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ORIGINAL RESEARCH

Annatto-Derived Tocotrienol Promotes Mineralization of MC3T3-E1 Cells by Enhancing BMP-2 Protein Expression via Inhibiting RhoA Activation and HMG-CoA Reductase Gene Expression

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Purpose: Annatto-derived tocotrienol (AnTT) has been shown to improve bone formation in animal models of osteoporosis and promote differentiation of pre-osteoblastic cells. However, the mechanism of action of AnTT in achieving these effects is unclear. This study aims to investigate the mechanism of action of AnTT on MC3T3-E1 pre-osteoblasts via the mevalonate pathway.

Methods: Murine pre-osteoblastic cells, MC3T3-E1, were cultured with the density of 1×10^4 cells/mL and treated with 4 concentrations of AnTT (0.001–1 μg/mL). Expression of HMG-CoA reductase (HMGR) gene was carried out using qPCR after treatment with AnTT for 21 days. RhoA activation and bone morphogenetic protein-2 (BMP-2) were measured using immunoassay after 9 and 15 days of AnTT treatment. Lovastatin was used as the positive control. Mineralized nodules were detected using Von Kossa staining after 21 days of AnTT treatment.

Results: The results showed that HMGR was up-regulated in the lovastatin group on day 9 and 21 compared to the control. Lovastatin also inhibited RhoA activation (day 9 and 15) and increased BMP-2 protein (day 15). On the other hand, AnTT at 0.001 μg/mL (day 3) and 0.1 μg/mL (day 21) significantly down-regulated HMGR gene expression compared to the control. On day 21, HMGR gene expression was significantly reduced in all groups compared to day 15. AnTT at 0.1 μg/mL significantly decreased RhoA activation on day 9 compared to the control. AnTT at 1 μg/mL significantly increased BMP-2 protein on day 15 compared to the control ($P<0.05$). Mineralized calcium nodules were more abundant in AnTT treated groups compared to the control on day 21.

Conclusion: AnTT suppresses the mevalonate pathway by downregulating HMGR gene expression and inhibiting RhoA activation, leading to increased BMP-2 protein in MC3T3-E1 cells. This explains the stimulating effects of AnTT on osteoblast mineralization.

Keywords: bone, osteogenic, osteoporosis, tocotrienol, vitamin E

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Introduction

Bone remodelling is regulated by osteoblast-mediated bone formation and osteoclast-mediated bone resorption. The imbalance of these two processes causes abnormalities in bone remodelling, which can produce a variety of bone disorders including osteoporosis.[1] Osteoporosis is a "silent" bone degenerative disorder

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characterized by low bone mass and deterioration of skeletal microarchitecture, leading to bone fragility.[2] Osteoporosis mainly affects postmenopausal women but it can also occur in men.[3,4] It is one of the most under-recognised non-communicable health conditions affecting developing countries with increasing elderly population.[5]

The current therapies for osteoporosis include anti-resorptive agents (bisphosphonates, calcitonin, denosumab, estrogen + progesterone) and anabolic agents (teriparatide).[6,7] These agents are effective against osteoporosis but they come with adverse side effects.[8,9] The preventive agents for osteoporosis are limited to calcium with or without vitamin D. This highlights a significant gap for pharmacological prevention of osteoporosis.

The mevalonate pathway, responsible for cholesterol synthesis, has been the target of drug intervention for osteoporosis.[10] Statins, traditionally known as cholesterol-lowering agents, have been found to promote bone formation in vitro and in vivo.[11–13] Statins regulate the mevalonate pathway by inhibiting the 3-hydroxy-3-methyl-glutaryl coenzyme-A (HMG-CoA) reductase (HMGR) enzyme, the rate-limiting enzyme for the mevalonate pathway, from converting HMG-CoA into mevalonate. These will subsequently suppress the synthesis of isoprenoids, such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). FPP and GGPP are involved in the protein prenylation process, whereby the isoprenoids are bound covalently to the C-terminal of small G-proteins or GTPase.[14] G-proteins consist of 5 families; the Rho/Rac/Cdc42, Ras, Rab, Sar1/Arf, and Ran families.[15] Rho is involved in statin-induced osteogenesis. A study by Harmey et al showed that Rho-Rho kinase inhibition stimulated differentiation and calcium nodule formation of mouse calvariae cells.[16] Inhibition of Rho kinase was shown to increase bone morphogenetic protein-2 (BMP-2) gene expression by pitavastatin in human osteoblasts.[17] However, this stimulatory effect was abolished by mevalonate or GGPP, indicating that these effects originated from inhibition of the mevalonate pathway.[17] BMP-2 plays a crucial role in the differentiation of human embryonic stem cells.[18] BMP signalling regulates transcription factors including runt-related factor 2 (Runx2) and osterix (OSX) involved in the formation of osteoblasts and expression of downstream genes involved in bone formation.[19]

Tocotrienol is a member of the vitamin E family, along with tocopherol. It can be found in palm oil, wheat germ, rice bran, barley, and annatto bean. Both tocotrienol and tocopherol contain 4 isomers, i.e. alpha (α), beta (β), delta (δ) and gamma (γ), depending on the side chains on the chromanol ring. Tocotrienol exerts powerful neuroprotective, antioxidant, anticancer and lipid-lowering properties, which distinguish it from tocopherol.[20] In addition, γ-tocotrienol preserved normal body composition and calcium content more effectively compared to α-tocopherol in dexamethasone-induced rats.[21] Both palm tocotrienol and annatto derived-tocotrienol have been reported to protect bone in various animal models of osteoporosis.[22] A previous study suggested the involvement of mevalonate pathway in the bone-sparing effects of γ-tocotrienol in ovariectomized mice.[23] Annatto derived-tocotrienol (AnTT) from seeds of achiote tree native to tropical America contains 90% δ- and 10% γ-tocotrienol.[24,25] In animal models of osteoporosis due to testosterone deficiency, AnTT was shown to prevent bone loss by increasing osteoblast number, osteoid volume and osteoid surface.[26] Combination of AnTT and lovastatin also increased bone formation, improved bone structure and bone strength in ovariectomized rats.[27,28] In cell culture studies, AnTT stimulated MC3T3-E1 differentiation and mineralization.[29] However, the mechanism of action behind this osteogenic activity is still unknown.

In the present study, the mechanism of action of AnTT on the mevalonate pathway, specifically on HMGR gene, RhoA prenylated protein and BMP-2 protein, in MC3T3-E1 cells was investigated. It is hypothesized that AnTT promotes bone mineralization via the mevalonate pathway, marked by decreased expression of HMGR gene, RhoA and increased BMP-2 proteins in MC3T3-E1 cells.

Materials and Methods

Chemicals

Minimum Essential Medium Eagle – Alpha Modification (α-MEM) was obtained from Invitrogen (Carlsbad, USA). Antibiotic-antifungal (AA), foetal bovine serum (FBS) and phosphate-buffered saline (PBS) were obtained from Gibco (Waltham, USA). Sodium phosphate, ascorbic acid and silver nitrate were obtained from Sigma-Aldrich Co. (St Louis, USA). Ethanol was obtained from HmbG Chemicals (Hamburg, Germany). Lovastatin was obtained from ChemFaces (Wuhan, China).

Experimental Treatments

Annatto-derived tocotrienol (AnTT) was a generous gift from American River Nutrition (Hadley, USA). AnTT was prepared based on a previous study.[29] Briefly, AnTT stock solution was dissolved in ethanol to a concentration of 5 mg/mL. From the stock solution, 25 μL AnTT was

added to 60 µL FBS and incubated overnight. On the following day, 90 µL differentiation media and 105 µL ethanol were added to the mixture. AnTT was diluted into 0.001, 0.01, 0.1 and 1 µg/mL in differentiation media. For the control group, the same amount of ethanol as the AnTT groups was used.

For the positive control group, 20 mg lovastatin was dissolved with 1 mL ethanol. Then, lovastatin was diluted into 5 µM in differentiation media. All treatments were freshly prepared every 3 days until the end of treatment.

Cell Culture

Murine calvarial pre-osteoblast cell line, MC3T3-E1, was purchased from American Type Culture Collection (ATCC) (ATCC No CRL-2594) (Manassas, USA). The cells were cultured in growth media (α-MEM supplemented with 10% AA and 10% FBS) at 37°C and 5% carbon dioxide. The cells were seeded at the density of 1×10^4 cells/mL growth media in all experiments. On the next day, the cells were treated with AnTT (0.001–1 µg/mL) and 5 µM lovastatin prepared in differentiation media (growth media + 3 mM sodium phosphate + 50 µg/mL ascorbic acid).

HMGR Gene Expression

Pre-osteoblast cells were seeded at a density of 5×10^4 in 6-well plate. On the following day, the cells were treated with AnTT (0.001–1 µg/mL) and 5 µM lovastatin for 3, 9, 15 and 21 days. At the end of each time-point, the cells were washed with PBS and lysed using TRI-Reagent (Molecular Research Centre, Inc., Cincinnati, OH, USA). The extracted RNA converted into cDNA with a thermal cycler (Techne, Staffordsire, UK) using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules CA, USA). Expression of targeted genes was quantified by CFX96 Touch™ Real-Time Detection System (Bio-Rad Laboratories Inc., Hercules CA, USA) under the amplification condition of 40 cycles, 10 s at 95 °C for denaturation and 30 s at 56 °C for annealing. The mice primers used were: β-actin, 5′-GAAGAGCTATGAGCTGCCTGA-3′ and 5′-GCACTGTGTTGGCATAGAGGT-3′; HMGR, 5′-TCTTTCCGTGCTGTGTTCTG-3′ and 5′-TTTTAACC CACGGAGAGGTG-3′ (First Base, Singapore Science Park II, Singapore).

RhoA Activation Assay

The RhoA activation assay measured the active form of RhoA in the cells. The cells were seeded in a 6-well plate at a density 5×10^4 and incubated overnight. Then, they were treated with AnTT (0.001–1 µg/mL) and 5 µM lovastatin for 9 and 15 days. The cells were harvested at the end of each time-point and assayed with G-LISA® RhoA Activation Assay Kit (Cat. BK124) (Cytoskeleton Inc. Denver, USA).

BMP-2 Protein

Murine preosteoblastic cells were seeded at a density of 5×10^4 in a 6-well plate. On the next day, the cells were treated with AnTT (0.001–1 µg/mL) and 5 µM lovastatin for 9 and 15 days. At the end of each time-point, the cells were washed with PBS. Then, the cells were lysed with freeze-thaw method 3 times in PBS. The supernatants were collected after quick centrifuged at high speed. The expression of BMP-2 was determined using an enzyme-linked immunosorbent assay (Cat. No. E-EL-M0193, Elabscience, Wuhan, China).

Assessment of Mineralization

To determine the effects of AnTT on mineralization, the extracellular matrix of the culture was assessed using Von Kossa staining. Cells were treated with AnTT for 3, 9, 15 and 21 days. At the end of each time-point, the cells were washed with deionised water and fixed with 10% buffered formalin for 10 min. Then, the cells were treated with 5% silver nitrate and incubated at room temperature for 1 hr under ultraviolet light. After the cells were washed with deionised water, positive staining for Von Kossa was visualized under the inverted microscope EVOS Cell Imaging System (Thermo Fisher Scientific).

Statistical Analysis

Statistical analysis was performed using SPSS software for Windows, version 20 (IBM Corporation, Armonk, NY, USA). The difference in the variables of interest among the study groups was analysed using one-way analysis of variance (ANOVA) with Turkey post hoc pairwise comparison. For gene expression analysis, mixed-design ANOVA with small effect analysis was used. A *P*-value less than 0.05 ($P<0.05$) was considered statistically significant.

Results

Effects of AnTT on HMGR Gene

In this study, MC3T3-E1 cells were treated with AnTT (0.001–1 µg/mL) and 5 µM lovastatin (positive control) for 3, 9, 15 and 21 days (Figure 1). There were significant time ($P<0.05$) and treatment ($P<0.05$) effects on HMGR gene expression. In terms of time, for the control group, HMGR gene expression was significantly reduced on day 9 and day

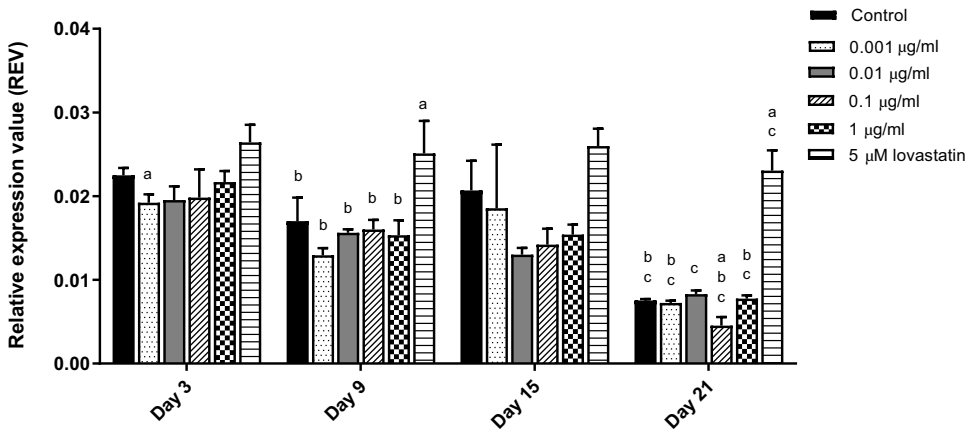

Figure 1 HMGR gene expression in MC3T3-E1 cells after AnTT treatment for day 3, day 9, day 15 and day 21. [a]Indicates a significant difference between the marked group compared to the control at the same time-point; [b]Indicates a significant difference between the marked group compared to its previous time-point; [c]Indicates a significant difference between the marked group compared to day 3. Data are expressed as mean±S.E.M.

21 compared to day 3 and day 15, respectively (P<0.05). HMGR gene expression for cells treated with AnTT at 0.001, 0.1 and 1 µg/mL reduced on day 9 and 21 compared to day 3 and 15, respectively (P<0.05). In terms of treatment, HMGR gene expression of cells treated with 0.01 µg/mL of AnTT reduced on day 9 compared to the control (P<0.05). HMGR gene expression was significantly decreased in all groups on day 21 compared to day 3 (P<0.05). For the lovastatin group, HMGR gene expression was up-regulated on day 9 and day 21 compared to the control (P<0.05). On day 3, 0.001 µg/mL AnTT significantly down-regulated HMGR gene expression compared to the control (P<0.05). On day 21, 0.1 µg/mL AnTT significantly down-regulated HMGR gene expression compared to the control (P<0.05).

Effects of AnTT on RhoA Activation

In order to confirm the involvement of the mevalonate pathway, RhoA activation assay was carried out to determine whether Rho was geranylgeranylated after AnTT treatment. MC3T3-E1 cells were treated with AnTT (0.001–1 µg/mL) and 5 µM lovastatin (positive control) for 9 and 15 days (Figure 2). RhoA activation was significantly decreased in the lovastatin group on day 9 and 15 compared to control (P<0.05). On day 9, RhoA

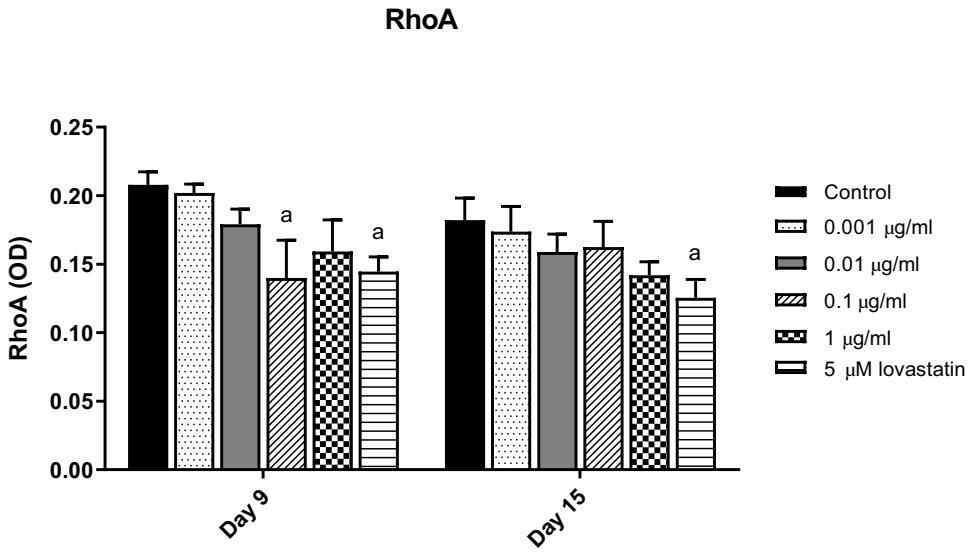

Figure 2 RhoA activation in MC3T3-E1 cells after AnTT treatment for day 9 and day 15. [a]Indicates a significant difference between the marked group compared to the control at the same time-point. Data are expressed as mean±S.E.M.

activation was significantly decreased in the 0.1 µg/mL AnTT group compared to the control ($P<0.05$).

Effects of AnTT on BMP-2 Protein

In this study, MC3T3-E1 cells were treated with AnTT (0.001–1 µg/mL) and 5 µM lovastatin (positive control) for 9 and 15 days (Figure 3). In terms of time, BMP-2 protein levels of the control, 0.001, 0.1, 1 µg/mL AnTT and lovastatin groups were significantly increased on day 15 compared to day 9 ($P<0.05$). In terms of treatment, BMP-2 protein level of the lovastatin group was significantly increased compared to the control on day 15 ($P<0.05$). On day 15, 1 µg/mL AnTT significantly increased BMP-2 protein compared to the control ($P<0.05$).

Effects of AnTT on Mineralization

AnTT (0.001–1 µg/mL) were treated for 9 and 15 days and stained using Von Kossa technique to determine the degree of mineralization (Figure 4). Positive staining for Von Kossa staining appeared in dark brown or black in the culture. On day 3 and 9, all groups were not stained. On day 15, positive staining appeared in all groups. On day 21, the cultures were stained more intensively in 0.01, 0.1 and 1 µg/mL AnTT groups compared to the control. These data indicated that AnTT promoted osteoblast mineralization.

Discussion

Osteoblasts play a crucial role in bone formation. Osteoblasts secrete proteins to form the bone matrix and later mineralise it. Previous studies showed that statins promote osteoblast differentiation via inhibition of the mevalonate pathway, which leads to suppression of prenylated proteins including RhoA. This will modulate signalling pathways including BMP-2, to promote bone formation. The present study showed that AnTT down-regulated HMG-CoA reductase gene expression, which leads to inhibition of RhoA prenylated protein. This indicated that AnTT, acting as a HMG-CoA reductase suppressor, inhibited the mevalonate pathway. Besides that, AnTT also increased BMP-2 protein level involved in the expression of transcription factors Runx2 and Osx critical in osteoblast differentiation. This translated to increased mineralization in osteoblast culture as observed in the current study. Therefore, AnTT could serve as a potential bone anabolic agent via inhibition of the mevalonate pathway.

In the present study, AnTT was found to down-regulate HMGR gene in preosteoblastic cells. A previous study demonstrated that γ- and δ-tocotrienol stimulated HMGR degradation, however only δ-tocotrienol completely blocked nuclear SREBP-2 processing in the SV589 human fibroblast cells.[30] The protein SREBP-2 is involved in the regulation of cholesterol biosynthesis enzymes including HMGR gene.[31] Another study showed that δ-tocotrienol down-regulated SREBP-2 target gene, including HMGR in Chinese-hamster ovary cell lines, CHO and prostate cancer cell lines, LNCaP.[32] These studies suggested that AnTT, which contains γ- and δ-tocotrienol,

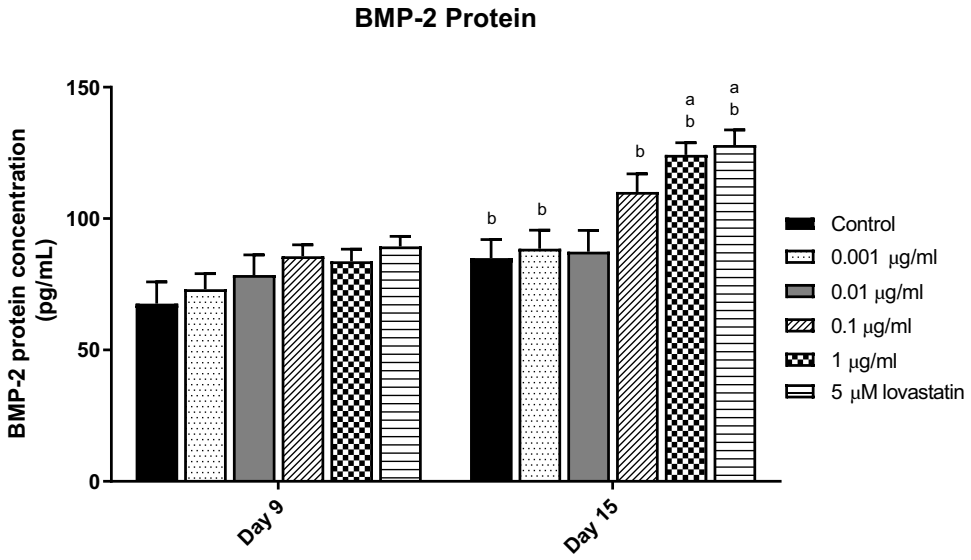

Figure 3 BMP-2 protein in MC3T3-E1 cell after AnTT treatment for day 9 and day 15. [a]Indicates a significant difference between the marked group compared to the control at the same time-point; [b]Indicates a significant difference between the marked group compared to its previous time-point. Data are expressed as mean±S.E.M.

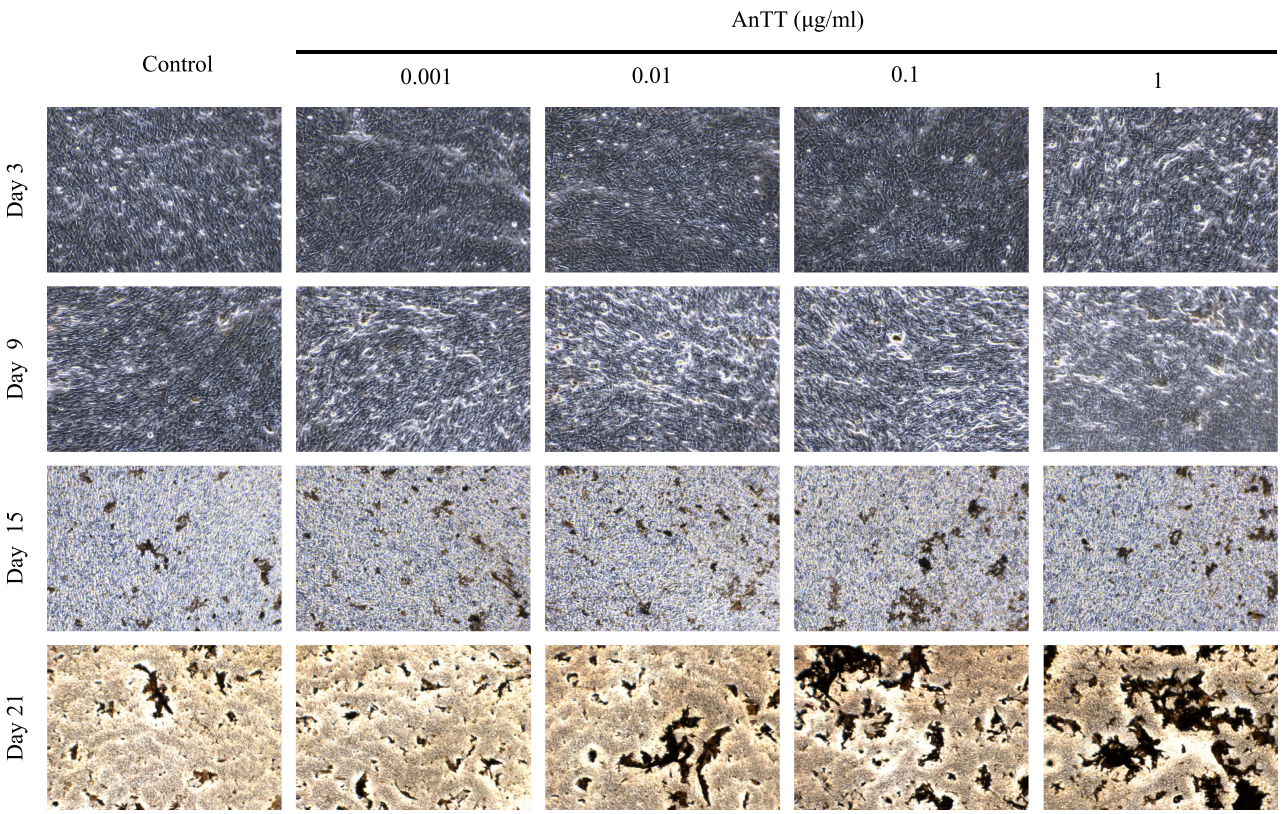

Figure 4 Von Kossa staining on MC3T3-E1 cells after AnTT treatment for day 3, day 9, day 15 and day 21.

could inhibit the mevalonate pathway by down-regulating HMGR at the transcription level.

This study also demonstrated that AnTT inhibited RhoA activation in MC3T3-E1 cells in a manner comparable to the positive control, lovastatin. A previous study showed that HMG-CoA inhibitor, simvastatin blocked HMG-CoA /GGPP/RhoA-dependent pathway in mouse embryonic stem cell lines.[33] Similarly, Ohnaka et al found that pitavastatin stimulated BMP-2 and osteocalcin gene by inhibiting Rho-kinase activity and inactivation of Rho via the mevalonate pathway in human osteoblasts.[17] In human airway smooth muscle cells, 50 μM γ-tocotrienol inhibited RhoA activation that leads to a reduction of cell proliferation and migration.[34] On the other hand, the combination of statin and γ-tocotrienol inhibited HMG-CoA reductase activity and RhoA activation in human colon cancer HCT116 and HT29 cells.[35] Since AnTT inhibited HMGR in this study, it is suggested that the production of isoprenoids and prenylated G-protein like RhoA would be suppressed.

The current study also showed that AnTT increased BMP-2 protein level in pre-osteoblastic cells in a manner comparable to lovastatin. Mevastatin and simvastatin were shown to activate BMP-2 promoter, leading to increased BMP-2 mRNA and protein expressions in human osteosarcoma cell line.[36] A previous animal study also showed that the combination of AnTT and lovastatin increased the expression of BMP-2 mRNA in the bones of ovariectomized rats.[37] This paper investigated the effects of annatto tocotrienol via BMP2 but it cannot be ruled out that it may act through other BMPs and transforming growth factor signaling in bone metabolism.[38–41]

In this study, AnTT promoted osteoblast mineralization in MC3T3-E1 cells. This validated the observation previously obtained in a cellular study, whereby AnTT enhanced differentiation and mineralization (marked by Alizarin Red staining) of MC3T3-E1 cells.[29] A study by Deng et al showed that γ-tocotrienol increased calcium nodule formation in mice bone marrow cells. This effect was abolished by co-treatment with mevalonate, implicating the involvement of mevalonate pathway in the mineralization induced by γ-tocotrienol.[23] In animal studies, AnTT alone and in combination with lovastatin were shown to improve the structural properties of femoral trabecular bone in ovariectomized rats.[28] Similarly, AnTT preserved trabecular bone microarchitecture in an animal model of osteoporosis induced by testosterone deficiency and metabolic syndrome.[37,42]

Several limitations should be acknowledged in this study. We only studied the mevalonate pathway partially, focusing on the upstream (HMGR) and down-stream (RhoA) section of the pathway. The effects of AnTT on the other steps of the pathway remain unknown, especially on the production of isoprenoids such, as FPP and GGPP. Inhibition of the mevalonate pathway by statins has been widely shown to deplete FPP and GGPP levels.[43] We also did not study the activity of HMGR to correlate with the gene expression level due to technical issue. Nevertheless, this is the first time the effects of AnTT on the mevalonate pathway were elucidated in pre-osteoblast cells. Annatto tocotrienol, which is shown in this study to promote mineralization by modulating BMP and HMG-CoA reductase, will likely to have multifaceted effects in other physiological and pathological context like cardiovascular diseases, neurodegenerative diseases or cancer.[44–49] Our work warrants further investigation of mechanism and synthetic modification to improve tocotrienols to enhance its pharmacological and therapeutic application in a disease-specific manner.

Conclusion

AnTT treatment down-regulates HMGR gene expression, thus inhibiting the mevalonate pathway, leading to reduced RhoA activation downstream of the mevalonate pathway. AnTT also increases BMP-2 protein expression. The suppression of mevalonate pathway may partially explain the anabolic effect of AnTT on osteoblast mineralization.

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Disclosure

The authors report no conflicts of interest in this work.

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