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Dual targets guided screening and isolation of Kukoamine B as a novel natural anti-sepsis agent from traditional Chinese herb *Cortex lycii*

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ABSTRACT

Treating sepsis remains challenging at present. Bacterial lipopolysaccharide (LPS) and bacterial DNA/CpG DNA are important pathogenic molecules and drug targets for sepsis. It is thus a promising strategy to treat sepsis by discovering agents that neutralize LPS and CpG DNA simultaneously. In this study, we present evidences of the biosensor based screening and isolation of active anti-sepsis fractions and monomers from traditional Chinese herbs using dual targets (LPS and CpG DNA) guided drug discovery strategy. Firstly, LPS or CpG DNA was immobilized on surfaces of cuvettes in the biosensor to establish a screening platform. Then, *Cortex lycii* with both highest affinities was selected out from one hundred and fourteen traditional Chinese herbs. In subsequent experiments, chromatography was utilized and coupled with the biosensor to purify fractions with a higher affinity for LPS and CpG DNA. In line with affinity assay, these fractions were shown to neutralize LPS and CpG DNA and inhibit their activity *in vitro* and *in vivo*. Lastly, the contributing monomer Kukoamine B (KB) was purified. KB neutralized LPS and CpG DNA *in vitro*. It inhibited TLR4, TLR9 and MyD88 mRNA expressions up-regulated by LPS and CpG DNA, and also attenuated the LPS and CpG DNA elicited nuclear translocation of NF-κB p65 protein in RAW264.7 cells. It also protected mice from lethal challenge of heat-killed *E. coli*, a mixture of LPS and CpG DNA. In conclusion, we presented a dual target guided discovery of a novel anti-sepsis agent KB from traditional Chinese herbs via combination of biosensor technology and chromatography methods.

1. Introduction

Sepsis is a systemic inflammatory response induced by infections. It has become the leading cause of death in critically ill patients. Treating sepsis remains challenging as no effective anti-sepsis drug is available. Bacterial lipopolysaccharide (LPS) and bacterial DNA/CpG DNA are two well recognized pathogenic factors for the induction of sepsis and thus regarded as important drug targets for sepsis. LPS and CpG DNA usually co-exist and act synergistically to activate myeloid differentiation primary response gene (88) (MyD88) dependent signaling pathway in macrophages, which leads to upregulation of proinflammatory responses and eventually causes sepsis [1–3]. As a result, their consequences will be effectively prevented or attenuated if they are simultaneously neutralized. Therefore, it is important to investigate and develop effective agents targeting on both LPS and CpG DNA to treat sepsis. Unfortunately, there has been no report of dual target based drugs discovery strategy for sepsis treatment by simultaneous neutralization of LPS and CpG DNA until now [4,5].

Traditional Chinese herbs have been widely used as common remedies for infectious and inflammatory diseases for thousands of years. They are focused and investigated with renewed interests and regarded as promising drug candidates in treating sepsis nowadays [6–9]. Some herbs were evaluated and reported to be with direct anti-LPS or anti-CpG DNA activities in previous studies [10–12]. However, it remains unclear whether there exist natural dual inhibitors for both of LPS and CpG DNA in the herbs and how to get these contributing monomers from the complicated herb extracts if they do exist [13].

Many efforts have been paid to separate active compounds from the herbal extracts. Nevertheless, there exist few reports on establishment of dual target guided separation methods and their application in discovery of natural anti-sepsis agents. Previously, a rapid screening technique for affinity assay of herbs was established by immobilizing LPS or CpG DNA separately on the reacting surfaces of a biosensor in our lab [12,14]. Evaluation of the binding abilities of herbs or agents for LPS or CpG DNA would be achieved in just a few minutes. Importantly, results of bioactivity assessments were in line

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with that of the affinity tests, suggesting the validity and feasibility of this separation technique [12,14].

Based on the above facts and investigation, the aim of this study is therefore to develop a dual target (LPS and CpG DNA) guided drug discover strategy to discover novel natural anti-sepsis fractions and monomers from traditional Chinese herbs The anti-LPS and anti-CpG DNA bioactivities of the isolated fractions and monomers were also evaluated *in vitro* and *in vivo*.

2. Materials and methods

2.1. Reagents

Lipopolysaccharide from *E. coli* O111:B4 (LPS), n-octyl-D-glucopyranoside (OG) and avidin were purchased from Sigma Chemicals (St. Louis, MO, USA). CpG DNA 1826 (5′-TCCATGACGTTCCTGAT GCT′-3, CpG DNA) were synthesized in SBS Genetech (Beijing, China). Solvents for HPLC use were all in HPLC grade and purchased from Honeywell International Inc. (Morristown, NJ US). Primers for RT-PCR were synthesized by Invitrogen Biotechnology (Shanghai, China). All other chemicals were at the best grade among those commercially available.

2.2. Traditional Chinese herbs

One hundred and fourteen Traditional Chinese Herbs (listed in Table 1) were purchased from the Chongqing Market of Traditional Chinese Herbs and authenticated in the Chongqing Academy of the Chinese Materia Medica (Chongqing, China).

2.3. Establishment of the dual target guided affinity screening method using biosensor technology

2.3.1. Immobilization of LPS and CpG DNA on the reacting surface of the biosensor

LPS and CpG DNA were immobilized on the reacting surfaces of cuvettes in the affinity biosensor according to the manufacturer's instructions (Thermo Labsystem, USA). LPS was immobilized on the non derivatised cuvette. Briefly, a non derivative cuvette was inserted and rinsed three times with 50 μl of PBS/OG [0.01 M PBS (pH7.4)/1.25% OG(w/v)] to form a lipid-coated surface. Then 30 μl of LPS (2 mg/ml dissolved in PBS/OG) was added into the cuvette and left until response is about at a plateau, after which 30 μl PBS was added twice without removing the LPS solution until the binding curve reached a plateau. Biotinylated CpG DNA was immobilized on the surface of a biotin cuvette. Briefly, the biotin cuvette was inserted and rinsed with 40 μl of PBST [0.01 M PBS (pH 7.4) + 0.05% tween 20 (v/v)] for equilibration. 20 μl of 2 mg/ml avidin was later added to the PBST in the cuvette and left for about 10 min. Then the free avidin was removed and biotinylated CpG DNA (400 μg/ml) was added. The deposition of CpG DNA last until the binding curve reached platform. The amounts of immobilized LPS and CpG DNA 1826 on the surfaces of the cuvettes were determined by subtracting the initial baseline levels from the final levels.

2.3.2. Affinity assay

1 μl of aqueous herbal extracts or the isolated fractions/monomers were added separately into the two cuvettes containing 49 μl PBS (0.02 M, pH 7.4) and the binding reaction last for about 3 min. The cuvettes were then rinsed with 50 μl PBS (0.02 M, pH 7.4) and 50 μl HCl (0.01 M) for regeneration and ready for the next cycle of affinity test. Data analyses were performed using the FASTplot software package (Thermo Labsystem, USA).

Table 1
One hundred and fourteen traditional Chinese herbs.

Name	Name	Name
Aspongopus	*Herba andrographis*	*Radix Gentianae Macrophyllae*
Bulbus Fritillariae Cirrhosae	*Herba Artemisiae Annuae*	*Radix Ilicis Pubescentis*
Cacumen platycladi	*Herba Artemisiae Scopariae*	*Radix isatidis*
Caulis akebiae	*Herba asari*	*Radix Paeoniae Rubra*
Caulis Piperis Kadsurae	*Herba chenopodii*	*Radix phytolaccae*
Caulis sargentodoxae	*Herba Equiseti Hiemalis*	*Radix platycodonis*
Cortex cinnamomi	*Herba Hedyotis Diffusae*	*Radix Puerariae Lobatae*
Cortex dictamni	*Herba houttuyniae*	*Radix pulsatillae*
Cortex fraxini	*Herba hyperici*	*Radix rhapontici*
Cortex lycii	*Herba Lespedezae Cuneatae*	*Radix Rumicis Crispi*
Cortex moutan	*Herba Lobeliae Chinensis*	*Radix Salviae Miltiorrhizae*
Cortex phellodendri	*Herba menthae*	*Radix sangusorbae*
Flos caryophylli	*Herba Patriniae Cum Radice*	*Radix scrophulariae*
Flos chrysanthemi	*Herba portulacae*	*Radix scutellariae*
Flos Chrysanthemi Indici	*Herba saxifragae*	*Radix semiaquilegiae*
Flos farfarae	*Herba Scutellariae Barbatae*	*Radix Sophorae Flavescentis*
Flos Lonicerae Japonicae	*Herba sedi*	*Radix stellariae*
Flos sophorae	*Herba Solani Nigri*	*Radix Stephaniae Tettandrae*
Folium Artemisiae Argyi	*Herba taraxaci*	*Radix tinosporae*
Folium isatidis	*Herba verbenae*	*Radix trichosanthis*
Folium Hibiscus Mutabilis	*Herba violae*	*Ramulus cinnamoni*
Folium nelumbinis	*Lasiosphaera Seu Calvatia*	*Rhizoma Alpiniae Officinarum*
Folium sennae	*Pericarpium granati*	*Rhizoma anemarrhenae*
Fructus arctii	*Plumula nelumbinis*	*Rhizoma bistortae*
Fructus Aurantii Immaturus	*Poria*	*Rhizoma bletillae*
Fructus bruceae	*Pseudobulbus Cremastrae Seu Pleiones*	*Rhizoma cimicifugae*
Fructus cannabis	*Radix Aconiti Lateralis Preparata*	*Rhizoma coptidis*
Fructus Chebulae Immaturus	*Radix ampelopsis*	*Rhizoma dioscoreae*
Fructus cnidii	*Radix arnbbiae*	*Rhizoma Dryopteris Crassirhizomatis*
Fructus corni	*Radix asparagi*	*Rhizoma Fagopyri Cymosi*
Fructus crataegi	*Radix bupleuri*	*Rhizoma Iridis Tectori*
Fructus forsythiae	*Radix clematidis*	*Rhizoma paridis*
Fructus gardeniae	*Radix Crotonis Crassifolii*	*Rhizoma phragmitis*
Fructus kochiae	*Radix dichroae*	*Rhizoma Smilacis Glabrae*
Fructus mume	*Radix Et Rhizoma Cynanchi Atrati*	*Semen cassiae*
Fructus sophorae	*Radix Et Rhizoma Tripterygii*	*Semen persicae*
Fructus Terminaliae Chebulae	*Radix Et Rhizoma Rhei*	*Spica prunellae*
Fructus tsaoko	*Radix gentianae*	*Spina gleditsiae*

2.4. Bioactivity assessments of the isolated fractions and the monomers

2.4.1. Cell culture

The murine macrophage-like cell line, RAW264.7 cells (purchased from ATCC Manassas, VA, USA) were cultured at 37 °C in a 5% CO2 humidified incubator and maintained in a DMEM medium supplemented with 10% endotoxin free fetal bovine serum (GIBCO, Grand Island, NY, USA), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were diluted with 0.4% trypan blue in PBS (0.1 mM, pH 7.4) and live cells were counted by a hemacytometer. The concentration of the cells was adjusted to 1×10^6/ml before stimulation by LPS and CpG DNA in all experiments.

2.4.2. LPS-neutralization assessment by the Limulus Amebocyte Lysate (LAL) test

The isolated fractions or the monomers were incubated with LPS (1 ng/ml) in equal volume at 37 °C for 30 min for the binding and neutralization process. Subsequently, 100 μl of each reacted LPS solutions was added to 100 μl of the quantitative LAL products

dissolved in LPS free water to allow a reaction at 37 °C for 60 min in an ATI 320-06 Kinetic Tube Reader (Lab Kinetics Ltd, UK). The Kinetic turbidimetric assay was utilized to measure the gel clotting formation of LAL products induced by the existence of non neutralized LPS.

2.4.3. Cytokine measurement using ELISA

RAW264.7 cells were treated with 100 ng/ml LPS or 10 μg/ml CpG DNA in the presence or absence of fractions and monomers. The supernatants were harvested at indicated time points and the production of TNF-α and IL-6 was measured using ELISA kit (R&D, Minneapolis, MN USA) according to the manufacturer's instruction.

2.4.4. mRNA expression detection using RT-PCR

2 ml of RAW 264.7 cells grown in 6-well plates were added with 100 ng/ml LPS or 10 μg/ml CpG DNA in the presence or absence of the monomer for 4 h. Total RNA of each group was isolated with TRIzol™ reagent according to the manufacturer's instructions. Semiquantitative RT-PCR was carried out to investigate the mRNA expression level of TLR4, TLR9 and MyD88 in different groups. 2 μg total RNA of each group was reverse transcribed with AMV reverse transcriptase. 1 μl cDNA from each sample was used as the template in the PCR reaction with the primer pairs of TLR4 (F: 5′-AAGGCATGGCATGGCTTACAC-3′, antisense, 5′-GGCCAATTTTGTCTCCACAGC-3′), TLR9 (F: 5′-TCGCTCAA-CAAGTACACGC-3′, R: 5′-GCTCTG CATCATCTGCCTC-3′) and MyD88 (F: 5′-ACTCGCAGTTTGTTGGATG-3′; R: 5′-CACCTGTAAAGGCTTCTCG-3′). Meanwhile, PCR reactions used as the internal control were carried out using GAPDH gene specific primer pairs (F: 5′-CTGCACCACCAACTGCT-TAG-3′, R: 5′-GTCTGGGATGGAAATTGTGA -3′) to estimate whether the equal amounts of cDNA among samples were used in the PCR reaction.

2.4.5. NF-κB p65 detection using western blot

5 ml of RAW 264.7 cells grown in 6-well plates was treated with 100 ng/ml LPS or 10 μg/ml CpG DNA in the presence or absence of the active monomer for 1 h. The nucleic protein extracted with the Nuclear Protein Extraction Buffer kit (Thermo Pierce, IL, US).was adjusted in an equal protein amount and separated by SDS PAGE and transferred onto PVDF membranes (Millipore, MA, US). Blots on the membranes were blocked in 5% dry skim milk and probed with the anti-NF-κB p65 and -GAPDH primary antibodies (1:200 dilutions, Santa Cruz, CA, US) at 4 °C overnight separately. The blots were then incubated with secondary goat anti-mouse IgG antibodies (1:2000 dilutions, Santa Cruz, CA, US) and developed with SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Pierce, IL, US) for chemiluminescence assay under a ChemiDoc XRS gel imaging system (Bio-Rad, USA).

2.4.6. Survival analysis of experimental sepsis mouse model

Kunming (KM) mice (4–6 week-old, weighing 18–20 g, male and female in equal number.) were obtained from the Experimental Animal Center of the Third Military Medical University (Chongqing, China) and housed under specific pathogen free conditions with free access to food and water. All animal experiments were performed in accordance with the National Guidelines for Animal Care and Use.

Heat-killed *E. coli* was prepared as described previously [12]. Total bacteria were diluted in sterile normal saline to 1.0×10^{10} colony forming unit (CFU)/ml before injection. Then the KM mice were randomly divided into different groups (10 or 20 mice per group) and injected intravenously with a single dosage of heat-killed *E. coli* or heat-killed *E. coli* with different doses of the fractions or monomers separated from *Cortex lycii*. The volume of a single injection was 0.2 ml per 20 g bodyweight. The mortality was observed for 7 days.

2.5. Preparation of aqueous extracts of traditional Chinese herbs

2.5.1. Preparation of aqueous extracts for screening

The crude drugs of 114 herbs were pulverized. 1 g of each powder was added with 10 ml distilled water, soaked for 2 h, and then boiled at 100 °C for 45 min. After filtration, the materials were centrifuged at 4000 ×*g* for 30 min and the supernatants, termed here as "aqueous extracts", were collected for affinity detection.

2.5.2. Preparation of aqueous extracts of C. lycii for isolation

2 kg of *C. lycii* were rinsed with distilled water, dried, mixed with 10,000 ml distilled water, soaked for 2 h, and then boiled at 100 °C for 45 min. The mixture was filtrated and centrifuged at 4000 ×*g* for 30 min. The supernatants were collected for separation.

2.6. Separation of the active fractions from C. lycii under dual target guide

2.6.1. Separation via macroporous adsorption resin chromatography

Aqueous extracts from *C. lycii* was obtained as described in Section 2.5.2. and isolated by macroporous adsorption resins with the following procedures. Briefly, 1000 g macroporous adsorption resins for chromatography use (Haiguang Chemical, Tianjin, China) were packed into a column and eluted with ethanol and distilled water before sampling. The aqueous extracts were loaded uniformly onto the upper part of the prepared resin column and eluted with distilled water and gradient ethanol (10%, 20%, 50% and 100%) successively. The eluted fractions (Fr. CL-1, -2, -3, -4, -5) were collected, concentrated by rotary-evaporation (BUCHI Rotavapor R205, Switzerland) and freeze dried for affinity test.

2.6.2. Separation via cationic exchange gel chromatography

The fraction identified with highest binding ability to LPS and CpG DNA was further isolated by cationic exchange column chromatography. 200 g of SP Sepharose Fast Flow (SPFF) cationic gels (Pharmacia, Uppsala, Sweden) was packed into a glass column which had been rinsed by distilled water and prepared for sampling on an AKTA Explorer 100 HPLC system (Pharmacia, Uppsala, Sweden). The active fraction dissolved in distilled water was eluted with distilled water, 0.3 M Na_2HPO_4 and 0.5 M Na_2HPO_4 successively, yielding three more purified fractions named Fr. CL-4a, CL-4b and CL-4c which were collected, concentrated and freeze dried respectively for bioactivity assessment.

2.7. Analytical and preparative purification of monomers

An Agilent Technology 1200 Series analytical HPLC system equipped with a binary pump, a degasser and a photodiode array detector was used for composition analysis of the active faction. The analysis was carried out with a ZORBAX SB-C18 analytical column (150 mm × 4.6 mm, 5 μm). The binary mobile phases consisted of 0.5‰ trifluoroacetic acid in water (solvent A) and acetonitrile (solvent B). The flow rate was at 1 ml/min. The active fraction (250 μg/ml) filtered by a 0.22 μm filter membrane was injected into the HPLC system (10 μl each time) and separated by isocratic elution (20% B in 30 min). Peaks were monitored at 280 nm and 320 nm with the diode array detector.

An Agilent Technology 1100 Series preparative HPLC system was used for the isolation of major monomers from active faction. The preparation was carried out with a Agilent ZORBAX SB-C18 PrepHT column (250 mm × 21.2 mm, 7 μm) The binary mobile phases consisted of 0.5‰ trifluoroacetic acid in water (solvent A) and acetonitrile (solvent B). The flow rate was at 10 ml/min. Fr. CL-4b was dissolved in 20% B (10 mg/ml) and filtered by a 0.22 μm filter membrane. The filtrate was injected into the preparative HPLC system (10 ml each time) and separated by isocratic elution (20% B in 60 min) into two major active monomers, which were concentrated by rotary-evaporation and freeze dried for activity assessment.

2.8. Structure determination of the active monomer

The purity of the active monomer prepared by HPLC was detected by HPLC with the diode array detector. Its chemical structure was identified later after analyzed by mass spectrometry and nuclear magnetic resonance (NMR) at the National Center of Biomedical Analysis (Beijing, China).

2.9. Statistical analysis

All tests are repeated at least three times and one representative result for each test is shown. Data except the survival analysis are all expressed as mean ± SD of duplicates. The Kaplan–Meyer log-rank test was used for the survival analysis of experimental sepsis mouse model. Student's *t* test was used for paired comparisons and the one-way ANOVA test or post hoc Bonferroni correction was used for multiple comparisons. Difference with the *p* value less than 0.05 or 0.01 was considered statistically significant.

3. Results

3.1. Immobilization of LPS and CpG DNA on cuvettes of the biosensor

The dual target guided affinity screening method was established via the immobilization of LPS and biotin labeled CpG DNA on the reacting surface of the biosensor. Both immobilizing curves fitted well with the standard curves presented in the protocols. The images of both resonance peaks were single and symmetrical, suggesting that the molecules of LPS and CpG DNA were uniformly immobilized on the surfaces of the cuvettes (Fig. 1A). The calculated amounts of immobilized LPS and CpG DNA were 781.63 arc seconds (as) and 196.39 as, equated to an immobilization concentration of 1.30 ng/mm^2 and 0.33 ng/mm^2, respectively (1 ng/mm^2 approximately equated to 600 as).

3.2. Dual targets guided screening of C. lycii from one hundred and fourteen of herbs

Aqueous extracts of the 114 herbs (Table 1) were prepared and their binding abilities were screened by the biosensor. 16 herbs and

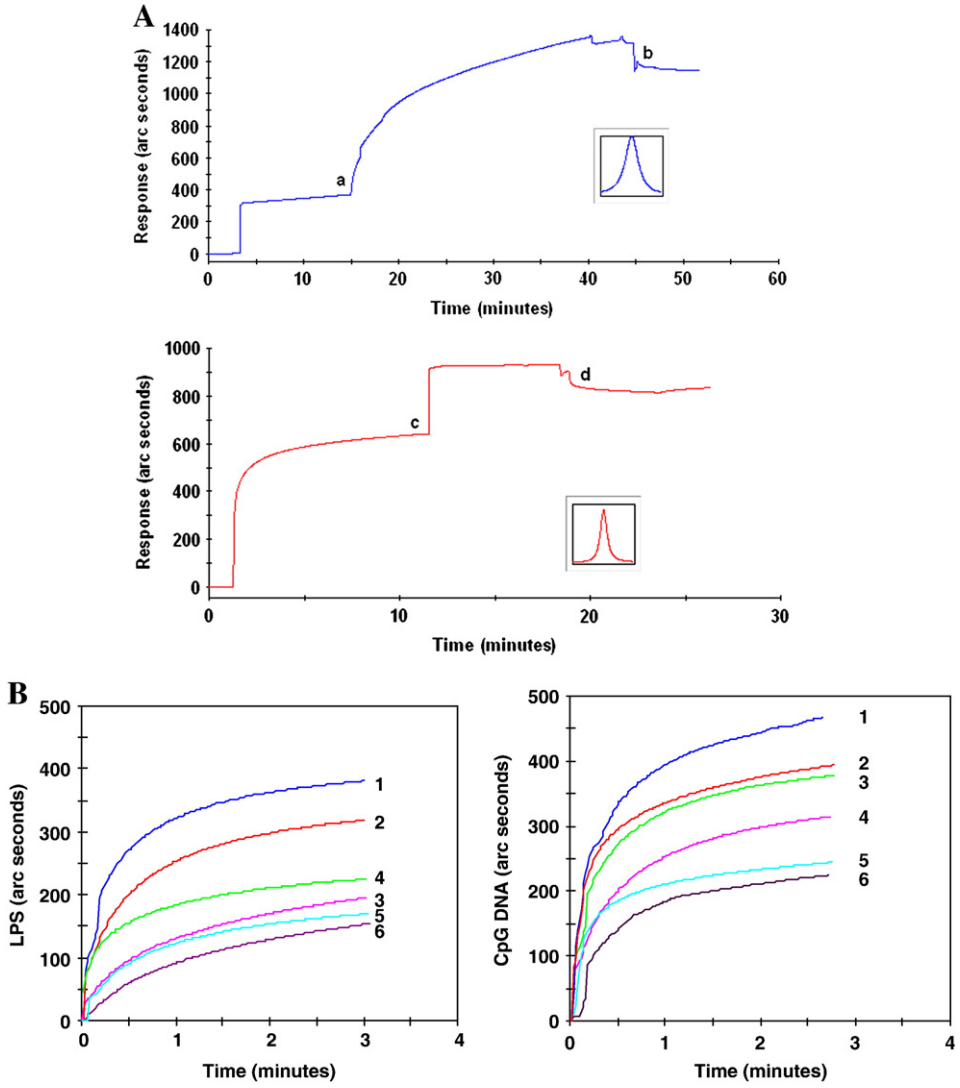

Fig. 1. Affinity screening of 114 traditional Chinese herbs. (A) For LPS immobilization, a non derivative cuvette was rinsed with 50 μl of PBS/OG (0.01 M PBS + 1.25% OG) to form a lipid-coated surface. Then LPS was added into the cuvette and left until response was about at a plateau. Addition of 60 μl PBS was followed without removing the LPS until the binding curve reached another plateau. The process was finalized after removal of excess LPS with PBS. For CpG DNA immobilization, the biotin cuvette was firstly rinsed with PBST. Then avidin was added in the cuvette to form an avidin layer. The excess avidin was removed and biotinylated CpG DNA was added for deposition for 10 min. Finally, the cuvette was balanced with PBST and left until the curve reached a platform. The amount of immobilized LPS or CpG DNA was calculated by subtracting from the baseline levels (a, c) to the final levels (b, d). (B) Six herbs (as listed above) were screened out from 114 herbs. The affinities for LPS and CpG DNA of their aqueous extracts (1 mg/ml) were detected by the biosensor.

22 herbs were found with high affinity for LPS or CpG DNA, respectively (Table 2). Among those herbs, *C. lycii*, *C. moutan*, *F. corni*, *R. anemarrhenae, R. Et Rhizama Rhei* and *R. scutellariae* were shown to exhibit high affinity for both LPS and CpG DNA (Fig. 1B). As possessing highest affinity for both LPS and CpG DNA, *C. lycii* was selected for further investigation in the subsequent experiments.

3.3. Dual target guided sequential separation and purification of active fractions from C. lycii

To obtain the active monomer from water extracts of *C. lycii*, macroporous adsorptive resins and cationic exchange gel column chromatography were sequentially used. Five fractions (Fr. CL-1, -2, -3, -4 and -5) were isolated from *C. lycii* by macroporous adsorptive resin column chromatography (Fig. 2A). Among these five fractions, Fr. CL-4 was screened out to possess the highest binding abilities for both LPS (397.52 as) and CpG DNA (454.56 as) determined by the biosensor (Fig. 2B). From CL-4, three fractions (Fr. CL-4a, -4b and -4c) with higher purity were isolated by cationic exchange gel column chromatography (Fig. 2C). Fr. CL-4b possessed the highest binding abilities for both LPS (640.54 as) and CpG DNA (1019.46 as) than Fr. CL-4a (37.95 as, 42.38 as) and Fr. CL-4c (298.71 as, 306.66 as) (Fig. 2D).

3.4. Identification of anti-LPS and anti-CpG DNA activities of Fr. CL-4b

To determine whether anti-LPS and anti-CpG DNA bioactivities of Fr. CL-4b were in line with its binding abilities, the anti-LPS and anti-CpG DNA abilities were examined. *In vitro*, the results showed that Fr. CL-4b could significantly neutralize LPS ($p<0.01$) (Fig. 3A) and inhibit TNF-α and IL-6 release from RAW 264.7 cells induced by LPS and CpG DNA ($p<0.05$ or $p<0.01$) (Fig. 3B), two representative proinflammatory cytokines for sepsis [15,16]. *In vivo*, Fr. CL-4b protected sepsis mice challenged with heat-killed *E. coli* (a mixture of LPS and CpG DNA) by reducing mortality from 90% to 50% ($p<0.05$) (Fig. 3C). These data demonstrated that Fr. CL-4b possessed both anti-LPS and anti-CpG DNA activities in line with its binding abilities. Thus, Fr. CL-4b was further identified as the active anti-sepsis fraction of *C. lycii*.

Table 2
Herbs posses high affinity for LPS and CpG DNA (RU >100 as).

LPS		CpG DNA	
Name	RU value (arc sec)	Name	RU value (arc sec)
Cortex lycii	384.68	***Cortex lycii***	471.88
Cortex moutan	336.31	***Cortex moutan***	390.70
Rhizoma anemarrhenae	203.45	***Fructus corni***	378.43
Fructus corni	197.13	*Cacumen platycladi*	318.86
Radix Et Rhizoma Rhei	196.32	***Rhizoma anemarrhenae***	309.56
Cortex cinnamomi	176.92	*Pericarpium granati*	276.55
Radix scutellariae	156.33	***Radix Et Rhizoma Rhei***	252.67
Folium Artemisiae Argyi	151.32	***Radix scutellariae***	243.24
Pericarpium granati	156.36	*Rhizoma coptidis*	186.36
Cacumen platycladi	134.58	*Cortex cinnamomi*	192.35
Radix ilicis Pubescentis	132.66	*Radix Rumicis Crispi*	188.65
Semen persicae	127.30	*Herba portulacae*	182.36
Fructus Chebulae Immaturus	122.25	*Fructus tsaoko*	181.32
Radix bupleuri	113.86	*Radix Ilicis Pubescentis*	180.69
Radix Rumicis Crispi	108.55	*Radix Paeoniae Rubra*	176.35
Fructus sophorae	104.24	*Folium Artemisiae Argyi*	168.36
		Herba Scutellariae Barbatae	165.55
		Herba hyperici	156.67
		Flos sophorae	135.26
		Fructus crataegi	133.65
		Fructus Terminaliae Chebulae	128.23
		Spica prunellae	123.67

3.5. Purification and bioactivity identification of the active monomer from Fr. CL-4b

Fr. CL-4b was subjected to the analytical HPLC system for the setup of the isocratic isolation method (Fig. 4A). Two major fractions (CL-4b$_1$ and CL-4b$_2$) were separated by HPLC as monitored by two wave lengths (280 nm and 320 nm). The purity of these two fractions was more than 99% as determined by HPLC with the diode array detector. So the fractions were considered to be single monomers. Then Fr. CL-4b was separated using the preparative HPLC system via this isocratic isolation method, and two major monomers named as CL-4b$_1$ and CL-4b$_2$ (Fig. 4B) were yielded.

Binding assay results showed that CL-4b$_2$ possessed a higher binding ability than CL-4b$_1$ for both LPS and CpG DNA (Fig. 5A). Only CL-4b$_2$ could significantly neutralize LPS (Fig. 5B) and significantly inhibited TNF-α releases from RAW 264.7 cells induced by both LPS and CpG DNA (Fig. 5C). Therefore, CL-4b$_2$ was reasonably determined as the major active monomer of *C. lycii* and received further structure and activity analysis.

3.6. Physicochemical properties and structure confirmation of CL-4b$_2$

CL-4b$_2$ was a pale yellow powder which was soluble in water and partially soluble in methanol. It was identified as an alkaloid compound as positive color reacted with Dragendorff's reagent (data not shown) [17]. The purity of CL-4b$_2$ was over 98% as determined by HPLC with the diode array detector (Fig. 6A). ^{13}C NMR and ^{1}H NMR spectra were the same of Kukoamine B (KB) as reported previous [18]. Till now, CL-4b$_2$ was determined as KB (Fig. 6B). Its content was about 0.1–0.5% in the crude drug of *C. lycii*, determined by HPLC (data not shown).

3.7. Bioactivity identification of KB in vitro and in vivo

To further confirm that KB is a dual inhibitor for LPS and CpG DNA, it was analyzed again in *in vitro* and *in vivo* experiments. *In vitro,* KB significantly inhibited TNF-α and IL-6 release from RAW 264.7 cells induced by both LPS and CpG DNA in a dose-dependent manner (Fig. 7). *In vivo*, pretreatment with 30 mg/kg KB 30 min before the challenged with heat-killed *E. coli* significantly increased the survival of mice. The survival rate rose from 0% to 40% ($p<0.01$) (Fig. 8). So, KB was verified from the above results as the major contributing component for the dual inhibitory activity of *C. lycii* against LPS and CpG DNA.

3.8. Inhibition of KB on important TLR4- and TLR9-mediated signal molecules

For the induction of cytokine release induced by LPS and CpG DNA, receptors (TLR4 and TLR9), signaling adaptors (like MyD88) and transcription factor (like NF-κB) are all sequentially involved. Therefore, in the present experiment, we first investigated the effect of KB on TLR4, TLR9 and MyD88 mRNA expressions up-regulated by LPS and CpG DNA in RAW 264.7 cells. The RT-PCR results showed that KB could inhibit mRNA expressions of TLR4, TLR9 and MyD88 up-regulated by LPS and CpG DNA (Fig. 9A). Additionally, KB also attenuated the LPS and CpG DNA elicited nuclear translocation of NF-κB p65 protein in RAW264.7 cells (Fig. 9B).

4. Discussion

In this study, we presented a dual target guided screening and isolation of natural anti-sepsis fractions and compounds via combination of biosensor technology and chromatography methods. Kukoamine B (KB) was discovered by this dual target guided separation method from traditional Chinese herb *C. lycii* and verified to be a double inhibitor for LPS and CpG DNA, two important pathogenic molecules for sepsis.

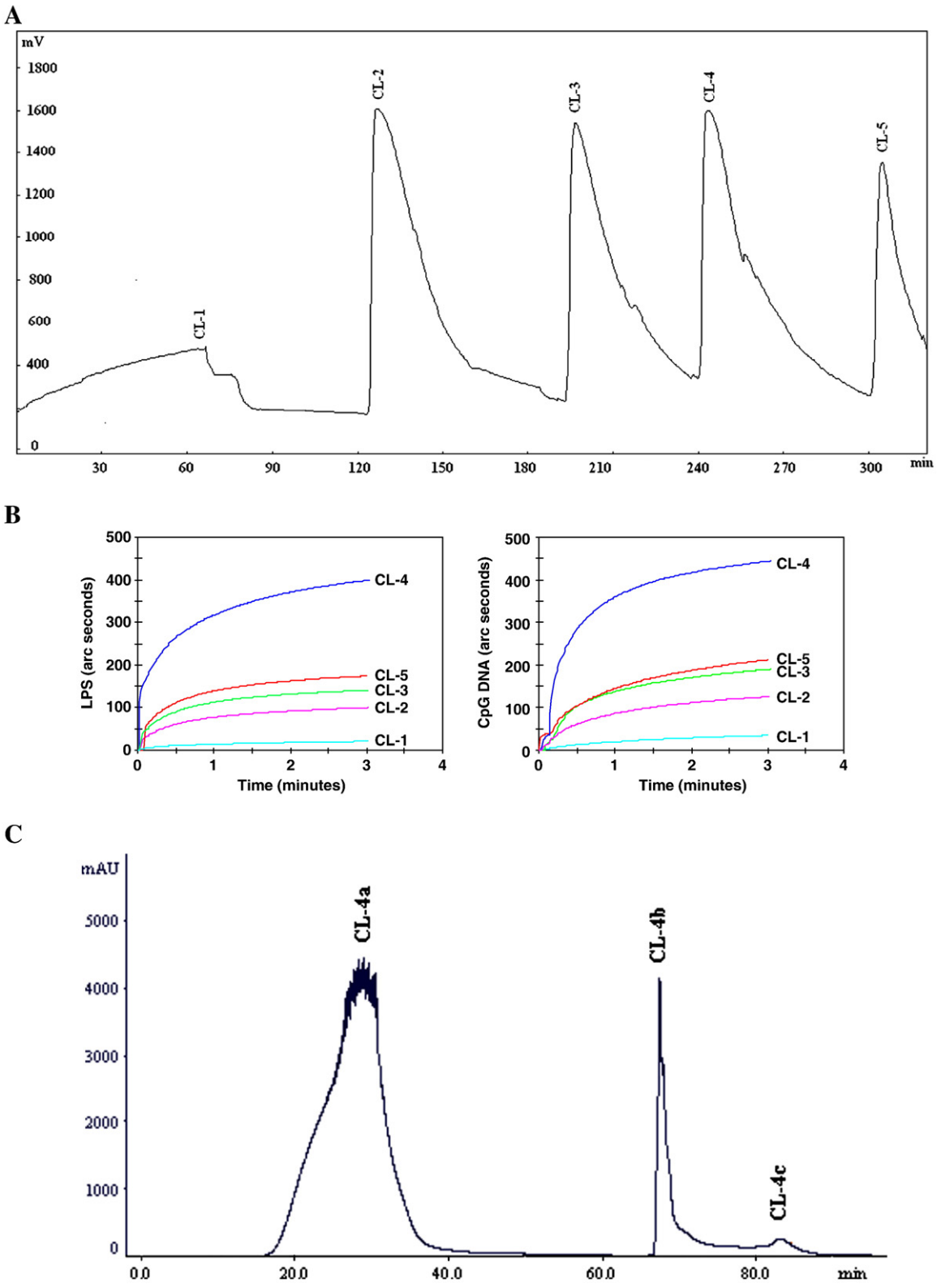

Fig. 2. Dual target guided fractionation of the aqueous extracts of *Cortex lycii*. (A). The aqueous extracts of *Cortex lycii* was subjected to macroporous resin column chromatography and eluted successively with water and ethanol (10%, 20%, 50% and 100%). Five fractions (Fr. CL-1, -2, -3, -4 and -5) were collected and frozen dried for affinity tests. (B). Fr. CL-1, -2, -3, -4 and -5 (200 μg/ml each) were added into the cuvettes immobilized with LPS or CpG DNA and incubated for approximately 3 min for affinity determination. (C). Fr. CL-4 was subjected onto cationic SPFF gels and separated by water, 0.3 M NaCl and 0.5 M NaCl successively into three fractions CL-4a, CL-4b and CL-4c. (D). Fr. CL-4a, 4b and 4c obtained from CL-4b by cationic exchange SPFF gel column chromatography were added into the two cuvettes (100 μg/ml each) for affinity determination. The heights of the binding curves represent their response strength and are thus positively related to their affinities to LPS and CpG DNA.

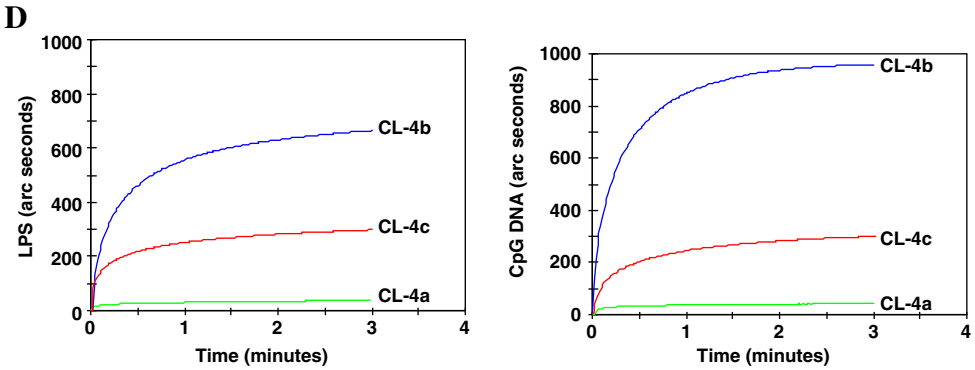

Fig. 2 (*continued*).

Despite considerable progress in genome- and proteome-based high-throughput screening methods and rational drug design, the number of successful single target drugs did not increase appreciably during the past decade. On the other hand, simultaneous inhibition of a small number of targets involved in the complicated process of a certain kind of disease seems to be more efficient than the complete inhibition of a single target [19,20].

LPS and CpG DNA are major contributing pathogenic factors and important drug targets for sepsis. They act as invasive triggering factors to induce subsequent events involved in sepsis, like activation of protein kinases/transcription factors and release of large quantities of proinflammatory cytokines. We have also known PAMPs such as LPS and CpG DNA could not only trigger sepsis by itself but also synergistically trigger sepsis. The anti-sepsis strategy will be more

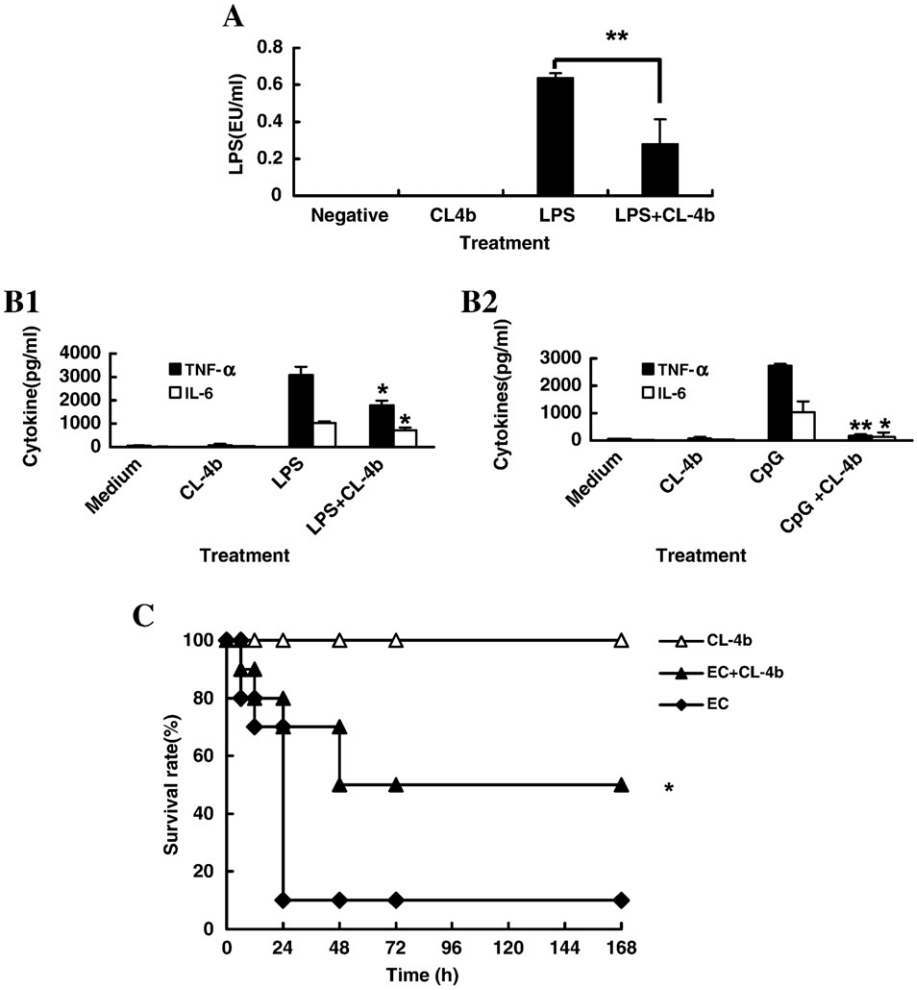

Fig. 3. Fr. CL-4b possesses anti-LPS/-CpG DNA activities *in vitro* and *in vivo*. (A). LAL test for LPS-neutralizing abilities of CL-4b *in vitro*. CL-4b (8 μg/ml) was pre-incubated with LPS (1 ng/ml) in an equal volume for 30 min and reacted with LAL reagents for 60 min. The gel clotting formation activated by the remaining free LPS was detected by the kinetic turbidimetric assay. **: $p<0.01$ for the comparison between groups indicated. (B). RAW 264.7 cells were incubated with 100 ng/ml of LPS (B1) or 10 μg/ml of CpG DNA (B2) in the presence or absence of CL-4b (200 μg/ml) for 24 h. TNF-α (black bars) and IL-6 (white bars) levels in the culture supernatants were detected by ELISA. Statistical differences between each groups were examined and noted as *: $p<0.05$ or **: $p<0.01$. (C). KM mice were intraperitoneally injected with 60 mg/kg of CL-4b alone (10 mice, △), 1.0×10^{11} CFU/kg body weight of heat-killed *E. coli* alone (10 mice, ◆), 1.0×10^{11} CFU/kg of heat-killed *E. coli* in combination with 60 mg/kg CL-4b (10 mice, ▲), respectively. *: $p<0.05$ compared with the heat-killed *E. coli* group. CpG DNA and heat-killed *E. coli* was abbreviated as CpG and EC, respectively.

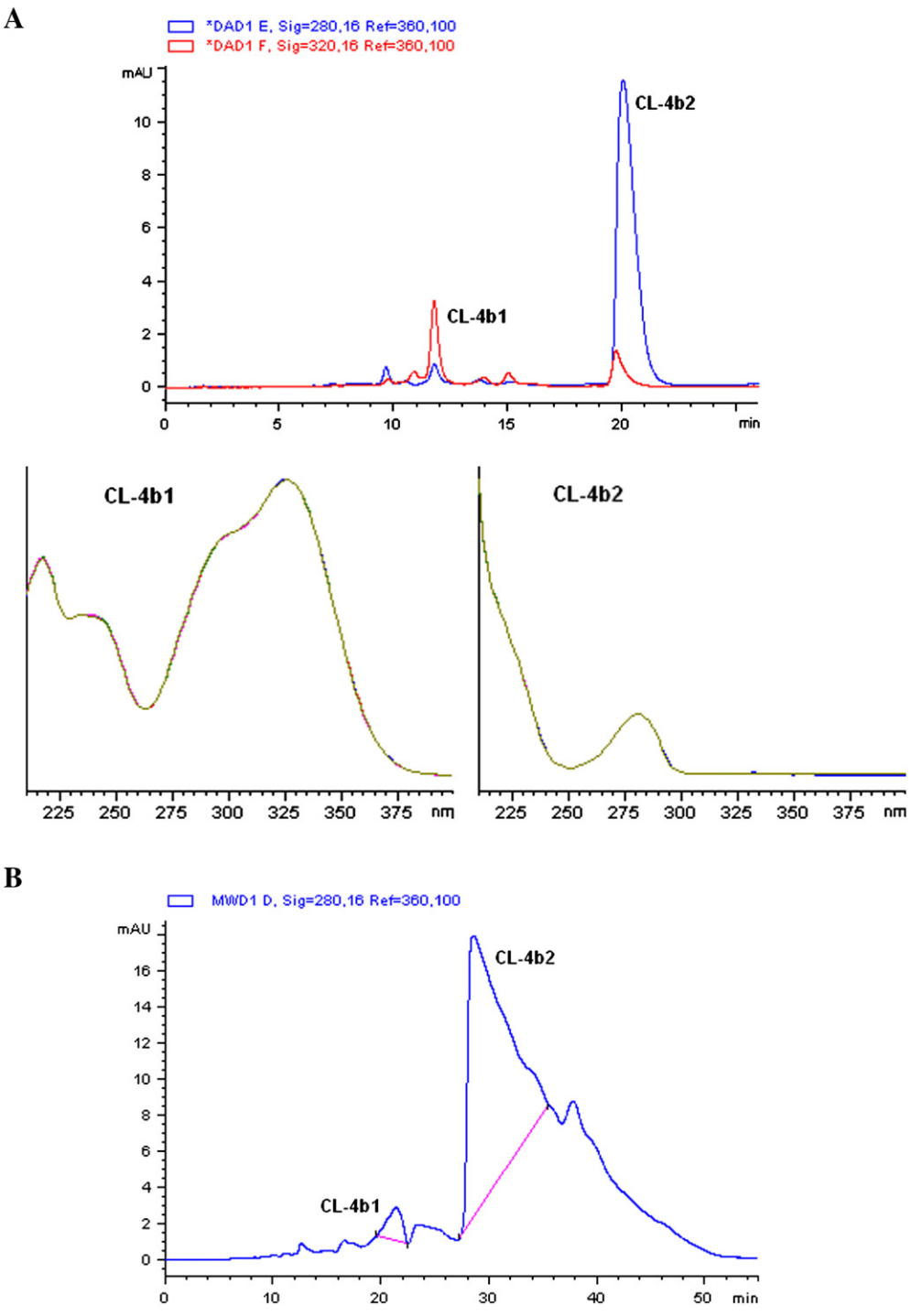

Fig. 4. Purification of two monomers from Fr. CL-4b via HPLC. (A). CL-4b (250 μg/ml) was subjected into the analytical HPLC system and separated by isocratic elution (20% B in 30 min) with 0.5‰ trifluoroacetic acid in water (solvent A) and acetonitrile (solvent B). The five points' purity analysis was performed by the diode array detector in the analytical HPLC system and the spectra of CL-4b$_1$ and CL-4b$_2$ were shown below the analytical graph. (B). CL-4b (10 mg/ml) was subjected into the preparative HPLC system and separated into two major constituents (CL-4b$_1$ and CL-4b$_2$) by isocratic elution (20% B in 60 min) with 0.5‰ trifluoroacetic acid in water (solvent A) and acetonitrile (solvent B).

effective if the triggering factors were selected directly and simultaneously as drug targets. Additionally, LPS and CpG DNA are bacterial components, the elimination of which will cause no unfavorable effects on the host itself. So it is necessary to develop a dual targeted anti-sepsis drug discovery strategy for sepsis by simultaneous neutralization of the two foreign pathogenic factors.

For a variety of reasons, renewed interests have been focused on studying and evaluating naturally existing products with long term therapeutic applications in critical patients [7,10,21,22]. Some traditional Chinese herbs examined with scientific rigor and modern technology in our lab or in other labs were shown to exert excellent anti-sepsis effects via probably direct antagonistic activities on LPS or CpG DNA [11,12,23]. However, it remains unknown whether the contributing compounds inside the herbs can act as a dual inhibitor for LPS and CpG DNA. These compounds are always not easy to discover as they are usually in small proportions and thus extraordinarily difficult

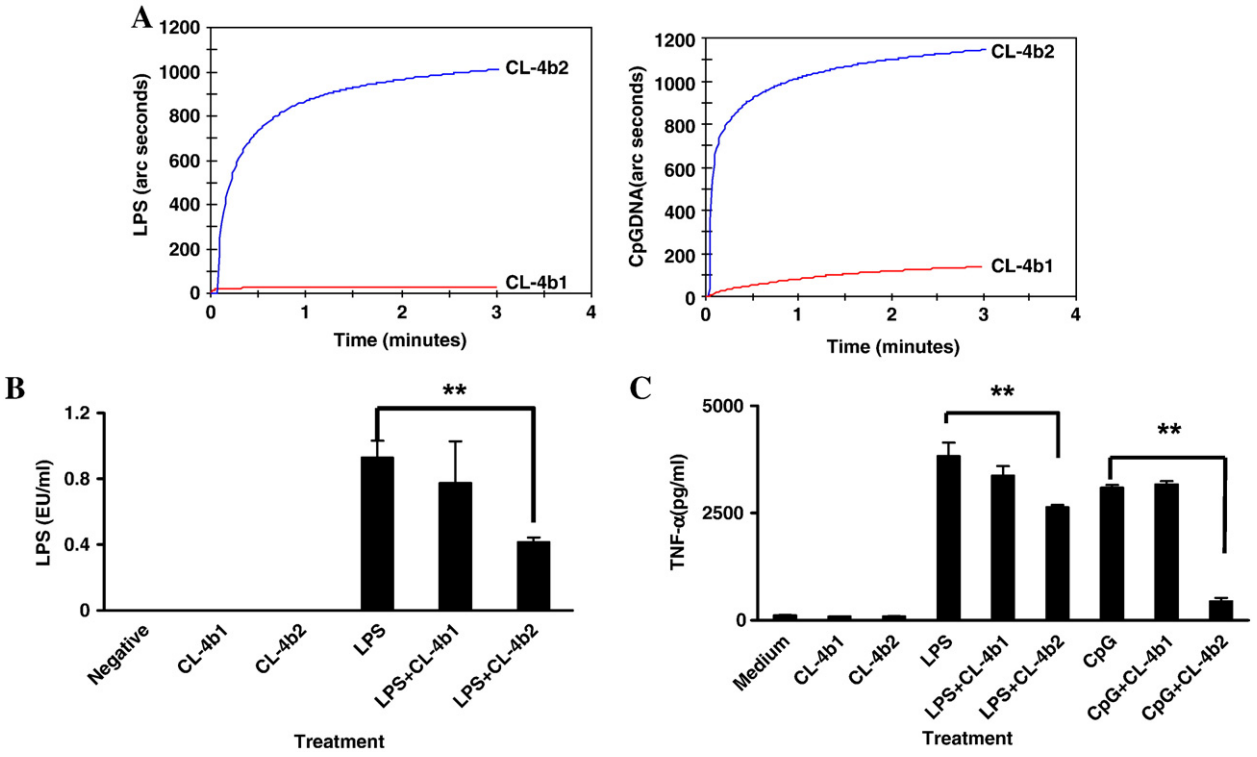

Fig. 5. Bioactivity identification of the active monomer. (A). CL-4b$_1$ and CL-4b$_2$ (50 μg/ml) were added into the cuvettes of the biosensor for affinity determination. Binding curves representing their affinities for LPS (left) and CpG DNA (right) were representative results of three independent experiments. (B). CL-4b$_1$ and CL-4b$_2$ (8 μg/ml each) was pre-incubated with LPS (1 ng/ml) for 30 min and reacted with LAL reagents for 60 min. The remaining free LPS was detected by the kinetic turbidimetric assay. ** represents $p<0.01$ for the comparison between groups indicated. (C). RAW 264.7 cells were incubated with 100 ng/ml LPS or 10 μg/ml CpG DNA in the presence or absence of CL-4b$_1$ or CL-4b$_2$ (both 100 μg/ml) for 24 h. The supernatants were collected and levels of TNF-α were detected by ELISA. ** represents $p<0.01$ for the comparison between groups indicated. CpG DNA was abbreviated as CpG in the figure.

to purify. Besides, no activity information could be provided via only the routine separation methods, it is therefore impossible to find LPS and CpG DNA targeting compounds efficiently. So it is necessary to develop a target guided separation method. Herein, we developed a dual target guided screening and fractionation method for the identification of active compounds with anti-LPS and anti-CpG DNA activities by a coupling biosensor with chromatography.

The affinity biosensor utilized in this study is a kind of an optical sensor based on the surface plasmon resonance (SPR) technique [24,25]. It is widely used as a label free method for the detections of interaction between immobilized molecules and sampling substances. To create the dual target guided affinity detection method, LPS and CpG DNA were immobilized on the reacting surfaces of the sensor on which samples were added and their affinities for LPS and CpG DNA were measured with short time intervals.

Traditional Chinese herbs with heat-clearing and detoxifying activity were always utilized to treat infection and inflammatory diseases. So one hundred and fourteen herbs of this kind were selected. Their affinities for LPS and CpG DNA were screened in this way. *C. lycii* was found with highest affinities for LPS and CpG DNA from these herbs. *C. lycii* is the root bark of Lycium Chinese Miller (solanaceae). There are several active ingredients including organic acids, alkaloids, flavanoids and cyclopeptides in *C. lycii* [26,27]. However, no report has shown the isolation and identification of active anti-sepsis monomers in this herb.

In this study, the biosensor platform was also utilized for the dual target guided isolation of an active anti-sepsis monomer in *C. lycii* by sequential chromatography separation. Macroporous adsorption resin is usually used in the first step for the elimination of inactive ingredients like proteins and polysaccharides [28]. The aqueous extracts of this herb were first isolated by macroporous adsorption resin chromatography columns as the initiating separation [29,30]. The fraction with highest binding affinities for LPS and CpG DNA was rapidly screened out via the biosensor and further purified by cationic exchange chromatography to three fractions. The active fraction named as Fr. CL-4b was shown with highest affinities for both LPS and CpG DNA. It was also verified *in vitro* and *in vivo* to neutralize LPS and CpG DNA and possess anti-sepsis activities, providing evidences that the binding affinity is in line with the inhibitory effects.

Reverse phase HPLC coupled with diode array detection was used for the final purification of monomers (CL-4b$_1$ and CL-4b$_2$). CL-4b$_2$ was identified with LPS/CpG DNA binding and neutralizing abilities. It was determined later as Kukoamine B (KB) by structural analysis and comparison with information published in previous data [16]. KB is a positively charged alkaloid compound. Its content in the crude drug of *C. lycii* is about 0.1–0.5%. So KB is a relatively rich component in *C. lycii*. After all purification process, only 40-50% of KB in the crude drug are extracted (data not shown).

The bioactivities of KB have not been elucidated till now. Herein, KB was identified as a novel anti-LPS and anti-CpG DNA compound of *C. lycii*. It inhibited the release of proinflammatory cytokines (TNF-α) induced by LPS or CpG DNA. The inhibitory effect of KB was further strengthened as it inhibited the upregulation of TLR4, TLR9 and MyD88 mRNA expression as well as the nuclear translocation of NF-kB p65, which were all subsequent events after LPS and CpG DNA stimulation. *In vivo*, simultaneous administration of KB protected mice from intravenous challenge of heat-killed *E. coli*, a mixture of LPS and CpG DNA. In addition, KB is still protective when it was given intravenously and *E. coli* was injected intraperitoneally (data not shown). So we think that the protective effects of KB on sepsis mice might probably due to its anti-LPS and CpG DNA activity instead of its directly binding *E. coli* in this study. However, the *in vivo* function of an agent is complicated. The *in vivo* interaction of KB with bacteria, LPS and other targets will be further studied in the subsequent work.

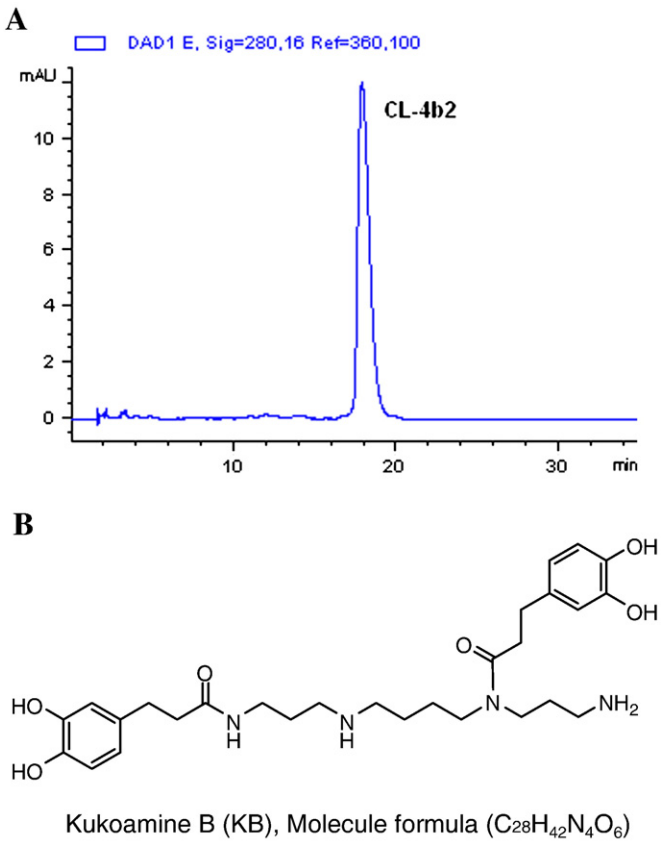

Fig. 6. Structure determination of CL-4b$_2$. (A). The prepared monomer CL-4b$_2$ (0.25 mg/ml) was injected into the analytical HPLC system. Purity verification was identified by diode array detection. (B). The structure and molecule formula of KB.

The active fractions and the monomer were found with higher affinity and stronger inhibitory activity for CpG DNA, although there is no direct report that presents explanation for this phenomenon. According to the results of systematically exploring of the relationship between variously elongated spermine, spermidine and norspermidine backbones by Anurupa, spermine (backbone of KB) may not be the optimal structure for LPS-binding, as the enhancement of LPS-neutralizing activity was observed paralleled to the increasing in the backbone length [31]. Therefore, we think it is probably due to the unique structure of KB that makes it more capable of neutralizing CpG DNA than LPS. However, this speculation needs further investigation to prove in the future.

In conclusion, our results demonstrate for the first time that the biosensor based dual target guided affinity screening technique

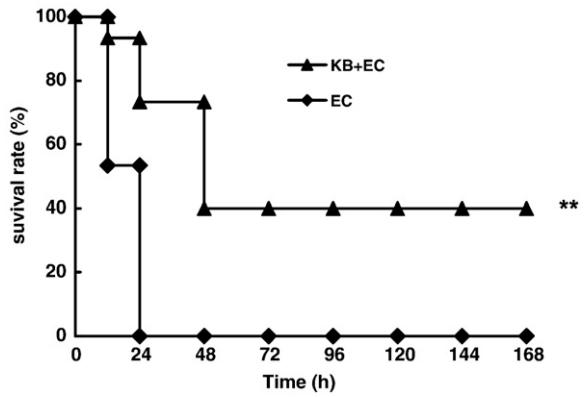

Fig. 8. KM mice were randomly divided into two groups (20 mice per group). Each group was treated with ◆: Heat-killed *E. coli* (1.0×10^{11} CFU/kg body weight); ▲: Combination of heat-killed *E. coli* (1.0×10^{11} CFU/kg body weight) and CL-4b$_2$ (30 mg/kg). Mortality of the mice was observed for 7 days. **: $p<0.01$ compared with the heat-killed *E. coli* group. Heat-killed *E. coli* was abbreviated as EC in the figure.

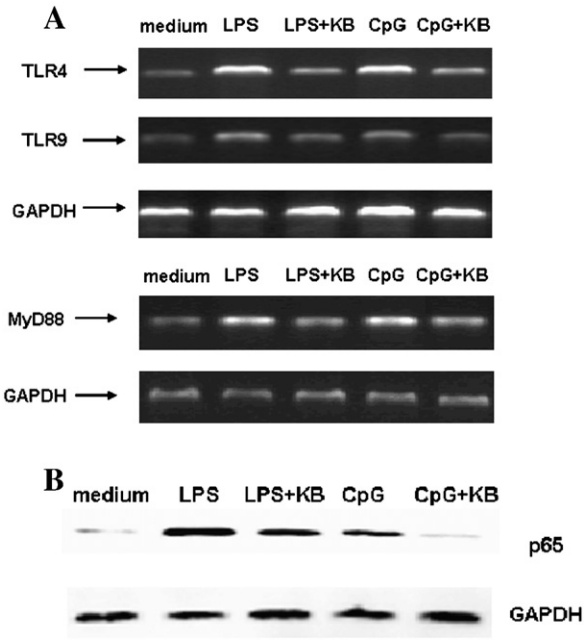

Fig. 9. Effects of KB on LPS and CpG DNA induced mRNA expression of receptors and mediators and translocation of p65. (A) RAW264.7 cells were each incubated with100 ng/ml LPS or 10 μg/ml CpG DNA in the presence or absence of KB (200 μM) for 4 h. The mRNA expression of TLR4, TLR9 and MyD88 was analyzed by RT-PCR. (B) RAW264.7 cells were each incubated with100 ng/ml LPS or 10 μg/ml CpG DNA in the presence or absence of KB (200 μM) for 1 h. Nuclear protein was extracted and NF-κB p65 protein was analyzed by western blot.

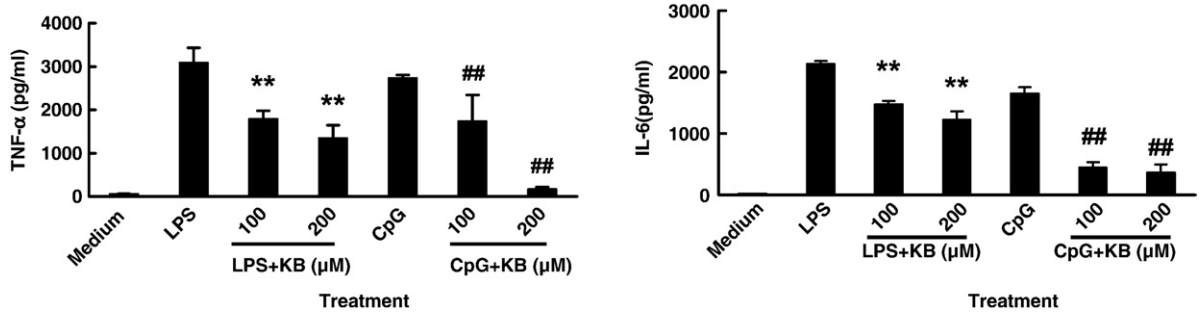

Fig. 7. KB reduces release of TNF-α and IL-6 from RAW 264.7 cells induced by LPS and CpG DNA. RAW 264.7 cells were incubated with 100 ng/ml LPS or 10 μg/ml CpG DNA in the presence or absence of KB (100, 200 μM) for 24 h. TNF-α and IL-6 in the supernatants were detected by ELISA. *: $p<0.05$ and **: $p<0.01$ represent statistical differences compared to groups of LPS. #: $p<0.05$ and ##: $p<0.01$ represent statistical differences compared to groups of CpG DNA. Differences were made among treatment groups in the presence or absence of KB.

coupled with chromatography is efficient for screening and isolating novel anti-sepsis monomers from traditional Chinese herbs. KB, the effective LPS/CpG DNA inhibitor isolated from *C. lycii* under the dual target guide, is worth further investigating as a promising drug candidate for the sepsis treatment.

Acknowledgements

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