Tricetin, a dietary flavonoid, suppresses benzo(a)pyrene-induced human non-small cell lung cancer bone metastasis

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Abstract. This is the first study to demonstrate that benzo(a)pyrene (BaP) was able to enhance the production of parathyroid hormone-related protein (PTHrP) by human non-small cell lung cancer H460 cells. Such effect would further contribute to bone metastasis of lung cancer by increasing osteoclastogenesis. This study is also the first to reveal that tricetin (TCN), a flavonoid derivative found in Myrtaceae pollen and *Eucalyptus* honey, was able to reverse BaP-mediated bone resorption activity of lung cancer cells. Human non-small cell lung cancer H460 cells were treated with BaP to generate conditioned medium. When osteoblasts were cultured with BaP-H460-CM, their expression of osteoclastogenesis activator macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL) was increased. BaP-H460-CM reduced the production of osteoprotegerin (OPG), an osteoclastogenesis inhibitor, in osteoblasts. Osteoclastogenesis and bone resorption activity of H460 cells were increased by BaP-H460-CM. With BaP-mediated PTHrP upregulation, IL-8 secretion in H460 cells was increased contributing to human non-small cell lung cancer-mediated osteoclast differentiation and bone resorption. Moreover, TCN suppressed BaP-mediated bone resorption. Therefore, TCN may be a novel agent for treatment of non-small cell lung cancer patients with bone metastasis.

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Introduction

Lung cancer is one of the common cancers in the world, and also one of the leading causes of cancer-related deaths worldwide (1-3). Lung cancer metastasizes to the skeletal system frequently. About 30-40% of lung cancer patients will develop bone metastasis during the progression of their disease, which results significant impact on the patients' quality of life, morbidity and survival (3-5). Bone metastasis from lung cancer usually causes osteolytic lesions characterized by increased osteoclast activity (3,4) and decreased osteoblast capacity (6-8). Parathyroid hormone-related protein (PTHrP), produced by lung cancer cells, will stimulate osteoblasts to express elevated levels of receptor activator of nuclear factor κB ligand (RANKL) and will stimulate osteoclastogenesis by binding to the receptor RANK and activating its downstream signaling pathways in hematopoietic osteoclast precursors (3,9). Thus, therapy targeting osteoclast/osteoblast interactions during lung cancer progression isimportant.

Polycyclic aromatic hydrocarbons (PAHs), are formed by the incomplete combustion of organic matter. Benzo(a) pyrene (BaP) is the most commonly measured and studied PAH. They usually present in the environment at detectable levels in many types of uncooked food, and cooking process could generate PAHs in food. Several studies have been conducted to determine the levels of exposure to PAHs from representative human diet and the proportion of the overall burden of environmental exposure to PAHs that is attributable to diet (10-13). Previous studies demonstrated the association between PAHs and an increased risk of respiratory tract cancer (14-16). Exposure to BaP enhances the invasion and metastasis of lung cancer cells and BALB/c 3T3 cells *in vivo* and *in vitro* (17-19). Moreover, BaP can enhance the expression

level of epithelial-mesenchymal transition-related genes (20) and can promote migration and invasion of lung cancer cells through upregulating Twist (21).

Tricetin (TCN) (5,7,3',4',5'-pentahydroxyflavone), a flavonoid derivative found in Myrtaceae pollen and *Eucalyptus* honey (22-24), possesses potent anti-inflammatory and anti-cancer activities (25-27). This study evaluated the effects of BaP in human non-small cell lung cancer bone metastasis and investigated the potential role of TCN against the effects from BaP on human non-small cell lung cancer.

Materials and methods

Chemicals. TCN was obtained from Extrasynthese (Genay, France), dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA), and stored at -20˚C. Control cultures received the carrier solvent (0.1% DMSO). All chemicals used were in their purest form available commercially.

Cell culture and conditioned medium. Human non-small cell lung cancer H460 cells were obtained from the American Type Culture Collection (HTB-177) (Manassas, VA, USA) and cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) (both from Gibco-BRL, Gaithersburg, MD, USA). Human primary osteoblasts were obtained from Lonza (Walkersville, MD, USA) and cultured in osteoblast medium (OBM) (Lonza).

To obtain the various conditioned media (CM), H460 cells ($2x10^6$/100 mm dish) were treated with various concentrations of BaP (Sigma-Aldrich) for 6 h. After treatment, the medium was replaced and the supernatant harvested and filtered (0.22 mm) after 24 h of incubation.

Measurement of secreted factors. Supernatants from osteoblasts and H460 cells were collected. Levels of osteoprotegerin (OPG), macrophage colony-stimulating factor (M-CSF), RANKL and IL-8 were assessed and quantified using the DuoSet enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA). PTHrP levels were determined by an ELISA kit (Abnova Corp., Taipei, Taiwan).

Isolation of $CD14^+$ monocytes and osteoclast differentiation. Monocytes were purified from peripheral blood mononuclear cells (PBMCs) obtained from healthy donors. Mononuclear cells were isolated from blood by Ficoll-Hypaque gradient (GE Healthcare UK, Ltd., Buckinghamshire, UK). $CD14^+$ monocytes were purified using $CD14^+$ monoclonal antibody-conjugated magnetic beads (MACS MicroBeads; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), according to the manufacturer's instructions. Osteoclasts were generated by culturing $CD14^+$ monocytes in medium containing 20% vehicle control-CM-cultured osteoblasts or BaP-H460-CM-cultured osteoblasts presented in 100 ng/ml M-CSF and 50 ng/ml RANKL (R&D Systems) for 14-21 days. The medium was replaced with fresh medium containing M-CSF and RANKL every 5 days.

Osteoclast formation was measured by quantifying cells positively stained by TRAP (Sigma-Aldrich). Osteoclasts were deemed TRAP-positive by light microscopy that revealed staining of multinuclear (>3 nuclei) cells. The TRAP-positive cells and the number of nuclei per TRAP-positive cells in each well were counted. The bone resorption activity of osteoclasts was assessed by a 48-well plate bone resorption assay (Cosmo Bio Co., Ltd., Tokyo, Japan), under the same culture conditions as described above.

The Institutional Review Board (IRB) of Kaohsiung Medical University Chung-Ho Memorial Hospital (Kaohsiung, Taiwan) approved the study protocol and all of the participants provided written informed consent in accordance with the Declaration of Helsinki (IRB nos.: KMUH-IRB-990345, KMUH-IRB-20110377 and KMUH-IRB-20130054).

Real-time polymerase chain reaction (RT-qPCR). The TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) was used for RNA isolation while cDNA was prepared using an oligo(dT) primer and reverse transcriptase (Takara Bio, Inc., Shiga, Japan) following standard protocols. The RT-qPCR was performed using SYBR-Green on the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Each PCR reaction mixture contained 200 nM of each primer, 10 μl 2X SYBR-Green PCR Master Mix (Applied Biosystems), and 5 μl cDNA and RNase-free water for a total volume of 20 μl. The RT-qPCR was conducted with a denaturation step at 95˚C for 10 min, then for 40 cycles at 95˚C for 15 sec, and 60˚C for 1 min. All PCRs were performed in triplicate and normalized to internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The relative expression level was determined using the $2^{-\Delta\Delta CT}$ method.

PTHrP knockdown. H460 cells were transfected with 20 nM non-target or PTHrP siRNA pool (Dharmacon, Inc., Lafayette, CO, USA) by DharmaFECT 4 Transfection Reagents, according to the manufacturer's instructions. After 24 h transfection, the medium was changed to whole medium and the cells were treated with BaP. The PTHrP changes were measured by RT-qPCR.

Statistical analysis. Data are expressed as means ± standard errors. Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences ($p<0.05$) between the means of the test groups were analyzed by Student's t-test.

Results

BaP induces PTHrP secretion in human non-small cell lung cancer. To investigate the effects of BaP on PTHrP secretion by human non-small cell lung cancer cells, BaP was added to the culture medium of H460 cells to the concentration of 10 μM for 6 h. After washing, cells were cultured with new medium for another 24 h, the conditioned medium of BaP-treated H460 (BaP-H460-CM) was harvested and PTHrP levels of these CM were assessed. After exposure to BaP, the production of PTHrP in human non-small cell lung cancer H460 cells was increased (Fig. 1A). H460 cells were also treated with different concentrations of BaP, the result revealed that BaP increased the production of PTHrP in H460 cells in a dose-dependent manner (Fig. 1B).

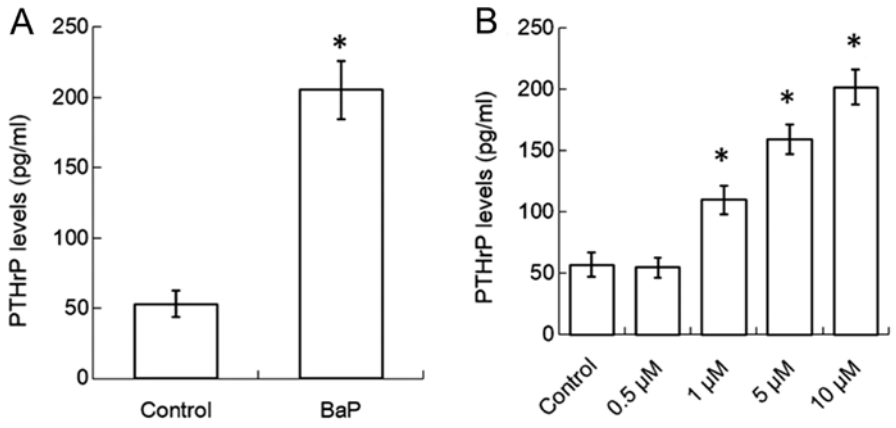

Figure 1. Benzo(a)pyrene (BaP) increased parathyroid hormone-related protein (PTHrP) expression in human non-small cell lung cancer. (A) The effects of 10 μM BaP on PTHrP levels in H460 cells. (B) BaP increased PTHrP expression in a dose-dependent manner. H460 cells treated with 10 μM BaP or various concentrations of BaP for 6 h. After washing and a 24-h culture, the conditioned media (CM) of BaP-treated H460 cells (BaP-H460-CM) were harvested, and PTHrP levels in these CM were then assessed by enzyme-linked immunosorbent assay (ELISA). Each value was the mean ± SD of three independent experiments. *P<0.05, significant difference between the control and test groups.

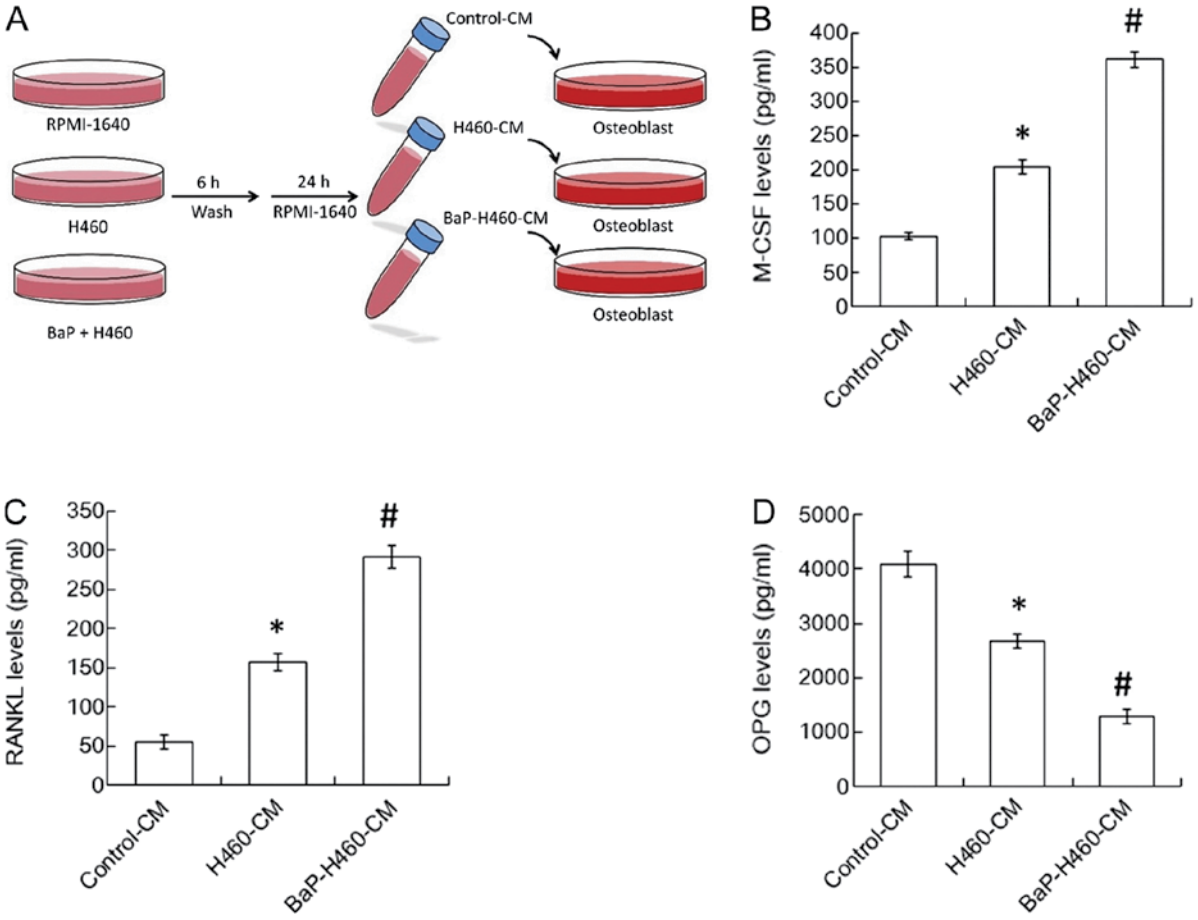

Figure 2. Benzo(a)pyrene (BaP) reinforces the interference of human non-small cell lung cancer in osteoblasts. (A) Flow chart of the production of control-CM, H460-CM, and BaP-H460-CM. BaP (10 μM) enhanced the stimulatory effect of H460 cells on the expression of (B) macrophage colony-stimulating factor (M-CSF) and (C) receptor activator of nuclear factor κB ligand (RANKL) in osteoblast. (D) BaP (10 μM) potentiated the inhibitory effect of human non-small cell lung cancer on osteoprotegerin (OPG) expression in osteoblasts. H460 cells treated with or without 10 mM BaP for 6 h. After washing and a 24-h culture, the conditioned media (CM) of non-treated or BaP-treated H460 cells (H460-CM and BaP-H460-CM) were harvested and stored at -80˚C. Osteoblasts were treated with various CM for 24 h. The levels of M-CSF, RANKL or OPG in the supernatants of osteoblasts were assessed by enzyme-linked immunosorbent assay (ELISA). Each value was the mean ± SD of three independent experiments. *Significant difference with control-CM treatment, #significant difference with H460-CM treatment, p<0.05.

Conditioned medium of BaP-treated H460 cells increases M-CSF and RANKL expression, and decreases OPG expression of osteoblasts. Osteoclastogenesis is regulated by PTHrP by altering the ratio of osteoclastogenesis activator (M-CSF

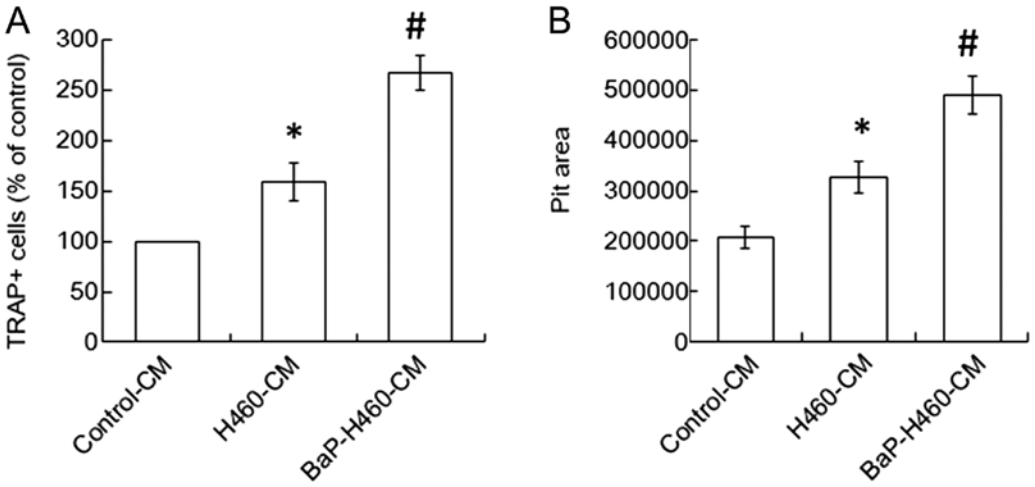

Figure 3. Benzo(a)pyrene (BaP) strengthens the stimulatory effect of human non-small cell lung cancer on osteoclastogenesis and bone resorption. (A) BaP (10 μM) increased the effect of H460 cells on osteoclastogenesis and (B) their bone resorption activity. Osteoclastogenesis was determined by TRAP staining (osteoclast: >3 nuclei, TRAP-positive). $CD14^+$ monocytes were treated with H460-CM and BaP-H460-CM (20%) cultured with macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL) for 14-21 days. Media were replaced every 5 days. Osteoclasts were then stained by TRAP. Multinucleated (>3 nuclei) TRAP-positive cells are defined as osteoclasts. The pit area was determined by AlphaEaseFC software. Each value was the mean ± SD of three independent experiments. *Significant difference with control-CM treatment, #significant difference with H460-CM treatment, $p<0.05$.

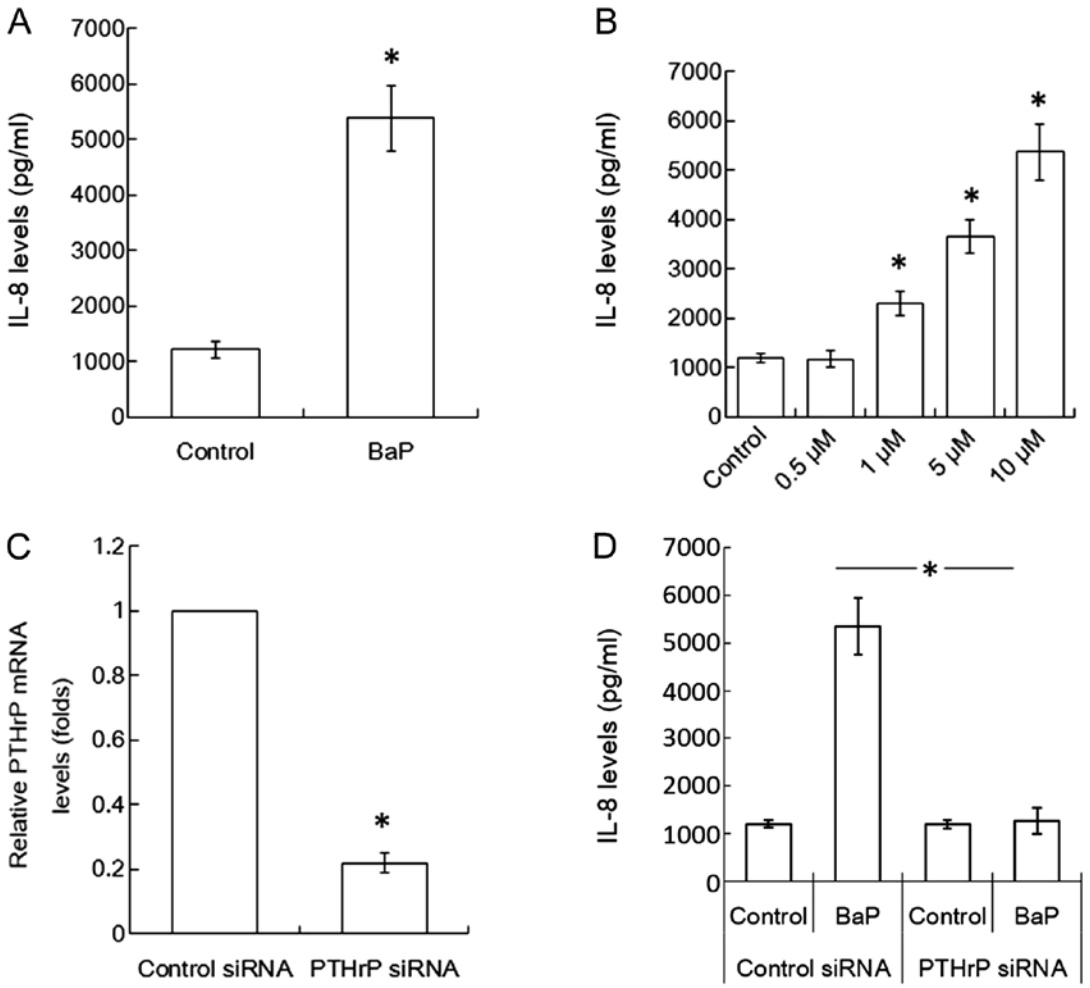

Figure 4. Benzo(a)pyrene (BaP) upregulates the expression of IL-8 by increasing parathyroid hormone-related protein (PTHrP) in human non-small cell lung cancer. (A) The effect of 10 μM BaP on IL-8 levels in H460 cells. (B) BaP upregulated IL-8 expression in a dose-dependent manner. (C) The efficiency of PTHrP siRNA transfection. (D) Inhibition of PTHrP decreased the upregulation of IL-8 by BaP in H460 cells. The IL-8 levels were assessed by enzyme-linked immunosorbent assay (ELISA), and the efficacy of siRNA was determined by real-time polymerase chain reaction (RT-qPCR). H460- or siRNA-transfected H460 cells treated with BaP (10 μM or various concentrations) for 24 h. PTHrP levels were determined by ELISA. Each value is the mean ± SD of three independent experiments. *$P<0.05$, significant difference between the control and test groups.

and RANKL)/inhibitor (OPG) produced by osteoblasts (28,29). To determine if BaP influences the interaction between non-small cell lung cancer cells and the secretion of M-CSF, RANKL, and OPG by osteoblasts, H460 cells were treated with 0.1% DMSO or 10 μM BaP for 6 h. Then BaP was removed by washing. H460 cells were cultured with fresh medium and the culture medium was collected thereafter (defined as control-CM, H460-CM, and BaP-H460-CM).

Human osteoblasts were cultured with the CM prepared to assess the effects of BaP on the interaction between non-small cell lung cancer and osteoblasts (Fig. 2A). H460-CM was found to be able to increase the M-CSF and RANKL expressions in osteoblasts and such stimulatory effect was further enhanced when H460 cells were pre-treated with BaP (Fig. 2B and C). In contrast, H460-CM decreased the OPG expression in osteoblasts and this inhibitory effect was strengthened when non-small cell lung cancer cells were exposed to BaP (Fig. 2D).

BaP increased human non-small cell lung cancer H460 cell-mediated osteoclastogenesis and bone resorption. To assess the effect of BaP on non-small cell lung cancer-mediated osteoclastogenesis, TRAP and bone resorption assays were tested. H460-CM increased osteoclastogenesis induced by H460 cells, and such effect was strengthened when H460 cells were pre-treated with BaP (Fig. 3A). Similarly, H460-CMs enhanced bone resorption activity and such enhancement further intensified once H460 cells were pre-treated with BaP (Fig. 3B).

PTHrP/IL-8 autocrine loop is involved in the stimulation of BaP on non-small cell lung cancer-mediated osteoclastogenesis. Since PTHrP was reported to be able to increase IL-8 expression of cancer cells (30), this study assessed whether BaP increased the effect of H460 cells on osteoclastogenesis is through the PTHrP/IL-8 loop or not. The level of IL-8 was higher in BaP-treated H460 cells than in the control group (Fig. 4A). Also, the effect of BaP to increase IL-8 expression from H460 cells was demonstrated to be in a dose-dependent (Fig. 4B).

To confirm the role of PTHrP in the upregulation of IL-8 induced by BaP, H460 cells were transfected with PTHrP siRNA. The expression of PTHrP mRNA in H460 cells was decreased ~78% when the cells were transfected with PTHrP siRNA (Fig. 4C). PTHrP silencing prevented the upregulation effect from BaP on the expression of IL-8 in H460 cells (Fig. 4D).

TCN suppresses BaP-mediated bone resorption. To access the effects of TCN on BaP-mediated non-small cell lung cancer bone metastasis, BaP-induced PTHrP and IL-8 secretion by non-small cell lung cancer H460 cells were tested again, both PTHrP and IL-8 secretion were decreased by 1 μM TCN treatment (Fig. 5A and B). Similarly, the CM of BaP-treated H460 cells enhanced RANKL upregulation in osteoblasts, and 1 μM TCN treatment blocked such upregulation (Fig. 5C). The activity of TCN on human non-small cell lung cancer-mediated interaction of osteoblasts and osteoclasts was further investigated (Fig. 6A). Osteoclastogenesis and bone resorption were both significantly decreased by TCN treatment (Fig. 6B and C).

Discussion

Bone metastasis is a devastating event for lung cancer patients because once it occurs, the morbidity and mortality will increase (31,32). Sone and Yano have demonstrated that several compounds, including bisphosphonates and reveromycin A, able to suppress the activity of osteoclast, are beneficial for the treatments of lung cancer patients with bone metastasis (33). However, 30-50% of lung cancer patients still developed new bone metastasis or skeletal complications while they are receiving such therapy, which emphasizes the necessity for new therapies (34,35). Evaluation of the differentiation of osteoclast is important in bone metastasis of lung cancer. Multimodality therapy is necessary to improve the efficacy of therapy against lung cancer bone metastasis (31-33). This is the first study to demonstrate that BaP increases the stimulatory effect of non-small cell lung cancer on osteoclastogenesis and osteoclastic bone resorption activity directly via PTHrP/IL-8 and by interfering in the osteoblast-osteoclast interaction.

PTHrP, a potent activator of osteoclastic bone resorption, is an important pathologic factor for hypercalcemia among cancer patients (36-38). PTHrP stimulates osteoclastogenesis by increasing RANKL expression and by reducing OPG expression in osteoblasts. However, it does not act directly on the precursors of the osteoclasts (39,40). RANKL binds with the RANK receptor of the precursors of osteoclasts and induces the formation of mature osteoclasts in the presence of M-CSF (41,42).

OPG is a soluble decoy receptor for RANKL, with the ability to decrease osteoclastogenesis (43). Increase of RANKL/OPG ratio by cancer-derived PTHrP results in osteoclastic bone resorption (3,44). This study shows that BaP upregulates the secretion of PTHrP in non-small cell lung cancer cells. Besides upregulation of PTHrP, BaP can reinforce the effects of non-small cell lung cancer on osteoblasts, including increased M-CSF and RANKL, and decreased OPG. These results suggest that BaP may worsen bone metastasis in non-small cell lung cancer.

Many studies have reported that non-small cell lung cancer expresses high levels of IL-8, which enhances both osteoclastogenesis and bone resorption (45,46). PTHrP has been reported to enhance osteoclastogenesis by increasing the expression of osteoclast stimulatory factors such as IL-8 (3,44). The present study demonstrates that BaP increases IL-8 expression in non-small cell lung cancer cells. Inhibition of PTHrP by siRNA transfection prevents the upregulation effects from BaP on IL-8 secretion. These results suggest that PTHrP is a major mediator involved in the stimulatory effect of BaP on IL-8 production. Furthermore, BaP enhances the stimulatory effect of non-small cell lung cancer on osteoclastogenesis and their bone resorption activity. Thus, the regulation of IL-8 expression by BaP through PTHrP is a key point in BaP-induced osteoclastogenesis and bone resorption.

Current therapy for bone metastases have limited efficacy and are only palliative. The side-effects of these treatments, renal toxicity and osteonecrosis of the jaw potentially will decrease the quality of life of these lung cancer patients (47,48). In addition, eliminating all BaP exposure may be impossible

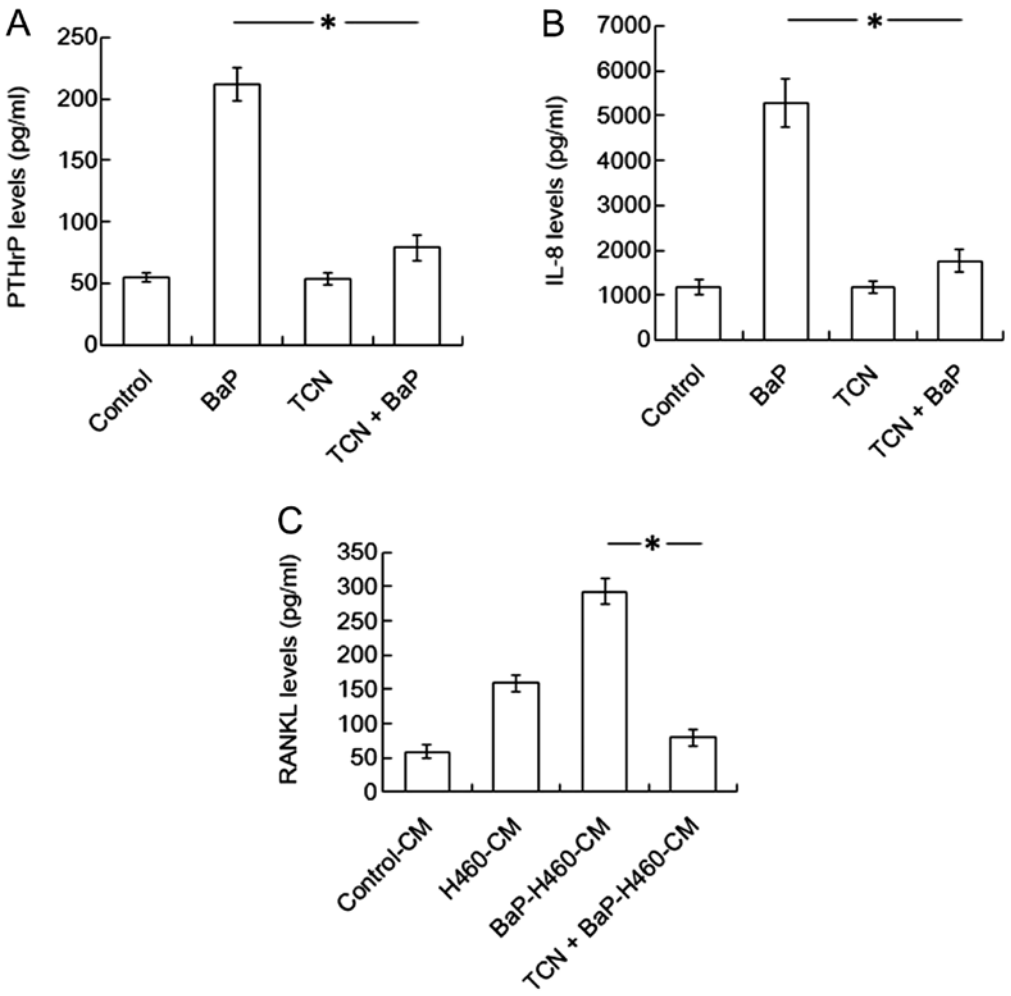

Figure 5. Tricetin (TCN) decreases the effects of benzo(a)pyrene (BaP) on parathyroid hormone-related protein (PTHrP), IL-8, and receptor activator of nuclear factor κB ligand (RANKL) expression. TCN (1 μM) decreased the upregulation of BaP on (A) PTHrP and (B) IL-8 in H460 cells. (C) TCN decreased the effects of BaP on RANKL expression of osteoblasts. For (A) and (B), H460 cells were pre-treated with TCN for 1 h, then incubated with BaP (10 μM) for another 6 h for PTHrP, or 24 h for IL-8 analysis. After washing and a 24-h culture, the culture media were collected and PTHrP levels were assessed by enzyme-linked immunosorbent assay (ELISA). (C), H460 cells were pre-treated with TCN for 1 h, then incubated with BaP (10 μM) for another 6 h. After washing and a 24-h culture, the culture media were collected, then added to osteoblasts for another 24 h. The levels of RANKL in the supernatants of osteoblasts were assessed by ELISA. Each value is the mean ± SD of three independent experiments. *P<0.05, or significant difference between the control and test groups.

because BaP is widely present in modern life. It is therefore important that strategies be developed for preventing bone metastasis in non-small cell lung cancer.

Our data show that TCN, a flavonoid derivative found in Myrtaceae pollen and *Eucalyptus* honey, exhibits effects to decrease PTHrP expression in non-small cell lung cancer H460 cells. Simultaneously, TCN also decreases IL-8 expression, resulting in the inhibition of H460-mediated osteoclastogenesis and bone resorption. Moreover, TCN also decreases the stimulatory effect from non-small cell lung cancer on RANKL espression of osteoblasts, suggesting that TCN may be a potential agent to prevent the aggravating effect from BaP on non-small cell lung cancer bone metastasis.

In conclusion, there are two novel findings in this study: it is the first study to demonstrate that BaP increases the stimulatory effect of human non-small cell lung cancer on osteoclastogenesis and their activity of osteoclast bone resorption directly by PTHrP/IL-8 and by interfering in the osteoblast-osteoclast interaction, and it is also the first to reveal that TCN, a flavonoid derivative found in Myrtaceae pollen and *Eucalyptus* honey, reverses BaP-mediated bone resorption (Fig. 7).

Acknowledgements

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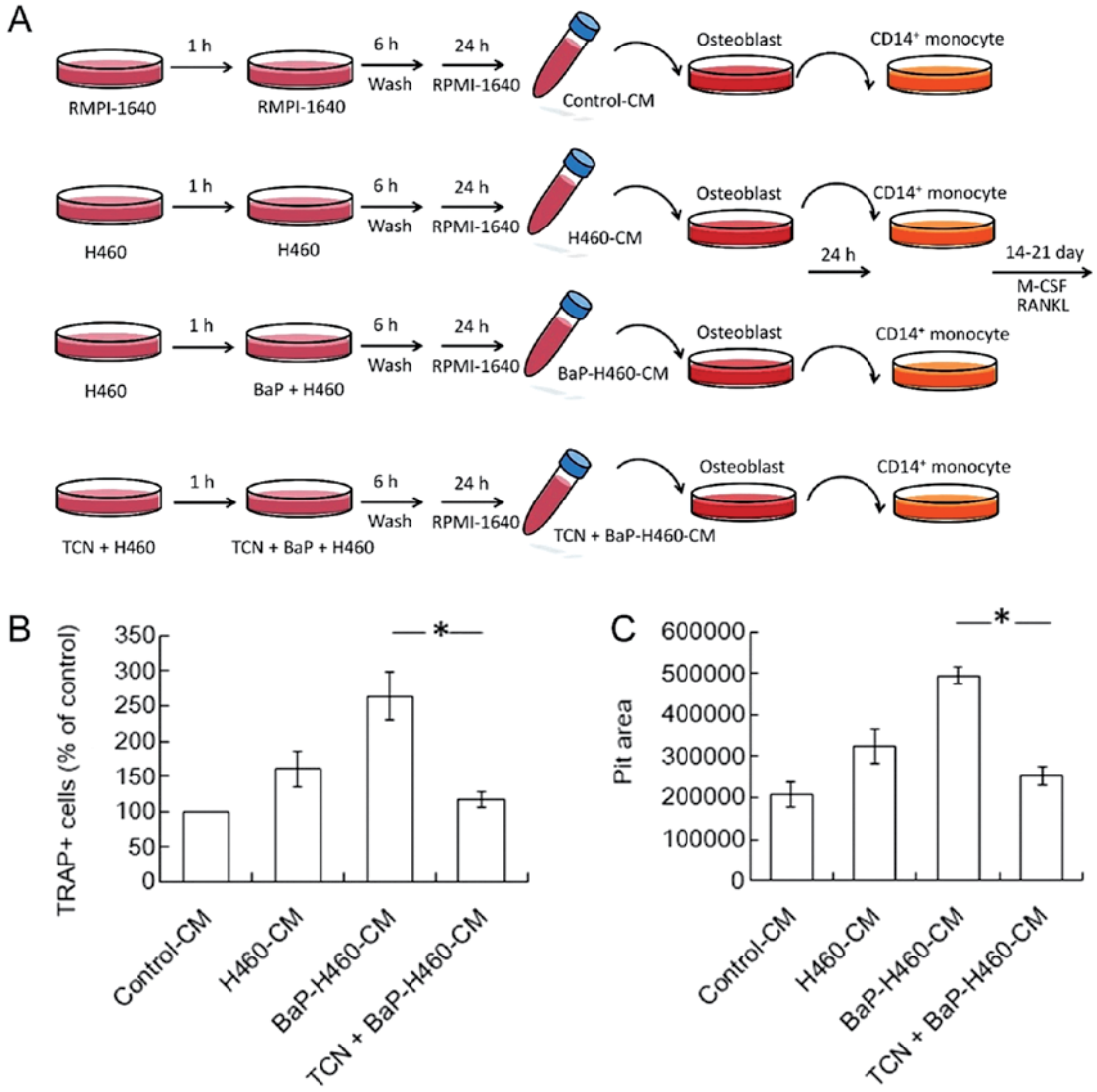

Figure 6. Tricetin (TCN) decreased the effects of benzo(a)pyrene (BaP) on human non-small cell lung cancer bone metastasis events. (A) Flow chart of production of control-CM, H460-CM, BaP-H460-CM, TCN + BaP-H460-CM or various osteoblast conditioned media (CM). TCN decreased the effects of BaP on (B) osteoclastogenesis and (C) bone resorption. For (B) and (C), H460 cells were pre-treated with TCN for 1 h, then incubated with BaP (10 μM) for another 6 h. After washing and a 24-h culture, the culture medium (20%) was collected, then added to CD14$^+$ monocytes presenting in macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL) for a 14-21 days. The osteoclastogenesis and bone resorption was conducted as described above. Each value is the mean ± SD of three independent experiments. *P<0.05, or significant difference between the control and test groups.

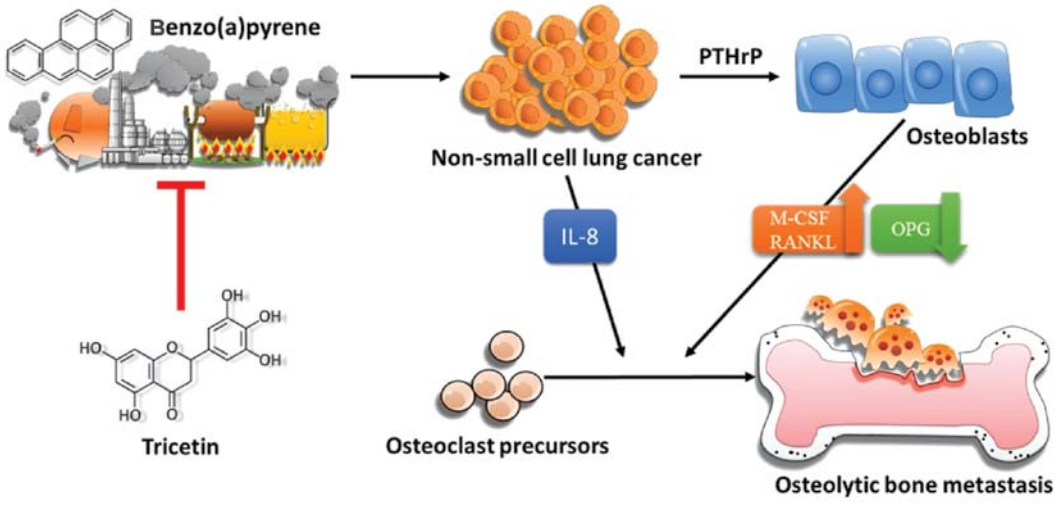

Figure 7. Scheme of proposed tricetin (TCN)-inhibited benzo(a)pyrene (BaP)-induced human non-small cell lung cancer bone metastasis.

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