production was found between resting PMs and cells treated with HC (5 μg/mL) or AO-I (10 μg/mL) alone. However, both HC and AO-I inhibited LPS-induction of TNF-α and IL-1β production in a dose-dependent manner. Respective IC50 values for HC and AO-I were of 2.3 μg/mL and 5.3 μg/mL for the inhibition of TNF-α production, and 1.8 μg/mL and 5.1 μg/mL for the inhibition of IL-1β production. Because at concentrations below 50 μg/mL, neither HC nor AO-I showed any cytotoxic effects (Fig. 8), the inhibition of LPS-induced cytokine production is not due to cell toxicity.

3.7. Effects of AO-I and HC on LPS-induced NO production

Over 24 h, resting state PMs released 360 ± 24 ng/mL of nitrite, a stable metabolite of NO. In contrast, NO production increased to 1123 ± 86 ng/mL nitrite after stimulation with LPS (Fig. 9). There was no significant difference in NO production between resting PMs and cells treated with either AO-I (10 μg/mL) or HC (5 μg/mL). However, HC inhibited LPS-induced NO production in a dose-dependent manner, with an IC50 value of 3.7 μg/mL. AO-I also inhibited LPS-induced NO production in a dose-dependent manner (IC50, 7.5 μg/mL). The inhibitory efficacy of AO-I was thus similar with that of HC.

4. Conclusions

Screening target components from medicinal plants is a laborious and time-consuming process for drug discovery. We have shown that the PM/CMC-offline-GC/MS method can be used for the rapid detection and enrichment of active components from a complex molecular mixture, and the technique permits fast and accurate molecular identification by mass spectrometry. In addition, the method also allows evaluation of receptor specificity through competition between ligands for PM/CMC retention. This method will enable rapid screening and identification of new active molecules in complex biological samples.

Acknowledgment

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