International Immunopharmacology 51 (2017) 114–123

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

# Boldine isolated from *Litsea cubeba* inhibits bone resorption by suppressing the osteoclast differentiation in collagen-induced arthritis

Hongyan Zhao[a,b,1], Huihui Xu[c,d,1], Senyan Qiao[e,1], Cheng Lu[f], Gui Wang[c,d], Meijie Liu[a,b], Baosheng Guo[g], Yong Tan[f], Dahong Ju[a,b], Cheng Xiao[c,*]

[a] *Experimental Research Center, China Academy of Chinese Medical Science, Beijing, China*
[b] *Institute of Basic Theory for Chinese Medicine, China Academy of Chinese Medical Science, Beijing, China*
[c] *Institute of Clinical Medicine, China-Japan Friendship Hospital, Beijing, China*
[d] *Beijing University of Chinese Medicine, Beijing, China*
[e] *Department of Pathology, The Third People's Hospital of Zhengzhou, Henan, China*
[f] *Institute of Basic Research in Clinical Medicine, China Academy of Chinese Medical Science, Beijing, China*
[g] *Institute for Advancing Translational Medicine in Bone & Joint Diseases, School of Chinese Medicine, Hong Kong Baptist University, China*

ARTICLE INFO

*Keywords:*
Boldine
OPG/RANKL/RANK signaling pathway
Collagen-induced arthritis
Osteoclast
Bone resorption
*Litsea cubeba*

ABSTRACT

*Objective:* To investigate the effect of boldine isolated from *Litsea cubeba* on collagen-induced arthritis (CIA) rats and explore the molecular mechanism predicted by network pharmacology.

*Material and methods:* CIA rats were orally administered with boldine. The bone destruction of paws was analyzed by histologic examination, tartrate-resistant acid phosphatase (TRACP) staining and micro-computed tomography. Prediction of signal pathway associated with boldine network molecules and CIA genes was applied by the network pharmacology analysis. The expressions of osteoprotegerin (OPG), receptor activator of nuclear factor-κB (RANK) and its ligand (RANKL) in the ankle were detected by immunohistochemistry. *In vitro* osteoclasts were cultured in the presence of variable doses of boldine and the RANK expressions were evaluated using Real-time polymerase chain reaction and western blot.

*Results:* Boldine reduced ankle swelling, alleviated pathological damage and significantly prevented bone destruction in CIA rats. Consistent with this, enzyme linked immunosorbent assay revealed boldine decreased serum TRACP5b levels and osteoclast number in the ankle region by TRACP staining from CIA rats. The network pharmacology analysis indicated that RANK signaling in osteoclasts was the most significant canonical pathway associated with boldine network molecules and CIA genes, which was verified by the increased expression of OPG, reduced expression of RANK, RANKL and RANKL/OPG in boldine-treated CIA rats. The *in vitro* study further confirmed that boldine inhibited osteoclastogenesis by inhibiting the RANKL/RANK signaling pathway.

*Conclusion:* Taken together, our study first indicates that boldine from *Litsea cubeba* suppresses osteoclastogenesis, improves bone destruction by down-regulating the OPG/RANKL/RANK signal pathway and may be a potential therapeutic agent for rheumatoid arthritis.

## 1. Introduction

Rheumatoid arthritis (RA) patients have a higher risk of fracture due to progressive bone deterioration and subsequent systemic osteoporosis during RA development. Current RA therapeutic agents are divided into glucocorticoids, non-steroidal anti-inflammatory drugs (NSAIDs), disease-modifying anti-rheumatic drugs (DMARDs) and biological drugs [1–3]. However, these clinical therapies have been limited because of their side effects and high cost of treatment [4,5]. *Litsea cubeba* (Lour.) Pers. (Lauraceae) has long been applied for the treatment of rheumatism, traumatic injury, common cold, stomach pain, vomiting and diarrhea and cerebral thrombosis in China. Researches showed that its extracts and compounds have many pharmacological activities such as antioxidant [6], antibacterial [7] and anti-inflammatory immunological activities [8]. Boldine is an aporphine isoquinoline alkaloid extracted from the root of *Litsea cubeba* and also possesses these properties, including antioxidant, anti-inflammatory and cytoprotective effects [9]. Our preliminary results also showed that boldine significantly improved the bone micro-architecture of the lumbar spine and proximal femur in collagen-induced arthritis (CIA) rats [10]. These

* Corresponding author.
*E-mail address:* xc2002812@126.com (C. Xiao).
[1] Hongyan Zhao, Huihui Xu and Senyan Qiao contributed equally to this work.

http://dx.doi.org/10.1016/j.intimp.2017.08.013
Received 9 July 2017; Received in revised form 14 August 2017; Accepted 16 August 2017
Available online 18 August 2017
1567-5769/ 

results suggested that boldine is at least partly responsible for the pharmacological function of *Litsea cubeba* and may have a potential role in the prevention of osteoporosis and bone resorption in arthritis.

Many studies have shown that osteoclasts play an important role in the pathogenesis of RA [11–13]. Osteoclasts, as the primary bone resorptive cells, are found in areas of pannus invasion into bone at the sites of osteoclasia in both animal models of arthritis and in patients with RA, these cells play an essential role in bone loss and bone destruction [14–17]. Moreover, our previous experiments also revealed that boldine have an inhibiting effect on differentiation and resorption function of osteoclasts induced from mouse monocyte RAW264.7 *in vitro* (Chinese article). We therefore hypothesized that the therapeutic effects of boldine may occur through the inhibition of bone resorption by osteoclasts.

How is bone resorption being inhibited? In recent years, network pharmacology provides new methods and opportunities for the discovery of bioactive components and biomarkers, and may be helpful to explore the scientific basis of numerous Chinese herbal medicines and formulae [18,19]. Therefore, we investigate the effect of boldine on CIA rats and explore its molecular mechanism predicted by network pharmacology approach.

## 2. Materials and methods

### 2.1. Materials

Boldine (purity > 98%) was provided by Professor Chai Xingyun (Beijing University of Chinese Medicine, China). The extraction method of boldine was described earlier [20].

### 2.2. Animals

Adult male Sprague-Dawley rats were purchased from the National Institutes for Food and Drug Control (Animal license number: SCXK (Beijing) 2009-0017), housed in groups of four and given seven days of feeding and housing adaptation. All rats were housed in barrier system in experiment animal center of China-Japan Friendship Hospital (The experimental animal room license number: SYXK (Beijing) 2010–0011). Environmental conditions were controlled in a temperature of 20 °C–22 °C, humidity of 45%–65% and a 12 h light/dark cycle (lights off at 20:00). Rats were housed in 545 × 395 × 200 mm cages and given with free access to standard rat chow (Feed production license number: Beijing (2014) 06054) and water. At the beginning of the experiments, rats weighed 277 ± 9 (mean ± SD) grams. Rats were monitored twice daily for health status. No adverse events were observed. The participants were blinded to the drug treatment while data processing. All procedures were carried out in accordance with the Laboratory Animal Management Regulations, 1988 (China) (amended 2011). This experiment was approved by the Research Ethics Committee of the Institute of Basic Theory of Traditional Chinese Medicine, China Academy of Chinese Medical Sciences, Beijing, China. All sections of this research adhere to the ARRIVE Guidelines for reporting animal research.

### 2.3. Induction of CIA and treatment

Fifty Sprague-Dawley rats were randomly divided into the normal control group ($n$ = 8) and the CIA model group ($n$ = 42) using the standard = RAND () function in Microsoft Excel. CIA was induced as previously described [21]. Briefly, bovine type II collagen (Chondrex) was emulsified with an equal amount of incomplete Freund's adjuvant (Chondrex), then rats were intradermally injected with 100 μg emulsion at the base of the tail. A booster with the same preparation was given after 7 days. On the 15th day after the primary immunization, the ankle joints were not swollen in 5 rats and the arthritis index is 1 point in 3 rats. Then the CIA rats (arthritis index ≥ 2) were randomly divided into 4 groups: the CIA group ($n$ = 9), the positive control group of methotrexate ($n$ = 9), the boldine low-dose group ($n$ = 8) and the boldine high-dose group ($n$ = 8). Afterwards, boldine low-dose group (2 mg/kg, daily), the boldine high-dose group (4 mg/kg, daily) and methotrexate group (3 mg/kg, twice a week) (Shanghai Sine Pharmaceutical, Shanghai, China) were both given by oral administration for a period of 28 days. The normal and CIA groups were given an equal volume of deionized water at the same time.

### 2.4. Arthritis assessment

Rats were assessed for disease severity every 2 days after the booster immunization. Arthritis severity was expressed as the arthritic index (AI) from 0 to 4 according to the following scale: 0, no signs of disease; 1, detectable arthritis with erythema in at least some digits; 2, significant redness and swelling; 3, severe redness and swelling from joint to digit; 4, maximal swelling with arthrokleisis. The maximum arthritic score per rat was 8 (4 points × 2 hind paws)

### 2.5. Histopathology and TRACP staining

On day 28 after administration, all rats were sacrificed *via* anesthesia after serum collection. The hind limbs, including the paws and ankles, were dissected and immediately fixed in 4% paraformaldehyde (PFA) for 48 h, decalcified in 10% EDTA for up to 1 month at 4 °C and embedded in paraffin. Tissue sections (5 μm) were stained with hematoxylin and eosin (H & E). The data were expressed as the mean bone destruction scores (infiltration of synovial fluid, pannus formation, synovial hyperplasia and destruction of cartilage and bone), which were based on a scale from 0 to 4 (0: absent; 1: weak; 2: moderate; 3: severe; 4: very severe) [22]. In order to identify osteoclasts, sections were stained with acid phosphatase Kit (Sigma) for TRACP staining. TRACP positive multinucleated cells containing > 3 nuclei were identified as osteoclasts and counted by light microscopy.

### 2.6. Micro-computed tomography (micro-CT) analysis

The fixed hind paws were placed in a centrifuge tube with physiological saline and scanned with a 1174 compact micro-CT (Bruker, Belgium). Images of 570 regions from the calcaneus to the tarsus were analyzed with bone volume (BV), bone surface (BS) and BS/BV as the histomorphometric parameters. All micro-CT analyses were performed according to international guidelines [23,24].

### 2.7. Serum TRACP5b measurements

Bone resorption and osteoclast activity were assessed by measuring serum levels of TRACP5b using ELISA kits (CUSABIO). Measurements were performed according to the manufacturer's instructions.

### 2.8. Network pharmacology approach for predicting the mechanism of boldine in CIA rats

We identified boldine target proteins in the PubChem and TCMSP databases (until October 12, 2016). Since the bio-information was possibly cross-referenced to other databases, such as the American National Center for Biotechnology Information (NCBI), we collected only boldine target proteins that were validated in biological assays in this study. We used the keyword “Rheumatoid arthritis” to search the RA genes in the NCBI Gene database (http://www.ncbi.nlm.nih.gov/gene) and the identified genes were filtered with variations in medical significance, as reported *via* Variation Viewer (until October 12, 2016).

Then we uploaded the boldine total protein targets and related-RA genes to Ingenuity Pathway Analysis platform (IPA, http://www.ingenuity.com). We use the term “focus molecules” as the molecules entering into IPA. The focus molecules were converted into a set of