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Suppression of NF- κ B Activation By Gentian Violet Promotes Osteoblastogenesis and Suppresses Osteoclastogenesis

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Abstract

Skeletal mass is regulated by the coordinated action of bone forming osteoblasts and bone resorbing osteoclasts. Accelerated rates of bone resorption relative to bone formation lead to net bone loss and the development of osteoporosis, a devastating disease that predisposes the skeleton to fractures. Bone fractures are associated with significant morbidity and in the case of hip fractures, high mortality. Gentian violet (GV), a cationic triphenylmethane dye, has long been used as an antifungal and antibacterial agent and is presently under investigation as a potential chemotherapeutic and antiangiogenic agent. However, effects on bone cells have not been previously reported and the mechanisms of action of GV, are poorly understood. In this study we show that GV suppresses receptor activator of NF- κ B ligand (RANKL)-induced differentiation of RAW264.7 osteoclast precursors into mature osteoclasts, but paradoxically stimulates the differentiation of MC3T3 cells into mineralizing osteoblasts. These actions stem from the capacity of GV to suppress activation of the nuclear factor kappa B (NF- κ B) signal transduction pathway that is required for osteoclastogenesis, but inhibitory to osteoblast differentiation and activity. Our data reveal that GV is an inhibitor of NF- κ B activation and may hold promise for modulation of bone turnover to promote a balance between bone formation and bone resorption, favorable to gain of bone mass.

Keywords

Bone formation; gentian violet; NF- κ B; osteoblast; osteoclast; osteoporosis

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CONFLICT OF INTEREST

The authors declare no financial conflicts of interests.

INTRODUCTION

Cytokines such as Tumor necrosis factor alpha (TNF α) that potently activates NF- κ B have a profoundly inhibitory effect on basal bone formation. In fact, bone formation in mice is extremely sensitive to even physiological TNF α concentrations and we have reported that genetic ablation of TNF α , or the p55 TNF α receptor, significantly increases peak bone mineral density (BMD) in mice, without effects on osteoclast formation or activity [1]. However, under inflammatory states and in estrogen deficiency, TNF α reaches concentrations capable of both suppressing bone formation and stimulating osteoclastic bone resorption. This effectively diminishes the natural compensatory increase in bone formation in response to increased bone resorption creating a differential between bone formation and resorption favorable to bone loss [2]. As a consequence the TNF α pathway has received much attention as a potential therapeutic target for inflammatory bone disease. Indeed, TNF α neutralization in mice is effective in mitigating bone loss associated with estrogen deficiency in ovariectomized mice [3]. TNF α antagonists are further able to reverse compromised bone regeneration potential associated with aging in an animal fracture repair model [4] through association of TNF α with its type I receptor [5].

It is now evident that much of the action of TNF α on bone cells is mediated through the NF- κ B signal transduction pathway and NF- κ B signal transduction plays key roles in the regulation of bone cells and in skeletal homeostasis. This stems from the fact that NF- κ B activation by receptor activator of NF- κ B ligand (RANKL), the key osteoclastogenic cytokine, is central to formation of osteoclasts, the cells that resorb bone [6, 7]. We and others have demonstrated that suppression of NF- κ B signaling using pharmacological antagonists is effective in suppressing osteoclastogenesis *in vitro* [1, 8–11] and in reducing the loss of BMD associated with ovariectomy in mice, a model of postmenopausal osteoporosis [12]. NF- κ B antagonists have further been demonstrated to suppress osteoclastogenesis and bone erosion in inflammatory arthritis [13].

By contrast, activation of NF- κ B in osteoblasts, the cells that build bone, is potently inhibitory to osteoblast differentiation and function [1, 14]. Indeed, pharmacological suppression of NF- κ B ameliorates ovariectomy induced bone loss in mice by increasing bone formation and reducing bone resorption [15]. Furthermore, conditional genetic ablation of NF- κ B signaling in osteoblasts stimulates bone formation, promoting accretion of BMD, and preventing osteoporotic bone loss induced by ovariectomy in adult mice [16].

Given this capacity of NF- κ B to differentially regulate osteoclast and osteoblast activities, agents capable of modulating this pathway may hold great promise for the development of dual anabolic and anti-catabolic pharmaceuticals. Interestingly, we have recently reported that several natural compounds, long believed to possess anabolic and/or anti-catabolic properties, may protect bone by antagonizing NF- κ B activation. Among these agents are vitamin K₂ [9], 17 β -estradiol [17], the carotene, p-hydroxycinnamic acid, the xanthophyll, beta-cryptoxanthin [18], honokiol, a component of Asian herbal teas used extensively in traditional Japanese and Chinese medicine [19], and the plant-derived flavonoid quercetin [20].

Gentian violet (GV) a triaminophenylmethane dye has been used extensively in medicine for a century and has potent anti-microbial action [21]. Recent studies further suggest angiogenic and anticancer properties of GV and there is now renewed interest in the medical applications of this dye [22, 23].

In this present study we examined the effects of GV on the differentiation of bone cells *in vitro* and report that GV exhibits anti-NF- κ B activity and like other NF- κ B antagonists can potentially augment osteoblast differentiation and mineralization but potentially suppresses osteoclast differentiation. GV may consequently hold promise for further development as an anti-osteoporotic and/or anti-inflammatory agent.

MATERIAL AND METHODS

Materials

α -Minimal essential medium (α -MEM) and antibiotics (penicillin and streptomycin) were purchased from Invitrogen Corp. (Carlsbad, CA). Fetal bovine serum (FBS) was from Hyclone. RANKL, TNF α , Transforming growth factor beta (TGF β) and bone morphogenetic protein -2 (BMP-2) were from R&D Systems (Minneapolis, MN). GV, tartrate resistant acid phosphatase (TRAP) staining kit and all other reagents were purchased from the Sigma-Aldrich Chemical Corporation, (St. Louis, MO) unless otherwise specified.

Cell Culture

The preosteoblastic cell line MC3T3-E1, clone 14 (MC3T3) and the osteoclast precursor cell line RAW264.7 were purchased from the American Type Culture Collection (Manassas, VA) and cultured as previously described [1, 9, 24].

Osteoblast Differentiation Assays and Alizarin RedS Staining

MC3T3 cells, were plated and cultured for 72 h in α -MEM (1.0 ml/well) containing 10% FBS in 12-well dishes at a density of (1.0×10^5 cells per well). Medium was aspirated and changed to mineralization medium (α -MEM supplemented with 10% FBS, L-ascorbic acid (100 μ g/ml) and 4 mM β -glycerophosphate) as previously described [1, 9, 24, 25]. GV was added at the indicated dose and cells replenished with fresh medium every 3 days. Between 14 and 18 days cells were rinsed with PBS and mineralization nodules visualized by fixing the cells in 75% ethanol for 30 minutes at 4°C followed by staining with Alizarin Red-S (40 mM, pH 6.2) for 30 minutes at room temperature. Excess stain was removed by copious washing with distilled water. Plates were imaged using a flatbed scanner (Epson Perfection V600 Photo).

Osteoclastogenesis Assays and TRAP Staining

Osteoclasts were generated as previously described [1, 9, 24]. Briefly the RAW264.7 osteoclast precursor cell line was cultured for 6 days in 96-well plates in α -MEM supplemented with 10% FBS and 100 IU/ml penicillin, and 100 μ g/ml streptomycin at a density of 1×10^4 cells/well. Osteoclast formation was induced by addition of RANKL (30 ng/ml) pre-incubated for 10 minutes with crosslinking anti-poly-histidine antibody (2.5 μ g/ml) per the manufacturers directions. GV was added in the range 0.01 μ M to 1 μ M. After

6 days of culture, the cells were fixed and stained for Tartrate resistant acid phosphatase (TRAP). Per convention, TRAP⁺ cells with three or more nuclei were defined as osteoclasts and were quantitated under light microscopy and 5 wells per group averaged. Representative wells were photographed under bright field microscopy using a Nikon Eclipse TE2000-S inverted microscope. Images were captured using a digital camera (QImaging Corp., Burnaby, BC, Canada).

NF- κ B Constructs and Luciferase Assays

The NF- κ B responsive reporter pNF- κ B-Luc (BD Biosciences) or pGL3-Smad, responsive to all R-Smads, was used as previously described by us [1, 9, 24]. Briefly, reporter pNF- κ B-Luc or pGL3-Smad plasmid was transfected into MC3T3 or RAW264.7 cells using Lipofectamine 2000 reagent (Invitrogen) in α -MEM without FBS and antibiotics. Five hours later the cells were seeded at a density of 1×10^5 cells/well in 96 well plates with 5 replicate wells per experimental condition, in α -MEM containing 10% FBS plus antibiotics. MC3T3 cells were treated with TNF α (1 ng/mL) or RAW264.7 cells treated with RANKL (30 ng/mL) to stimulate NF- κ B activity while parallel groups received GV at the indicated dose. Cells were extracted with passive lysis buffer (Promega Corporation, Madison WI) 24 hours later, and luciferase activity measured using the Luciferase Assay System of Promega, on a microplate luminometer (Turner Designs, Sunnyvale, CA, USA).

Real-Time RT-PCR for Osteoblastic Gene Expression

Total RNAs were extracted from MC3T3 cells using Trizol Reagent and real-time RT-PCR performed on an ABI Prism 7000 instrument (Applied Biosystems, Foster City, CA) and expression of the osteoblast related genes bone sialoprotein, type I collagen, osteocalcin, Osterix and Runx2 was quantified by real-time RT-PCR and normalized to 18S mRNA using published primer sets as previously described [26]. Briefly, amplification reactions were performed using SYBR Green PCR Master Mix (Applied Biosystems) with the following primer sets: 18S mRNA, 5'-ATTCG AACGTCTGCCCTATCA-3' (forward) and 5'-GTCACC CGTGGTCACCATG-3' (reverse); osteocalcin, 5'-GCC TTCATGTCCAAGCAGGA-3' (forward) and 5'-GCGC CGGAGTCTGTTCACCTA-3' (reverse); Osterix, 5'-GTG TTAGTAACCTGGCCGGG-3' (forward) and 5'-CATT GGACTTCCCCCTTCTTG-3' (reverse); Runx2, 5'-CT GTGGTTACCGTCATGGCC-3' (forward) and 5'-GGA GCTCGGCGGAGTAGTTC-3' (reverse); and type I collagen, 5'-CCCTACTCAGCCGTCTGTGC-3' (forward) and 5'-GGGTTCTGGGCTGATGTACC-3' (reverse). Dissociation curves revealed a single product in all cases. Changes in relative gene expression between control and treated groups were calculated using the 2^{-C_t} method of Livak and Schmittgen [27] with normalization to 18S.

Apoptosis Assays

Apoptosis was quantified in RAW264.7 cells by flow cytometry using the Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA). Briefly, RAW264.7 cells were treated with a dose range of GV (0.01 μ M to 1 μ M). At 1, 3 and 7 days of treatment, cells were washed twice with cold PBS and then recovered by gentle scraping and concentrated by centrifugation (1600 RPM for 5 minutes) in a 5 ml flow tube. Cell pellets were resuspended in 1X binding buffer at a concentration of 1×10^6 cells/ml. 1×10^5 cells were

stained with 5 μ l of Annexin V-PE and 5 μ l 7-AAD for 15 min at RT (~25°C) in the dark. After staining cells were diluted with 400 μ l of 1X binding buffer and analyzed by flow cytometry within 1 hr using an Accuri flow cytometer (BD Immunocytometry Systems, San Jose, CA). Cells positive for Annexin V but negative for 7-AAD were defined as apoptotic.

Statistical Analysis

Statistical significance was determined using GraphPad InStat version 3 for Windows XP (GraphPad Software Inc. La Jolla, CA). Simple comparisons involved Student t tests. Multiple comparisons were performed by one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons post-test for parametric data. Statistical analysis of apoptosis assays involved two-way ANOVA with Bonferroni post-test. Gaussian distribution was assessed using the Kolmogorov and Smirnov test. $P < 0.05$ was considered statistically significant.

RESULTS

GV promotes mineralization of osteoblasts differentiated from MC3T3 precursors and alleviates the suppressive effect of TNF α on osteoblast mineralization

GV is now under investigation for anticancer properties; however its actions *in vivo* are poorly understood. In this study we examined the effect of GV on bone forming osteoblasts using an *in vitro* culture system. When cultured under permissive conditions in mineralization medium MC3T3-E1 (MC3T3) osteoblast precursors spontaneously differentiate into functional osteoblasts capable of depositing a mineralized bone matrix. Using Alizarin Red-S staining to visualize calcium deposition, weak mineralization becomes detectable at around 14 days of culture and reaches a peak (saturation) at around 18–21 days. MC3T3 were differentiated into osteoblasts in the presence or absence of a dose range of GV (0.001 μ M, 0.01 μ M, 0.1 μ M or 0.5 μ M) in mineralizing medium for 14 days and stained for calcium deposition with Alizarin Red-S. The data show (Fig. 1A) that GV dose dependently amplified mineralization between 0.001 and 0.1 μ M. Mineralization was still elevated relative to control at 0.5 μ M but reduced from the 0.1 μ M dose, possibly due to overgrowth of the cells or weak toxic effects at high dose. We and others have demonstrated that TNF α is a potent inhibitor of osteoblastic differentiation *in vivo* and *in vitro* and potently diminishes basal peak bone mineral density in rodents under physiological conditions [1].

We thus cultured MC3T3 cells for 18 days in the presence or absence of TNF α (5 ng/ml), used to suppress mineralization, as occurs *in vivo*, and examined the capacity of GV to ameliorate this action. Addition of GV between 0.001 μ M and 0.1 μ M dose dependently alleviated the repressive effect of TNF α on mineralization (Fig. 1B).

GV promotes differentiation of MC3T3 precursors along an osteoblast trajectory

Because GV may enhance osteoblast function through specific effects on the mineralization process or act by enhancing the differentiation of osteoblasts, we next quantified osteoblast-related gene expression in MC3T3 cells following GV treatment. Runx2 and Osterix are two key osteoblast transcription factors that are required for osteoblast differentiation.

Interestingly, Osterix expression, quantified by real-time RT-PCR was potently upregulated by GV (0.1 μ M) after just 6 hr of treatment and remained elevated for at least 24 hr (Fig. 2A). Runx2 expression was also significantly induced at 6 hr but had returned to baseline by 24 hr (Fig. 2B). We further quantified expression of 3 key osteoblast related gene products type I collagen, bone sialoprotein and osteocalcin. Both type I collagen, and bone sialoprotein expression was significantly elevated at 24 hr of GV treatment (Fig. 2C, D) while osteocalcin, an osteoblast product expressed late in differentiation was not significantly changed at this early time point (Fig. 2E).

Taken together these data suggest a dominant effect of GV on early osteoblast commitment and differentiation.

GV suppresses the differentiation of RAW264.7 osteoclast precursors into mature osteoclasts

Bone homeostasis is regulated principally by the coordinated action of osteoblastic bone formation and osteoclastic bone resorption. To examine the effect of GV on osteoclast formation, RAW264.7 osteoclast precursors were differentiated into mature osteoclasts by stimulation with RANKL in the presence or absence of a dose range of GV (0.01, 0.1, 0.5 and 1 μ M). GV significantly reduced osteoclast formation in the range 0.01 to 0.1 μ M, as quantitated in Fig. (3A) with representative photomicrographs presented in Fig. (3B). At 0.5 μ M and above GV was toxic to the precursors suppressing their proliferation over 7 days of culture, but no toxicity was observed at doses up to at least 0.1 μ M (Fig. 3C).

GV suppresses early osteoclast differentiation and fusion in vitro

RAW264.7 cells typically differentiate into TRAP positive mononucleated pre-osteoclasts within 3 days of RANKL treatment and then fuse into mature multinucleated osteoclasts over next 3–5 days. In order to determine at which step GV acts to inhibit osteoclast formation GV (0.1 μ M) was added at day 1 of 7 days culture, day 3 of 7 days culture or at day 5 of 7 days culture. When present for all 7 days of culture (added at day 1) GV potently inhibited osteoclast formation (Fig. 4A). Addition of GV at day 3 led to a partial suppression of osteoclast formation, while GV addition at day 5 of seven days culture had no effect. These data suggest that GV inhibits early osteoclast commitment and differentiation without significant effect on the later fusion stages. Representative photomicrographs are presented in Fig. (4B) for each condition.

GV induces apoptosis at high, but not low bioactive, dose

In order to differentiate an anti-osteoclastogenic effect of GV from a cytotoxic effect we quantified apoptosis in RAW264.7 cells cultured in the presence or absence of a dose range of GV (0, 0.01, 0.1, 0.5 or 1 μ M) for 7 days. Apoptosis was quantified at 24 h (1 day), 3 days and 7 days of culture by Annexin V flow cytometry. Doses of GV up to 0.1 μ M, failed to elicit any increase in apoptosis following 1, 3 or 7 days of treatment (Fig. 5). By contrast, GV doses of 0.5 and 1 μ M induced a significant increase in the rate of apoptosis, by 3 and 7 days of treatment.

GV suppresses basal and TNF α -induced NF- κ B activity in MC3T3 cells

The mechanisms of GV action on bone cells are unknown. However, because GV was able to neutralize the inhibitory effect of TNF α on MC3T3 mineralization, and suppress RANKL-induced osteoclastogenesis, both NF- κ B dependent processes, we speculated that GV might antagonize activation of the NF- κ B signal transduction pathway. We consequently examined the activity of GV on basal and TNF α -induced NF- κ B activation using an NF- κ B luciferase reporter, a reagent that quantifies the net total effect of NF- κ B signaling cascades over an extended period of time (24 hr). GV significantly suppressed basal NF- κ B activity between 0.1 and 1 μ M (Fig. 6A) and TNF α -induced NF- κ B activation between 0.1 and 1 μ M (Fig. 6B).

GV alleviates the suppressive actions of TNF α on BMP-2- and TGF β -induced Smad activation in MC3T3 cells

One mechanism by which TNF α suppresses osteoblast differentiation is through blunting Smad activation by bone morphogenetic proteins (BMP) such as BMP-2 and by TGF β an osteoblast commitment and recruitment factor. To test the effect of GV on Smad activation we transfected MC3T3 osteoblast precursors with a Smad-luciferase reporter construct responsive to all R-Smad species [1]. GV had no direct effect on basal Smad-activation (Fig. 7A) and TGF β - (Fig. 7B) and BMP2-stimulated (Fig. 7C) Smad-activation at doses previously observed to promote osteoblast differentiation (0.01 and 0.1 μ M) but suppressed Smad activity at 0.5 and 1 μ M, doses likely toxic to the cells. Importantly GV significantly reversed the suppressive activity of TNF α on TGF β (Fig. 8A) and BMP-2 (Fig. 8B)-induced Smad activation, at GV doses between 0.1 and 0.5 μ M, consistent with an inhibitory effect of GV on TNF α -induced NF- κ B activation.

GV suppresses basal and RANKL-induced NF- κ B activity in RAW264.7 cells

Given the capacity of GV to suppress RANKL-induced osteoclast formation and to suppress NF- κ B activation by TNF α in MC3T3 cells we further examined whether GV antagonizes RANKL-induced NF- κ B activation in osteoclast precursors. GV modestly, but significantly, suppressed basal NF- κ B activity in RAW264.7 cells at 1 μ M (Fig. 9A) and RANKL-induced NF- κ B activation between 0.01 and 1 μ M (Fig. 9B). Although the higher doses 0.5 and 1 μ M may represent toxic effects, significant repression of NF- κ B activation was seen at low non-toxic doses of GV (0.01 and 0.1 μ M).

DISCUSSION

In this study we demonstrate a new action of GV, an *in vitro* capacity to upregulate osteoblastic differentiation and mineralization and to suppress osteoclast formation by antagonizing NF- κ B activation.

GV has long been employed as an antimicrobial agent. When applied externally GV has potent antiseptic properties. However, GV has also been used orally as an anti-helminthic agent and as a blood additive to prevent transmission of protozoan parasites during allogeneic blood transfusions, with apparent low toxicity [28]. Intravenous GV has further been used successfully to treat extreme septicemia [29]. While chronic high dose

administration of GV in mice has been reported to cause carcinogenicity [30], there is no evidence of any case of cancer ever being definitively associated with pharmacological dosages of GV in humans [31]. Recently, there has been renewed interest in the use of GV as an antitumor [22] and antiangiogenic agent [23].

In our study GV was effective in promoting osteoblast mineralization in the range 0.01 to 0.1 μM . At higher dose (0.5 μM) mineralization was reduced, a possible consequence of cell overgrowth, rather than overt toxicity, given the effectiveness of this same dose in reversing the inhibitory effect of $\text{TNF}\alpha$ on $\text{TGF}\beta$ and BMP2-induced Smad activation (Fig. 4). Doses of 1 μM , however, were observed to mediate a non-specific suppressive effect in the Smad activity assays suggesting a generalized toxic effect.

Interestingly, a very low dose of GV (0.001 μM) was found to be effective in reversing $\text{TNF}\alpha$ -mediated suppression of mineralization, while a higher dose (0.1 μM) was required to significantly suppress $\text{TNF}\alpha$ -induced NF- κB in the reporter assays. A likely explanation is that because the cultures are refreshed with medium and replenished with cytokine every 3 days, the bioactivity of $\text{TNF}\alpha$ likely declines over the intervening 3 day period. By contrast the short duration (24 h) reported assays likely maintain a higher bioactivity of $\text{TNF}\alpha$ thus necessitating a higher dose of GV to reverse $\text{TNF}\alpha$ -induced NF- κB . Although different experimental conditions likely account for this difference, we can however not rule out a NF- κB -independent activity of GV that may contribute to the anabolic activity.

In contrast to MC3T3 cells, doses of GV of 0.5 μM and above halted normal cell proliferation in RAW264.7 cells although doses of up to at least 0.1 μM were effective in significantly reducing osteoclast formation (and NF- κB activation) without impeding the proliferation of the low density cultures which as expected had expanded to fill the culture wells by 7 days in the absence of RANKL. Because we could not differentiate an anti-osteoclastogenic effect of GV mediated through NF- κB suppression from an overt toxic effect we further performed an apoptosis assay. The data revealed that 0.01 and 0.1 μM concentrations of GV had not toxic effects on RAW264.7 cells however, at 0.5 and 1 μM , GV significantly increased the rate of apoptosis.

Taken together our data suggest an *in vitro* therapeutic range of between 0.01 and 0.1 μM for osteoclast suppression and between 0.1 and 0.5 μM for osteoblast differentiation.

While the molecular function of NF- κB in osteoclastogenesis has been well studied [6, 32–38], how NF- κB signaling acts to downregulate osteoblast differentiation is poorly understood. Our data show that GV promoted early differentiation of osteoblast precursors as evidenced by significant increases in the two key transcription factors, Runx2 and Osterix. Runx2 and Osterix play critical roles in osteoblast differentiation, and many key osteoblast gene products including alkaline phosphatase, osteopontin, bone sialoprotein, and type I collagen require Runx2 for their expression [14]. Consistent with this temporal relationship, Runx2 gene expression revealed a significant early induction within just 6 h of GV stimulation. Expression of type I collagen and bone sialoprotein, genes distal to Runx2, were induced by 24 h of GV treatment. Osteocalcin is associated with fully differentiated mineralizing osteoblasts and is a late marker of the osteoblast phenotype. Consistent with

this concept osteocalcin gene expression was not induced within the early stages of differentiation. Although Runx2 and Osterix are induced early on initiating differentiation, the final events associated with mineralization are only observed by approximately 14 days of culture.

One major mechanism of GV action appears to involve the intersection of NF- κ B with the Smad signaling pathway. TGF β - and BMP-induced Smad signaling respectively play important roles in the early commitment and differentiation of osteoblasts [39, 40]. NF- κ B signaling in osteoblasts intersects with and disrupts Smad signaling by promoting production of Smad7, an inhibitor of TGF β and BMP-induced R-Smad activation [41]. TNF α further antagonizes BMP signaling by upregulating Smad ubiquitination regulatory factor 1 (Smurf1), promoting proteasomal degradation of bone morphogenetic signaling proteins [42]. Consistent with this data we have reported that multiple suppressors of NF- κ B activation including the carotenoid beta-cryptoxanthin [43], the biphenol honokiol [19], vitamin K₂ [9], quercetin [20], zinc [24] and a pharmacological NF- κ B inhibitor based on the NEMO binding domain [1] are all capable of rescuing the inhibitory effect of TNF α on BMP2 and/or TGF β -induced Smad activation.

Although we recently demonstrated that vitamin K₂ suppresses NF- κ B activation by stabilization of I κ B α [9], the molecular mechanism by which GV suppresses NF- κ B activation remains to be determined. Our gene expression studies however, demonstrate that GV leads to early expression of Runx2 (Cbfa1), a key osteoblast transcription factor [44], as well as a potent and sustained (for at least 24 hr) induction of Osterix (Sp7) expression [45], another crucial osteoblast transcription factor. Induction of differentiation was further validated by induction of the osteoblastic gene products bone sialoprotein and type I collagen.

In conclusion, we demonstrate that GV possesses anti-NF- κ B activity and may have promise for development into an antiosteoporotic agent capable of promoting new bone formation while simultaneously reducing bone resorption. GV may further exhibit other generalized anti-inflammatory activities by virtue of its anti-NF- κ B activity and may be useful in the amelioration of multiple inflammatory states.

Acknowledgments

M.N. Weitzmann was involved in study conception and design, analysis and interpretation of data, and wrote the paper. M. Yamaguchi was involved in study conception and design, acquisition of data, analysis and interpretation of data. T. Vikulina performed acquisition of data. J.L. Arbiser was involved in study conception and contributed important reagents. All authors reviewed the manuscript.

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References

1. Li Y, Li A, Strait K, Zhang H, Nanes MS, Weitzmann MN. Endogenous TNF α lowers maximum peak bone mass and inhibits osteoblastic Smad activation through NF- κ B. *J Bone Miner Res.* 2007; 22:646–655. [PubMed: 17266397]

2. Weitzmann MN, Pacifici R. Estrogen deficiency and bone loss: an inflammatory tale. *J Clin Invest.* 2006; 116:1186–1194. [PubMed: 16670759]
3. Kimble RB, Bain S, Pacifici R. The functional block of TNF but not of IL-6 prevents bone loss in ovariectomized mice. *J Bone Miner Res.* 1997; 12:935–941. [PubMed: 9169353]
4. Wahl EC, Aronson J, Liu L, et al. Restoration of Regenerative Osteoblastogenesis in Aged Mice: Modulation of TNF. *J Bone Miner Res.* 2010; 25:114–123. [PubMed: 19580462]
5. Wahl EC, Aronson J, Liu L, et al. Direct bone formation during distraction osteogenesis does not require TNF α receptors and elevated serum TNF α fails to inhibit bone formation in TNFR1 deficient mice. *Bone.* 2010; 46:410–417. [PubMed: 19772956]
6. Boyce BF, Yao Z, Xing L. Functions of nuclear factor kappaB in bone. *Ann N Y Acad Sci.* 2010; 1192:367–375. [PubMed: 20392262]
7. Vaira S, Alhawagri M, Anwisye I, Kitaura H, Faccio R, Novack DV. RelA/p65 promotes osteoclast differentiation by blocking a RANKL-induced apoptotic JNK pathway in mice. *J Clin Invest.* 2008; 118:2088–2097. [PubMed: 18464930]
8. Hall TJ, Schaublin M, Jeker H, Fuller K, Chambers TJ. The role of reactive oxygen intermediates in osteoclastic bone resorption. *Biochem Biophys Res Commun.* 1995; 207:280–287. [PubMed: 7857277]
9. Yamaguchi M, Weitzmann MN. Vitamin K2 stimulates osteoblastogenesis and suppresses osteoclastogenesis by suppressing NF-kappaB activation. *Int J Mol Med.* 2011; 27:3–14. [PubMed: 21072493]
10. Feng R, Anderson G, Xiao G, et al. SDX-308, a nonsteroidal anti-inflammatory agent, inhibits NF-kappaB activity, resulting in strong inhibition of osteoclast formation/activity and multiple myeloma cell growth. *Blood.* 2007; 109:2130–2138. [PubMed: 17095620]
11. Clohisy JC, Hirayama T, Frazier E, Han SK, Abu-Amer Y. NF-kB signaling blockade abolishes implant particle-induced osteoclastogenesis. *J Orthop Res.* 2004; 22:13–20. [PubMed: 14656654]
12. Strait K, Li Y, Dillehay DL, Weitzmann MN. Suppression of NF-kappaB activation blocks osteoclastic bone resorption during estrogen deficiency. *Int J Mol Med.* 2008; 21:521–525. [PubMed: 18360699]
13. Dai S, Hirayama T, Abbas S, Abu-Amer Y. The IkappaB kinase (IKK) inhibitor, NEMO-binding domain peptide, blocks osteoclastogenesis and bone erosion in inflammatory arthritis. *J Biol Chem.* 2004; 279:37219–37222. [PubMed: 15252035]
14. Nanes MS. Tumor necrosis factor- α : molecular and cellular mechanisms in skeletal pathology. *Gene.* 2003; 321:1–15. [PubMed: 14636987]
15. Alles N, Soysa NS, Hayashi J, et al. Suppression of NF-kappaB increases bone formation and ameliorates osteopenia in ovariectomized mice. *Endocrinology.* 2010; 151:4626–4634. [PubMed: 20810563]
16. Chang J, Wang Z, Tang E, et al. Inhibition of osteoblastic bone formation by nuclear factor-kappaB. *Nat Med.* 2009; 15:682–689. [PubMed: 19448637]
17. Yamaguchi M, Weitzmann MN. The estrogen 17 β -estradiol and phytoestrogen genistein mediate differential effects on osteoblastic NF-kappaB activity. *Int J Mol Med.* 2009; 23:297–301. [PubMed: 19148557]
18. Yamaguchi M, Weitzmann MN. The bone anabolic carotenoids p-hydroxycinnamic acid and beta-cryptoxanthin antagonize NF-kappaB activation in MC3T3 preosteoblasts. *Mol Med Report.* 2009; 2:641–644.
19. Yamaguchi M, Arbiser JL, Weitzmann MN. Honokiol stimulates osteoblastogenesis by suppressing NF-kappaB activation. *Int J Mol Med.* 2011; 28:1049–1053. [PubMed: 21887456]
20. Yamaguchi M, Weitzmann MN. Quercetin, a potent suppressor of NF-kappaB and Smad activation in osteoblasts. *Int J Mol Med.* 2011; 28:521–525. [PubMed: 21769418]
21. Berrios RL, Arbiser JL. Effectiveness of gentian violet and similar products commonly used to treat pyoderma. *Dermatol Clin.* 2011; 29:69–73. [PubMed: 21095530]
22. Perry BN, Govindarajan B, Bhandarkar SS, et al. Pharmacologic blockade of angiopoietin-2 is efficacious against model hemangiomas in mice. *J Invest Dermatol.* 2006; 126:2316–2322. [PubMed: 16741507]

23. Zhang X, Zheng Y, Fried LE, et al. Disruption of the mitochondrial thioredoxin system as a cell death mechanism of cationic triphenylmethanes. *Free Radic Biol Med*. 2011; 50:811–820. [PubMed: 21215310]
24. Yamaguchi M, Weitzmann MN. Zinc stimulates osteoblastogenesis and suppresses osteoclastogenesis by antagonizing NF-kappaB activation. *Mol Cell Biochem*. 2011; 355:179–186. [PubMed: 21533765]
25. Sugimoto E, Yamaguchi M. Anabolic effect of genistein in osteoblastic MC3T3-E1 cells. *Int J Mol Med*. 2000; 5:515–520. [PubMed: 10762655]
26. Li JY, Tawfeek H, Bedi B, et al. Ovariectomy disregulates osteoblast and osteoclast formation through the T-cell receptor CD40 ligand. *Proc Natl Acad Sci U S A*. 2011; 108:768–773. [PubMed: 21187391]
27. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-Delta Delta C(T))} Method. *Methods*. 2001; 25:402–408. [PubMed: 11846609]
28. Docampo R, Moreno SN. The metabolism and mode of action of gentian violet. *Drug Metab Rev*. 1990; 22:161–178. [PubMed: 2272286]
29. Hinton D. Results of the intravenous use of gentian violet in cases of extreme septicaemia. *Ann Surg*. 1925; 81:687–692. [PubMed: 17865227]
30. Littlefield NA, Blackwell BN, Hewitt CC, Gaylor DW. Chronic toxicity and carcinogenicity studies of gentian violet in mice. *Fundam Appl Toxicol*. 1985; 5:902–912. [PubMed: 4065463]
31. Arbiser JL. Gentian violet is safe. *J Am Acad Dermatol*. 2009; 61:359. [PubMed: 19615548]
32. Franzoso G, Carlson L, Xing L, et al. Requirement for NF-kappaB in osteoclast and B-cell development. *Genes Dev*. 1997; 11:3482–3496. [PubMed: 9407039]
33. Iotsova V, Caamano J, Loy J, Yang Y, Lewin A, Bravo R. Osteopetrosis in mice lacking NF-kappaB1 and NF-kappaB2. *Nat Med*. 1997; 3:1285–1289. [PubMed: 9359707]
34. Boyce BF, Xing L, Franzoso G, Siebenlist U. Required and nonessential functions of nuclear factor-kappa B in bone cells. *Bone*. 1999; 25:137–139. [PubMed: 10423039]
35. Romas E, Gillespie MT, Martin TJ. Involvement of receptor activator of NFkappaB ligand and tumor necrosis factor-alpha in bone destruction in rheumatoid arthritis. *Bone*. 2002; 30:340–346. [PubMed: 11856640]
36. Xing L, Bushnell TP, Carlson L, et al. NF-kappaB p50 and p52 expression is not required for RANK-expressing osteoclast progenitor formation but is essential for RANK- and cytokine-mediated osteoclastogenesis. *J Bone Miner Res*. 2002; 17:1200–1210. [PubMed: 12096833]
37. Novack DV, Yin L, Hagen-Stapleton A, et al. The IkappaB function of NF-kappaB2 p100 controls stimulated osteoclastogenesis. *J Exp Med*. 2003; 198:771–781. [PubMed: 12939342]
38. Vaira S, Johnson T, Hirbe AC, et al. RelB is the NF-kappaB subunit downstream of NIK responsible for osteoclast differentiation. *Proc Natl Acad Sci U S A*. 2008; 105:3897–3902. [PubMed: 18322009]
39. Janssens K, Ten Dijke P, Janssens S, Van Hul W. Transforming Growth Factor- β 1 to the Bone. *Endocr Rev*. 2005; 26:743–774. [PubMed: 15901668]
40. Wan M, Cao X. BMP signaling in skeletal development. *Biochem Biophys Res Commun*. 2005; 328:651–657. [PubMed: 15694398]
41. Eliseev RA, Schwarz EM, Zuscik MJ, O'Keefe RJ, Drissi H, Rosier RN. Smad7 mediates inhibition of Saos2 osteosarcoma cell differentiation by NFkappaB. *Exp Cell Res*. 2006; 312:40–50. [PubMed: 16259979]
42. Guo R, Yamashita M, Zhang Q, et al. Ubiquitin ligase Smurf1 mediates tumor necrosis factor-induced systemic bone loss by promoting proteasomal degradation of bone morphogenetic signaling proteins. *J Biol Chem*. 2008; 283:23084–23092. [PubMed: 18567580]
43. Yamaguchi M, Weitzmann MN. The bone anabolic carotenoid beta-cryptoxanthin enhances transforming growth factor-beta1-induced SMAD activation in MC3T3 preosteoblasts. *Int J Mol Med*. 2009; 24:671–675. [PubMed: 19787201]
44. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell*. 1997; 89:747–754. [PubMed: 9182762]

45. Nakashima K, Zhou X, Kunkel G, et al. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell*. 2002; 108:17–29. [PubMed: 11792318]

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Fig. (1).

GV promotes osteoblast differentiation and mineralization and alleviates the suppression of osteoblast mineralization induced by TNF α *in vitro*. (**A**) MC3T3 cells were cultured in the presence (+) or absence (-) of mineralizing medium (MM) with a dose range of GV (0, 0.001, 0.01, 0.1 or 0.5 μ M), and (**B**) GV (0, 0.001, 0.01, or 0.1 μ M) with or without TNF α (5 ng/ml). Calcium deposition was visualized by Alizarin Red S staining at 14 days (**A**) or 18 days (**B**) of culture. Each data point was performed in duplicate. All wells within each experiment were from the same plate but were digitally separated and reorganized for clarity with non-contiguous wells separated by a white space to indicate this fact.

Fig. (2).

GV induces expression of key osteoblastic transcription factors and gene products in MC3T3 cells. Gene expression of the key osteoblastic transcription factors (**A**) Osterix and (**B**) Runx2 were quantified by Real-time RT-PCR at 6 and 24 hr after stimulation with GV (0.1 μM) in MC3T3 cells cultured in mineralizing medium. The osteoblastic gene products (**C**) type I collagen, (**D**) bone sialoprotein and (**E**) osteocalcin were quantified at 24 hr under the same conditions. *p<0.001, **p<0.001, ***p<0.001 vs untreated control (0 μM GV). Data are presented as Mean ± SEM of 5 replicate wells per data point processed independently and is representative of 2 independent experiments.

Fig. (3).

GV suppresses osteoclast differentiation *in vitro*. **(A)** RAW264.7 cells were treated with RANKL (30 ng/ml) in the presence or absence of a dose range of GV (0, 0.01, 0.1, 0.5 or 1 μM) for 7 days and TRAP stained. TRAP+ multinucleated cell (3 or more nuclei) were quantitated and averaged for 5 independent wells for each data point. Data are representative of 2 independent experiments. *** $P < 0.001$ relative to no GV (grey bar). **(B)** Photomicrographs of a representative field for each condition presented in panel A were taken under bright field microscopy at 200 X magnification. **(C)** RAW264.7 cells were cultured with a dose range of GV (0, 0.1 or 0.5 μM) in the absence of RANKL and cultures photographed after 7 days under bright field microscopy at 200 X magnification.

Fig. (4).

GV suppresses early osteoclast differentiation and fusion *in vitro*. **(A)** RAW264.7 cells were cultured in the presence or absence of RANKL (30 ng/ml) for 7 days and TRAP stained. GV (0.1 μM) was added to some wells for 7 of 7 days, the last 5 of 7 days or the last 3 of the 7 days of culture. Cells TRAP+ multinucleated cell (3 or more nuclei) were quantitated and averaged for 5 independent wells for each data point. Data are representative of 4 independent experiments. ***P < 0.001 relative to RANKL with no GV (grey bar). **(B)** Photomicrographs of a representative field for each condition presented in panel **A** were taken under bright field microscopy at 100 X magnification.

Fig. (5).

GV induces apoptosis at high, but not low bioactive dose. RAW264.7 cells were cultured in the presence or absence of a dose range of GV (0, 0.01, 0.1, 0.5 or 1 μM) for 7 days and apoptosis quantified at 24 h (1 day), 3 days and 7 days of culture by Annexin V flow cytometry. Apoptotic (Annexin V positive, 7-AAD negative) cells were quantitated and 3 independent wells run individually and averaged for each data point. *P < 0.05 or ****P < 0.0001 relative to respective 0 μM GV control for each time point.

Fig. (6).

GV suppresses basal and TNFα-induced NF-κB activation in osteoblast precursors. **(A)** pNF-κB-Luc, an NF-κB activity reporter plasmid was transfected into MC3T3 cells and cultures treated with GV (0, 0.1, 0.5, and 1 μM). *p<0.05, ***p<0.001 vs Control (white bar). **(B)** MC3T3 cells were transfected with pNF-κB-Luc, and cells treated with GV (0, 0.1, 0.5, and 1 μM) in the presence or absence of TNFα (1 ng/ml). *p<0.05, ***p<0.001 vs TNFα stimulated (grey bar). Data are presented as Mean ± SD of 5 replicate wells per data point and is representative of 2 independent experiments.

Fig. (7).

Effect of GV on basal or TGF β or BMP2-induced Smad activity. MC3T3 cells were transfected with pGL3-Smad luciferase reporter plasmid, and the effect of GV (0, 0.01, 0.1, and 1 μ M) on (A) basal Smad-induced luciferase activity. (B) Effect of GV (0, 0.01, 0.1, 0.5 and 1 μ M) on (B) TGF β -stimulated, and (C) BMP2-stimulated Smad-activation. ** $p < 0.01$ or *** $p < 0.001$ vs TGF β or BMP2-stimulated (grey bar). Data are presented as Mean $\pm$ SD of 5 replicate wells per data point and is representative of 2 independent experiments.

Fig. (8).

GV alleviates TNFα-induced suppression of TGFβ-induced Smad activation. MC3T3 cells were transfected with pGL3-Smad luciferase reporter plasmid, and cells treated with (A) TGFβ (1 ng/ml) or (B) BMP2 (0.5 μg/ml), in the presence or absence of GV (0.01, 0.1, 0.5, or 1 μM) and/or TNFα (1 ng/ml). *p<0.05, **p<0.01, ***p<0.001 vs Control (grey bar) or as indicated. Data are presented as Mean ± SD of 5 replicate wells per data point and is representative of 2 independent experiments.

Fig. (9).

GV suppresses RANKL-induced NF-κB activation in osteoclast precursors. pNF-κB-Luc, an NF-κB activity reporter plasmid was transfected into RAW264.7 cells and cultures treated with (A) GV (0, 0.01, 0.1 and 1 μM). * $p < 0.05$, vs Control (white bar). (B) RAW264.7 cells were transfected with pNF-κB-Luc, and cells treated with GV (0, 0.1, 0.5, 1, and 1 μM) in the presence or absence of RANKL (30 ng/ml). *** $p < 0.001$ vs RANKL stimulated (grey bar). Data are presented as Mean $\pm$ SD of 5 replicate wells per data point and is representative of 2 independent experiments.