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A Triterpene Glycoside from Black Cohosh that Inhibits Osteoclastogenesis by Modulating RANKL and TNFα Signaling Pathways

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SUMMARY

Osteoporosis is a major age-related source of morbidity and mortality. Increased bone resorption mediated by osteoclasts is central to its pathogenesis. Cytokines, particularly RANKL and TNFα, are often increased under pathologic conditions, leading to enhanced osteoclastogenesis. Black cohosh (*Actaea/Cimicifuga racemosa* L), a popular herbal supplement for the treatment of menopausal symptoms, was recently shown to have the beneficial effect of preventing bone loss. Here, we demonstrate that 25-acetylcimigenol xylopyranoside (ACCX), a triterpenoid glycoside isolated from black cohosh, potently blocks in vitro osteoclastogenesis induced by either RANKL or TNFα. This blockage of osteoclastogenesis elicited by ACCX results from abrogation of the NF-κB and ERK pathways induced by either RANKL or TNFα, respectively. Importantly, this compound attenuates TNFα-induced bone loss in vivo. Therefore, ACCX represents a potential lead for the development of a new class of antiosteoporosis agents.

INTRODUCTION

Osteoporosis is a major age-related heath problem that is characterized by decreased bone density and increased risk of fractures. It is especially common in woman after menopause and is initiated by estrogen deficiency. The underlying pathogenesis of reduced bone mass in osteoporosis is an imbalance of osteoclast-mediated bone resorption relative to osteoblast-mediated bone formation, resulting in a net loss of bone [1–4].

Although postmenopausal osteoporosis is epidemic, the choices of treatments are far from ideal. Although estrogen-replacement therapy can prevent postmenopausal osteoporosis [5, 6], it increases the risk for breast cancer, stroke, heart attack, and blood clots, and it is no longer used for long-term treatment [7, 8]. Drugs that either block osteoclast resorption, such as bisphosphonates, or increase bone formation, such as PTH, are proposed as alternatives. Bisphosphonates, however, are poorly absorbed from the gastrointestinal (GI) tract and have been associated with GI adverse events [9]; whereas, PTH, a therapeutic peptide, cannot be given orally, and concern about osteosarcoma has led to a recommendation of a 2 year maximum treatment course [10]. Therefore, the search for other alternative treatment options is of considerable scientific and public interest.

Natural products (NPs) have been a successful source of therapeutic agents and drug leads. The roots and rhizomes of black cohosh have a long history of medicinal use by native North Americans for treatment of gynecological disorders and rheumatism [11]. In the past several decades, nutraceutical products made from an alcoholic extract of the roots and rhizomes of black cohosh extract have been used primarily as dietary supplements for the treatment of hot flashes, profuse sweating, nervous irritability, and other menopausal symptoms in the United States and Europe. Several randomized clinical trials document positive effects of black cohosh extract on menopausal symptoms [12–14]. Interestingly, animal studies with the rat ovariectomy model of osteoporosis suggest that extracts of black cohosh exhibit protective effects on estrogen-deficiency-induced bone loss [15, 16]. The mechanism of action of black cohosh on bone cells and the responsible active components, however, remain elusive.

Despite considerable efforts in seeking plant-derived estrogen-like substances, namely, phytoestrogens, in black cohosh, growing evidence suggests that this plant does not possess phytoestrogens [13, 16–21]. Therefore, black cohosh may confer its bone loss-protection effects through a novel mechanism rather than the estrogenic pathway. The accumulated phytochemical studies reveal that black cohosh contains structurally diversified cycloartane triterpene glycosides and aromatic acids as the

major constituents [11, 22–24]. Although cycloartane triterpene glycosides have cytotoxicity or antiproliferation activity against cancer cells [25–29] and aromatic acids have antioxidant activity [30], none of them has been investigated for their effects on bone cells. Thus, identification of the active component for the protective effects of black cohosh on bone loss and the delineation of the molecular mechanism of action merit further investigation.

Osteoclasts play a central role in pathological bone loss, including postmenopausal osteoporosis [3, 4]. Loss of estrogen markedly increases the generation, function, and lifespan of osteoclasts [3, 4]. Despite a variety of mechanisms through which estrogen deficiency induces bone loss, upregulation of the osteoclastogenic cytokines RANKL and TNFα plays a significant role [31, 32]. The positive effects of black cohosh extract on bone loss prompted us to investigate the hypothesis that the bioactive components of black cohosh directly target osteoclasts to prevent bone loss.

Here, we report that 25-acetylcimigenol xylopyranoside (ACCX), a cycloartane triterpenoid glycoside isolated from black cohosh, potently blocks osteoclastogenesis induced by either RANKL or TNFα, with inhibition of the NF-κB and ERK pathways. Importantly, the efficacy of this compound on attenuation of TNFα-induced bone loss is verified in vivo. Because RANKL and TNFα are important mediators of bone loss in several pathological conditions, including inflammatory osteolysis and postmenopausal osteoporosis, ACCX represents a promising therapeutic agent.

RESULTS

ACCX Dampens Osteoclastogenesis In Vitro

Initially, all of the 46 compounds that we isolated from black cohosh were screened to evaluate their effects on in vitro osteoclastogenesis. Bone marrow macrophages (BMMs) were induced to generate osteoclasts in cell culture plates by the addition of M-CSF and RANKL in the presence or absence of the tested compounds at 100 μM. Compounds that showed inhibition of osteoclastogenesis were tested again at concentrations ranging from 5 to 50 μM. Among the compounds tested, ACCX (Figure 1A) is the most potent inhibitor of osteoclastogenesis, with an IC_{50} of ~5 μM (Figure 1B), followed by cimigenol, actein, and cimiaceroside B, with IC_{50} values of 25, 42, and 45 μM, respectively, while others showed no activity (IC_{50} > 100 μM). Therefore, we chose ACCX for further investigation. This compound is one of the major components of black cohosh and comprises about 0.1%–0.5% of an ethanolic extract of black cohosh. ACCX significantly impairs osteoclast formation in a dose-dependent fashion (Figure 1B). The blockage of osteoclastogenesis by ACCX is not due to potential toxicity of this compound because cells preexposed to ACCX for 24 hr exhibit a normal capacity to generate osteoclasts after removal of this compound (Figure 1B).

To determine at which stage ACCX inhibits osteoclastogenesis, ACCX was added to osteoclast differentiation cultures beginning at days 0–4. This compound potently inhibited osteoclastogenesis when added during the first 2 days, while exposure of precursor cells to ACCX at later stages was not effective in the prevention of osteoclastogenesis (Figure 1C). Since the precursors exposed to RANKL for 3 days have already committed to osteoclast differentiation, it is reasonable to suggest that ACCX blocks osteoclast differentiation but cannot reverse the differentiation process once cells have committed to the osteoclast lineage.

Effects of ACCX on Proliferation and Cell Death of BMMs

Data presented thus far establish that ACCX blocks osteoclastogenesis. Given that impaired osteoclastogenesis in the presence of ACCX may reflect decreased proliferation or elevated cell death of the precursor cells, we next examined the effects of ACCX on proliferation and cell death of BMMs. ACCX at 10 μM, which completely blocks osteoclastogenesis, only caused moderate inhibition of proliferation in the presence of M-CSF alone or with RANKL, although greater inhibition can be induced when higher doses of ACCX are used (Figure 2A). Furthermore, despite the fact that higher doses elicited cell death, ACCX induced no or minimal cell death at doses up to 10 μM (Figure 2B).

ACCX Alters RANKL-Induced Signaling Events

Osteoclast differentiation from BMMs is induced by RANKL, whereas M-CSF promotes proliferation of osteoclast precursors and survival of osteoclasts and their precursors [1, 2, 33]. Upon binding to its receptor RANK, RANKL rapidly activates the NF-κB pathway via activating IKK, which phosphorylates IκBα and targets it for proteasomal degradation and leads to translocation of NF-κB from the cytosol to the nucleus to bind its target sequence and activate transcription of the NF-κB target genes [1, 2]. Simultaneously, RANKL activates MAPKs, including ERK, p38, and JNK [1, 2]. More distally, RANKL upregulates the only known osteoclastogenic transcription factor, NFAT2, and a number of osteoclast-specific proteins, such as Cathepsin K, β3 integrin, and c-Src.

To test whether ACCX blocks osteoclastogenesis via altering RANKL signals, we examined the effects of ACCX on these downstream events. ACCX blocks RANKL-induced phosphorylation and degradation of IκBα, indicating inhibition of the NF-κB pathway (Figure 3A). Furthermore, the blockage of IκBα degradation by ACCX is dose and time dependent (Figures 3B and 3C). Moreover, NF-κB DNA-binding activity is also inhibited by ACCX (Figure 3D), providing further support that ACCX inhibits the NF-κB pathway. RANKL-induced phosphorylation of ERK is also inhibited by ACCX (Figure 3E). On the other hand, p38 activation is prolonged (Figure 3E). Additionally, the peak of JNK activation is blunted, although the basal level of activation is moderately increased (Figure 3E). Consistent with impaired osteoclastogenesis in the presence of ACCX, this compound eliminates expression of NFAT2, Cathepsin K,

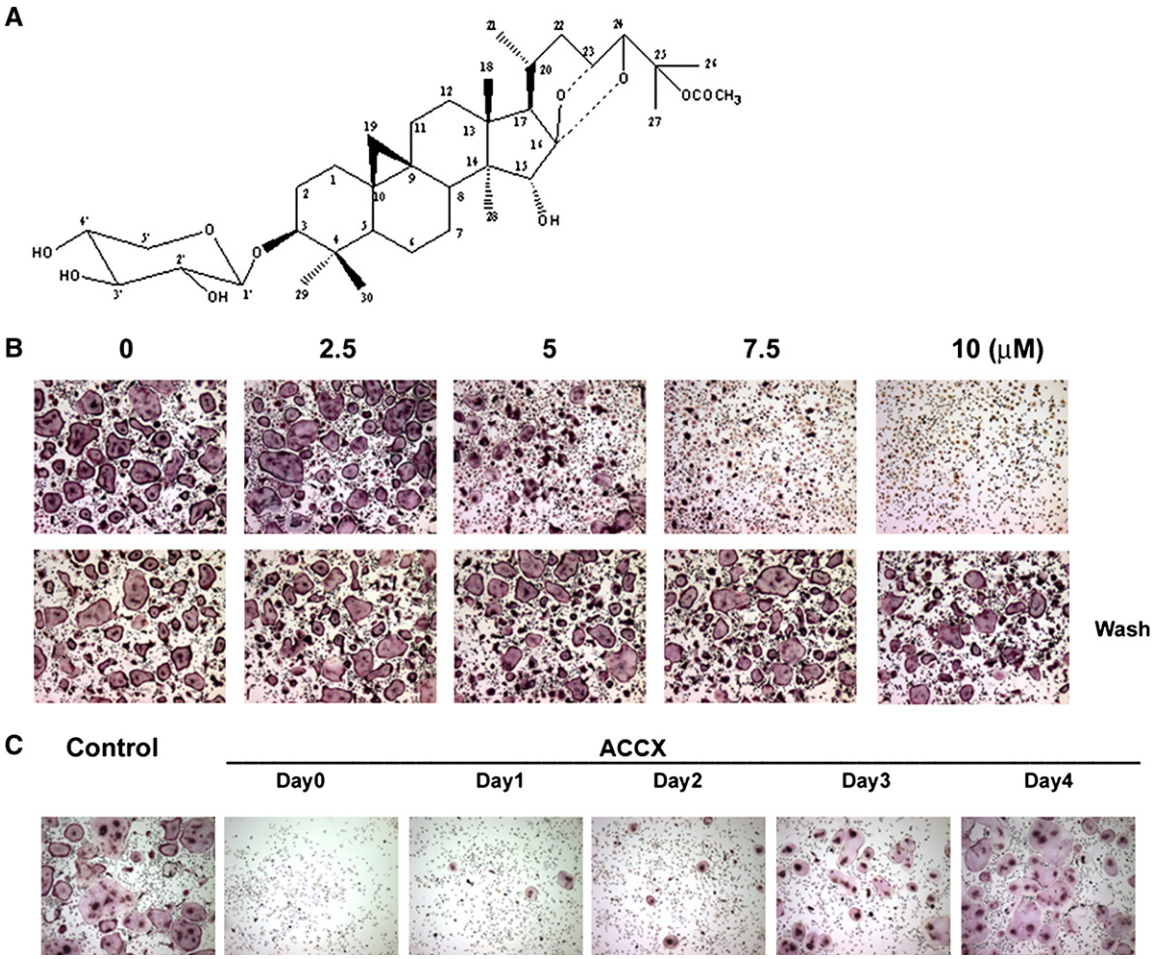

Figure 1. ACCX Inhibits Osteoclastogenesis In Vitro

(A) Structure of ACCX.

(B and C) BMMs were grown in the presence of 10 ng/ml M-CSF and 100 ng/ml RANKL for 4 days to generate mature osteoclasts. ACCX was supplied to the medium at the indicated concentration for (B) 4 days (upper panels), for (B) 1 day at the beginning before being withdrawn for the remaining 3 days (lower panels), or (C) at 10 μM at the indicated time. Cells were stained for TRAP.

and β3 integrin and reduces expression of c-Src (Figure 3F), providing further evidence that osteoclast differentiation is arrested by ACCX.

TNFα-Induced In Vitro Osteoclastogenesis Is Blocked by ACCX

TNFα is the critical inflammatory cytokine that leads to inflammation-induced osteoclastogenesis and bone loss [34–36]. This cytokine is upregulated in estrogen-deficient mice and is essential for the bone loss induced by estrogen deficiency [32, 37]. Addition of ACCX at 10 μM completely blocks osteoclast differentiation induced by TNFα (Figure 4A).

To gain insights into how ACCX dampens TNFα-induced osteoclastogenesis, we examined the effects of ACCX on TNFα-induced activation of the NF-κB pathway and MAPKs, including ERK, p38, and JNK. Similar to its effects on RANKL signaling, ACCX inhibits TNFα-induced activation of ERK, while it constitutively activates p38 and JNK, blunts peak JNK activation, and prolongs p38 phosphorylation (Figure 4B). Furthermore, this compound blocks phosphorylation and degradation of IκBα induced by TNFα (Figure 4C). Given that activated NF-κB stimulates the transcription of a number of target genes, including *TNFα*, *IκBα*, and *TLR2*, we examined whether inhibition of the NF-κB pathway by ACCX leads to downregulation of these genes. Indeed, ACCX did prevent both RANKL- and TNFα-induced transcription of endogenous *TNFα*, *IκBα*, and *TLR2*, as revealed by RT-PCR analysis (Figure 4D).

ACCX Reduces In Vivo Osteoclastogenesis and Bone Resorption Induced by TNFα

To evaluate the effects of ACCX on osteoclastogenesis in vivo, we injected mice daily with TNFα and ACCX or its solvent DMSO for 5 days. Given that ACCX is not water soluble and that DMSO-dissolved ACCX can form precipitates when diluted in water at more than 200 μM, we

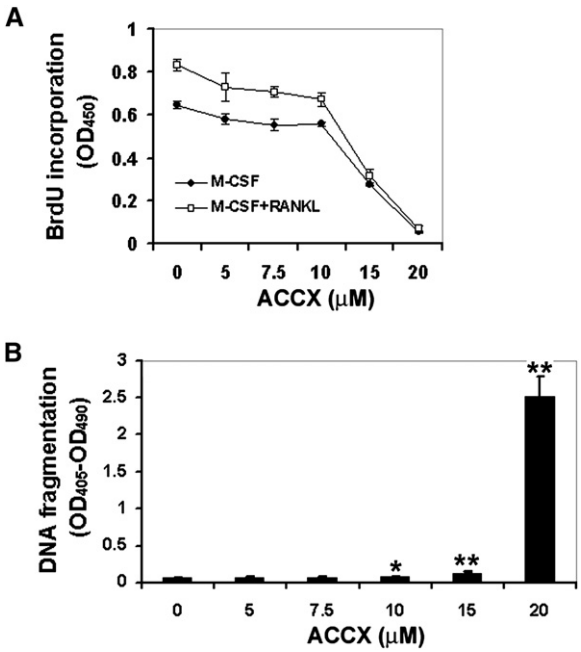

Figure 2. Effects of ACCX on Proliferation and Cell Death of BMMs

(A) BMMs were grown in the presence of 10 ng/ml M-CSF with or without 100 ng/ml RANKL for 24 hr. Cells were simultaneously exposed to ACCX at the indicated concentrations. Cells were labeled with BrdU for 6 hr and were then assayed for BrdU incorporation (*$p < 0.01$ versus nontreated).

(B) BMMs were cultured with 10 ng/ml M-CSF and were exposed to ACCX at the indicated concentration for 24 hr. DNA fragmentation was assayed by cell death ELISA (*$p < 0.05$, **$p < 0.0005$ versus nontreated; the error bars show standard deviations).

chose the dose of 12 mg/kg/mouse to ensure solubility of ACCX in vivo. Mice not injected or injected with DMSO alone were used as controls for basal levels of osteoclastogenesis. DMSO alone did not impact osteoclastogenesis (Figures 5A and 5B). TNFα increased osteoclastogenesis (Figures 5A and 5B) irrespective of ACCX. The extent of osteoclastogenesis induced by TNFα, however, was significantly reduced in the mice injected with ACCX (Figures 5A and 5B).

DISCUSSION

Osteoclasts play a key role in menopausal osteoporosis. Estrogen deficiency enhances the genesis and activity of osteoclasts, resulting in an unbalanced increase in bone resorption [2–4]. Recent studies have established that black cohosh, a widely used herbal medicine to alleviate menopausal symptoms, reduces bone loss in the ovariectomized rat model [15, 16]. These observations prompted us to investigate whether a component of black cohosh is capable of blocking osteoclastogenesis. We established that a triterpenoid glycoside, ACCX, inhibits osteoclastogenesis induced by RANKL in a dose-dependent manner. This compound only shows moderate inhibition of proliferation, and induction of cell death elicited by this compound occurs at doses that completely eliminate osteoclastogenesis. Most importantly, ACCX directly targets differentiation of osteoclasts from their precursors, as demonstrated by the fact that ACCX-treated cells completely lack osteoclast-specific marker proteins, such as Cathepsin K and β3 integrin.

Osteoclastogenesis is induced by RANKL under physiological conditions [1, 2]. Although the exact mechanism by which RANKL induces osteoclastogenesis is not completely understood, it is known that RANKL-induced activation of the NF-κB and MAPK pathways as well as upregulation of NFAT2 expression are required for osteoclastogenesis [38–41]. We find that ACCX eliminates RANKL-induced activation of the NF-κB pathway, as demonstrated by a lack of phosphorylation and degradation of IκBα, an absence of NF-κB DNA-binding activity, and an absence of RANKL-induced expression of the NF-κB-dependent genes. Although the exact targets of ACCX on the NF-κB pathway remain to be determined, our data suggest that the potential targets are upstream of IκBα phosphorylation. The importance of the NF-κB pathway in osteoclastogenesis is underscored by the findings that mice lacking both NF-κB p50 and p52 or mice deficient in IKKβ postnatally are osteopetrotic [42–44]. Furthermore, peptide inhibitors of IKK are effective in preventing osteoclastogenesis and bone loss in a mouse model of arthritis [45, 46]. Therefore, it is reasonable to suggest that inhibition of the NF-κB pathway is likely responsible for ACCX-elicited blockage of osteoclastogenesis.

RANKL-induced activation of MAPKs is also modulated by ACCX. This triterpene dampens RANKL-induced activation of ERK. Given the positive role of ERK activation in cell proliferation [47], ACCX-elicited inhibition of ERK activation is likely responsible for the reduced proliferation of osteoclast precursors in the presence of the compound. RANKL also transiently activates JNK and p38, which is important for osteoclastogenesis [1, 2]. ACCX does not block these pathways. Instead, ACCX activates p38 in the absence of RANKL and prolongs p38 activation. On the other hand, ACCX at an effective dose for blocking osteoclastogenesis only increases basal activation of JNK, while attenuating peak activation of JNK. High doses of ACCX prolong JNK activation (data not shown). A number of studies suggest that prolonged activation of JNK leads to cell death, whereas transient activation of JNK leads to proliferation [48–50]. The observation that ACCX potently increases cell death at high doses is in agreement with the notion that constitutive activation of JNK leads to cell death.

TNFα is the primary inflammatory cytokine responsible for bone loss occurring in various pathological conditions [51]. The finding that TNFα-deficient mice are protected from estrogen-deficiency-induced bone loss establishes the critical role of TNFα in this circumstance as well [32]. TNFα stimulates osteoclastogenesis indirectly via increased expression of M-CSF, IL-1, and RANKL in

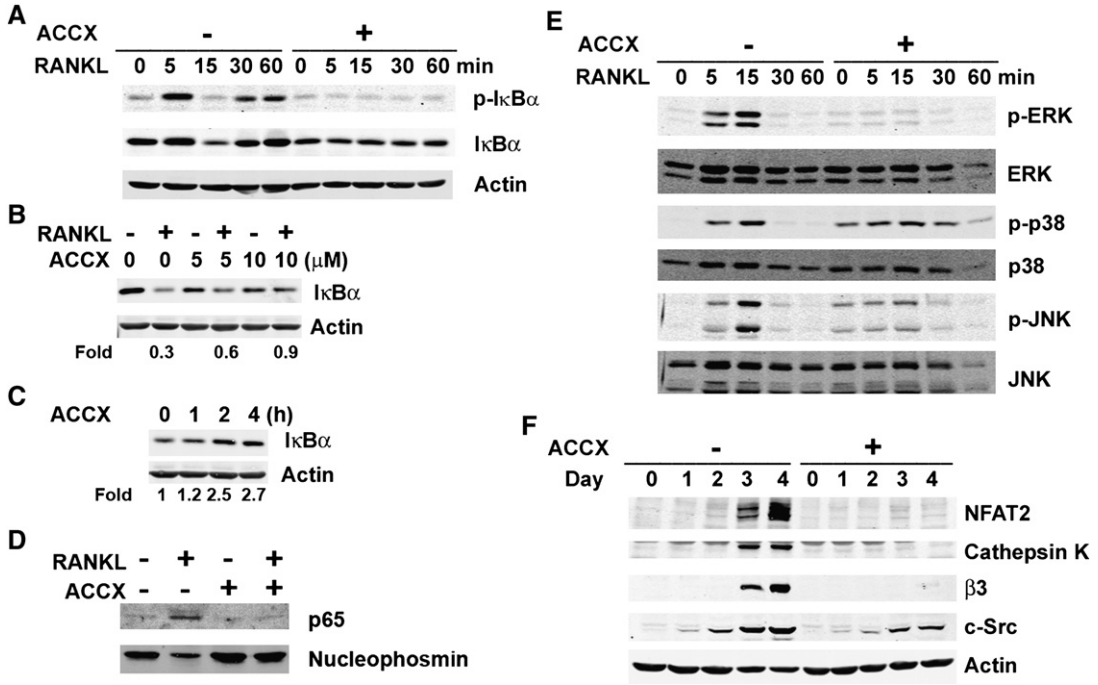

Figure 3. ACCX Alters RANKL Signaling Events and Suppresses Osteoclast Gene Markers

(A) BMMs were grown in serum-free medium for 6 hr with or without exposure to 10 μM ACCX for 4 hr. Cells were subsequently stimulated with 100 ng/ml RANKL for the indicated time periods. Cell lysates were subjected to immunoblotting with antibodies as indicated.

(B) BMMs were treated in a manner similar to that described in (A), but with indicated doses of ACCX, and were stimulated with RANKL for 15 min.

(C) BMMs were treated with 20 μM ACCX for the indicated time periods and were stimulated with RANKL for 15 min.

(D) Cells were treated with or without 20 μM ACCX for 4 hr, followed by RANKL (100 ng/ml) stimulation for 0 or 30 min. Nuclear extracts were allowed to bind biotin-labeled oligonucleotides bearing a κB site. Bound proteins were pulled down with streptavidin beads and were immunoblotted with a p65 antibody. Nucleophosmin in nuclear extracts served as an input control.

(E) Similar to (A).

(F) BMMs were cultured with 20 ng/ml M-CSF and 100 ng/ml RANKL with or without 10 μM ACCX. Cell lysates were collected at the indicated time periods and were subjected to immunoblotting with antibodies as indicated.

stromal/osteoblast cells, while it also directly facilitates osteoclast differentiation from BMMs primed with RANKL [36, 52–54]. We found that ACCX blocks TNFα-induced osteoclastogenesis in vitro. Similar to the effect on RANKL signaling, ACCX eliminates TNFα-induced activation of the NF-κB and the ERK pathways while leading to prolonged activation of p38 and increased basal activation of JNK. Most importantly, ACCX reduces TNFα-induced osteoclastogenesis in vivo.

Our study clearly establishes that a component of black cohosh, ACCX, directly targets osteoclastogenesis from BMMs. Our data, however, do not exclude the possibility that other mechanisms or other less potent components from black cohosh may also contribute synergistically to the beneficial skeletal effects of this natural product. In fact, we find that the constituent cimigenol is also active in the blockage of osteoclastogenesis, although it is much less potent than ACCX. Interestingly, the structure of cimigenol is very similar to that of ACCX (without the acetyl group and sugar in cimigenol), whereas other analogs, cimigenol xylopyranoside (CX, missing the 25-acetyl group with respect to ACCX) and 25-acetylcimigenol (AC, without the xylose with respect to ACCX), are inactive, revealing subtle yet strict requirements for optimal activity. On the other hand, black cohosh extracts were shown to stimulate production of the decoy receptor for RANKL, osteoprotegrin (OPG), in osteoblasts and, therefore, to indirectly impair osteoclastogenesis [55]. The responsive component of black cohosh responsible for upregulation of OPG, however, remains to be determined.

SIGNIFICANCE

Black cohosh is one of the most popular dietary botanicals for treatment of menopausal symptoms. An alcoholic extract of this herb shows the beneficial effect of preventing bone loss in vivo. The mechanism of action and the responsible active component, however, need to be identified. The current discovery is significant in several respects. First, to our knowledge, ACCX is the first component in black cohosh that is proven to exhibit potent inhibition of osteoclastogenesis both in vitro and in vivo, providing a scientific rationale at the molecular level for the claim that black cohosh

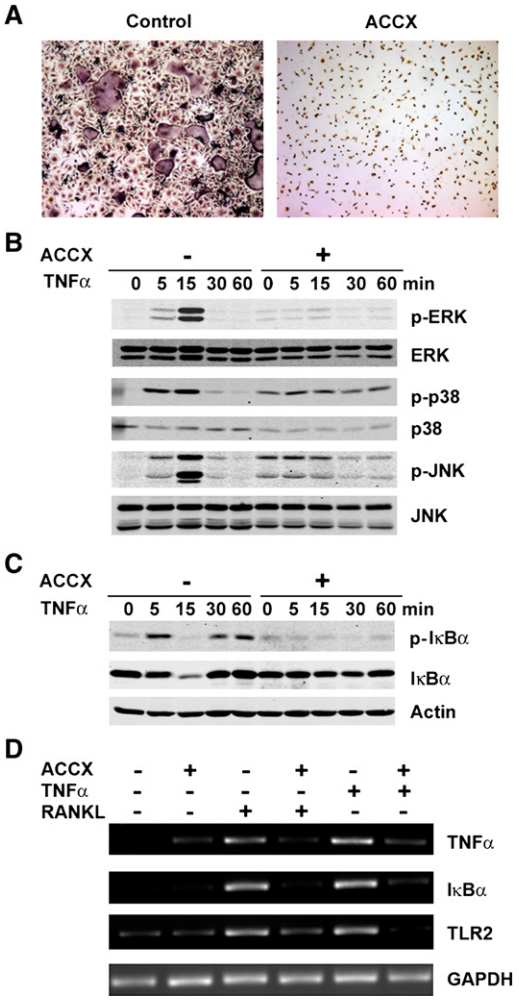

Figure 4. ACCX Blocks TNFα-Induced Osteoclastogenesis in Vitro and Alters TNFα- Elicited Signaling Events

(A) BMMs were cultured with 50 ng/ml M-CSF and 50 ng/ml TNFα with or without 10 μM ACCX for 5 days. Cells were stained for TRAP.

(B and C) BMMs were grown in serum-free medium with or without exposure to 10 μM ACCX for 4 hr. Cells were stimulated with 50 ng/ml TNFα for the indicated time periods. Cell lysates were analyzed by immunoblotting with indicated antibodies.

(D) BMMs pretreated with or without 10 μM ACCX for 4 hr were stimulated with 200 ng/ml RANKL or 50 ng/ml TNFα for 30 min. Levels of *TNFα*, *IκBα*, and *TLR2* mRNA were determined by RT-PCR. *GAPDH* mRNA served as loading controls.

can offer effective prevention of postmenopausal osteoporosis/bone loss. Second, ACCX is a novel inhibitor of the NF-κB pathway, representing a New Chemical Entity (NCE) targeting NF-κB pathways. This compound also targets the MAPK pathway. Modulating multiple targets may provide better therapeutic intervention to alleviate menopausal osteoporosis and other inflammatory bone erosion, rendering ACCX as a chemical tool for studies of osteoporosis biology and as a potential lead for the development of a new class of antiosteoporosis agents.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies

Antibodies for phospho-IκBα, phospho-JNK, JNK, p38, phospho-p38, ERK, phospho-ERK, and β3 were purchased from Cell Signaling Technology. Antibodies for p65, NFAT2, IκBα, and Cathepsin K were obtained from Santa Cruz Biotechnology, Inc. The anti-β-actin antibody was obtained from Sigma-Aldrich. The antibody for nucleophosmin was obtained from J. Weber (Washington University, St. Louis, MO). The monoclonal antibody for c-Src was a gift of Dr. A. Shaw (Washington University, St. Louis, MO). Recombinant human M-CSF was generously provided by Dr. D.H. Fremont (Washington University, St. Louis, MO). Murine recombinant GST-RANKL was previously described [56].

Isolation of Triterpenoid Glycosides from Black Cohosh and Structure Confirmation of ACCX

The pooled ethanolic extracts of the rhizomes of *Cimicifuga racemosa* (10 kg, purchased from Indiana Botanical Garden, Hobert, IN) were concentrated under vacuum until most of the organic solvents were removed. The residue (1 kg) was suspended in warm water (4 l, 60°C) and then partitioned successively with petroleum ether, ethyl acetate, and n-BuOH (4 l × 4 each). The ethyl acetate extract (500 g) was subjected to column chromatography on silica gel (2 kg, 160–200 mesh) and eluted with petroleum ether-acetone (5:1–1:1) to give four fractions. Each fraction was further separated by using alternatively normal-phase and reverse-phase Chromatorex C18 silica gel chromatography to afford 46 compounds, including 32 triterpenoid glycosides, 6 aromatic acids, and 8 others whose structures were determined by using a combination of mass spectroscopy and 1D- and 2D-NMR techniques. ESI mass spectra were recorded with a Thermo-Finnigan LCQ^{DECA} spectrometer (see the Supplemental Data available with this article online). NMR spectra were obtained with a Bruker Avance 600 spectrometer. The physical and spectral data of ACCX ([23*R*, 24*S*] 25-O-acetyl-cimigenol-3-O-β-D-xylopyranoside) are as follows: colorless needles, mp 240–242°C, $[\alpha]_D^{20}$: +38° (*c* 0.1044, MeOH); ESI-MS *m/z*: 685 $[M+Na]^+$, 661 $[M-H]^-$; Molecular formula $C_{37}H_{58}O_{10}$. ^{1}H-NMR (600 MHz, pyridine-d_5) δ: 3.49 (1H, d, *J* = 11.3 Hz, 3α-H), 4.23 (1H, s, 15β-H), 1.11 (3H, s, 18-Me), 0.26, 0.50 (each 1H, d, *J* = 2.6 Hz, 19-H), 0.83 (3H, d, *J* = 9.4 Hz, 21-Me), 4.56 (1H, d, *J* = 9.2 Hz, 23-H), 4.08 (1H, s, 24-H), 1.93 (3H, s, 25-OAc), 1.65 (3H, s, 26-Me), 1.63 (3H, s, 27-Me), 1.16 (3H, s, 28-Me), 1.30 (3H, s, 29-Me), 1.03 (3H, s, 30-Me), 4.83 (1H, d, *J* = 7.1 Hz, 1′-H), 4.01(1H, t, *J* = 8.1 Hz, 2′-H), 4.12 (1H, t, *J* = 8.5 Hz, 3′-H), 4.19 (1H, dd, *J* = 8.3, 4.1 Hz, 4′-H), 3.70 (1H, t, *J* = 10.9 Hz, 5′-H), 4.32 (1H, dd, *J* = 4.9, 10.7 Hz, 5′-H); ^{13}C-NMR (150 MHz, pyridine-d_5) δ: 32.4 (C-1), 30.9 (C-2), 88.5 (C-3), 41.3 (C-4), 47.2 (C-5), 21.0 (C-6), 26.3 (C-7), 48.6 (C-8), 20.0 (C-9), 26.4 (C-10), 26.7 (C-11), 34.0 (C-12), 41.8 (C-13), 47.6 (C-14), 80.1 (C-15), 112.4 (C-16), 59.4 (C-17), 19.5 (C-18), 30.9 (C-19), 23.9 (C-20), 19.5 (C-21), 37.9 (C-22), 71.7 (C-23), 86.7 (C-24), 83.1 (C-25), 22.2 (C-26), 23.4 (C-27), 11.8 (C-28), 25.7 (C-29), 15.4 (C-30), 170.1(-$\underline{C}OCH_3$), 21.5 (-CO$\underline{C}H_3$) (the corresponding chemical shift is denoted by the underlined carbon), 107.5 (C-1′), 75.5 (C-2′), 78.6 (C-3′), 71.2 (C-4′), 67.1 (C-5′). The identity of ACCX and other compounds was achieved by establishing that their NMR data are nearly identical with those reported in the literature [11, 22–24].

Cell Culture

Whole bone marrow cells were isolated from flushing the long bones of 4- to 8-week-old C57BL/6 mice (Jackson Laboratory). These cells were grown in α-MEM with 10% FBS and 1:10 CMG14-12 culture supernatant [57] containing 1.2 μg/ml M-CSF for 3 days to generate bone marrow macrophages (BMMs). To generate osteoclasts, 50 ng/ml M-CSF and 100 ng/ml RANKL or 50 ng/ml TNFα were added to α-MEM medium containing 10% FBS. Osteoclasts were stained for tartrate-resistant acid phosphatase (TRAP) as per the manufacturer's instructions (Sigma-Aldrich).

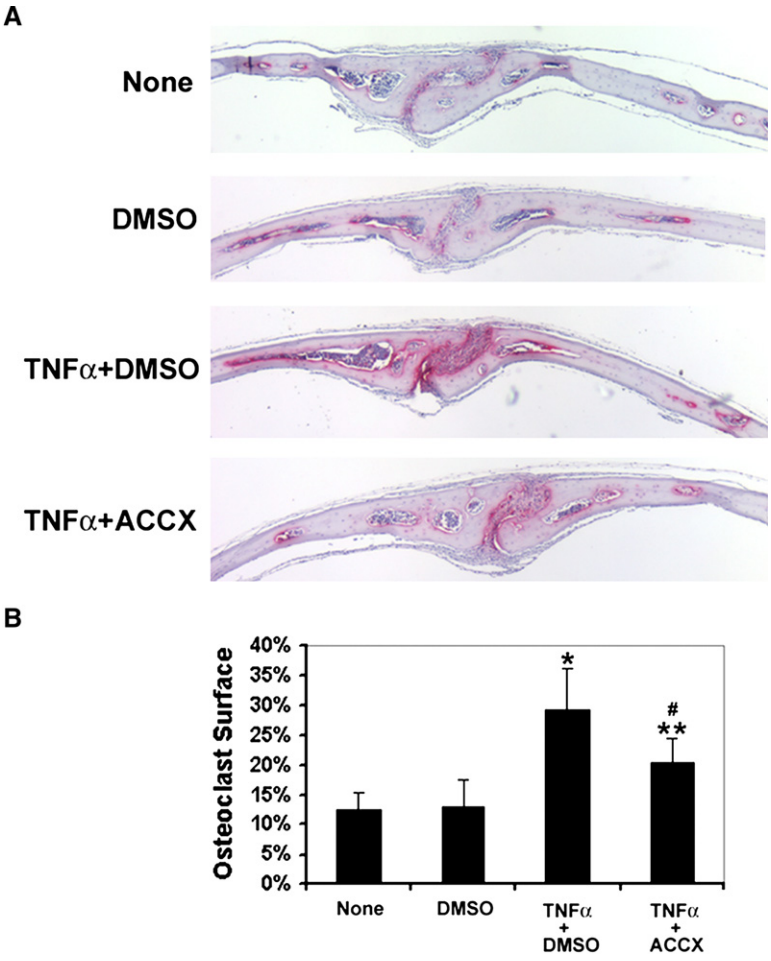

Figure 5. In Vivo Osteoclastogenesis Induced by TNFα Is Reduced by ACCX

Mice were injected daily with DMSO or ACCX with or without supracalvaria injection of TNFα for 5 days and were sacrificed the following day.

(A) Histological sections of calvaria from these mice were stained for TRAP activity.

(B) Histomorphometric quantitation of the percentage of bone surface covered by osteoclasts in calvaria of each group of mice (*$p < 0.005$, **$p < 0.02$, versus DMSO; #$p < 0.04$, versus TNFα + DMSO; the error bars show standard deviations).

Western Blot Analysis

Cultured cells were washed twice with ice-cold PBS and were lysed in a buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM NaF, and 1× protease inhibitor mixture. Cell lysates were clarified by centrifugation at 6000 rpm for 5 min. Western blot analysis was carried out by using 20–40 μg total proteins as previously described [54].

Proliferation Assay and Cell Death ELISA Assay

BMMs were plated in 96-well plates at 1 $\times 10^4$ cells/well and were grown for 1 day. Cells were labeled with BrdU for the last 6 hr of culture. The BrdU ELISA assay was carried out as recommended by using the cell proliferation biotrak ELISA system (Amersham Biosciences). Cell death was analyzed by using the cell death detection $ELISA^{PLUS}$ kit (Roche Applied Science) as per the manufacturer's instructions. Quadruplicate samples were used. All experiments were repeated with satisfactory results.

RT-PCR

RNA was isolated by using RNeasy kits (QIAGEN Sciences). First-strand cDNA was generated from 1 μg total RNA by using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) as recommended by the manufacturer. One fifth of the RT reaction product, 45 μl PCR SuperMix (Invitrogen), and 0.5 μM primers for the genes to be tested were amplified in a PCR Express Thermal Cycler (HYBAID). The cDNA was denatured at 94°C for 5 min and was subsequently subjected to various amplification cycles comprised of 94°C for 40 s, 60°C for 50 s, and 72°C for 60 s. The primers (listed in the 5′ to 3′ direction) used were as follows: (1) *TNFα*, AATGGCCTC CCTCTCATCAGTTCT and TGAGATAGCAAATCGGCTGACGGT; (2) *IκBα*, CAAGTGGAGTGGAG TCTGCAGGTTGTT and GCCTGGACTC CATGAAGGAC; (3) *TLR2*, GGCTTCCTCTTGGCCTGGAG and GGA GACTCTGGAAGCAGGCG; (4) *GAPDH*, ACTTTGTCAAGCTCATTTCC and TGCAGCGAACTTTATTGATG.

NF-κB DNA-Binding Assay

BMMs were treated with or without 20 μM ACCX for 4 hr, followed by RANKL (100 ng/ml) stimulation for 0 or 30 min. Cells were collected in hypotonic buffer (10 mM HEPES [pH 7.6], 1.5 mM $MgCl_2$, 1 mM KCl, 5 mM NaF, 1 mM Na_3VO_4, 1 mM DTT, 1× protease inhibitor cocktail [Roche Applied Science]) and were lyzed by the addition of NP-40 (0.1%). After centrifugation at 10,000 × g for 30 s at 4°C, nuclear pellets were washed with hypotonic buffer, collected again by centrifugation, and resuspended in high-salt buffer (400 mM NaCl in hypotonic buffer). Biotin-labeled oligonucleotides for NF-κB-binding sites [58] were purchased from Invitrogen and were conjugated to streptavidin agrose beads (Sigma). A total of 20 μg nuclear proteins were mixed with 10 μl agarose beads in binding buffer (30 mM NaCl, 10 mM Tris [pH 7.4], 1 mM EDTA, 5% glycerol, 1 mg/ml BSA, 1 mM DTT, 1× protease inhibitor cocktail) in the presence of 1 μg poly dIdC (Pharmacia) for 45 min at room temperature. Beads were washed three times with binding buffer and resuspended in SDS-PAGE loading

buffer (Cell Signaling). Proteins were analyzed by immunoblotting with p65 antibody.

TNFα-Induced In Vivo Osteoclastogenesis and Histological Analysis

C57BL/6 mice (6 weeks old) were purchased from the Jackson Laboratory and were housed in the animal care unit at Washington University School of Medicine. Mice were intraperitoneally injected daily with 50 μl DMSO or ACCX dissolved in DMSO (12 mg/kg/mouse) with or without supracalvaria injection of TNFα (3 μg in PBS/mouse) for 5 days. Each group had 5–10 mice. All animals were sacrificed on day 6. Calvaria taken from these mice were sequentially fixed in 10% buffered formalin for 24 hr, decalcified in 14% EDTA (pH 7.2) for 7 days, dehydrated in graded alcohol, cleared through xylene, and embedded in paraffin. Paraffin sections were stained histochemically for TRAP. Histomorphometric quantitation was performed by using the Bioquant System (BIOQANT Image Analysis). All animal experiments were approved by the Animal Studies Committee of Washington University School of Medicine.

Supplemental Data

Supplemental Data include structures of compounds 25-acetylcimigenol xylopyranoside (ACCX), cimigenol xylopyranoside (CX), cimigenol (C), acetylcimigenol (AC), actein, and cimiaceroside B, as well as NMR data of ACCX, and are available at http://www.chembiol.com/cgi/content/full/14/7/860/DC1/.

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