

# Syringetin suppresses osteoclastogenesis mediated by osteoblasts in human lung adenocarcinoma

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**Abstract.** Bone metastasis in lung cancer results in an unfavorable outcome for patients by not only impairing the quality of life, yet also increasing the cancer-related death rates. In the present study, we discuss a novel treatment strategy that may benefit these patients. Human CD14<sup>+</sup> monocytes treated with macrophage-colony stimulating factor (M-CSF)/receptor activator of nuclear factor  $\kappa$ B ligand (RANKL) differentiated into osteoclasts, whereas syringetin (SGN), a flavonoid derivative found in both grapes and wine, suppressed the osteoclastogenesis *in vitro* in a dose-dependent manner. In addition, SGN inhibited osteoclast formation induced by human lung adenocarcinoma A549 and CL1-5 cells. The associated signaling transduction pathway in osteoclastogenesis and SGN inhibition was found to be via the AKT/mammalian target of rapamycin (mTOR) signaling pathway. Blocking AKT and mTOR by respective inhibitors significantly decreased lung adenocarcinoma-mediated osteoclastogenesis. Moreover, SGN regulated the lung adenocarcinoma-mediated interaction between osteoblasts and osteoclasts by suppressing the stimulatory effect of lung adenocarcinoma on M-CSF and RANKL production in osteoblasts, and reversing the inhibitory effect of the lung adenocarcinoma on OPG production in osteoblasts. The present study has two novel findings. It is the first to illustrate lung adenocarcinoma-mediated interaction between osteoblasts and osteoclasts, leading to osteolytic bone metastasis. It also reveals that SGN, a flavonoid derivative, directly inhibits osteoclastogenesis and reverses lung adenocarcinoma-mediated osteoclastogenesis. In conclusion, the present study suggests that SGN, a natural compound, prevents and treats bone metastasis in patients with lung cancer.

## Introduction

Lung cancer is the leading cause of cancer-related mortality, with an extremely poor prognosis worldwide (1-3). It has also been reported that ~30-40% of patients with lung cancer eventually develop bone metastasis, not only impairing quality of life but also increasing the incidence of cancer-related death (3-5). Osteolytic metastasis develops in certain types of cancer, such as lung cancer, and is featured as upregulated osteoclast activity and downregulated osteoblast capacity, resulting in bone destruction (6-8). Therefore, novel therapeutic strategies for preventing and treating bone metastasis in lung cancer should be developed.

As is well known, cancer cells secrete various factors which enhance osteoclastic resorption, and the release of certain factors from the skeletal matrix has been proven to enhance bone metastasis (9,10). There are several factors contributing to cancer cell migration to bone, such as stromal-derived factor-1, monocyte chemotactic protein 1, and receptor activator of nuclear factor  $\kappa$ B ligand (RANKL). Other factors enhancing cancer cell growth within bone, such as transforming growth factor- $\beta$  (TGF- $\beta$ ) and parathyroid hormone (PTH)-related protein, have also been identified (11-13). Takiguchi *et al* reported that CXCL14 promoted bone metastasis through enhancement of cancer cell tropism to the bone and/or recruitment of bone marrow cells around metastatic cancer cells (14). Our recent study illustrated that lung cancer-derived IL-8 increased osteoclastogenesis via the phospholipase D signaling transduction pathway (15).

Grapes are a rich source of polyphenols, including stilbene and flavonol derivatives (16). Stilbenes, vine phytoalexins, are associated with the beneficial effects of drinking wine. Resveratrol, pterostilbene and piceatannol are naturally occurring stilbene derivatives (17). Previous studies have revealed that resveratrol and pterostilbene possess antioxidant, anti-inflammatory and anticancer properties (18-24). Moreover, even freeze-dried residue from red wine significantly inhibits bone resorption (25). Red grapes are rich in flavonols, a ubiquitous class of flavonoids, which along with other derivatives of flavonoids such as myricetin, laricitrin and syringetin (SGN),

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possess antioxidant effects (26-28). SGN (3,5,7,4'-tetrahydroxy-3',5'-dimethoxyflavone), a flavonoid derivative, exists in both grapes and wine (27-29). The SGN content of red grapes is 3.22% (27). Syringetin-3-*O*-glycoside is the major derivative present in red grapes, with syringetin-3-glycosides coexisting with corresponding free aglycones released by hydrolysis in wine (30). In a colorectal epithelial adenocarcinoma cell model, SGN was shown to inhibit cellular proliferation by decreasing cyclooxygenase-2 and cyclin D1 expression (31). Our previous study indicated that SGN stimulated osteoblast differentiation via the bone morphogenetic protein-2 (BMP2)/extracellular signal-regulated kinase 1/2 (ERK1/2) signaling transduction pathway (32). The present study demonstrated that SGN inhibited human lung adenocarcinoma A549 and CL1-5 cell-mediated osteoclastogenesis through the downregulation of the AKT/mammalian target of rapamycin (mTOR) signaling pathway. Moreover, SGN downregulated macrophage-colony stimulating factor (M-CSF) and RANKL expression in A549-CM-stimulated osteoblasts and inhibited osteoblast-mediated osteoclastogenesis.

## Materials and methods

**Chemicals.** SGN and laricitrin were purchased from Extrasynthese (Genay, France); resveratrol, pterostilbene, piceatannol and myricetin were obtained from Sigma Chemical (St. Louis, MO, USA) (Fig. 1). These were dissolved in dimethylsulfoxide (DMSO) (Sigma Chemical) and stored at -20°C. Control cultures received the carrier solvent (0.1% DMSO). All other chemicals used were of the purest form available commercially.

**Cell culture.** Human lung adenocarcinoma A549 cells were obtained from the American Type Culture Collection (CCL-185) (Manassas, VA, USA) and cultured in F-12K medium containing 10% fetal bovine serum (FBS) (both from Gibco-BRL, Gaithersburg, MD, USA). CL1-5 human lung adenocarcinoma cells were generously provided by Dr Pan-Chyr Yang (Department of Internal Medicine, National Taiwan University Hospital) (33,34), and cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin (both from Gibco-BRL). Human primary osteoblasts were cultured in osteoblast growth medium (OBM) (both from Lonza, Walkersville, MD, USA). For conditioned medium (CM) collection, the A549 and CL1-5 cells were treated with vehicle (0.1% DMSO) or various concentrations of SGN for 12 h. After washing, fresh culture medium was added and cultured for another 24 h. The supernatants were collected, filtered (0.22-μm) and identified as A549-CM, SGN-A549-CM, CL1-5-CM and SGN-CL1-5-CM. Osteoblasts were cultured with A549-CM, SGN-A549-CM, CL1-5-CM or SGN-CL1-5-CM (20%) for another 24 h, then the supernatants were collected and filtered (0.22-μm). These supernatants were grouped as osteoblast-CM (OB-CM), A549-OB-CM, SGN-A549-OB-CM, CL1-5-OB-CM and SGN-CL1-5-OB-CM. All condition media were frozen and stored at -80°C, with a single thawing for study.

**Osteoclast differentiation.** Human peripheral blood, obtained from healthy adult volunteers, was collected in syringes

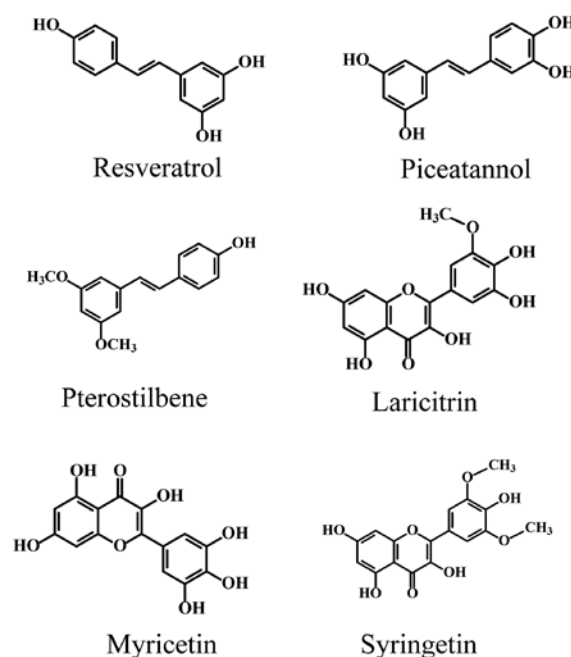


Figure 1. Chemical structure of resveratrol, piceatannol, pterostilbene, laricitrin, myricetin and syringetin (SGN).

containing 1,000 U/ml of preservative-free heparin. Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation using Ficoll-Hypaque, and re-suspended in RPMI-1640 medium supplemented with 10% heat-inactivated FBS. The PBMCs were then plated and incubated overnight at 37°C. CD14<sup>+</sup> monocytes were isolated using CD14<sup>+</sup> mAb-conjugated magnetic beads (MACS MicroBeads; Miltenyi Biotec, Ltd., Bergisch Gladbach, Germany) according to the manufacturer's protocol. The purity of CD14<sup>+</sup> cells was 94-97%. Monocytes were grown in culture medium containing vehicle or tested compounds preset at 200 ng/ml human M-CSF and 100 ng/ml human RANKL (R&D Systems, Minneapolis, MN, USA) for 14-21 days. The medium was replaced every 5 days. The Institutional Review Board of the Kaohsiung Medical University Chung-Ho Memorial Hospital approved the study protocol, and all participants provided written informed consent in accordance with the Declaration of Helsinki. Osteoclast formation was measured by quantifying cells positively stained by tartrate-resistant acid phosphatase (TRAP) (Sigma-Aldrich, St. Louis, MO, USA). Briefly, the cells were fixed with formaldehyde for 30 min and then stained with naphthol AS-BI phosphate and a tartrate solution for 1 h at 37°C, followed by counterstaining with a hematoxylin solution. Osteoclasts were determined to be TRAP-positive staining multi-nuclear (>3 nuclei) cells by light microscopy. The total number of TRAP-positive cells and the number of nuclei/TRAP-positive cell in each well were counted.

**Bone resorption assay.** CD14<sup>+</sup> monocytes were plated into a calcium phosphate apatite-coated 48-well plate for the bone resorption assay (Cosmo Bio Co., Ltd., Tokyo, Japan) in the same culture conditions as previously described. After a 14-day culture, each well was washed with saline. A solution of 5% sodium hypochlorite was left in the well for 5 min to detach the cells. The pit area in each well was determined

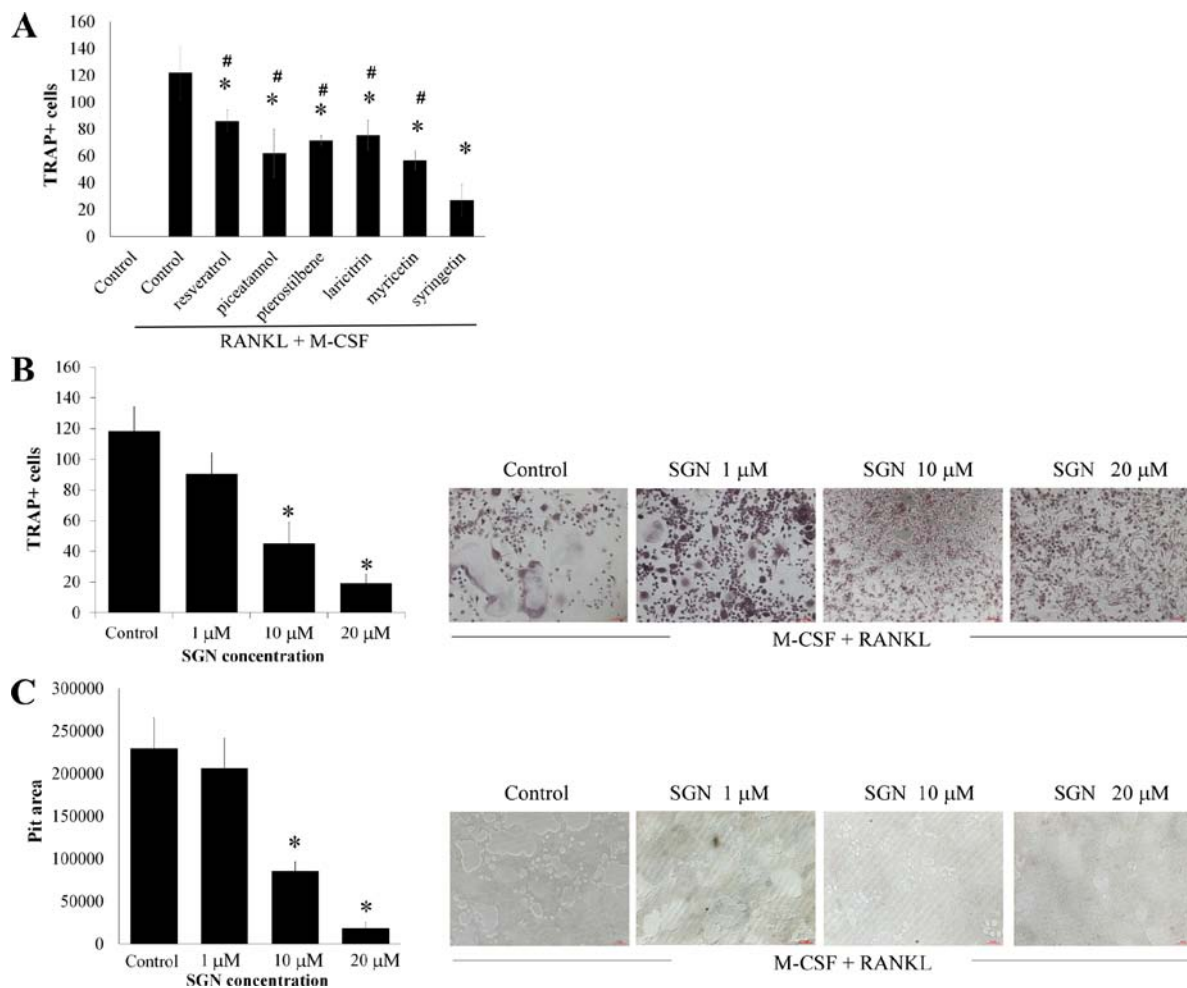


Figure 2. SGN decreases osteoclastogenesis. (A) The effects of various compounds on osteoclastogenesis. SGN decreased (B) osteoclast differentiation and (C) bone resorption. CD14<sup>+</sup> monocytes were treated with vehicle (0.1% DMSO) or various test compounds (20  $\mu$ M) or various concentrations of SGN (1, 10 and 20  $\mu$ M) in the presence of RANKL (100 ng/ml) and M-CSF (200 ng/ml) for 21 (TRAP analysis) or 14 days (bone resorption assay). Osteoclast cells were stained for TRAP activity, and bone resorption activity was determined by the bone resorption assay kit. The pit areas were determined by AlphaEase FC software. Each value is the mean  $\pm$  SD of 3 independent experiments. \* $p$ <0.05, significant difference with controls; # $p$ <0.05 significant difference with SGN, as analyzed by the Student's t-test. SGN, syringetin; DMSO, dimethylsulfoxide; RANKL, receptor activator of nuclear factor  $\kappa$ B ligand; TRAP, tartrate-resistant acid phosphatase; M-CSF, macrophage-colony stimulating factor.

by AlphaEaseFC software (Alpha Innotech Corporation, San Leandro, CA, USA).

**Immunoblotting.** CD14<sup>+</sup> monocytes were pre-treated with vehicle control (0.1% DMSO) or SGN (20  $\mu$ M) for 1 h or otherwise indicated times (time-dependent manner), and then M-CSF (200 ng/ml)/RANKL (100 ng/ml) was added for 0.5, 1 and 6 h. The expression of various proteins was assessed by immunoblotting. The cells were lysed on ice for 15 min by M-PER lysis reagent (Thermo Fisher Scientific, Waltham, MA, USA). The cell lysate was centrifuged at 14,000  $\times$  g for 15 min, and the supernatant fraction was collected for immunoblotting. Equivalent amounts of protein were resolved by SDS-PAGE (6-8%) and transferred to polyvinylidene difluoride membranes. After blocking for 1 h in 5% non-fat dry milk in TBS, the membrane was incubated with the primary Ab for 1-16 h (1 h for GAPDH; 16 h for phosphorylated AKT, mTOR, 4EBP1 and p70S6K; and total protein of AKT, mTOR, 4EBP1 and p70S6K). The membrane was then treated with the appropriate peroxidase-conjugated secondary Ab, and the immunoreactive proteins were detected using an ECL

kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. All antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). To quantify immunoblot images on unsaturated bands, densitometric analysis was performed using AlphaEaseFC software.

**Enzyme-linked immunosorbent assay (ELISA).** M-CSF levels were assessed by the M-CSF ELISA kit (R&D Systems). The RANKL and OPG levels in the osteoblasts were quantified using the DuoSet ELISA (R&D Systems).

**Statistical analysis.** Data are expressed as means  $\pm$  SD. Statistical comparisons were carried out using analysis of variance. Significant differences ( $p$ <0.05) between two test groups were analyzed by the Student's t-test.

## Results

**SGN exhibits a direct inhibitory effect on osteoclast differentiation and bone resorption activity.** To explore whether the novel agents inhibit osteoclast development, the effects on

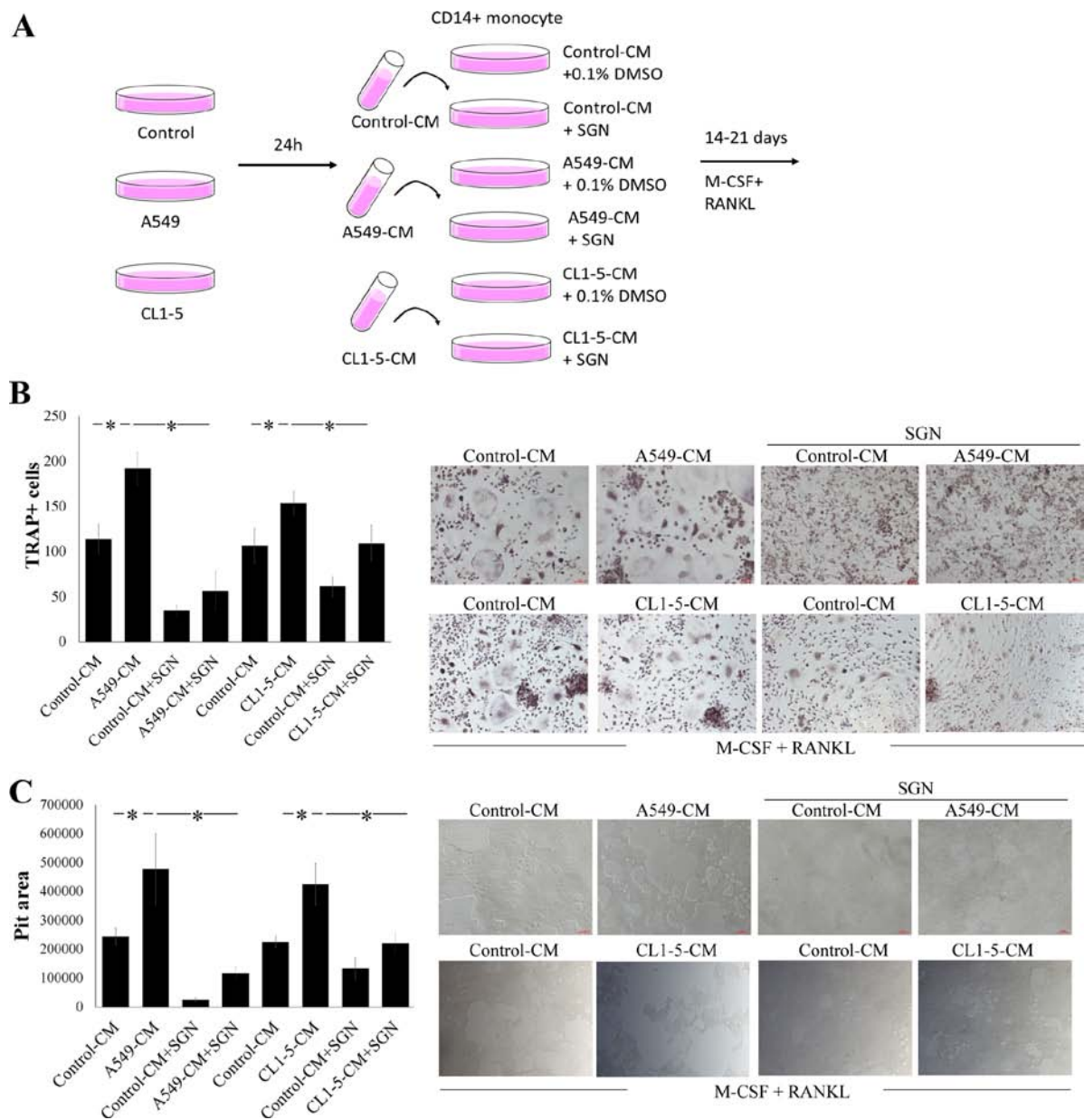


Figure 3. SGN inhibits human lung adenocarcinoma cell-mediated osteoclastogenesis. (A) Flow chart of the production of the control-, A549- and CL1-5-CM. SGN (20  $\mu$ M) decreased (B) A549-mediated osteoclast differentiation and (C) bone resorption. CD14<sup>+</sup> monocytes were treated with vehicle control (0.1% DMSO) or SGN (20  $\mu$ M) in RPMI-1640 medium containing 20% of control-CM, A549-CM or CL1-5-CM for 21 (TRAP analysis) or 14 days (bone resorption assay). All media contained RANKL (100 ng/ml) and M-CSF (200 ng/ml). The media were replaced every 5 days. Osteoclast cells were stained for TRAP activity, and bone resorption activity was determined by the bone resorption assay kit. Each value is the mean  $\pm$  SD of 3 independent experiments. The pit areas were determined by AlphaEase FC software. \* $p$ <0.05, significant difference with two tested groups, as analyzed by the Student's t-test. SGN, syringetin; DMSO, dimethylsulfoxide; TRAP, tartrate-resistant acid phosphatase; RANKL, receptor activator of nuclear factor  $\kappa$ B ligand; M-CSF, macrophage-colony stimulating factor.

osteoclast differentiation by various polyphenols, including resveratrol, piceatannol, pterostilbene, laricitrin, myricetin and SGN were assessed. Treatment of the CD14<sup>+</sup> monocytes with M-CSF and RANKL caused formation of numerous TRAP-positive multi-nucleated osteoclasts (Fig. 2A), whereas osteoclast differentiation was significantly inhibited by resveratrol, piceatannol, pterostilbene, laricitrin, myricetin and SGN (20  $\mu$ M). SGN showed the most significant inhibition of osteoclast differentiation in comparison with resveratrol, piceatannol, pterostilbene, laricitrin and myricetin. Consequently, we selected SGN as the model for exploring the mechanism of lung adenocarcinoma-related osteoclastogen-

esis. First, SGN treatment inhibited osteoclast differentiation in a dose-dependent manner (Fig. 2B). SGN treatment also substantially reduced bone resorption in a dose-dependent manner (Fig. 2C). These findings suggest that SGN inhibits osteoclast differentiation and reduces bone resorption activity *in vitro*.

*SGN inhibits human lung adenocarcinoma-mediated osteoclastogenesis.* Next, we evaluated the effects of SGN on lung adenocarcinoma-mediated osteoclastogenesis (Fig. 3A). The results showed that A549- and CL1-5-CMs markedly promoted osteoclastogenesis (Fig. 3B). However, the



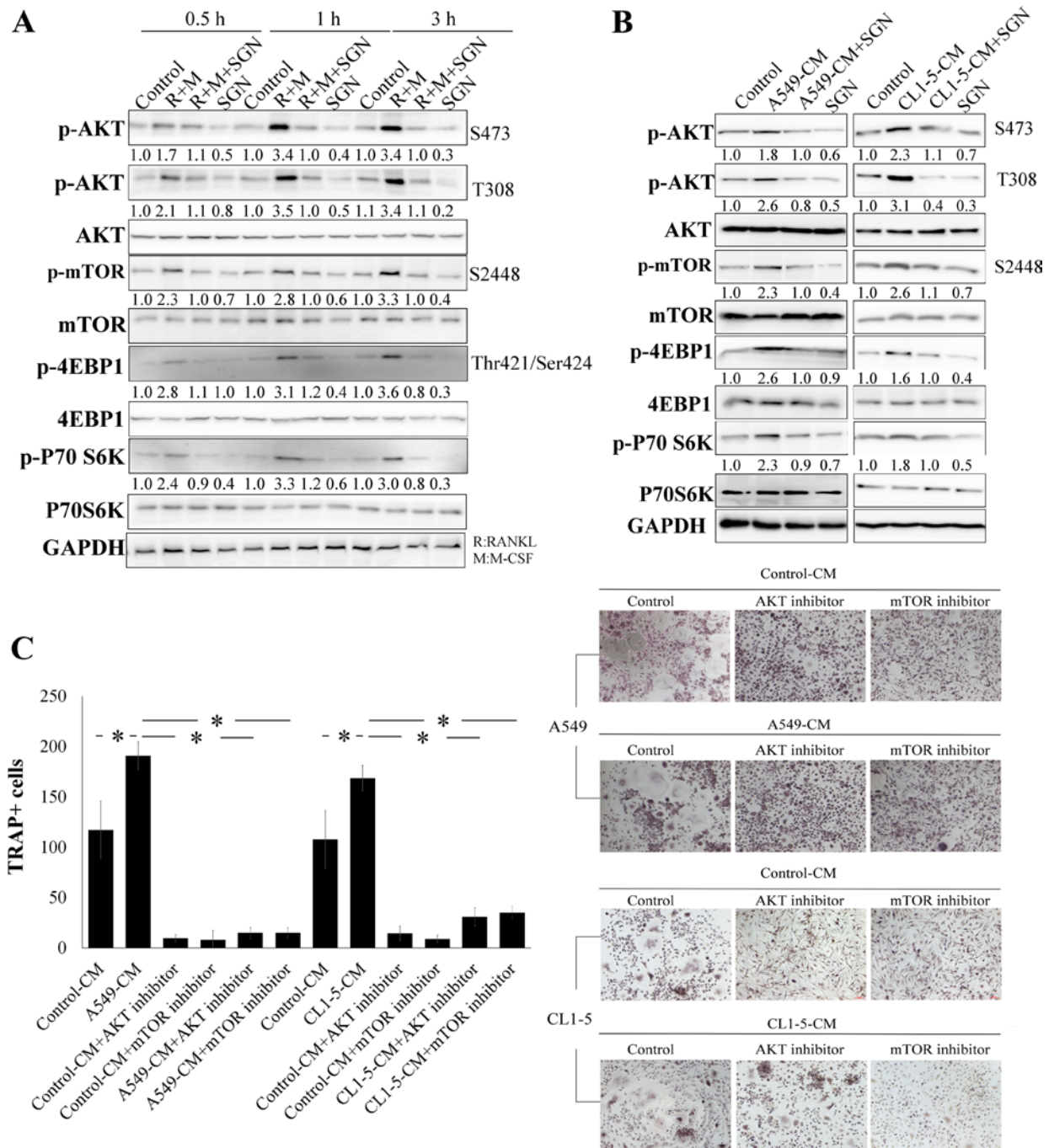


Figure 4. SGN suppresses osteoclastogenesis by inhibiting AKT/mTOR signaling. (A) SGN (20  $\mu$ M) reduced M-CSF/RANKL-mediated AKT and mTOR signaling activation. CD14<sup>+</sup> monocytes were pre-treated with vehicle control (0.1% DMSO) or SGN (20  $\mu$ M) for 1 h and then M-CSF (200 ng/ml)/RANKL (100 ng/ml) were added for 0.5 h or the indicated times (in a time-dependent manner). The expression of various proteins was assessed by immunoblotting. (B) SGN decreased AKT/mTOR activation induced by A549- and CL1-5-CM. CD14<sup>+</sup> monocytes were pre-treated with vehicle control (0.1% DMSO) or SGN (20  $\mu$ M) for 1 h and then control-CM, A549-CM or CL1-5-CM (20%) containing M-CSF (200 ng/ml)/RANKL (100 ng/ml) was added for 0.5 h or the indicated times (time-dependent manner). The expression of various proteins was assessed by immunoblotting. (C) AKT (AKT inhibitor IV) and mTOR (rapamycin) inhibitors decreased osteoclast differentiation. CD14<sup>+</sup> monocytes were treated with vehicle control (0.1% DMSO), AKT (AKT inhibitor IV, 0.1  $\mu$ M) and mTOR inhibitor (rapamycin, 0.5  $\mu$ M) in RPMI-1640 medium containing 20% of various control-CM, A549-CM and CL1-5-CM for 21 days (TRAP analysis) or 14 days (bone resorption assay). All media contained RANKL (100 ng/ml) and M-CSF (200 ng/ml). Each value is the mean  $\pm$  SD of 3 independent experiments. \* $p$ <0.05, significant difference between two tested groups, as analyzed by the Student's t-test. R, RANKL; M, M-CSF; SGN, syringetin.

osteoclastic effect induced by human lung adenocarcinoma A549- and CL1-5-CMs was markedly inhibited by SGN. Furthermore, A549- and CL1-5-CMs enhanced the bone resorption activity of the osteoclasts, yet this effect was also reduced by SGN (Fig. 3C). Both results indicate that SGN

suppresses human lung adenocarcinoma A549 and CL1-5 cell-mediated osteoclast differentiation and bone resorption activity.

*SGN suppresses osteoclastogenesis by inhibiting AKT/mTOR.* To clarify the molecular mechanism involved in

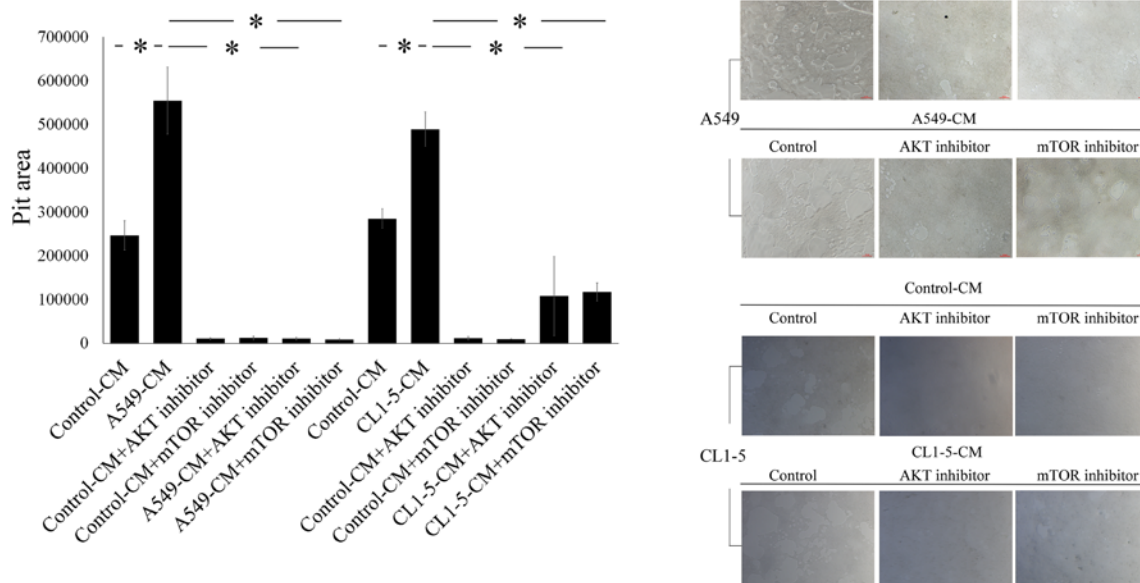
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Figure 4. Continued. (D) AKT (AKT inhibitor IV) and mTOR (rapamycin) inhibitors decrease bone resorption. CD14<sup>+</sup> monocytes were treated with vehicle control (0.1% DMSO), AKT (AKT inhibitor IV, 0.1  $\mu$ M) and mTOR inhibitor (rapamycin, 0.5  $\mu$ M) in RPMI-1640 medium containing 20% of various control-CM, A549-CM and CL1-5-CM for 21 days (TRAP analysis) or 14 days (bone resorption assay). All media contained RANKL (100 ng/ml) and M-CSF (200 ng/ml). Each value is the mean  $\pm$  SD of 3 independent experiments. The pit areas were determined by AlphaEase FC software. \* $p$ <0.05, significant difference between two tested groups, as analyzed by the Student's t-test. RANKL, receptor activator of nuclear factor  $\kappa$ B ligand; M-CSF, macrophage-colony stimulating factor; mTOR, mammalian target of rapamycin.

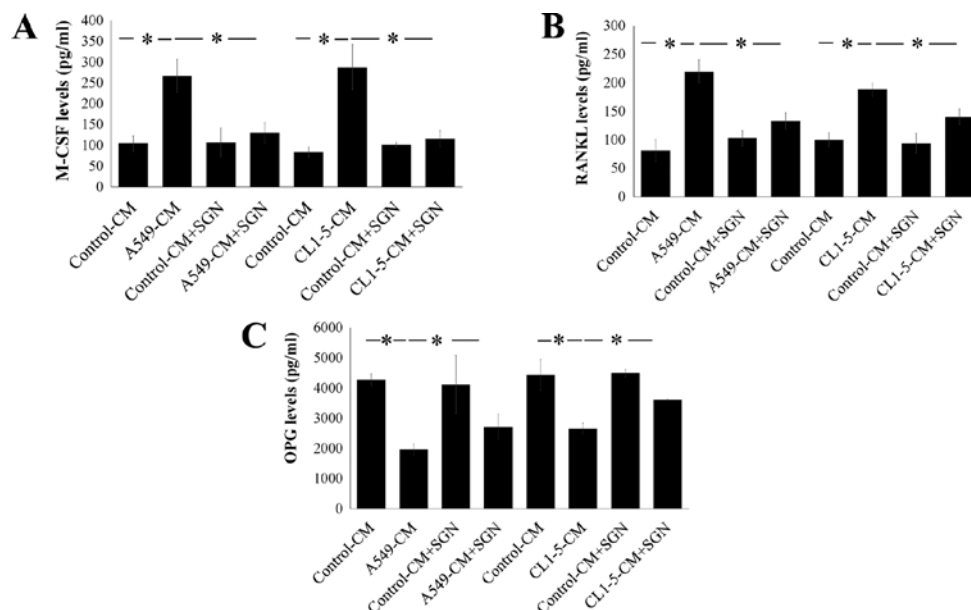


Figure 5. SGN attenuates the upregulation of M-CSF and RANKL in osteoblasts after lung adenocarcinoma stimulation. SGN (20  $\mu$ M) decreased the stimulatory effect of lung adenocarcinoma on the production of (A) M-CSF and (B) RANKL, and reversed the suppressive effect of lung adenocarcinoma on the production of (C) OPG in osteoblasts. A549 and CL1-5 cells were treated with vehicle (0.1% DMSO) or SGN (20  $\mu$ M) for 12 h. After washing, fresh medium was added and cultured for another 24 h. The supernatants were collected, filtered (0.22  $\mu$ m) and identified as A549-CM, A549-CM+SGN, CL1-5-CM and CL1-5-CM+SGN. Osteoblasts were cultured with A549-CM, A549-CM+SGN, CL1-5-CM and CL1-5-CM+SGN (20%) for another 24 h, and the supernatants were then collected. Levels of M-CSF, RANKL and OPG in the supernatants of osteoblasts were assayed by M-CSF, RANKL and OPG ELISA kits, respectively. Each value is the mean  $\pm$  SD of 3 independent experiments. \* $p$ <0.05 significant difference between two tested groups, as analyzed by the Student's t-test. SGN, syringetin; RANKL, receptor activator of nuclear factor  $\kappa$ B ligand; M-CSF, macrophage-colony stimulating factor.

SGN osteoclastogenesis, signaling transduction pathways were investigated. Treatment of M-CSF/RANKL increased the phosphorylation of AKT and mTOR. The downstream

targets of mTOR, p70S6K and 4EBP1 were also activated by M-CSF/RANKL, suggesting that the AKT/mTOR signaling transduction pathway was activated during osteoclastogenesis.

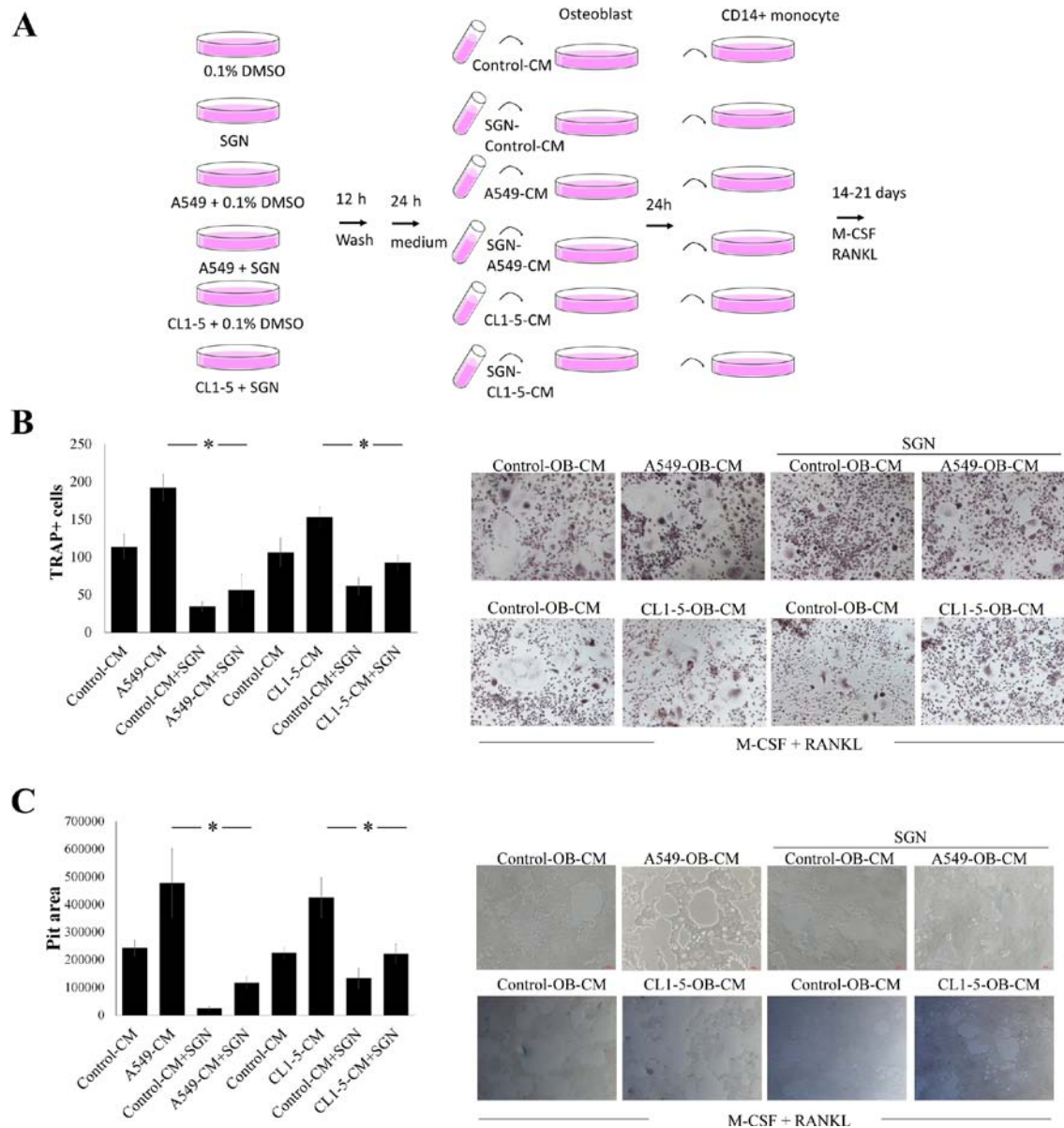


Figure 6. SGN reduces the stimulatory effect of lung adenocarcinoma on osteoblast-mediated osteoclastogenesis. (A) Flow chart of production of control-CM, A549-CM, SGN-A549-CM, CL1-5-CM and SGN-CL1-5-CM, or various osteoblast conditioned media. SGN (20  $\mu$ M) reduced (B) osteoclast differentiation and (C) bone resorption activity induced by lung adenocarcinoma-stimulated osteoblasts. A549 and CL1-5 cells were treated with vehicle (0.1% DMSO) or SGN (20  $\mu$ M) for 12 h. After washing, fresh medium was added and cultured for another 24 h. The supernatants were then collected and filtered (0.22  $\mu$ m). Osteoblasts were cultured with control-CM, SGN-control-CM, A549-CM, SGN-A549-CM, CL1-5-CM and SGN-CL1-5-CM (20%) for another 24 h, and the supernatants were collected and filtered (0.22  $\mu$ m). These supernatants were grouped as osteoblast-CM (OB-CM), SGN-OB-CM, A549-OB-CM, SGN-A549-OB-CM, CL1-5-OB-CM and SGN-CL1-5-OB-CM. CD14<sup>+</sup> monocytes were treated with various CMs of osteoblasts containing RANKL (100 ng/ml) and M-CSF (200 ng/ml) for 21 days (TRAP analysis) or 14 days (bone resorption assay). All media contained RANKL (100 ng/ml) and M-CSF (200 ng/ml). Osteoclast cells were stained for TRAP activity, and bone resorption activity was determined by the bone resorption assay kit. Each value is the mean  $\pm$  SD of 3 independent experiments. The pit areas were determined by AlphaEase FC Software. \* $p$ <0.05, significant difference between two tested groups, as analyzed by the Student's t-test. SGN, syringetin; RANKL, receptor activator of nuclear factor  $\kappa$ B ligand; M-CSF, macrophage-colony stimulating factor.

The osteoclastogenetic inducers (M-CSF and RANKL) of AKT/mTOR expression were suppressed by SGN (Fig. 4A). Furthermore, both A549- and CL1-5-CMs activated AKT/mTOR expression (Fig. 4B). Through treatment with both AKT inhibitor IV and mTOR inhibitor (rapamycin) we further supported the role of AKT/mTOR in osteoclast differentiation and bone resorption (Fig. 4C and D). Both AKT and mTOR inhibitors also suppressed A549 and CL1-5-mediated osteoclastogenesis (Fig. 4C and D). In short, SGN not only inhibited M-CSF/RANKL-mediated AKT/mTOR activation,

yet also reduced the reinforcing effect of lung adenocarcinoma on this signaling transduction pathway (Fig. 4B). These results indicate that SGN inhibits the AKT/mTOR signaling transduction pathway, which is critical in osteoclastogenesis induced by both M-CSF/RANKL and lung cancer cells.

*SGN attenuates the stimulatory effect of human lung adenocarcinoma on M-CSF and RANKL expression in osteoblasts.* Osteoblasts play a critical role in assisting bone metastasis by increasing M-CSF and RANKL expres-



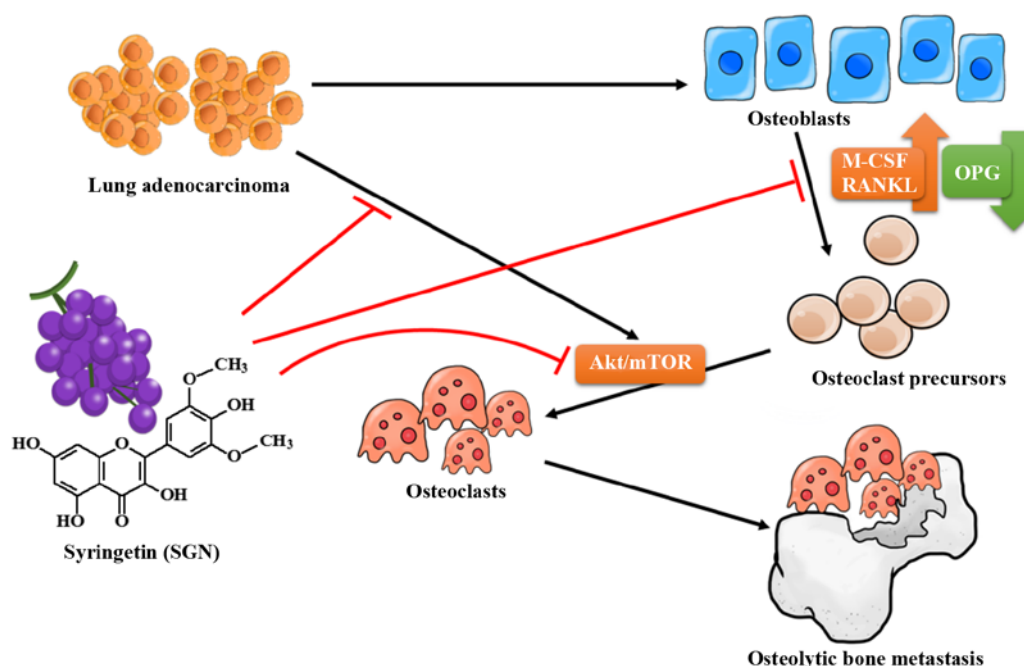


Figure 7. Scheme of proposed SGN-inhibited osteoclastogenesis. SGN not only blocks M-CSF/RANKL-induced AKT/mTOR activation, yet also prevents lung adenocarcinoma-induced osteolytic bone metastasis by decreasing the stimulatory effect of lung adenocarcinoma on osteoclasts, along with lung adenocarcinoma-mediated interaction of osteoblasts and osteoclasts on this signaling pathway. RANKL, receptor activator of nuclear factor  $\kappa$ B ligand; OPG, osteoprotegerin; M-CSF, macrophage-colony stimulating factor; mTOR, mammalian target of rapamycin.

sion and decreasing OPG expression (35). To verify this, we examined the effect of SGN on secretions of M-CSF, RANKL and OPG in osteoblasts by lung adenocarcinoma, A549- and CL1-5-CMs, which markedly increased M-CSF and RANKL expression in the osteoblasts (Fig. 5A and B). On the other hand, A549- and CL1-5-CMs decreased OPG expression in the osteoblasts (Fig. 5C). SGN attenuated the stimulatory effect of lung adenocarcinoma on the expression of M-CSF and RANKL, and reversed the effect of lung adenocarcinoma on the suppression of OPG expression in the osteoblasts (Fig. 5A-C).

*SGN reduces the enhancing effect of human lung adenocarcinoma on osteoblast-mediated osteoclastogenesis.* The role of SGN in human lung adenocarcinoma-mediated interaction between osteoblasts and osteoclasts was further investigated (Fig. 6A). When compared with unstimulated osteoblasts, human lung adenocarcinoma A549 and CL1-5 cell-treated osteoblasts enhanced osteoclastogenesis. Although lung adenocarcinoma A549 and CL1-5 cells promoted osteoblast-mediated osteoclast differentiation and bone resorption, this effect was altered by SGN (Fig. 6B and C). These findings suggest that SGN inhibits the osteoblasts in lung adenocarcinoma-mediated osteolytic bone metastasis.

## Discussion

Bone metastasis in lung cancer patients is a devastating event, since once it occurs, it markedly worsens morbidity and mortality rates (36,37). Sone and Yano demonstrated that compounds, bisphosphonates and reveromycin A, which potentially suppress osteoclast activity, were of benefit in

treating lung cancer bone metastasis (38). Despite modern treatment strategies, 30-50% of patients still develop new bone metastases, skeletal complications and disease progression, emphasizing the necessity for novel therapies (39,40). Osteoclastic metastasis characterized by lung cancer bone metastasis and osteoclast differentiation could be a target for treatment strategy. Multi-modality therapy may be required for lung cancer bone metastasis (36-38). In the present study, SGN, a flavonoid derivative found in grapes and wine, was first investigated to determine its suppressive effects on osteoclast differentiation and bone resorption activity, stimulated either by osteoclastogenic factors or lung cancer cells. Furthermore, SGN was also found to inhibit the pro-osteoclastic effect in osteoblasts stimulated by lung cancer cells. This suggests that SGN possesses therapeutic potential in treating lung adenocarcinoma bone metastasis (Fig. 7).

The balance between osteoblasts and osteoclasts can be interrupted by metastasized cancer cells (6). Osteoblasts play vital roles in regulating skeleton physiology, since they are not only precursors of osteocytes, yet also influence osteoclast differentiation (41,42). Osteoblasts promote osteoclast differentiation from osteoclast precursors by producing M-CSF and RANKL (43). In contrast, osteoblasts inhibit osteoclastogenesis by secreting OPG, a decoy receptor for RANKL, to block the binding of RANKL to its receptors on pre-osteoclasts (44). Cancer cells modify the pattern of M-CSF, RANKL and OPG expression in osteoblasts, facilitating cancer cell-associated osteolytic bone metastasis (45). In the present study, human lung adenocarcinoma A549 and CL1-5 cells upregulated M-CSF and RANKL expression yet downregulated OPG expression in osteoblasts, thereby increasing osteoclast differentiation and bone resorption



activity. This upregulation of M-CSF and RANKL by human lung adenocarcinoma cells was inhibited by SGN, resulting in lower rates of lung cancer-associated osteoclastogenesis. The present study revealed that SGN reversed the dysregulated interaction between osteoblasts and osteoclasts in the skeletal microenvironment of a lung adenocarcinoma model.

Furthermore, AKT is known as an important mediator of proliferation, survival and differentiation in a variety of cell types (46). It is involved in regulating survival and differentiation of osteoclasts, and its deficiency in osteoclasts leads to impaired bone resorption (47,48). mTOR, a downstream molecule of AKT, is also known to regulate osteoclast survival (49). Inhibition of the mTOR pathway by rapamycin reduced the number of TRAP-positive multinucleated osteoclasts in the chondro-osseous junction in rats (50). In the present study, osteoclast differentiation induced by M-CSF/RANKL increased activation of the AKT and mTOR signaling transduction pathway. Inhibitors of both AKT and mTOR significantly inhibited osteoclast differentiation and bone resorption activity, suggesting that the AKT/mTOR pathway plays a crucial role in osteoclastogenesis. Moreover, human lung adenocarcinoma A549 and CL1-5 cells increased the activation of AKT/mTOR signaling, resulting in enhanced osteoclastogenesis. SGN not only blocked M-CSF/RANKL-induced AKT/mTOR activation, yet also prevented the reinforcing effect of lung adenocarcinoma on this signaling transduction pathway. These data suggest that SGN is an AKT/mTOR inhibitor, targeting both the induction of osteoclast differentiation and the cancer-induced activation of AKT/mTOR.

In conclusion, SGN has protective potential against human lung adenocarcinoma-mediated bone destruction by directly decreasing cancer cell-mediated osteoclast differentiation and bone resorption, and by restoring the balance of osteoblast-osteoclast interaction. Taken together, SGN possesses dual ameliorating effects on lung adenocarcinoma-associated osteolytic bone metastasis.

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