A Novel Multifunctional Therapeutic Strategy using P7C3 as a Countermeasure Against Bone Loss and Fragility in an Ovariectomized Rat Model of Postmenopausal Osteoporosis

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Introduction. Osteoporosis is a common, systemic, chronic, and progressive metabolic bone disease characterized by the gradual deterioration of bony architecture and composition, leading to decreased bone density and an increased risk of fractures. By 2060, it is projected that one in four Americans will be elderly (≥65 years), the number of our oldest-old (≥85 years) will triple, and the country will add 500,000 centenarians. Consequently, the prevalence of osteoporosis, the incidence of fragility fractures, and the risk of adverse health outcomes will also undoubtedly increase. Presently, no available pharmacological or non-pharmacological interventions definitively manage or prevent osteoporosis, as all have their disadvantages. Thus, there is an urgent need to develop effective prevention and treatment strategies to mitigate this major public health issue. Here, we explore the small molecule pool 7 compound 3 (P7C3) as a novel strategy for reducing progressive bone loss and fragility following the onset of ovariectomy (OVX)-induced osteoporosis in a rat model.

Methods. The elemental composition, and chemical analysis of P7C3 was carried out using X-ray photoelectron spectroscopy. Osteoclast maturation and activity was quantified using RANKL and TRAP analyses. hBMSC metabolic activity, morphology, osteogenic, and adipogenic differentiation were assessed using ALP, Alizarin Red, and qRT-PCR *in vitro*. Thirty female Sprague Dawley rats 8-9 weeks of age (~200g) underwent ovariectomy and were randomly assigned into one of five experimental groups (n=6): (i) sham control, (ii) OVX-operated, (iii) OVX-operated + DMSO vehicle control, (iv) OVX-operated + 17β-Estradiol, and (v) OVX-operated + P7C3 at a dose of 20 mg/kg. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Central Florida (protocol 2023-24) and were carried out following the guidelines of the American Veterinary Medical Association. Differences in body weight were recorded weekly. Tibiae were nanoCT scanned, biomechanical strength assessed (ultimate, fracture, and yield stress), and histological sections through the distal femur were examined using immunohistochemical techniques and staining ($RANKL^+$, and $TRAP^+$ cells). Bone marrow adiposity as well as serum levels of CTX-1 were quantified, and serum proteomics evaluated. Finally, alterations in the metatranscriptomic analyses of the gut microbiota (GM) were assessed. Statistical analysis was carried out using GraphPad Prism (version 8.0, US) and groups compared using the nonparametric Mann-Whitney test. p values < 0.05 were considered significant.

Results. Our *in vitro* analyses showed that P7C3 was non-cytotoxic and significantly upregulated both hBMSC osteogenic differentiation, as well as the deposition of bone mineral by mature osteoblasts. Controlling the adipo-osteogenic lineage commitment of BMSCs in favor of osteogenesis at the expense of adipogenesis, is considered a promising new strategic approach to promote bone regeneration and repair. To this end, treatment with P7C3 led to increased osteogenic differentiation parallel to a decrease in adipogenic differentiation. This was evidenced by increased ALP staining and mineralization, together with decreased lipid droplet staining and expression of adipogenesis-related genes. P7C3 also significantly decreased osteoclast maturation from osteoclastic progenitor cells as well as their activity. Further, our *in vivo* results reveal P7C3 inhibited osteoclastic activity, bone marrow adiposity, and whole-body weight gain, leading to maintained bone area, architecture, and mechanical strength, despite ovariectomy. The mechanistic novelty is based on P7C3 significantly increasing serum protein levels of MSP, IL-5, PDGF-BB, LIF, with downregulation of IL-1 R6, Galectin-3, and TNFRSF11A (RANK), as well as tyrosine kinase receptor modulation (including, FGFR3, IGF-1R, Ryk, and EphB2). Together, several pivotal biomolecules involved in inflammation resolution, bone, and adipose homeostasis, and osteoporosis were targeted. Notably, and in the GM, P7C3 increased the relative abundance of *Porphyromonadaceae* bacterium, an anti-inflammatory bacterial species reportedly able to directly alter adipo-osteogenic interactions within the host, and Candidatus Melainabacteria, also a lipid regulator. Levels of *Ruminococcaceae* bacterium, an important contributor to metabolic and immune function, and where low levels are associated with the elderly, frail, as well as heightened osteoclastic activity in an OVX animal model, was also increased by P7C3. To the best of our knowledge, this is first evidence of an intervention that drives against bone loss by contributing to a reduction in the expression of RANK, as well the use of P7C3 as a novel method to attenuate osteoporosis.

Discussion. We have identified a promising therapeutic approach for mitigating the loss in biomechanical strength, microarchitectural structure, and bone area caused by OVX-induced osteoporosis using P7C3, an aminopropyl carbazole anti-apoptotic agent. Data suggests a pivotal characteristic is in modulating adipo-osteogenic lineage commitment, and in regulating JAK/STAT, NF-κB and Wnt/β-catenin signaling. Further, P7C3 altered %abundance within the GM, which may also have beneficially contributed to favorable regulation of inflammation, bone, and adipose metabolism. Consistent with previous studies, our results provide further validation for the safety and effectiveness of P7C3 as a therapeutic agent, as we did not observe any histological indications of toxicity in vital organs including the heart, liver, spleen, lung, brain, or kidney up to 13 weeks following treatment.

Significance. Presently, no available pharmacological or non-pharmacological interventions definitively manage or prevent osteoporosis and this study reveals P7C3 as an undiscovered, multifunctional, and versatile therapeutic strategy to prevent the pathological progression of OVX-induced bone loss and frailty in a rodent model.