Emodin Successfully Inhibited Invasion of *Brucella abortus* Via Modulating Adherence, Microtubule Dynamics and ERK Signaling Pathway in RAW 264.7 Cells

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The aim of this work is to investigate the protective efficacy of emodin, an active, naturally-occurring anthraquinone derivative of several traditional Chinese herbs, against *Brucella abortus* infection in macrophages. *Brucella* were incubated with different concentrations of emodin and showed that bacterial survival rates were markedly reduced in a dose-dependent manner at increasing incubation time points. Through bacterial infection assay, the highest non-cytotoxic concentration of emodin demonstrated attenuated invasion of *Brucella* into macrophages, however it did not inhibit the growth of these pathogens within the host cells. On the other hand, emodin effectively decreased the number of bacteria that adhered to host cells, which indicated its potential as an anti-adhesin agent. Furthermore, using immunoblotting and FACS assay for detecting MAPK signaling proteins and F-actin polymerization, respectively, the results showed that the emodin-incubated cells displayed modest reduction in the phosphorylation levels of ERK1/2 and inhibition of F-actin polymerization as compared to control cells. These findings indicate the potential use of emodin as a naturally-occurring alternative method for the prevention of animal brucellosis although this requires confirmation of safe clinical doses.

Keywords: *Brucella abortus*, emodin, antibacterial, invasion

Introduction

Brucella (B.) abortus is a facultative intracellular pathogen that resists killing by neutrophils, replicates within macrophages and persists as a chronic infection with diverse pathological manifestations including arthritis, endocarditis and meningitis in humans, and spontaneous abortion in domestic animals [1]. New cases of brucellosis in humans are reported to be approximately half a million annually, not reflecting the total number of cases worldwide since the disease remains undiagnosed or misdiagnosed [2].

The intracellular lifestyle of *Brucella* facilitates successful evasion of the host immune response and protection from antimicrobial compounds [3]. No approved human vaccine is available for brucellosis and the patients infected with *Brucella* require a prolonged course of treatment using

multiple antimicrobial agents that are often harmful due to incidence of relapses as well as adverse effects that include hepatotoxicity and gastric damage [2]. Therefore, there is an urgent necessity for developing improved, safe and effective alternative treatment strategies for the disease.

Emodin (1,3,8-trihydroxy-6-methylanthraquinone), a naturally-occurring anthraquinone derivative and an active ingredient in traditional Chinese herbs *Rheum palmatum*, *Polygonum cuspidatum*, *Polygonum multiflorum*, *Aloe vera* and *Cassia obtusifolia*, has been demonstrated to possess a wide range of pharmacological benefits including antiviral, antibacterial, antiallergenic, antiosteoporotic, antidiabetic, anti-inflammatory and antitumor effects [4]. Despite these benefits of emodin, the compound has not been studied for its potential as an effective treatment for brucellosis. Consequently, the present study focuses on the effects of

emodin on the survival of *B. abortus*, and the pathogen's ability to invade and subsequently replicate within a murine macrophage cell line of RAW 264.7 cells.

Materials and Methods

Emodin Preparation

Emodin was purchased from Sigma-Aldrich (USA). The powder was dissolved in dimethyl sulfoxide (DMSO) to the concentration of 1 mg/ml and then diluted to different concentrations in phosphate-buffered saline solution (PBS, pH 7.4).

Bacterial Strain

B. abortus 544 (ATCC 23448), a smooth, virulent *B. abortus* biovar 1 strain used in the present study, is a standard wild-type cultivated in Brucella broth or on agar (1.5% agar) (Becton Dickinson, USA) and grown in broth at 37°C with shaking until the stationary phase was reached. Serial dilutions on agar plates were performed to assess the number of viable bacteria.

Cell Culture

RAW 264.7 cells (ATCC, USA) were grown at 37°C in 5% CO₂ atmosphere in RPMI 1640 containing 10% (v/v) heat-inactivated fetal bovine serum (FBS) with or without antibiotics (100 U/ml penicillin, and 100 µg/ml streptomycin) depending on the experiment. All the reagents were purchased from Gibco (USA). Fresh culture with PBS was used as a control in all in vitro assays performed.

Cell Viability

An overnight culture of RAW 264.7 cells at a concentration of 1×10^5 cells/well in a 96-well culture plate was incubated in the presence of emodin (3, 1.5, 0.75, 0.3, and 0.15 µg/ml) for 48 h and cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) cleavage assay as previously described [3]. The highest non-cytotoxic concentration of emodin (0.3 µg/ml) was used in the succeeding experiments.

Bacterial Survival

Brucella at a concentration of 1×10^4 colony-forming units (CFU)/ml were added to different concentrations of emodin (3, 6, and 15 µg/ml) in PBS incubated at 37°C for 0, 2, 4, 6, and 24 h. Bacterial survival rates were expressed as a percentage of the survival rate of the treated sample relative to the control sample set to 100%.

Bacterial Infection

A bacterial invasion assay was performed as previously described [3]. Briefly, an overnight culture of RAW 264.7 cells at a concentration of 1×10^5 cells/well in a 96-well culture plate was pre-incubated with emodin or PBS for 4 h. The cells were washed with PBS, incubated in fresh medium (RPMI 1640 with 10% heat-inactivated FBS) and then infected with *B. abortus* at multiplicities

of infection (MOIs) of 10. The cells were centrifuged at 150 ×g for 10 min and incubated at 37°C in 5% CO₂ for 0 or 30 min. At different time points, the cells were washed and incubated in fresh medium containing gentamicin (30 µg/ml) for 30 min to kill adhered and remaining extracellular bacteria. The cells were then washed again, lysed with distilled water, and the cell lysates were spread onto Brucella agar plates in triplicate. In a separate procedure for intracellular replication assay, cells were infected with *B. abortus*, washed and then incubated with fresh medium containing gentamicin and emodin or PBS for 2, 24, and 48 h. Bacterial infection, washing, lysing and plating procedures were done in the same manner as in the invasion assay.

Bacterial Adherence

An overnight culture of RAW 264.7 cells at a concentration of 1×10^6 cells/well in a 12-well culture plate with 18-mm diameter glass coverslips (Fisher Scientific, USA) was pre-incubated with emodin or PBS for 4 h. Forty minutes before infection, the cells were treated with cytochalasin D (500 µg/ml) to inhibit bacterial internalization. Infection of cells for 30 min was done in the same manner as in the bacterial invasion assay. After washing, cells were fixed with 4% paraformaldehyde at 37°C for 30 min, permeabilized with cold methanol (−20°C) for 10 sec, incubated with anti-*B. abortus* polyclonal rabbit serum (1:500) at 37°C for 1 h and then with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG) (1:500, Sigma-Aldrich, USA) at 37°C for 1 h. Random selection of one-hundred macrophages was done to determine the number of bacteria that adhered to these cells.

Fluorescence Microscopy

RAW 264.7 cells were prepared and incubated with emodin or PBS as in the bacterial adherence assay. In parallel, negative control cells were prepared for comparison. After washing, cells were infected with FITC (Sigma-Aldrich, USA)-labelled *B. abortus* for 10 min as previously described [3]. The cells were then fixed, permeabilized with 0.1% Triton X-100 for 10 min, blocked with 2% goat serum in PBS for 30 min and stained with 0.1 µM rhodamine-phalloidin (Cytoskeleton, Inc., USA) for 30 min at 22°C. A laser scanning confocal microscope (Olympus FV1000, Japan) and FV10-ASW Viewer 3.1 software were used to capture and process fluorescence images.

Flow Cytometry

An overnight culture of RAW 264.7 cells at a concentration of 1×10^8 cells/well in a 6-well culture plate was pre-incubated with emodin or PBS for 4 h. A similar procedure was done as previously described [3]. The F-actin content was then measured using a BD FACSVerse flow cytometer (BD Biosciences, USA).

Western Blot

RAW 264.7 cells were prepared and pre-incubated with emodin or PBS as in the flow cytometry for F-actin content determination.

After washing, the cells were incubated at 4°C overnight using ice-cold radioimmunoprecipitation assay (RIPA) buffer with 1% protease inhibitor cocktail. The cell lysates were collected and the protein concentration was measured using the Bradford protein assay (Bio-Rad, USA). Proteins were separated by SDS-PAGE and electrically transferred onto Immobilon-P membranes (Millipore, USA). The membranes were incubated with mitogen-activated protein kinases (MAPKs) (ERK1/2, JNK and p38 α) as previously described [3], exposed to a Molecular Imager ChemiDoc XRS+ system machine (Bio-Rad, USA) and the immunoblot signals were quantified using NIH ImageJ software. In addition, we determined the effect of ERK activator, MEK-1 (Santa Cruz Biotechnology, Inc., Germany) on the internalization of *Brucella* in emodin-incubated cells. The same procedures were performed as in the bacterial invasion assay wherein after pre-incubation of macrophages with emodin, the cells were incubated with MEK-1 (3 μ g/well) for 45 min prior to infection.

Statistical Analysis

The results are expressed as the means with standard deviation (SD) for replicate experiments. Statistical analysis was performed using GraphPad InStat software version 3 (GraphPad Software, Inc., USA). Student's *t*-test or one-way ANOVA was used to make statistical comparisons between the groups. Differences of *p* < 0.05 were considered to be statistically significant.

Results

Effects on Cell Viability and Bacterial Survivability

RAW 264.7 cells were incubated with different concentrations of emodin (3, 1.5, 0.75, 0.3, 0.15 μ g/ml) for 48 h. The OD value (cell survival) at higher concentrations

Fig. 1. Incubation of *B. abortus* with different concentrations of emodin (0, 3, 6, and 15 μ g/ml) at 0, 2, 4, 6, and 24 h. Data represent mean $\pm$ SD of at least two replicates.

(3, 1.5, and 0.75 μ g/ml) decreased, which indicated cytotoxic effect. The highest non-cytotoxic concentration (0.3 μ g/ml) without significant effect on the cell viability was used in the succeeding experiments. In addition, survival rates of *Brucella* were reduced significantly in higher concentrations of emodin (3, 6, and 15 μ g/ml) over increasing time of incubation (Fig. 1), indicating that emodin has antibacterial effect against *Brucella* in a dose-dependent manner.

Effects on Bacterial Invasion and Intracellular Growth

Invasion of *Brucella* into macrophages was determined at

Fig. 2. The effects of emodin on the efficiency of (A) bacterial invasion at 0 and 30 min post-infection time and (B) intracellular replication of *B. abortus* at 2, 24, and 48 h post-incubation.

Data represent the mean $\pm$ SD of at least two replicates. Statistically significant differences relative to the untreated control are indicated by asterisks (**p* < 0.05; ***p* < 0.01).

indicated times after pre-incubation with emodin. The number of CFU was markedly reduced in emodin-incubated cells as compared to control cells at 0 ($p < 0.05$) and 30 min ($p < 0.01$) post-infection (Fig. 2A). However, this pattern was not evident in the intracellular growth of *Brucella* within macrophages where the number of CFU in the emodin-incubated cells was not significantly different from that of the control cells (Fig. 2B). These data indicated that emodin has an inhibitory effect on the phagocytosis of *Brucella* into macrophages but no influence on the bacterial replication when the pathogens are inside the host cells.

Effects on Bacterial Phagocytosis and Adherence

Since emodin significantly inhibited the entry of *Brucella* into macrophages, we evaluated its effect on actin polymerization, which is involved in the phagocytic uptake of microbes. In this study, the fluorescence microscopy examinations revealed reduced formation of lamellipodia,

filopodia and microspikes in emodin-incubated cells as compared to control cells after exposure to *Brucella* (Fig. 3A), indicating an inhibition in the F-actin polymerization in these cells and this was confirmed by quantification of F-actin content using flow cytometry. The results showed a significant reduction in the F-actin content in emodin-incubated cells as compared to control cells (Fig. 3B). On the other hand, we assessed the involvement of emodin on the pathogen's adherence to host cells by fluorescence microscopy. In the emodin-incubated cells, the number of bacteria adhered was significantly reduced (34 ± 7.5) as compared to control cells (81 ± 11.09), showing a reduction rate of $58.07 \pm 9.8\%$ ($p < 0.05$) (Fig. 4). Taken together, these findings indicated that emodin attenuated F-actin polymerization, which is a concern in the phagocytosis of *Brucella* in macrophages [5], and effectively attenuated attachment of these pathogens on the surface membrane of macrophages that could explain the reduction in the

Fig. 3. The effects of emodin on the modulation of F-actin polymerization using (A) fluorescence microscopy (scale bars = 5 μm) and (B) flow cytometry analysis.

Data represent mean $\pm$ SD of at least two replicates. Statistically significant differences relative to the untreated control are indicated by asterisks (* $p < 0.05$; ** $p < 0.01$).

Fig. 4. The effects of emodin on the adherence of *B. abortus* in RAW 264.7 cells.

Data represent mean $\pm$ SD of at least two replicates. Statistically significant differences relative to the untreated control are indicated by asterisks (* $p < 0.05$).

phagocytosis of *Brucella*.

Effects on Phagocytic Signals

Mitogen-activated protein kinase (MAPK) plays an important role in the phagocytic microbial entry mechanism including phagocytosis and actin cytoskeleton remodeling [5, 6]. The phosphorylation level of ERK1/2 in emodin-incubated cells at 30 min post-infection was reduced by 1.29-fold compared to control cells (Fig. 5). These findings suggested that the inhibitory effects of emodin on the phagocytosis of *Brucella* and F-actin polymerization in RAW 264.7 cells could be due to restraint of MAPKs, particularly ERK1/2.

Since emodin exclusively repressed phosphorylation of ERK1/2 and did not affect phosphorylations of JNK and p38, we treated emodin-incubated cells with ERK activator (MEK-1) to determine the effects in the invasion of *Brucella* in these cells. The results showed that internalization of *Brucella* was increased at 0 ($p < 0.01$) and 30 min ($p < 0.05$) post-infection as compared to emodin-incubated cells, although slightly decreased compared with the PBS-incubated cells but not significantly (Fig. 2A), which suggested that the mechanisms involved in the attenuation of bacterial invasion by emodin could be ERK-dependent.

Discussion

Brucella spp. are considered as major veterinary pathogens that can cause serious disease in humans with annually

Fig. 5. The effects of emodin on the phosphorylation level of ERK1/2 signaling in RAW 264.7 cells.

(A) Western blot images and (B) the phosphorylation levels of ERK1/2 shown are representative of at least three independent experiments. Statistically significant differences relative to the untreated control are indicated by asterisks (* $p < 0.05$).

reported new cases of about 500,000 around the globe and yet no patient-friendly treatment or effective vaccines are available for humans [7, 8]. These pathogens undergo several interactions with host cells as they invade and replicate within professional phagocytic cells, undertaking evasion of the killing mechanisms within macrophages, modulation of host immune response and dissemination to preferred tissues through cellular tropism – leading to chronicity and a difficult-to-treat form of infection [9, 11]. Currently, treatment of brucellosis using traditional plants and natural products has been massively reported as effective alternative control of this disease. The previous study proved that the Chinese herbs such as *Coptis chinensis*, *Radix paeoniae rubra*, *Galla chinensis*, and *Cortex phellodendri* possessed inhibitory effect against *Brucella melitensis* strains [10]. Moreover, the study by Lee (2011) elucidated that *Galla Rhois*, which has long been applied in traditional Korean and Oriental medicine, showed potential for use in

the prevention and treatment of brucellosis while also stating that some components of *Galla Rhois* had inhibitory effects on the growth of *Clostridium* spp., *Escherichia coli*, and *Salmonella typhimurium* [12].

Most bacterial mechanisms for engaging with host cells strictly depend on their adhesion to host cells [13]. *Brucellae* are known to bind to distinct phagocytic or unknown receptors in macrophages that subsequently lead to zipper-like phagocytosis or through lipid raft microdomains requiring F-actin polymerization and dynamic rearrangement of the actin cytoskeleton – which are essential in the uptake of microbes [9]. Since there is an alarming increase in drug-resistant bacteria and bacterial adhesion is one of the initial stages of infectious processes [14], one attractive approach is the use of natural agents that can effectively interfere with the ability of these pathogens to adhere to target cells. Emodin has been shown to significantly inhibit formation of biofilm in *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*, likely via penetration into the biofilm and interference with the quorum sensing (QS) system, leading to bacterial detachment and dispersal from the surface [15]. Furthermore, emodin has been reported to disrupt lipid rafts in the membrane of CD14-negative endothelial cells by depleting cholesterol [16]. Similarly, our results showed that emodin effectively inhibited the adherence of *Brucella* into the surface membrane of macrophages probably due to its ability to disrupt lipid rafts, or through competition of common receptors like those of *Brucella*, although this needs to be proven.

The antibacterial efficacy of emodin has been demonstrated against several microorganisms including *Bacillus subtilis*, *Staphylococcus aureus*, *Helicobacter pylori* and *Haemophilus parasuis* [17–20]. It has been observed that the antibacterial activity of emodin was concentration-dependent and attributed to its ability to destroy cell membrane integrity, increase membrane permeability and DNA damage [20]. Similarly, the antibacterial efficacy of emodin was observed in a concentration-dependent manner at increasing incubation periods. These results suggest that emodin is a viable candidate for treating animal brucellosis although it requires systematic animal toxicology experiments for the confirmation of safe clinical doses.

F-actin polymerization, which is an essential part of the entry mechanisms in phagocytosis of pathogens, has been identified to be involved in the ingestion of *Brucella* in epithelial cells and macrophages [21]. Emodin was reported to markedly inhibit the epidermal growth factor-induced formation of filopodia and lamellipodia [22]. This is similar to our present study in which the incubation of RAW 264.7

cells with emodin resulted in reduced formation of filopodial structures and F-actin content upon exposure to *Brucella*. On the other hand, the activation of the MAPK cascade also plays an important role in the phagocytosis of bacteria and remodeling of the actin cytoskeleton [13, 23]. The effect of emodin on MAPK signaling pathways was previously investigated and showed that it did not affect the activation of MAPK p38 and JNK but markedly inhibited the activation of ERK1/2 in rat C6 glioma cell line [24]. Furthermore, emodin effectively attenuated phosphorylation of ERK in HepG2 cells [25]. These results were in agreement to our present study where incubation of RAW 264.7 cells with emodin notably inhibited ERK1/2 phosphorylation levels. This suggests that the inhibitory effect of emodin on *Brucella* invasion into macrophages could be attributed to downregulation of MAPK. In conclusion, emodin provides protection against *Brucella* invasion into macrophages, suggesting that the use of emodin, possibly as a feed supplement, may provide a natural alternative option in the prevention of animal brucellosis although further validation of its therapeutic use in animals is still required.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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