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# Reversal of loss of bone mass in old mice treated with mefloquine

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ABSTRACT

Aging is accompanied by imbalanced bone remodeling, elevated osteocyte apoptosis, and decreased bone mass and mechanical properties; and improved pharmacologic approaches to counteract bone deterioration with aging are needed. We examined herein the effect of mefloquine, a drug used to treat malaria and systemic lupus erythematosus and shown to ameliorate bone loss in glucocorticoid-treated patients, on bone mass and mechanical properties in young and old mice. Young 3.5-month-old and old 21-month-old female C57BL/6 mice received daily injections of 5 mg/kg/day mefloquine for 14 days. Aging resulted in the expected changes in bone volume and mechanical properties. In old mice mefloquine administration reversed the lower vertebral cancellous bone volume and bone formation; and had modest effects on cortical bone volume, thickness, and moment of inertia. Mefloquine administration did not change the levels of the circulating bone formation markers P1NP or alkaline phosphatase, whereas levels of the resorption marker CTX showed trends towards increase with mefloquine treatment. In addition, and as expected, aging bones exhibited an accumulation of active caspase3-expressing osteocytes and higher expression of apoptosis-related genes compared to young mice, which were not altered by mefloquine administration at either age. In young animals, mefloquine induced higher periosteal bone formation, but lower endocortical bone formation. Further, osteoclast numbers were higher on the endocortical bone surface and circulating CTX levels were increased, in mefloquine- compared to vehicle-treated young mice. Consistent with this, addition of mefloquine to bone marrow cells isolated from young mice led to increased osteoclastic gene expression and a tendency towards increased osteoclast numbers in vitro. Taken together our findings identify the age and bone-site specific skeletal effects of mefloquine. Further, our results highlight a beneficial effect of mefloquine administration on vertebral cancellous bone mass in old animals, raising the possibility of using this pharmacologic inhibitor to preserve skeletal health with aging.

## 1. Introduction

A combination of intrinsic factors, including elevated oxidative stress, increased endogenous glucocorticoid action and low sex steroids [1], and reduced physical activity, lead to bone loss and increased risk of fractures with advanced age. This not only results in considerable morbidity, but also elevated mortality following, for example, hip fractures [2]. As the skeleton ages, there is an imbalance in bone remodeling with bone resorption prevailing over bone formation, resulting in a net loss of bone mass [3]. In addition, bone mechanical properties decrease with aging. Factors associated with aging, have opposite effects on osteoclasts and osteoblasts, resulting in increased differentiation/activity of the bone resorbing cells whereas bone forming cells exhibit increased apoptosis and reduced activity. Osteocyte apoptosis and empty lacunae are also increased with aging and their accumulation is associated with defective bone material properties [4, 5]. Further, osteoclasts accumulate in areas nearby apoptotic osteocytes, but not in the vicinity of empty lacunae, suggesting that dying osteocytes secrete factors that signal for osteoclast recruitment [6–11].

Mefloquine is a member of the quinine-derived family of drugs that also includes chloroquine and hydroxychloroquine, commonly used in patients to treat malaria and systemic lupus erythematosus [12]. As a group, the quinine-derived family of drugs are inhibitors of autophagy [13]. In addition, it has been shown that mefloquine inhibits membrane

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channels such as pannexin 1 (Panx1) [14, 15], L-type calcium channels [16], potassium channels [17], volume-regulated and calcium-activated chloride channels [18], ATP-sensitive potassium channels [19], and connexin channels [20]. Studies in patients have shown that hydroxychloroquine administration results in attenuated spinal bone loss when the drug is administered alongside glucocorticoids [21], an agent that induces bone loss and increases fragility [22]. Additionally, quinine-derived drugs increased spinal and hip bone mineral density in patients with systemic lupus erythematosus [23, 24]. Studies have shown that chloroquine reduces osteoclast numbers in young growing rodents and prevents overiectomy-induced bone loss in older, 8–9-month-old mice [25]. Whether this kind of drug alters the consequences of aging on the skeleton was heretofore unknown.

We hypothesized that by reducing osteoclast numbers mefloquine would ameliorate the deleterious skeletal effects of aging. To test this, we treated young and old mice with mefloquine daily for 2 weeks. Unexpectedly, we found that short-term mefloquine administration did not decrease osteoclastogenesis in old mice and actually increased osteoclast number/activity in young mice. Further, in aged mice mefloquine increased vertebral cancellous bone formation and mass in a sclerostin-independent manner. In addition, our results suggest that mefloquine administration might prevent the consequences of aging on bone strength. These findings raise the possibility of using this pharmacologic inhibitor to improve the cancellous bone mass and strength with aging.

## 2. Materials and methods

### 2.1. Mice and treatment

3.5- (young, $n = 8$–9/group) and 21-month-old (old, $n = 10$/group) C57BL/6 female mice were obtained from National Institute on Aging (NIA) and administered daily intraperitoneal injection of vehicle (1.5% ethanol) or 5 mg/kg/day of mefloquine (BioBlocks Inc., San Diego, CA, USA, cat.# QU024-1) for 14 days [26]. Mice were assigned an ID number and the age and treatment were recorded in a database. Investigators performing endpoint measurements were only given the mouse IDs, thus blinded to treatment and age. Mice were randomized and assigned to each experimental group based on matching spinal BMD. Animals were sacrificed 4–6 h after receiving the last injection. Mice (5/cage) were fed a regular diet (Envigo, Indianapolis, IN) and water ad libitum, and maintained on a 12 h light/dark cycle. All experiments were carried out as planned, with no adverse effects resulting from treatments. The mice received intraperitoneal injections of calcein (30 mg/kg; Sigma-Aldrich, Saint Louis, MO, USA) and alizarin red (50 mg/kg; Sigma) 7 and 2 days before sacrifice, respectively, to allow for dynamic histomorphometric measurements [27].

### 2.2. Micro-computed tomography (μCT) analysis

Lumbar vertebrae (L4) and femora were dissected, cleaned of soft tissue and wrapped in PBS-soaked gauze and frozen at −20 °C until imaging [28]. Bones were scanned using 50 kV source, 120 mA, 151 milliseconds integration time, and 10 μm voxel resolution on a μCT-35 (Scanco Medical AG, Brüttisellen, Switzerland). Scans were reconstructed and analyzed using manufacturer software. The following parameters were obtained for the cancellous bone of the lumbar vertebrae: trabecular bone volume per total volume (BV/TV, %), trabecular number (Tb.N, mm-1), trabecular thickness (Tb.Th, mm), and trabecular spacing (Tb.Sp, mm). For cortical bone of the femoral diaphysis, the following parameters were obtained: bone area/total area (%), marrow cavity area ($mm^2$), cortical thickness (mm), and polar moment of inertia (Ip, mm4). Nomenclature is reported in accordance with suggested guidelines for μCT [29].

### 2.3. Bone histomorphometry

Lumbar vertebrae (L1-L3) and femora and were dissected and fixed in 10% neutral buffered formalin [28]. Dynamic histomorphometric analysis of unstained methyl methacrylate embedded L1-L3 vertebra longitudinal sections, avoiding the primary spongiosa, and femoral mid-diaphysis cross-sections was performed using an epifluorescence microscope. Static histomorphometric analysis was performed on decalcified, paraffin-embedded femoral mid-diaphysis cross-sections (for osteoclasts) and undecalcified plastic-embedded L1-L3 vertebra longitudinal sections (for osteoclasts and osteoblasts). Sections were stained for TRAP/Toluidine blue and von Kossa/McNeal in order to visualize osteoclasts and osteoblasts, respectively. Histomorphometric analysis was performed using the OsteoMeasure high resolution digital video system (OsteoMetrics Inc., Decatur, GA, USA). The terminology and units used are those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research (ASBMR) [29].

### 2.4. Biomechanical testing

Three-point bending testing of the femoral mid-diaphysis was performed following previously published protocols [30]. Briefly, bones were thawed to room temperature, hydrated in 0.9% saline, and loaded to failure at 2 mm/min with force versus displacement data collected at 10 Hz using a servo-hydraulic test system (TestResources Inc., Shakopee, MN, USA). Femora were loaded to failure in an anterior–posterior direction with the upper contact area at the mid-diaphysis (50% total bone length) and the bottom contact points centered around this point and separated by 8 mm. Whole bone mechanical properties (load, displacement, stiffness, energy) were derived from the load-displacement curves. Cross-sectional moment of inertia and anterior–posterior diameter were determined by μCT and were used to calculate estimated material-level properties, as previously described [30].

### 2.5. Immunohistochemistry for active caspase-3

Decalcified, paraffin-embedded femoral mid-diaphysis cross-sections were deparaffinized, treated with 3% $H_2O_2$ to inhibit endogenous peroxidase activity, blocked and then incubated with rabbit monoclonal anti-active caspase-3 antibody (1:75; Thermo Fisher Scientific In., Rockford, IL, USA, cat.#PA5-23921) [31]. Sections were then incubated with anti-rabbit biotinylated secondary antibody followed by avidin conjugated peroxidase (Vectastain Elite ABC Kit; Vector Laboratories Inc., Burlingame, CA, USA). Colour was developed with a diaminobenzidine substrate chromogen system (Acros Organics, New Jersey, USA). Cells expressing the protein of interest are stained in brown, whereas negative cells are green-blue. Nonimmune IgG was used as a negative control. One section from each mouse at 400× magnification was evaluated.

### 2.6. RNA extraction and real-time PCR (qPCR)

Total RNA was isolated using TRIzol (Invitrogen, Grand Island, NY, USA), as previously published [32]. Reverse transcription was performed using a high-capacity cDNA kit (Applied Biosystems, Foster City, CA, USA). qPCR was performed using the Gene Expression Assay Mix TaqMan Universal Master Mix and an ABI 7900HT real-time PCR system. The house-keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used. Primers and probes were commercially available (Applied Biosystems, Foster City, CA, USA) or were designed using the Assay Design Center (Roche Applied Science, Indianapolis, IN, USA). Relative expression was calculated using the ΔCt method [33].