Effect of emodin on chondrocyte viability in an *in vitro* model of osteoarthritis

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Abstract. Emodin is an anthraquinone isolated from the Chinese herb Radix et Rhizoma Rhei and has been used to treat various diseases for centuries. The aim of the present study was to investigate the effect of emodin on the inflammatory mediators in rat chondrocytes. In the present study, chondrocytes were isolated from rats, cultured and harvested when they reached generation P3. Cells were treated with different doses of emodin (10, 20, and 30 μg/ml) followed by interleukin 1β (IL-1β, 10 ng/ml). Control cells were either untreated or treated with IL-1β alone. An enzyme-linked immunosorbent assay was used to measure levels of nitric oxide (NO) and prostaglandin E2 (PGE2). Reverse transcription-quantitative polymerase chain was performed to measure levels of matrix metallopeptidase (MMP)-3 and -13 mRNA. The expression of MMP-3, MMP-13, extracellular-signal regulatory kinase (ERK)1/2, phosphorylated ERK1/2 and β-catenin proteins were detected by western-blot analysis. The results demonstrated that treatment with emodin treatment reduced the cytotoxicity of IL-1β and inhibited the expression of NO and PGE2 in rat chondrocytes. Furthermore, emodin inhibited the expression of MMP3 and MMP13, and the phosphorylation of various proteins involved in the ERK and Wnt/β-catenin pathway. Therefore, emodin is able to promote the proliferation of chondrocytes by inhibiting the ERK and Wnt/β-catenin pathway and downregulating the expression of a series of inflammatory mediators in chondrocytes.

Introduction

Osteoarthritis (OA) is a common chronic joint disease in elderly and middle-aged people and in China, the incidence of OA is increasing rapidly due to the ageing population. Performing physically strenuous jobs, which is common for people in less developed areas of China, is closely associated with the development of OA (1). Indeed, the incidence of knee osteoarthritis in elderly females is higher in China (30-45%) than in the US (20-35%) (2). Degenerative changes of the articular cartilage, cartilage destruction and secondary subchondral bone hyperplasia are the primary pathological features of OA, and the clinical manifestations of this disease include joint pain, joint deformity and dysfunction (1).

Several studies have been performed to investigate the roles of cytokines in pathogenesis of OA. It has been demonstrated that interleukin (IL)-1β serves an important role during the inflammatory response to cause cartilage tissue injury in the body (3,4). Chondrocytes respond to IL-1β by secreting various inflammatory mediators, including cytokines, chemokines, matrix metalloproteinases (MMPs), nitric oxide (NO) and prostaglandin E2 (PGE2) (5-7). These factors serve crucial roles in the degeneration of cartilage matrix and destruction of articular cartilage during the development of OA and have therefore been selected as potential targets to treat OA (8). Emodin is a type of anthraquinone isolated from the Chinese herb Radix et Rhizoma Rhei and has been reported to exhibit anti-inflammatory, antibacterial and antitumor action (9). Emodin has been reported to exhibit therapeutic effects in various diseases. For example, emodin inhibits the effect of glucocorticoids, which may in turn alleviate insulin resistance and reduce the severity of type 2 diabetes (9). Furthermore, emodin has been reported to have an anti-cancer effect in human pancreatic cancer (10) and exhibit neuroprotective effects against glutamate toxicity (11). In addition, it has been reported that emodin may suppress lipopolysaccharide (LPS)-induced pro-inflammatory responses and the activation of nuclear factor (NF)-κB (12), meaning that it may be used to treat inflammatory disorders.

The aim of the current study was to investigate the effect of emodin on the inflammatory mediators involved in the development of osteoarthritis. The results may enable the development of novel diagnostic and therapeutic strategies to treat osteoarthritis.

Materials and methods

Isolation and culture of rat chondrocytes. A total of 8 Sprague Dawley rats (8 weeks old, 250-300 g; 4 males and 4 females) were purchased from Guangdong Medical Laboratory Animal

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Center [Foshan, China; Certificate of Quality no. SCXK (Guangdong) 2015-0019]. Rats were kept in a quiet environment at 25˚C, in strict compliance with their circadian rhythm (30-70% relative humidity; 12 h light-dark cycle) for 1 week before operation. All rats were had *ad libitum* access to food and water. Rats were sacrificed and knee joints were cut in the middle following disinfection with iodine tincture and 75% ethanol. The articular cartilage of the femoral condyle, trochlear and patella were collected and placed in phosphate-buffered saline (PBS) containing double antibiotics (100 U/ml penicillin and 100 g/ml streptomycin). Tissues were washed 3 times with PBS containing double antibiotics and blood were removed using sterile surgical instruments. Cartilage tissue was cut using ophthalmic scissors and placed in a sterile bottle. Following magnetic stirring, 0.25% trypsin was added to completely digest cartilage tissue. Subsequently, centrifugation was performed at 300 x g for 8 min at room temperature and the supernatant was discarded. Then, 4 ml Dulbecco's Modified Eagle's medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) was mixed with cells. Cells were inoculated into a 25-cm^2 flask and maintained at 37˚C in an incubator containing 5% CO_2. The culture medium was replenished every three days and cells were observed using an inverted microscope. In the test group, chondrocytes collected following 3 subcultures (each, 37˚C for 24) were treated with 10, 20 or 30 μg/ml emodin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 2 h. Subsequently, 10 ng/ml IL-1β (Sigma-Aldrich; Merck KGaA) was added to the cells and they were incubated for 24 h (13). Treatment with LPS (1 μ/m; Sigma-Aldrich; Merck KGaA) and the ERK inhibitor SCH772984 (10 nM, Sigma-Aldrich; Merck KGaA) was administered at room temperature for 2 h prior to subsequent experiments.

All experiments on animals were performed in accordance with the Declaration of Helsinki and the present study was approved by the Animal Ethics Committee of the Chinese Medicine Hospital of Xinjiang Uygur Autonomous Region (Urumqi, China).

The detection of NO and PGE2. Chondrocyte culture medium was centrifuged at 1,800 x g (room temperature) for 15 min to collect supernatant, and levels of NO and PGE2 were determined using ELISA kits (cat. no. KGE001 and KGE004B, respectively; R&D Systems, Inc., Minneapolis, MN, USA). Positive control, negative control and blank control wells were all used. The positive and negative controls were included in the kits, and diluent buffer was used as the blank control. Optical density (OD) values were measured at 450 nM using a microplate reader (Thermo Fisher Scientific, Inc.). Serum levels of NO and PGE2 were then calculated using a standard curve plotted using standard samples produced from the aforementioned ELISA kits.

MTT assay. A total of $2x10^3$ cells/well were inoculated in a 96-well plate following incubation with the different concentrations of emodin (0, 10, 20 and 30 μg/ml) and incubation at 37˚C in 5% CO_2 for 96 h. Subsequently, 20 μl MTT solution (5 mg/ml; Sigma-Aldrich; Merck KGaA) was added. Following incubation for a further 4 h, the supernatant was then removed and 100 μl dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) was added. OD values at 490 nm were measured using a microplate reader.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Following cell culture for 7 days, cells were digested with trypsin and collected following centrifugation (1,800 x g for 10 min at room temperature). TRIzol reagent (Thermo Fisher Scientific, Inc.) was used to extract total RNA from cells. RNA purity and concentration were detected using a NanoDrop ND-2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Inc.) and reverse transcription was performed using SuperScript III Reverse Transcriptase kit (Thermo Fisher Scientific, Inc.). Reverse transcription conditions were as follows: 25˚C for 5 min, 55˚C for 20 min and 80˚C for 20 min. The SYBR®-Green Real-Time PCR Master mix (Thermo Fisher Scientific, Inc.) and cDNA were then used in the qPCR reaction system. The following primers were used for qPCR: MMP-3 forward, 5'-GCATTGGCTGAGTGA AAGAGACTGTATC-3' and reverse, 5'-ATGATGAACGAT GGACAGATGA-3'; MMP-13 forward, 5'-AGTAGTTCCAAA GGCTACAACTTGTTT-3' and reverse, 5'-GGAGTGGTC AAGCCCTAAGGA-3'; and GAPDH forward, 5'-ATGACA ACTCCCTCAAGAT-3' and reverse, 5'-GATCCACAACGG ATACATT-3'. qPCR was performed on an CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The thermocycling conditions were as follows: 95˚C for 50 sec, followed by 40 cycles of 95˚C for 12 sec and 60˚C for 35 sec, and 72˚C for 5 min. Cq values were processed using the $2^{-\Delta\Delta Cq}$ method and the relative expression level of each gene was normalized to the endogenous control GAPDH.

Western blotting. Total protein was extracted from chondrocytes using RIPA Lysis and Extraction buffer (Thermo Fisher Scientific, Inc.) and protein quantification was performed using a bicinchoninic assay kit. A total of 30 μg protein/lane was subjected to 10% SDS-PAGE and were then transferred to PVDF membranes. Membranes were blocked with 5% skimmed milk at room temperature for 3 h. Following washing, membranes were incubated with the following primary antibodies overnight at 4˚C of MMP3 (1:1,000; cat. no. ab53015; Abcam, Cambridge, UK), MMP13 (1:1,000; cat. no. ab39012; Abcam), ERK1/2 (1:1,000; cat. no. ab196883; Abcam), p-ERK1/2 (1:1,000; cat. no. ab214362; Abcam), β catenin (1:1,000; cat. no. ab16051; Abcam) and GAPDH (1:1,000; cat. no. ab9485; Abcam). Following three washes with TBST (15 min/wash), membranes were incubated with horseradish peroxidase conjugated anti-rabbit immunoglobulin G (1:1,000; cat. no. ab6721; Abcam) for 1 h at room temperature. Following three washes with TBST (15 min/wash), enhanced chemiluminescence solution (Thermo Fisher Scientific, Inc.) was added to detect the signals. The relative expression level of each protein was normalized to the expression of the endogenous control GAPDH using Image-J V1.49 software (National Institutes of Health, Bethesda, MA, USA).

Statistical analysis. Data are expressed as mean ± standard deviation and were analyzed by SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA). Comparisons among multiple groups

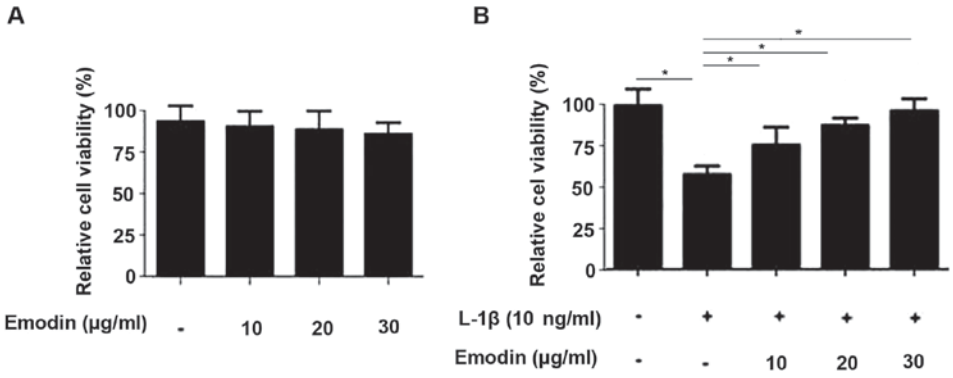

Figure 1. Effect of emodin on cytotoxicity of IL-1β in rat chondrocytes. (A) An MTT assay was used to detect the cytotoxicity of emodin (0, 10, 20 and 30 μg/ml) in chondrocytes. (B) The cytotoxicity of IL-1β (10 ng/ml) was reduced by pretreatment with different concentrations of emodin in a dose-dependent manner. *P<0.05. IL-1β, interluekin 1β.

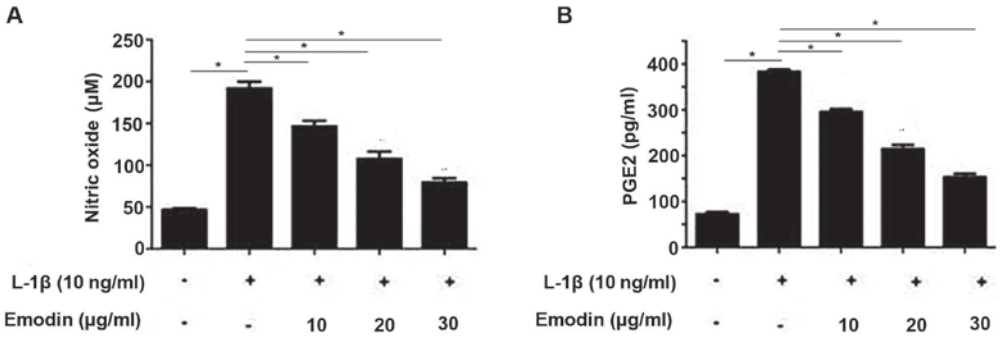

Figure 2. Effect of different concentrations of emodin on NO and PGE2 levels. (A) Effect of different concentrations of emodin on the production of NO. (B) Effect of different concentration of emodin on the production of PGE2. *P<0.05. NO, nitric oxide; PGE2, prostaglandin E2.

were conducted using one-way analysis of variance followed by a least-significant difference test. P<0.05 was considered to indicate a statistically significant difference.

Results

Emodin reduces the cytotoxicity of IL-1β in rat chondrocytes. The cytotoxicity of emodin was determined using an MTT assay. The results demonstrated that 10, 20 and 30 μg/ml emodin was not toxic to rat chondrocytes (Fig. 1A). The treatment of chondrocytes with IL-1β significantly reduced cell viability (P<0.05; Fig. 1B). However, this reduction in chondrocyte viability was significantly reversed following pretreatment with emodin (P<0.05) in a dose-dependent manner.

Emodin inhibits NO and PGE2. NO and PGE2 are closely associated with IL-1β signaling in chondrocytes (14). The inhibitory effect of emodin on IL-1β expression is may determined by measuring NO and PGE2 levels. Following the addition of IL-1β, NO and PGE2 levels were significantly increased compared with the control group (P<0.05; Fig. 2A and B). However, pretreatment with emodin significantly decreased NO and PGE2 levels in a dose-dependent manner (P<0.05).

Emodin inhibits the expression of MMP1 and MMP13. High expression levels of MMP proteins stimulate cartilage degradation (15). In the present study, MMP-3 and MMP13 levels in rat chondrocytes were determined by RT-qPCR and western blotting. It has been demonstrated that IL-1β is able to induce the expression of MMP3 and MMP13 (6) and this was also identified in the present study. The expression of MMP3 and MMP13 were significantly increased following the addition IL-1β (P<0.05; Fig. 3). However, cells the expression of MMP3 and MMP13 significantly decreased in cells pretreated with emodin compared with those treated with IL-1β alone. These decreases occurred in a dose-dependent manner.

Emodin inhibits the activation of the extracellular-signal regulatory kinase (ERK) pathway induced by LPS. The ERK signaling pathway regulates the proliferation and differentiation chondrocytes and serves a key role in the growth and repair of articular cartilage (16). It has also been demonstrated that LPS is able to induce the phosphorylation of ERK1/2 to activate ERK signaling pathway (16) and the results of the current study confirmed that this is the case. As shown in Fig. 4, Compared with untreated cells, the phosphorylation levels of ERK1/2 were significantly increased in cells treated with LPS (P<0.05). Treatment with the ERK inhibitor SCH772984 significantly reversed the increase in phosphorylation levels of ERK1/2 induced by LPS. Furthermore, treatment with different concentrations of emodin also significantly reduced the increased phosphorylation levels of ERK1/2 induced by LPS in a dose-dependent manner, indicating that emodin inhibits the signal transduction of the ERK pathway.

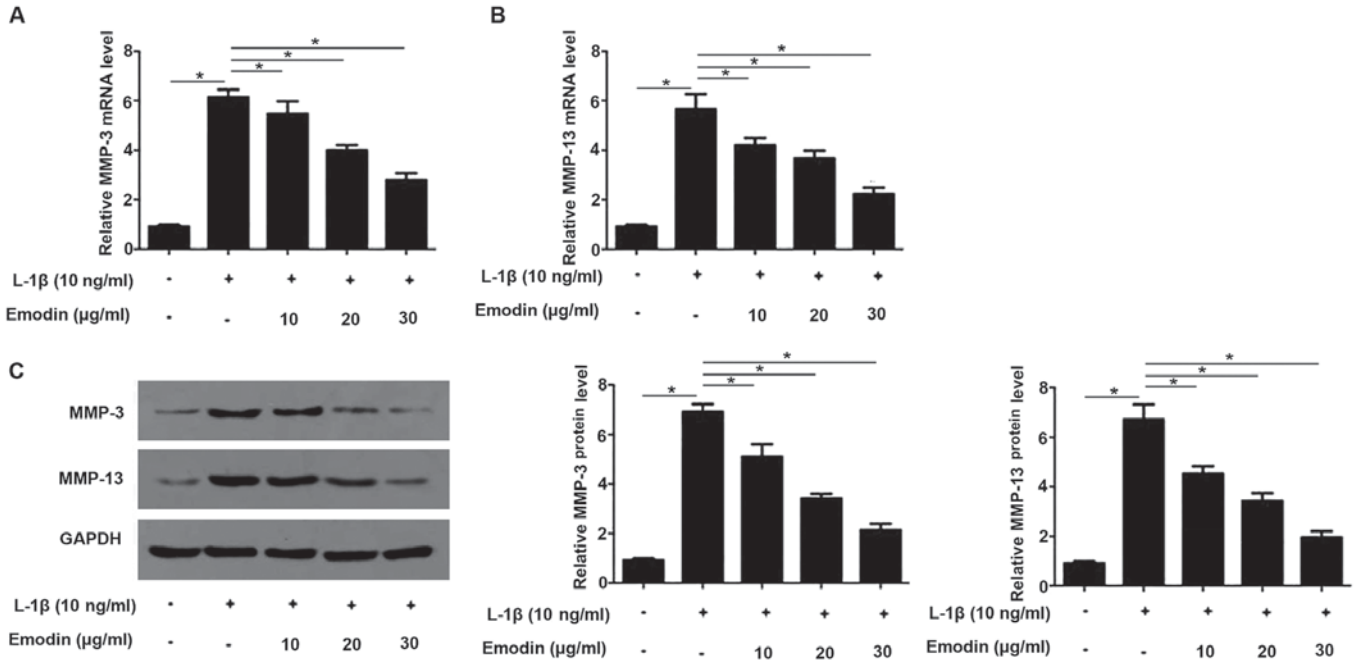

Figure 3. Effects of emodin on the expression of MMP-3 and MMP-13 mRNA and protein. (A) The expression of MMP-3 mRNA was decreased by different concentrations of emodin. (B) The expression of MMP-13 mRNA was decreased by different concentrations of emodin. (C) The expression of MMP-3 and MMP-13 proteins were inhibited by different concentration of emodin. *P<0.05. MMP, matrix metalloproteinase. IL, interleukin.

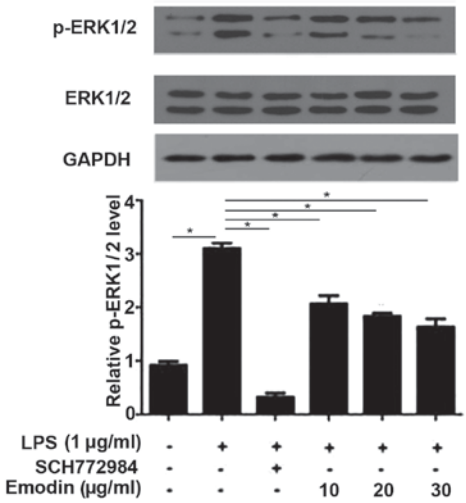

Figure 4. Effect of emodin on the phosphorylation of ERK1/2. *P<0.05. p-, phosphorylated; ERK, extracellular-signal regulatory kinase; LPS, lipopolysaccharide.

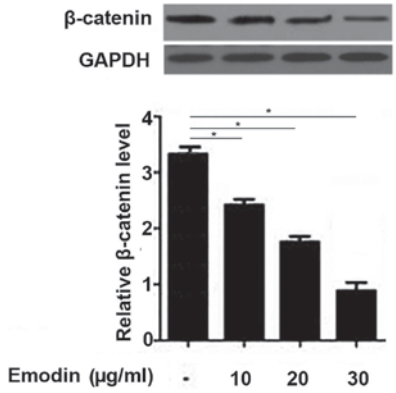

Figure 5. Effect of emodin on β-catenin expression. *P<0.05.

Emodin inhibits the Wnt/β-catenin pathway in rat chondrocytes. The Wnt/β-catenin pathway serves a pivotal role during the development of OA (17); therefore, the current study investigated the effects of emodin on β-catenin expression. The results demonstrated that the expression of β-catenin in the chondrocytes was significantly downregulated following treatment with emodin in a dose-dependent manner (Fig. 5). These data suggest that emodin may treat OA by inhibiting the Wnt/β-catenin pathway.

Discussion

It has been demonstrated that emodin not only exhibits neuroprotective activity (11) but also inhibits LPS-induced pro-inflammatory responses and the activation of NF-κB (12). However, its underlying mechanism of action remains unknown. In the current study, the effects of emodin on inflammatory mediators in the chondrocytes of rats were investigated. The results demonstrated that emodin reduced the cytotoxicity of IL-1β in rat chondrocytes in a concentration-dependent manner. These results are consistent with the results of a previous study demonstrating that emodin inhibited the IL-1β-induced inflammatory response by suppressing the NF-kB signaling pathway (12).

MMP genes are highly expressed in the articular cartilage and various cytokines, including IL-1 and tumor necrosis factor (TNF)-α are also produced in the articular cartilage. It has been demonstrated that excessive production of NO in OA may be associated with the IL-1 receptor (IL-1Ra) (18). Autocrine IL-1β released by the OA-affected cartilage is able to regulate NO and PGE2 production (19). The results of the current study demonstrated that emodin decreases NO and

PGE2 levels. Furthermore, NO and PGE2 levels decreased following treatment with emodin in a dose-dependent manner. The results are consistent with those of a previous study, which reported that emodin treatment could inhibit the expression of TNF-α and IL-6 in plasma and the production of PGE2 in the synovial tissues of mice (20). As the major enzymes involved in the degradation of the extracellular matrix, MMP family members serve pivotal roles in resisting compressive forces and maintaining the tensile properties of tissue (21,22). Previous studies determined that MMP-3 and MMP-13 are important genes that may be targeted to treat OA (22,23). Furthermore, it has been demonstrated that the expression of MMP-3 and MMP-13 are significantly increased in response to IL-1 and TNF-α in OA (23,24). The results of the current study are in accordance with those of previous studies, which demonstrated that emodin inhibited MMP-3 and MMP-13 expression and that this degree of inhibition was positively associated with the concentration of emodin (25,26).

The ERK pathway serves a critical function in regulating chondrocyte proliferation by regulating the expression of associated genes (27) and Wnt/β-catenin is a key factor in the development of OA. In the present study, emodin significantly reduced the phosphorylation level of ERK1/2 and the expression of β-catenin in chondrocytes in a dose-dependent manner. These data suggest that emodin may inhibit the development of OA by inhibiting the ERK and Wnt/β-catenin pathways.

In conclusion, the results of the current study suggest that emodin may improve OA by reducing the cytotoxicity of IL-1β, downregulating the expression of MMP-3 and MMP-13 and inhibiting the ERK and Wnt/β-catenin pathways. The results indicate that emodin may be used as a novel therapeutic strategy to treat patients with OA.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

ZFL, RF and QM designed the experiments, whilst ZFL and YL performed them. LL, ZQL, YD and RF analyzed the data. RF wrote the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

Animal experiments were performed in accordance with the Declaration of Helsinki and the present study was approved by the Animal Ethics Committee of the Chinese Medicine Hospital of Xinjiang Uygur Autonomous Region (Urumqi, China).

Consent for publication

Not applicable.

Competing interests

Authors declare that they have no competing interests.

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