Cistanche deserticola polysaccharide attenuates osteoclastogenesis and bone resorption via inhibiting RANKL signaling and ROS production

Dezhi Song^{1, 2, 3, 5, #}, **Zhen Cao**^{4, 5, #}, **Zaibing Liu**⁶, **Jennifer Tickner**⁵, **Heng Qiu**⁵, **Chao Wang**⁵, **Kai Chen**⁵, **Ziyi Wang**⁵, **Shiwu Dong**^{4, *}, **Jiake Xu**^{5, *}

¹Research Centre for Regenerative Medicine, Guangxi Medical University, Nanning, 530021, Guangxi, China

²Guangxi Key Laboratory of Regenerative Medicine, Guangxi Medical University, Nanning, 530021, Guangxi, China

³Department of Microbiology, Guangxi Medical University, Nanning, 530021, Guangxi, China

⁴Department of Biomedical Materials Science, Third Military Medical University, Chongqing 400038, China

⁵School of Biomedical Sciences, University of Western Australia, Perth, WA 6009, Australia

⁶Department of Radiology, People's Hospital of Zhangqiu District, Jinan, 250200, Shandong, China

Running title: CDP inhibits RANKL-induced osteoclastogenesis

#Co-first author: Dezhi Song and Zhen Cao contributed equally to this work

***Correspondence:**

Shiwu Dong

Department of Biomedical Materials Science, School of Biomedical Engineering, Third Military Medical University, Gaotanyan Street No.30, Chongqing 400038, China, Email: dongshiwu@tmmu.edu.cn

Jiake Xu

School of Biomedical Sciences, University of Western Australia, Perth, Western Australia 6009, Australia,

E-mail: jiake.xu@uwa.edu.au

Abstract:

Osteoporosis is a metabolic disease characterized by osteopenia and bone microstructural deterioration. Osteoclasts are the primary effector cells that degrade bone matrix and their abnormal function leads to the development of osteoporosis. Reactive oxygen species (ROS) accumulation during cellular metabolism promotes osteoclast proliferation and differentiation, therefore, playing an important role in osteoporosis. *Cistanche deserticola* polysaccharide (CDP) possesses anti-tumor, anti-inflammatory, and anti-oxidant activity. However, the impact of CDP on osteoclasts is unclear. In this study, tartrate-resistant acid phosphatase (TRAcP) staining, immunofluorescence, RT-PCR, and western blot were utilized to demonstrate that CDP inhibited osteoclastogenesis and hydroxyapatite resorption. In addition, CDP also inhibited the expression of osteoclast maker genes including *Ctsk*, *Mmp9* and *Acp5* and had no effect on RANK expression. Mechanistic analyses revealed that CDP increases the expression of antioxidant enzymes to attenuate RANKL-mediated ROS production in osteoclasts and inhibits NFAT and mitogen-activated protein kinase (MAPK) activation. These results suggest that CDP may represent a candidate drug for the treatment of osteoporosis caused by excessive osteoclast activity.

Keywords:

Bone resorption; *Cistanche deserticola* polysaccharide; MAPK; Osteoclast; Reactive oxygen species

Introduction

The balance between bone formation, mediated by osteoblasts, and bone resorption, mediated by osteoclasts, plays a vital role in maintaining bone metabolic homeostasis (Manolagas, 2000). When bone resorption exceeds bone formation, osteoporosis occurs, which is characterized by reduced bone mass and bone microstructural damage (Ikeda, 2008). Osteoporosis is a common disease in elderly individuals and postmenopausal women and its pathogenesis has not been fully elucidated (Cooper and Melton, 1992). Estrogen deficiency is a primary cause of osteoporosis (Manolagas et al., 2013). In addition, reactive oxygen species (ROS) can be induced by RANKL and are associated with osteoclast formation (ref), and thus may contribute to the development of osteoporosis (Manolagas, 2010). Some studies have found that Nrf2-antioxidant deficiency increases ROS levels and promotes receptor activator of nuclear factor kappa-B ligand (RANKL)-induced osteoclast differentiation (Hyeon et al., 2013). Therefore, reducing ROS production during osteoclast differentiation should be evaluated as a therapeutic strategy for the treatment of osteoporosis.

Osteoclasts are derived from the monocyte/macrophage hematopoietic lineage and are the only multinucleated cells that perform bone resorption (Teitelbaum, 2000). Therefore, research involving osteoclast formation is of great significance in developing effective treatments for bone metabolism diseases (Lorenzo, 2017). Macrophage colony stimulating factor (M-CSF) and RANKL, produced by osteoblasts and activated T cells, are important cytokines that regulate osteoclastogenesis (Kim and Kim, 2016; Teitelbaum and Ross, 2003). RANKL induces the expression of nuclear factor of activated T-cells (NFATc1), which is a critical transcription factor active during osteoclast formation (Ishida et al., 2002). Activated NFATc1 promotes the expression of

osteoclast marker genes such as tartrate-resistant acid phosphatase (TRAcP) and cathepsin K (CTSK) that regulate osteoclastogenesis and osteoclast function (Balkan et al., 2009; Crotti et al., 2008).

Cistanche deserticola polysaccharide (CDP) is isolated from the fleshy stems of *Cistanche* and possesses immune regulation, anti-tumor, anti-aging, and other pharmacological effects (Guo et al., 2016; Jia et al., 2012). CDP had an inhibitory effect on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in mouse microglial cells (BV-2 cells) (Nan et al., 2013). In addition, a phenylethanoid-rich extract (ECD) of *Cistanche* enhanced the swimming capacity of mice by decreasing muscle damage, delaying lactic acid accumulation, and improving energy storage (Cai et al., 2010). However, the effects of CDP on osteoclast function and activity remain unknown.

In this study, we demonstrated that CDP inhibits RANKL-induced osteoclast differentiation and bone resorption. The underlying mechanism was that CDP enhances the expression of antioxidant enzymes to attenuate ROS production, and then suppresses RANKL-activated NFAT and MAPK signaling cascades. These results suggest that CDP might be used to treat osteoporosis caused by excessive osteoclastic bone resorption.

75 **Materials and methods**

76 *Materials*

77 CDP (purity>98%) was purchased from Solarbio (Beijing, China) and prepared at a stock concentration of 1
78 mM in phosphate buffered saline (PBS). Antibodies specific for c-Fos, CTSK, GSR, TRX1, NOS2, TRAF6,
79 RANK, NFATc1, ERK, JNK, p38, phosphorylated (p)-ERK, p-p38, p-JNK, and β -actin were obtained from
80 Santa Cruz Biotechnology (San Jose, CA). Antibodies to V-ATPase d2 were produced as previously described
81 (Feng et al., 2009). The
82 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and
83 luciferase assay system were obtained from Promega (Sydney, Australia). Recombinant macrophage colony
84 stimulating factor (M-CSF) was purchased from R&D Systems (Minneapolis, MN). Recombinant
85 GST-rRANKL protein was expressed and purified as previously described (Xu et al., 2000).

86

87 *Cell culture*

88 RAW264.7 cells (mouse macrophage cells) were obtained from American Type Culture Collection (ATCC,
89 Manassas, VA) and cultured in α -modified minimal essential medium (α -MEM; Thermo Fisher Scientific,
90 Scoresby, Australia) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml
91 penicillin, and 100 μ g/ml streptomycin (complete medium). Bone marrow derived monocytes (BMMs) were
92 isolated from 6-week-old C57BL/6J mice, which were euthanized according to procedures approved by the
93 Animal Ethics Committee of the University of Western Australia (RA/3/100/1244). Long bones were dissected
94 free of soft tissues and the bone marrow was flushed from the femur and tibia, which was then cultured in
95 complete medium in the presence of M-CSF (50 ng/ml).

96

97 *Osteoclastogenesis assay*

98 BMMs were plated into 96-well culture plates at a density of 6×10^3 cells/well and treated with complete
99 medium containing M-CSF (50 ng/ml) and GST-rRANKL (100 ng/ml), in the presence or absence of varying
100 concentrations of CDP. The cell culture medium was changed every 2 days. After 5 days, cells were fixed with
101 4% paraformaldehyde for 10 min, washed three times with PBS, and then stained for TRAcP enzymatic activity
102 using the leucocyte acid phosphatase staining kit (Sigma-Aldrich, Sydney, Australia), following the
103 manufacturer's procedures. TRAcP-positive multinucleated cells (> 3 nuclei) were identified as osteoclasts.

104

105 *Cytotoxicity assays*

106 BMMs were seeded into 96-well plates at 6×10^3 cells/well and left overnight to adhere. The following day, the
107 cells were incubated with varying concentrations of CDP. After a further 48 h, MTS solution (20 μ l/well) was
108 added and incubated with cells for 2 h. The absorbance at 490 nm was determined with a microplate reader
109 (Multiscan Spectrum; Thermo Labsystems, Chantilly, VA).

110

111 *Immunofluorescent staining*

112 BMMs were seeded at a density of 6×10^3 cell/well in the presence of M-CSF (50 ng/ml) overnight. Cells were
113 then stimulated with M-CSF and GST-rRANKL (100 ng/ml) until mature osteoclasts formed. The osteoclasts
114 were then treated with varying concentrations of CDP for 48 h before fixing with 4% paraformaldehyde,
115 permeabilizing with 0.1% Triton X-100/PBS, and blocking with 3% BSA in PBS. Prepared cells were

incubated with rhodamine-conjugated phalloidin for 45 min in the dark to stain for F-actin. Cells were then washed with PBS, nuclei counterstained with DAPI, and mounted with cover slips for confocal microscopy.

Hydroxyapatite resorption assay

To measure osteoclast activity BMMs (1×10^5 cells/well) cultured on 6-well collagen-coated plates (BD Biocoat; Thermo Fisher) were stimulated with GST-rRANKL (100 ng/ml) and M-CSF (50 ng/ml) until mature osteoclasts were generated (Zhou et al., 2016). Cells were then gently detached from the plate using cell dissociation solution (Sigma-Aldrich) and equal numbers of mature osteoclasts were seeded onto individual wells in hydroxyapatite-coated 96-well plates (Corning Osteoassay; Corning, NY). Mature osteoclasts were incubated in medium containing GST-rRANKL and M-CSF with or without CDP at the indicated concentrations. After 48 h, half of the wells were immunohistochemically stained for TRAcP activity, as described above, to assess the number of multinucleated cells per well. The remaining wells were bleached for 10 min to remove cells and allow measurement of the resorbed areas. Resorbed areas were photographed under standard light microscopy and ImageJ software (National Institutes of Health, Bethesda, MD) was used to quantify the percentage area of hydroxyapatite surface resorbed by the osteoclasts.

Luciferase reporter assays

To investigate NFATc1 transcriptional activation, RAW 264.7 cells were stably transfected with an NFATc1-responsive luciferase reporter construct (Cheng et al., 2017; van der Kraan et al., 2013). Transfected cells were cultured in 48-well plates at a density of 1.5×10^5 cells/well and pretreated with various concentrations of CDP for 1 h. Following pre-treatment, cells were stimulated with GST-rRANKL (100 ng/ml)

for 24 h and luciferase activity measured using the luciferase reporter assay system according to the manufacturer's protocol (Promega).

Quantitative RT-PCR analysis

Total RNA was isolated from cells using Trizol reagent according to the manufacturer's protocol (Thermo Fisher Scientific). cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase with 1 µg of RNA template and oligo-dT primers. Polymerase chain reaction amplification of specific sequences was performed using the following program: 94°C for 5 min, followed by 30 cycles of 94°C for 40 sec, 60°C for 40 sec, and 72°C for 40 sec, and a final extension step of 5 min at 72°C. The detailed information of specific primers is shown in Table 1. Relative mRNA levels were calculated by normalization to expression of the housekeeping gene *Hmbs*.

Western blotting

BMMs were cultured in complete medium with M-CSF in 6-well plates and stimulated with GST-rRANKL (100 ng/ml) for the stated times. Cells were lysed in radioimmunoprecipitation (RIPA) lysis buffer and proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Silverwater, Australia). The membranes were blocked in 5% skim milk for 1 h and then probed with various specific primary antibodies with gentle shaking overnight at 4°C. Membranes were washed and subsequently incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. Antibody reactivity was then detected with enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech, Piscataway, NJ) and visualized with an Image-quant LAS 4000 (GE Healthcare).

158

159 *Intracellular ROS detection*

160 Intracellular ROS levels were detected using a 2',7'-dichlorofluorescein diacetate (DCFDA) cellular ROS
161 detection assay kit (Abcam, Melbourne, Australia). BMMs (5×10^3 cells/well) were cultured in 96-well plates
162 and treated with RANKL (100 ng/ml), M-CSF (50 ng/ml), and CDP for 72 h. Intracellular ROS levels were
163 measured using DCFDA, which oxidizes into fluorescent DCF in the presence of ROS. Cells were washed in
164 Hank's buffer and incubated in the dark for 30 min with 10 μ M DCFH-DA. Images were obtained using
165 confocal microscopy.

166

167 *Statistical Analyses*

168 All data are representative of at least three experiments performed in triplicate, unless otherwise indicated. Data
169 are expressed as mean $\pm$ SD. One-way ANOVA followed by Student-Newman-Keuls post hoc tests was used to
170 determine the significance of differences between results, with $p < 0.05$ regarded as significant

171

Results

CDP inhibits RANKL-induced osteoclastogenesis and osteoclast fusion

To determine if CDP can suppress RANKL-induced osteoclast formation, we first performed an osteoclastogenesis assay using mouse bone marrow-derived monocytes (BMMs) (Liu et al., 2013; Song et al., 2016). BMMs were treated with RANKL and M-CSF for 5 days with increasing concentrations of CDP. CDP reduced the number of TRAcP-positive multinucleated cells when the concentration of CDP reached 5 μ M or higher (Figure 1A, 1B). To evaluate CDP toxicity and confirm that these results were not a reflection of cell death or number, we performed an MTS assay. BMMs were treated with RANKL and M-CSF for 48 h with varying dosages of CDP. CDP had no effect on BMM proliferation at the concentration of 15 μ M or less (Figure 1C).

To test the effects of CDP on osteoclast fusion, osteoclasts were induced with RANKL and M-CSF treatment, with or without varying doses of CDP. Osteoclasts were stained with rhodamine-phalloidin and DAPI to assess the number of nuclei per osteoclast (Figure 2A). Both osteoclast number and the average number of nuclei per osteoclast decreased following CDP treatment (5-10 μ M) (Figure 2B, 2C). Therefore, CDP had inhibitory effects on RANKL-induced osteoclastogenesis and osteoclast fusion in a dose-dependent manner.

CDP attenuates RANKL-induced osteoclastic hydroxyapatite resorption activity

A hydroxyapatite resorption assay was performed to detect the effect of CDP on osteoclast function (Figure 3A). After a 24 h incubation, the number of osteoclasts per well did not change while the hydroxyapatite resorption area was significantly decreased by treatment with 5 and 10 μ M CDP, when compared to control

groups (Figure 3B, 3C). These results demonstrate that CDP has strong inhibitory effects on both osteoclast formation and osteoclast resorption activity without any cytotoxic effects.

CDP inhibits osteoclast marker gene expression

To further investigate the inhibitory effects of CDP on osteoclastogenesis and osteoclastic bone resorption, BMMs were treated with RANKL and M-CSF for 5 days with varying concentrations of CDP. RT-PCR was then performed to detect the expression of osteoclast marker genes. Expression of *Nfatc1*, a crucial transcription factor during osteoclastogenesis, was inhibited by CDP in a dose-dependent manner (Figure 4A). In addition, CDP (5 and 10 μ M) down-regulated expression of bone resorption-related genes, including *Mmp9*, *Ctsk*, and *Acp5* (Figure 4B-D).

CDP suppresses NFATc1 activity and downstream protein expression

To examine the effect of CDP on RANKL-induced NFATc1 activity, a luciferase reporter assay was performed. Treatment with CDP, at concentrations of 5 μ M and higher, significantly inhibited RANKL-induced NFATc1 activity (Figure 5A). In addition, western blotting showed that CDP significantly suppressed expression of NFATc1 and c-Fos in BMMs treated with RANKL and M-CSF for 3 and 5 days (Figure 5B). Moreover, the expression of osteoclast function-related proteins, such as V-ATPase-d2 and CTSK, was down regulated in the presence of CDP compared to control groups. However, CDP had no effect on RANK expression (Figure 5B).

CDP promotes the expression of antioxidant enzymes to scavenge ROS production during RANKL-induced osteoclastogenesis

To explore the underlying mechanism of CDP-dependent osteoclastogenesis inhibition, BMMs were treated with RANKL (100 ng/ml) and M-CSF (50 ng/ml) together with PBS or CDP for 72 h. We examined the effect of CDP on intracellular ROS production stimulated by RANKL. Intracellular ROS levels were increased by RANKL treatment, which was attenuated by CDP (5 and 10 μ M) (Figure 6A). Both the number of ROS-positive cells and the intensity of ROS staining were decreased by CDP treatment in a dose-dependent manner (Figure 6B, 6C). Besides, from the results of western blot, we found that CDP promoted thioredoxin (TRX1) and glutathione reductase (GSR) expression while suppressed the expression of inducible nitric oxide synthase (NOS2) in BMMs which were treated with RANKL and M-CSF for 3 days (Figure 6D).

In order to further explore if CDP suppressed osteoclast differentiation through reducing ROS production. We then treated BMMs with peroxide (10 μ M) to imitate the high ROS status in cells. BMMs were induced by RANKL and M-CSF for 3 days and the results of western blot and RT-PCR showed that peroxide promoted NFATc1 and c-Fos expression compared with the control groups. Consistent with the results in Figure 5, the expression of NFATc1 and c-Fos was suppressed by CDP while peroxide rescued the inhibitory effect of CDP (Figure 6E, 6F). These data indicated that CDP repressed the expression of NFATc1 and c-Fos by scavenging ROS production.

CDP represses MAPK pathways during RANKL-induced osteoclastogenesis

Next, we investigated the effect of CDP treatment on RANKL-mediated TRAF6 expression and MAPK pathway activation. Following incubation in serum-free medium for 2 h, BMMs were stimulated with RANKL, with or without CDP, for 60 min. Stimulation with CDP (10 μ M) had no effect on TRAF6 expression and

235 attenuated phosphorylation of JNK2 and ERK1/2 at 10 and 20 min (Figure 7). Additionally, p38
236 phosphorylation was significantly inhibited by CDP treatment for 60 mins compared to control groups (Figure
237 7). These data reveal that CDP suppresses RANKL-induced MAPK signaling pathways, consistent with its
238 inhibitory effect on osteoclast formation and activity.
239

Discussion

Cistanche, known as "desert ginseng," has attracted much attention recently for its ability to modulate immunity and act protectively during aging and oxidative stress (Jia et al., 2012; Snytnikova et al., 2012). Phenylpropanoid-substituted diglycosides, the major active components of *Cistanche*, have been shown to inhibit nitric oxide (NO) activity in macrophages (Ahn et al., 2017). In addition, a *Cistanche* extract reduced oxidative stress in reperfused myocardium following ischemia, and played a significant role in the inhibition of apoptotic pathways leading to cardioprotection (Yu et al., 2016). As an important component of *Cistanche*, CDP has a variety of pharmacological functions. Our current study found that CDP repressed RANKL-activated osteoclast differentiation and activation by attenuating ROS production as well as NFAT and MAPK activation.

As an acid phosphatase, TRAcP exists in a variety of cells and is abundant in osteoclasts and alveolar macrophages (Snipes et al., 1986). TRAcP is a characteristic enzyme of osteoclasts and its expression is closely related to osteoclast function, regarded as an indicator of osteoclast activity and bone resorption (Minkin, 1982). In our study, CDP inhibited the number of TRAcP-positive cells, indicating that RANKL-induced osteoclastogenesis was blocked by CDP. Degradation of bone matrix by osteoclasts depends on cathepsin K (CTSK) and MMPs (Gruber, 2015). Here, CDP significantly down-regulated the expression of osteoclast functional genes such as *Mmp9*, *Ctsk*, and *Acp5*.

Osteoclast differentiation and function are regulated by multiple signaling pathways (Boyle et al., 2003). After binding to RANK, RANKL recruits adaptor protein TRAF6 to activate the expression of NFATc1 which is an

important transcription factor for osteoclast formation, affecting osteoclast-specific gene expression, including TRAcP and CTSK (Feng, 2005; Takayanagi et al., 2002). In this study, we found that CDP had no effect on RANK and TRAF6 expression in osteoclasts. However, CDP inhibited RANKL-induced NFATc1 activation during osteoclastogenesis of BMMs. Besides, it was demonstrated that ROS, produced by mitochondria in the process of delivering electrons, promoted the proliferation and differentiation of osteoclasts and regulated bone matrix degradation (Ha et al., 2004). A recent study found that RANKL induced Bach1 nuclear import and attenuated Nrf2-mediated antioxidant enzyme production, thereby augmenting intracellular ROS expression and osteoclastogenesis in mice (Kanzaki et al., 2017). In addition, increased intracellular ROS generation by homocysteine enhanced osteoclast formation and activity (Koh et al., 2006). Our study found that CDP reduced ROS accumulation in osteoclasts by inhibiting NOS2 expression and promoting the expression of antioxidant enzymes, such as TRX1 and glutathione reductase. Besides, we treated BMMs with peroxide to enhance intracellular ROS accumulation. The results that increased ROS could improve NFATc1 expression revealed that ROS was in the upstream of NFATc1. We also found that peroxide rescued the inhibitory effect of CDP on NFATc1 expression. So, our data suggest that CDP suppresses ROS accumulation to inhibit NFATc1 expression, then to repress osteoclast formation and function.

ERK, JNK, and p38 belong to the MAPK family which is also involved in the regulation of osteoclast differentiation (Seger and Krebs, 1995). RANKL activates the MAPK pathway by increasing the phosphorylation of ERK, JNK, and p38 (Mizukami et al., 2002). We demonstrated that CDP repressed the RANKL-mediated phosphorylation of key proteins in the MAPK pathway, thereby contributing to the inhibitory effect of CDP on the expression of osteoclast marker genes. To our knowledge, this is the first study

282 showing the inhibitory effects of CDP on ROS production, NFAT and MAPK activation, representing novel
283 mechanisms of action of CDP *in vitro*.

284

285 In summary, we demonstrated that CDP attenuates osteoclastogenesis and hydroxyapatite resorption, and the
286 expression of osteoclast marker genes including *Ctsk*, *Mmp9* and *Acp5*. CDP was able to suppress
287 RANKL-mediated ROS production, as well as NFAT and mitogen-activated protein kinase (MAPK)
288 activation (Figure 8). Collectively, our results suggest that CDP may represent a candidate drug for the
289 treatment of osteoclast related conditions accompanied by ROS overproduction.

290

291 **Competing Interests**

292 The authors declare that they have no conflicts of interest with the contents of this article.

293

294 **Acknowledgments**

295 This work was supported by the Nature Science Foundation of China (81572164), the National Key
296 Technology Research and Development Program of China (2017YFC1103300), the Natural Science
297 Foundation of Guangxi Province (2016GXNSFAA380295) and the University Science and Technology
298 Research Project of Guangxi Province (KY2015YB054). It is also supported in part by Guangxi Scientific
299 Research and Technology Development Plan Project (GKG13349003, 1598013-15), Western Australia
300 Medical & Health Research Infrastructure Fund, Arthritis Australia foundation, The University of Western
301 Australia (UWA) Research Collaboration Awards, and the Australian Health and Medical Research Council
302 (NHMRC, No 1107828, 1027932).

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Figure legends

Figure 1. CDP inhibits RANKL-induced osteoclastogenesis. (A) Representative images of BMMs stained for TRAcP following CDP treatment at different concentrations. Scale bar represents 200 μ m. (B) Quantification of osteoclast numbers (TRAcP positive ≥ 3 nuclei). (C) Survival of BMMs in the presence of CDP as assessed by MTS cell viability assay. Data represent mean $\pm$ SD. Significant differences between the treatment and control groups are indicated as ** ($p < 0.01$), or *** ($p < 0.001$).

Figure 2. CDP suppresses RANKL-induced osteoclast fusion. (A) Representative confocal images of osteoclasts stained for F-actin and DAPI (nuclei). Scale bar represents 200 μ m. (B) Quantification of osteoclasts in each field. (C) Average number of nuclei per osteoclast in each field. Data represent mean $\pm$ SD. Significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

Figure 3. CDP attenuates RANKL-induced osteoclastic hydroxyapatite resorption activity. (A) Representative images of TRAcP-stained osteoclasts (upper panels) and the assay surface after removal of osteoclasts (middle, 4X mag; bottom, 10X mag of boxed area). Scale bar represents 200 μ m. (B) Quantification of osteoclasts in each well. (C) Quantification of bone resorption area in each well. Data represent mean $\pm$ SD. Significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

Figure 4. CDP inhibits osteoclast marker gene expression. BMMs were treated with M-CSF (50 ng/ml) and GST-rRANKL (100 ng/ml) in the presence or absence of the indicated concentrations of CDP. Gene expression was normalized to *Hmbs*. Relative mRNA expression levels of *Nfatc1* (A), *Mmp9* (B), *Ctsk* (C),

and *Acp5* (TRAcP) (D) during osteoclastogenesis are shown. Data represent mean $\pm$ SD. Significant differences between the treatment and control groups are indicated as ** ($p < 0.01$) or *** ($p < 0.001$).

Figure 5. CDP suppresses NFATc1 activity and downstream protein expression. (A) Luciferase activity in RANKL-stimulated RAW 264.7 cells transfected with an NFATc1 luciferase construct. Transfected cells were pre-treated with the indicated concentrations of CDP and subsequently stimulated with GST-rRANKL (100 ng/ml) for 24 h. (B) Representative western blot images of c-Fos, NFATc1, V-ATPase-d2, CTSK, RANK, and ACTB (β -actin) from BMMs induced with RANKL (100 ng/ml) and M-CSF (50 ng/ml) in the presence of CDP. The ratio of c-Fos, NFATc1, V-ATPase-d2, RANK and CTSK relative to ACTB was determined. Data represent mean $\pm$ SD. Significant differences between the treatment and control groups are indicated as *** ($p < 0.001$).

Figure 6. CDP scavenges ROS production during RANKL-induced osteoclastogenesis. (A) Representative images of ROS-positive BMMs during osteoclastogenesis treated with varying concentrations of CDP. Scale bar represents 200 μ m. (B) Quantification of ROS-positive cell numbers in each field. (C) Quantification of the average fluorescence intensity per cell. (D) Representative western blot images of NOS2, TRX1, GSR, and ACTB (β -actin) from BMMs induced with RANKL (100 ng/ml) and M-CSF (50 ng/ml) in the presence of CDP. The ratio of NOS2, TRX1, and GSR relative to ACTB was determined. (E) Representative western blot images of NFATc1, c-Fos, and ACTB (β -actin) from BMMs induced with RANKL (100 ng/ml) and M-CSF (50 ng/ml) in the presence of CDP or peroxide for 3 days. The ratio of NFATc1 and c-Fos relative to ACTB was determined. (F) Relative mRNA expression levels of *Nfatc1* and *Fos* against *Hmbs*. Data represent

mean $\pm$ SD. Significant differences between the treatment and control groups are indicated as * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$).

Figure 7. CDP represses MAPK pathways during RANKL-induced osteoclastogenesis. BMMs were induced with RANKL (100 ng/ml) and M-CSF (50 ng/ml) in the presence of CDP for 60 min. Western blots were probed with specific antibodies to TRAF6, ACTB, p-JNK1/2, JNK, p-ERK1/2, ERK, p-p38, and p38. The quantification of normalized expression intensity of TRAF6 and phosphorylated proteins relative to ACTB and unphosphorylated proteins was determined. The data in the figures represent the mean $\pm$ SD. Significant differences between the treatment and control groups are indicated as * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$).

Figure 8. Schematic diagram of CDP function in osteoclast differentiation. CDP suppresses RANKL induced ROS production, as well as NFATc1 and MAPK activation, hence inhibits osteoclastogenesis.

Figure 5

Figure 6

Figure 7

Figure 8