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Chondroprotective potential of bioactive compounds of *Zingiber cassumunar* Roxb. against cytokine-induced cartilage degradation in explant culture

Rujirek Chaiwongsa¹, Siriwan Ongchai¹*, Siriwan Tangyuenyong², Prachya Kongtawelert¹, Ampai Panthong³ and Vichai Reutrakul⁴

¹Thailand Excellence Center for Tissue Engineering and Stem Cells, Department of Biochemistry and Center of Excellence for Innovation in Chemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand.
²Equine Clinic, Department of Companion and Wildlife Animal Clinic, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai 50200, Thailand.
³Department of Pharmacology and Center of Excellence for Innovation in Chemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand.
⁴Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Mahidol University, Bangkok 10400, Thailand.

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The active compounds, cis-3-(2',4',5'-trimethoxyphenyl)-4-((E)-2''',4''',5'''-trimethoxystyrly)cyclohex-1-ene (Compound C) and (E)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol (Compound D), have been identified in hexane fraction of *Zingiber cassumunar* Roxb., the medicinal plant which has been used for pain relief in arthritis including osteoarthritis (OA) and rheumatoid arthritis (RA). It was therefore interesting to investigate the chondroprotective activity of these compounds in vitro. Articular cartilage explants were cultured in the culture media containing 7 ng/ml of interleukin-1β (IL-1β), in the presence or absence of Compound C or Compound D at the concentration range of 1 to 100 µM. It was found that these compounds at concentrations of 10 and 100 µM significantly inhibited the IL-1β-induced cartilage degeneration by conserving the content of the cartilage matrix biomolecules such as collagen and uronic acid (UA) within the cartilage explants; and also resulted in the decline of releasing sulfated glycosaminoglycans and hyaluronic acid (HA) into the culture media. The increase in activities of matrix metalloproteinase-2 (MMP-2) and MMP-13 caused by IL-1β was significantly diminished by Compound C and Compound D. Additionally, the results showed no significant difference between the two active constituents and diacerein, an anti-arthritic agent used in OA, in those activities at a concentration of 100 µM. This is a pioneering evidence that indicate the potential chondroprotective property of the *Z. cassumunar* active compounds.

Key words: *Zingiber cassumunar*, chondroprotective activity, cartilage explant, rheumatoid arthritis, osteoarthritis, cis-3-(2',4',5'-trimethoxyphenyl)-4-((E)-2''',4''',5'''-trimethoxystyrly)cyclohex-1-ene, (E)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol.

INTRODUCTION

Cartilage is a connective tissue that serves as a "cushion" between the bones of the joints. In normal articular cartilage, the collagen network of the extracellular matrix is important for the biological function of cartilage. The aggregate complex of hyaluronic acid (HA)-proteoglycans is trapped within the network. Sulfated glycosaminoglycans (S-GAGs), the carbohydrate part of proteoglycan, consist of disaccharide repeating units of uronic acids (UA) and hexosamines. The progressive loss of these matrix biomolecules is the main cause of
degenerative joint diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA) (Svetlana et al., 2007). These disorders cause joint pain and disability in elderly people. Interleukin-1β (IL-1β) is one of the most potent pro-inflammatory cytokine that involves in the destruction of cartilage (Mark et al., 2005). It induces the synthesis and release of matrix metalloproteinases (MMPs) (Tetlow et al., 2001; Phitak et al., 2009). These enzymes play a major role in the breakdown of cartilage matrix biomolecules, particularly proteoglycan and collagen which eventually causes the permanent loss of cartilage tissue and mechanical properties in degenerative joint diseases. Among MMPs, MMP-13 (collagenase-3) appears to be a key enzyme in the degradation of the collagenous network in OA and RA as it has the highest activity against collagen type II (Li et al., 2011). MMP-13 has been found highly expressed in the cartilage of the human OA patient (Li et al., 2011). MMP-2, classified as gelatinase, has been reported to be increased in the synovial fluid of OA (Volk et al., 2003; Zrimešek et al., 2007). The decrease in breakdown of cartilage matrix biomolecules has been found after the inhibition of MMP-2 expression induced by IL-1β (Mark et al., 2005). Diacerein, the commercial drug possessing anti-inflammatory and anti-arthritic activities, clearly demonstrates that it directly inhibits IL-1β-induced inflammation and cartilage degradation by the down regulation of pro-catabolic genes including MMP-13 (Tamura and Ohmori, 2001; Boileau et al., 2008). Alternatively, several medicinal plants used for the treatment of joint pain in folk medicine have been reported to possess anti-inflammatory and anti-arthritic activities such as Alpinia galanga (Phitak et al., 2009), Zingiber officinale (Haghighi et al., 2005) and Phyllanthus emblica (Sumantran et al., 2008).

Zingiber cassumunar Roxb., a tropical ginger distributed in Southeast Asia, has been widely used in traditional medicine for the treatment of various conditions such as muscular or joint pain, rheumatism, asthma and inflammation (Bhuiyan et al., 2008). Several compounds isolated from hexane fraction of this plant may implicate protective activity against cytokine-induced cartilage degradation. This study will be the pioneering attempt to reveal the chondroprotective activity of Z. cassumunar.

The purpose of the present study was to investigate the chondroprotective potential of Compound C and Compound D against cartilage degradation induced by IL-1β using the cartilage explant model. This technique is clearly demonstrated as an appropriate in vitro system for the study of cartilage metabolism and investigation of the chondroprotective activity (Mark et al., 2005; Chan et al., 2006; Sumantran et al., 2008; Macrory et al., 2009; Phitak et al., 2009; Harlan et al., 2011).

MATERIALS AND METHODS

The active constituents of Z. cassumunar, Compound C and Compound D, were provided from Professor Vichai Reutrakul, Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Mahidol University, Bangkok 10400, Thailand. They were purified and characterized by the method reported by Amatayakul et al. (1979) and Tuntiwachwuttikul et al. (1981). Diacerein was a generous gift from TRB Chemedica (Switzerland). Reagents for explant culture were from Gibco® Cell Culture Media, Invitrogen, USA. IL-1β was purchased from R&D Systems, Minneapolis, Minnesota, USA. All other chemicals utilized were of analytical grade.

Cartilage explants culture and treatments

Cartilage explants were prepared by a method described elsewhere (Phitak et al., 2009). In brief, articular cartilage was dissected from the metacarpophalangeal joints of porcine obtained from a local slaughter house within 6 h after the animal was slaughtered. The explants were incubated in a serum-free Dulbecco’s modified Eagle’s media containing 200 units/ml of penicillin and 200 µg/ml of streptomycin at 37°C, 5% CO₂. After 24 h incubation, media were collected and stored at -20°C for analysis of the release of cartilage matrix biomolecules, HA and S-GAGs, and designated as the day 0 samples. In order to investigate effect of those active compounds on the release of HA and S-GAGs, the explants were treated for 3 days with recombinant human IL-1β to induce cartilage degradation, in absence or presence of Compound C or Compound D at the concentration ranging from 1 to 100 µM. The 3 weeks treatment period was conducted for analysis the remaining of UA and collagen in the explant tissues. In parallel treatment, diacerein (1 to 100 µM), a commercial anti-OA drug, was used as a standard agent. Treatments were performed in triplicate using tissue from one animal donor. At the end of the incubation, the media and explants were harvested and stored at -20°C for analysis of the matrix degradation.

Abbreviations: Compound D, (E)-4-(3',4'-dimethoxyphenyl)but-3-ene-1-ol; Compound C, cis-3-(2', 4', 5'-trimethoxyphenyl) - 4 - { (E) - 2''', 4''', 5'''-trimethoxyxystyril)cyclohex-1-ene (Compound C) (Pongprayoon et al., 1996). The chemical structures of those two compounds are shown in Figure 1. Currently, there is little information regarding the biological activities of these active compounds. Interestingly, our preliminary data of ethanol and hexane extracts of Z. cassumunar rhizomes has shown the chondroprotective activity against IL-1β-induced cartilage degradation (Chaiwongsa, 2004). These led us to suspect that the anti-inflammatory effectiveness of the active constituents of this plant may implicate protective activity against cytokine-induced cartilage degradation. This study will be the pioneering attempt to reveal the chondroprotective activity of Z. cassumunar.

*Corresponding author. E-mail: ongchai.submission@gmail.com. Tel: +66 53 894188. Fax: +66-53-894188.
Determination of the cartilage matrix degradation

In the explant model, degradation of the cartilage matrix was investigated using the method clearly reported by Ong-Chai et al. (2008). The release of S-GAGs and HA in the media was analyzed by dimethylmethylen blue (DMMB) assay and enzyme-linked immunosorbent assay (ELISA) technique, respectively. The explants were digested with papain prior to the determination of UA content by colorimetric assay. Hydroxyproline assay was used for collagen content analysis in the explants. Briefly, a portion of papain-digested samples was further hydrolyzed with 6 N HCl at 90°C for 14 h, then 60°C for 24 h. Samples (50 µl) were combined with 50 µl of freshly prepared oxidizing solution for 5 min of incubation at room temperature. They were mixed with 100 µl Ehrlich’s reagent prior to incubation for 45 min at 60°C. Samples were extrapolated against hydroxyproline standards at the concentration range of 0 to 10 µg/ml. Absorbance of the mixtures was determined at 570 nm using the Titertek Multiskan M340 multiplate reader. The changes of cartilage matrix biomolecules were estimated by the calculation:

% Change of S-GAGs and HA release = [100 × (Day 3 medium - Day 0 medium) / Day 0 medium]

Collagen or UA content (% of control) = [100 × (Day 3 treated sample) / (Day 3 control)]

Assays for enzyme activity

The assay for MMP-2 activity in the conditioned medium by electrophoretic gelatin zymography was conducted as described by Phitak et al. (2009). MMP-13 activity was analyzed by fluorogenic assay as described by Netzel-Arnett et al. (1991). The culture media (40 µl) were pipetted into a 96-well black plate. Then 50 µl of the assay buffer containing 50 mM Heps, 200 mM NaCl, 1 mM CaCl2, 0.01% (v/v) Brij-35, pH 7.3 was added. The latent enzyme was activated by amino phenyl mercuric acetate for 1 h at 37°C. Fluorogenic substrate solution (Calbiochem, Germany) (10 µl) was added into the plates followed by incubation at 37°C for 3 h. The reaction mixtures were read at 325 nm for excitation and 390 nm for emission using BioTek Synergy H4.

Cytotoxicity assay

The toxicity effect of the test compounds on the cartilage explant system were determined by a colorimetric assay, based on the measurement of lactate dehydrogenase (LDH) activity in the culture medium (Chotjumlong et al., 2010). The culture media of cartilage explants were conducted according to the manufacturer’s instruction by comparing the amount of LDH in the samples with the untreated control. The explants treated with 10 mM H2O2 were used as the positive control.

Statistical analysis

All statistical analyses were performed by using Microsoft Excel 2000 or the SPSS 11.5 for windows software package. Data were expressed as means ± SD of triplicate independent experiments. Statistically significant values were compared by using one-way analysis of variance (ANOVA). Values of P<0.05 were considered significant.

RESULTS

Inhibitory effects of Compounds C and D on the release of S-GAGs and HA

A cartilage-specific chondroitin sulfate proteoglycan, aggrecan, consists of a core protein and numbers of S-GAGs chains. The aggregate of aggregcans and HA are present in the collagen network of cartilage tissue. In order to investigate the effects of the active compounds on the release of S-GAGs and HA, porcine cartilage explants were cultured in the conditioned media containing 7 ng/ml IL-1β, in the presence or absence of Compound C and Compound D. After 3 days of incubation, the culture media were evaluated for S-GAGs and HA. The results showed high releases of S-GAGs and HA in the culture media treated with IL-1β, when compared to the control. Compound C and Compound D at doses of 10 and 100 µM significantly suppressed the
Effect of active compounds of *Z. cassumunar* and diacerein on the release of S-GAGs induced by IL-1β. The porcine cartilage explants were cultured in the stress condition induced by IL-1β (7 ng/ml), within or without Compound C, Compound D and diacerein (1 to 100 µM), or left untreated as control. After 3 days of incubation, the culture medium samples were assayed by DMMB assay. The release of S-GAGs into the culture medium is expressed as a percentage relative to the control. Data are expressed as mean ± SD of three independent experiments, and the significant differences were tested by one-way ANOVA at \( P<0.01 \) (**). 

a, b, Significant different from the control and the IL-1β treated group, respectively; ns, no significant different from the IL-1β treated group.

Compounds C and D suppressing the loss of collagen and UA from the cartilage tissues

In the present study, the cartilage depletion induced by IL-1β was also analyzed by quantitative determination of collagen and UA content remained in the explant tissue. UA is one of the most important monosaccharide derivative monomers of glycosaminoglycans of proteoglycans and HA. At the end of the treatment as previously mentioned, the explants were digested with papain and further analyzed for the amounts of collagen and UA which normalized to the dry weight of the tissues. After 3 days of the culture under a stressed condition induced by IL-1β, the results showed great losses of UA and collagen indicated by a significantly lower content of these molecules in the explant than in those of the control. In co-treatments with the active compounds of *Z. cassumunar*, it was found that these compounds were able to counteract the destructive effects of IL-1β as indicated by the dose-dependent increase of the contents of both collagen and UA in the test explants. These results were similar to those of the diacerein treatments. In the analysis of the collagen content, however, the statistical significance of the increase was found at the highest concentration of Compound D and diacerein, but not at that of Compound C (Figures 4 and 5).

Compounds C and D reduced MMPs activities in the culture media

To assay the release of enzymes involved in cartilage degradation, the culture media harvested on day 3 of the treatment were analyzed for MMP-2 and MMP-13 activities. The gelatin zymography indicating MMP-2 activity in total active form is shown in Figure 6. In the IL-1β treated condition, the results showed a significant increase in MMP-2 activity. The highest concentrations of the active compounds and diacerein were able to significantly reduce the activity of MMP-2. Figure 7 showed the total activity of MMP-13 determined by fluorogenic assay. Similarly to its effect on MMP-2, IL-1β caused a dramatic increase in MMP-13 activity which was reduced by both active compounds at the concentrations of 10 and 100 µM. Diacerein seemed to be more potent when compared to the active compounds as its effectiveness occurred at the lower concentration of...
Figure 3. Effect of active compounds of *Z. cassumunar* and diacerein on the release of HA induced by IL-1β. The porcine cartilage explants were cultured in the stress condition induced by IL-1β (7 ng/ml), within or without Compound C, Compound D and diacerein (1 to 100 µM) or left untreated as control. After 3 days of incubation, the culture medium samples were analyzed by competitive inhibition ELISA assay. The release of HA into the culture medium is expressed as a percentage relative to the control. Data are expressed as mean ± SD of three independent experiments, and the significant differences were tested by one-way ANOVA at P<0.01 (**). a, b, Significant different from the control and the IL-1β treated group, respectively; ns, no significant different from the IL-1β treated group.

Figure 4. The reverse effect of active compounds of *Z. cassumunar* and diacerein on IL-1β-induced the loss of UA content from cartilage explants. The porcine cartilage explants were cultured in the stress condition induced by IL-1β (7 ng/ml), within or without Compound C, Compound D and diacerein (1 to 100 µM) or left untreated as control. After 3 days, the explants were harvested and analyzed for the remaining of UA by colorimetric assay as previously described. The remaining of UA in cartilage explants is expressed as a percentage relative to the control. Data are expressed as mean ± SD of three independent experiments, and the significant differences were tested by one-way ANOVA at P<0.05 (*) and P<0.01 (**). a, b, Significant different from the control and the IL-1β treated group, respectively; ns, no significant different from the IL-1β treated group.
Figure 5. The reverse effect of active compounds of *Z. cassumunar* and diacerein on IL-1β-induced the loss of collagen content from cartilage explants. The porcine cartilage explants were cultured in the stress condition induced by IL-1β (7 ng/ml), within or without Compound C, Compound D and diacerein (1 to 100 µM) or left untreated as control. After 21 days of incubation, the explants were harvested and analyzed for the remaining of collagen by colorimetric assay as previously described. The remaining of collagen in cartilage explants is expressed as a percentage relative to the control. Data are expressed as mean ± SD of three independent experiments, and the significant differences were tested by one-way ANOVA at P<0.05 (*). a, b. Significant different from the control and the IL-1β treated group, respectively; ns, no significant different from the IL-1β treated group.

1 µM.

**Cytotoxicity test of Compound C and Compound D**

To detect the cytotoxicity effect of the test compounds, the explants were treated with each of agents used in the present study. The release of LDH into the culture medium indicated a cellular loss of integrity, leading to cell death. No significant increase in LDH activity was observed neither in IL-1β treated alone, nor concomitantly treated with Compound C, Compound D and diacerein (Figure 8).

**DISCUSSION**

Pro-inflammatory cytokines, especially IL-1β trigger the onset of cartilage degradation by the induction of catabolic proteins including enzymes involve in the initiation and progression of the diseases (Daheshia and Yao, 2008). It has been reported that IL-1β induced the release of matrix sulfated proteoglycans and significantly inhibited sulfated proteoglycan synthesis, together with diminished glycosaminoglycan sulfate content in the extracellular matrix of the explants (Stabellini et al., 2003). These events are involved in the destructive effect of IL-1β on the up-regulation of the synthesis and release of MMP-1, -2, -3, -9 and -13 from chondrocytes (Tetlow et al., 2001; Phitak et al., 2009). These lead to the increase in destruction and release on both collagen and proteoglycans from the cartilage, as indicated by less remaining content of collagen and UA in the tissue, consequently with increasing the level of S-GAGs and HA in the culture medium (Stabellini et al., 2003; Phitak et al., 2009).

In the present study, the increase in activities of MMP-2 and MMP-13 was found in the culture medium of cartilage explants after treatment with IL-1β. This indicated that the addition of IL-1β into the explants culture was able to simulate the onset of cartilage degradation as previously reported (Stabellini et al., 2003). The result was also corresponding correlation with the dramatic elevations of S-GAGs and HA in the medium and the great losses of UA and collagen content of the cartilage tissue. In co-treatment with diacerein, the anti-arthritic drug, the results showed that this agent counteracted the destructive effects of IL-1β, resulting in the decline of cartilage degradation including MMP-2 and MMP-13 similarly to the previous report (Boileau et al.,
Figure 6. Zymographic analysis of MMP-2 in the culture medium (A) and optical density values of MMP-2 relative to the control group (B). The porcine cartilage explants were cultured in the stress condition induced by IL-1β (7 ng/ml), within or without Compound C, Compound D and diacerein (1-100 µM) or left untreated as control. After 3 days of incubation, the culture medium samples were analyzed for MMP-2 activity. The gelatin zymogram shown is representative of three independent experiments. The bar graph is expressed as mean ± SD of three independent experiments. The significant differences were tested by one-way ANOVA at P<0.05 (*) and P<0.01 (**). a, b, Significant different from the control and the IL-1β treated group, respectively; ns, no significant different from the IL-1β treated group.

Among the several arylbutanoids of *Z. cassumunar*, (E)-1-(3',4'-dimethoxyphenyl)butadiene (DMPBD), has been shown to possess potent anti-inflammatory activity. It has been reported that anti-inflammatory activity occurs via cyclooxygenase and lipoxygenase pathways (Jeenanapongsa et al., 2003). These pathways are also the targets of nonsteroidal anti-inflammatory drugs (NSAIDs), resulting in blocking the synthesis of prostaglandins and onset of inflammation. However, there are only a few NSAIDs drugs that claimed to have the chondroprotective activity (Kenneth, 2011). Although diacerein has been claimed as an anti-inflammatory agent, this drug is not included in the group of NSAIDs. It is classified as symptomatic slow acting and chondroprotective drug for OA treatment (Provvedini and Cohen, 2002). In contrast to NSAIDs, diacerein actually diminishes the production and activity of the pro-inflammatory, pro-catabolic cytokine IL-1β (Moldovan et al., 2000). These actions have no significant impact on the prostaglandin biosynthesis (A'lvarez-Soria et al., 2008). Unlike NSAIDs, therefore, it may not be surprising that this drug does not cause gastrointestinal bleeding (Annil et al., 2006).
Figure 7. Effect of active compounds of Z. cassumunar and diacerein on IL-1β-induced MMP-13 activity. The porcine cartilage explants were cultured in the stress condition induced by IL-1β (7 ng/ml), within or without Compound C, Compound D and diacerein (1 to 100 µM) or left untreated as control. After 3 days of incubation, the culture medium samples were analyzed for total activity of MMP-13 by fluorogenic assay as previously described. The results are expressed as a percentage relative to the control. Data are expressed as mean ± SD of three independent experiments, and the significant differences were tested by one-way ANOVA at P<0.05 (*) and P<0.01 (**). a, b, Significant different from the control and the IL-1β treated group, respectively; ns, no significant different from the IL-1β treated group.

Compound C and Compound D, the major lipophilic constituents of hexane extract of Z. cassumunar rhizome, have been reported to exhibit potent topical anti-inflammatory activity by inhibition of the edema formation in 12-O-tetradecanoylphorbol-13 acetate-induced rat ear edema (Pongprayoon et al., 1996). The results have shown that the anti-inflammatory activity of Compound C is much stronger than that of Compound D. According to the high performance liquid chromatography (HPLC) quantification of these compounds from the methanolic extract of Z. cassumunar, however, the content of both Compound C and Compound D in the dry powdered rhizomes has been reported to be approximately only 1 to 2% w/w (Paramapojn et al., 2009). Nevertheless, in the short term study (4 weeks), the topical cream containing 14% of the essential oil has the greater effectiveness than dlofenac gel in treatment of the patients suffering from mild to moderate degree of knee OA (Srirochana, 2010). This cream has demonstrated a significant reduction of joint pain and improvement in the daily living activities of the patients (P<0.05). This may suggest not only the anti-inflammatory activity, but also the chondro-beneficial activity of some active constituents in the essential oil, especially Compound C and Compound D. This may include the other major constituents such as terpinen-4-ol and DMPBD. Therefore, further study should be continued to explore the chondroprotective potential of these active chemicals. These scientific evidences will raise the value of Z. cassumunar for
therapeutic use in degenerative joint diseases.

Conclusion

The present study revealed the potential in vitro chondroprotective activity of Compound C and Compound D. Therefore, the clinical effectiveness of Z. cassumunar may not only relieve pain and reduce inflammation but also protect the cartilage degradation induced by pro-inflammatory cytokine. Our results indicated the potential of Z. cassumunar in the treatment of degenerative joint diseases.

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