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9-Demethoxy-medicarpin promotes peak bone mass achievement and has bone conserving effect in ovariectomized mice: Positively regulates osteoblast functions and suppresses osteoclastogenesis

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ABSTRACT

We report a new bone anabolic and anti-catabolic pterocarpan 9-demethoxy-medicarpin (DMM) for the management of postmenopausal osteoporosis. DMM promoted osteoblast functions via activation of P38MAPK/BMP-2 pathway and suppressed osteoclastogenesis in bone marrow cells (BMCs). In calvarial osteoblasts, DMM blocked nuclear factor kappaB (NFκB) signaling and inhibited the mRNA levels of pro-inflammatory cytokines. DMM treatment led to increased OPG (osteoprotegrin) and decreased transcript levels of TRAP (tartarate resistant acid phosphatase), RANK (receptor activator of NFκB) and RANKL (RANK ligand) in osteoblast–osteoclast co-cultures. Immature female SD rats administered with DMM exhibited increased bone mineral density, bone biomechanical strength, new bone formation and cortical bone parameters. Ovx mice administered with DMM led to significant restoration of trabecular microarchitecture and had reduced formation of osteoclasts and increased formation of osteoprogenitor cells in BMCs. DMM exhibited no uterine estrogenicity. Overall, these results demonstrate the therapeutic potential of DMM for the management of postmenopausal osteoporosis.

1. Introduction

Osteoporosis and related bone metabolic diseases have emerged as leading health care issues worldwide. Osteoporosis is characterized by low bone mass and/or micro-architectural deterioration of bone tissue resulting in the bone fragility and a consequent increment in fracture risks. The overall weakening of bone that results in osteoporosis is caused by a shift in the equilibrium of bone remodeling in which bone formation by osteoblasts is overtaken by the resorption of bone by osteoclasts. Therefore new agents that may stimulate bone anabolic action of osteoblast and/or retard the function of osteoclast in osteoporotic patients are indeed a need of today's world (Allen et al., 2010; Kanakamani and Tandon, 2008; Rodan and Martin, 2000).

Current drugs to rectify the functioning of osteoblast–osteoclast imbalance are divided into two distinct mechanistic classes. The first class is the anti-resorptives, which directly attenuate osteoclast activity (Lopez, 2000), thereby slowing the progression of resorption. This class is exemplified by bisphosphonates (Francis, 1995; Sato et al., 1999) selective estrogen receptor modulators (SERMs) like raloxifene (Mitlak and Cohen, 1995) and estrogens hormone replacement therapy (HRT) (Turner et al., 1994). All of these anti-resorptive agents have proven efficacy in humans for the management of osteoporosis, but are associated with serious side effects and offer little in terms of stimulating the formation of new bone (Cianferotti et al., 2013). The second mechanistic class for the treatment of osteoporosis is the osteogenic (bone forming) agents, which promote osteoblast activity allowing these cells to keep pace with or outperform bone resorbing osteoclasts (Bilezikian, 2008; Deal, 2009). These therapies include parathyroid hormone (PTH) (Nycomed) and its N-terminal 1–34 amino acid fragment teriparatide (Lilly) (Berg et al., 2003; Eriksen and Robins, 2004; Girotra et al.,

Abbreviations: DMM, 9-demethoxy-medicarpin; BMD, bone mineral density; MAR, mineral apposition rate; BFR, bone formation rate; NFkB, nuclear factor kappa B; OPG, osteoprotegrin; TRAP, tartarate resistant acid phosphatase; RANK, receptor activator of NFkB; SERM, selective estrogen receptor modulators; PTH, parathyroid hormone; MAPK, mitogen activated protein kinases; ALP, alkaline phosphatase; OVx, ovariectomized; TFSP, tibio-fibula separating point; μ-CT, micro computed tomography; B.Ar, cortical mean cross section; BV/TV, percentage bone volume; Cs.Th, cross sectional cortical thickness; T.Ar, periosteal area; T.Pm, periosteal perimeter; BMCs, bone marrow cells; M-CSF, macrophage-colony stimulating factor; TNF, tumor necrosis factor; RANKL, receptor activator of nuclear factor κB ligand; ER, estrogen receptor; Osx, osterix; OCN, osteocalcin.

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2006; Mohan et al., 2000). Maximum duration for teriparatide is recommended to 2 years because of osteosarcoma risk. Also use of teriparatide is limited to severe osteoporosis because of the high cost of the treatment.

Strontium ranelate (Protelos/Osseor) (Marie, 2007) has a dual mode of action, whereby it increases bone formation and decreases resorption, however the exact mechanism is not known. The treatment of peripheral osteoporosis (TROPOS) trial showed a reduction of non-vertebral fracture and hip fracture in women at high risk of hip fractures. Long term treatment was safe and associated with sustained increase in BMD. However it is contraindicated in patients at high risk of thromboembolic events. Other new therapeutic targets include cathepsin K inhibitors like odanacatib and antisclerostin antibodies (Kawai et al., 2011).

Nature is a phenomenal source of biologically active molecules such as flavonoids and iso-flavonoids, which are plant estrogens and have bone-conserving effects in the settings of estrogen deficiency-induced and aging-induced bone loss (Coxam, 2008). Based on these facts, ipriflavone, an isoflavone synthesized from the soy isoflavone daidzein, was developed for the treatment of osteoporosis (Tsuda et al., 1986). Ipriflavone had been shown to increase the bone's calcium retention, inhibits bone breakdown, promotes the activity of bone-building cells, and reduces the pain of osteoporotic fractures (Alexandersen et al., 2001). It has been approved for the treatment of involutional osteoporosis in few European countries and in Japan with the trade names Iprosten, Osteofix, and Osten.

During the drug development program for the management of osteoporosis, some of us revealed osteoprotective effects of extracts of the stem-bark of *Butea monosperma* in ovariectomized rats (Bhargavan et al., 2009; Pandey et al., 2010; Tyagi et al., 2010). Activity-guided analysis of the extracts revealed the presence of four methoxyisoflavonoids and a pterocarpan medicarpin, which were found to stimulate osteoblast functions via various mitogen activated protein kinases (MAPK) (Pandey et al., 2010; Tyagi et al., 2010). Of these compounds, medicarpin promoted enhancement of peak bone mass in female 21d old Sprague–Dawley rats (Bhargavan et al., 2012; Tyagi et al., 2012). Medicarpin possesses a hydroxy group at position 3 and a methoxy substituent at position 9 on the pterocarpan scaffold. In order to understand the role of 9-methoxy substituent on the bone-anabolic action of medicarpin, we recently devised a convenient general methodology for the synthesis of natural and synthetic pterocarpans and a protocol to resolve their respective enantiomers and determined their absolute configuration (Goel et al., 2012, 2013, 2014).

In this paper, we report synthesis of a new pterocarpan, 9-demethoxy-medicarpin (DMM), which exhibits bone anabolic and anti-catabolic activity. We further assess its efficacy on peak bone mass achievement in growing female SD rat model by assessing various skeletal parameters like bone mineral density, bone biomechanical strength, bone microarchitecture and new bone formation rate. Furthermore, we determine the bone conserving efficacy of DMM in Ovx female Balb/c mice model.

2. Materials and methods

2.1. Materials

Cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA). All fine chemicals were purchased from Sigma Aldrich (St. Louis, MO). BMP-2 ELISA kit was purchased from R&D Systems. ECL kit was purchased from Amersham Pharmacia, USA. All antibodies for western blot analysis were obtained from Cell Signaling Technologies, USA. BrdU ELISA kit was procured from Roche (USA). HPLC grade acetonitrile and isopropanol were purchased from Sisco Research Laboratories (SRL) Pvt. Limited (Mumbai, India); n-hexane, ammonium acetate and glacial acetic acid AR was obtained from E-Merck Limited (Mumbai, India).

2.2. Methods

2.2.1. Culture of calvarial osteoblasts

Rat calvarial osteoblasts were obtained following our previously published protocol (Bhargavan et al., 2009). Briefly, calvaria from 1- to 2-day-old Sprague–Dawley rats were pooled. After surgical isolation from the skull and the removal of sutures and adherent mesenchymal tissues, calvaria were subjected to five sequential digestions (10–15 min) at 37 °C in a solution containing 0.1% dispase and 0.1% collagenase P. Cells released from the second to fifth digestions were pooled, centrifuged, resuspended and plated in T-25 cm^2 flasks in α-MEM containing 10% FCS and 1% penicillin/streptomycin (complete growth medium).

2.2.2. Osteoblast differentiation

For the measurement of alkaline phosphatase (ALP) activity, osteoblasts at ~80% confluence were trypsinized and 2×10^3 cells/well were seeded in 96-well plates. Cells were treated with different concentrations of DMM for 48 h in α-MEM supplemented with 5% charcoal treated FCS, 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid and 1% penicillin/streptomycin (osteoblast differentiation medium). At the end of incubation period, total ALP activity was measured using p-nitrophenylphosphate (PNPP) as substrate and quantitated colorimetrically at 405 nm.

2.2.3. Mineralization of bone marrow derived osteoblast cells

For mineralization studies, bone marrow cells were cultured in medium consisting of α-MEM, supplemented with 10% fetal bovine serum, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate. Cells were cultured with and without DMM for 21 days at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air, and the medium was changed every 48 h. After 21 days, the attached cells were fixed in 4% formaldehyde for 20 min at room temperature and rinsed once in PBS. After fixation, the specimens were processed for staining with 40 mM Alizarin Red-S, which stains areas rich in nascent calcium. BMP2 was used as a positive control. For quantification of alizarin red-S staining, 800 μl of 10% (v/v) acetic acid was added to each well, and plates were incubated at room temperature for 30 min with shaking. The monolayer, now loosely attached to the plate, was then scraped with a cell scraper and transferred with 10% (v/v) acetic acid to a 1.5-ml tube. After vortexing for 30 s, the slurry was overlaid with 500 μl mineral oil (Sigma–Aldrich), heated to exactly 85 °C for 10 min, and transferred to ice for 5 min. The slurry was then centrifuged at $20{,}000 \times g$ for 15 min and 500 μl of the supernatant was removed to a new tube. Then 200 μl of 10% (v/v) ammonium hydroxide was added to neutralize the acid. In some cases, the pH was measured at this point to ensure that it was between 4.1 and 4.5. OD (405 nm) of 150 μl aliquots of the supernatant were measured in 96-well format using opaque-walled, transparent-bottomed plates.

For measuring BMP-2 production from osteoblasts, 5 x10^3 cells/well were seeded in 24-well plates. Cells were exposed to 100 pM concentration of DMM for 48 h with or without inhibitors in α-MEM media supplemented with 5% FCS, 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid. At the end of incubation, supernatants were collected for determination of BMP-2 by ELISA as per the manufacturer's instructions. For inhibitor studies, cells were pre-treated with inhibitors 30 min prior to compound treatment.

2.2.4. Total RNA isolation and qPCR

Total RNA was extracted from isolated CD_4^+ T cells and B220+ cells of all the in vivo groups using Trizol (Invitrogen). cDNA was synthesized from 1 μg total RNA with the Revert AidTM H Minus