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The epigenetically active small chemical N-methyl pyrrolidone (NMP) prevents estrogen depletion induced osteoporosis

Bebeka Gjoksi [a,b], Chafik Ghayor [a], Barbara Siegenthaler [a,c], Nisarat Ruangsawasdi [a], Marcy Zenobi-Wong [b], Franz E. Weber [a,c,d,*]

[a] University Hospital, Division of Cranio-Maxillofacial and Oral Surgery and University of Zurich, Center of Dental Medicine, Oral Biotechnology & Bioengineering, Plattenstrasse 11, 8032 Zürich, Switzerland
[b] Cartilage Engineering + Regeneration Laboratory, ETH Zurich, Zurich, Switzerland
[c] Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Switzerland
[d] CABMM, Center for Applied Biotechnology and Molecular Medicine, University of Zurich, Zurich, Switzerland

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ABSTRACT

Currently, there are several treatments for osteoporosis however; they all display some sort of limitation and/or side effects making the need for new treatments imperative. We have previously demonstrated that NMP is a bioactive drug which enhances bone regeneration in vivo and acts as an enhancer of bone morphogenetic protein (BMP) in vitro. NMP also inhibits osteoclast differentiation and attenuates bone resorption.

In the present study, we tested NMP as a bromodomain inhibitor and for osteoporosis prevention on ovariectomized (OVX) induced rats while treated systemically with NMP. Female Sprague–Dawley rats were ovariectomized and weekly NMP treatment was administrated 1 week after surgery for 15 weeks. Bone parameters and related serum biomarkers were analyzed. 15 weeks of NMP treatment decreased ovariectomy-induced gained weight in average by 43% and improved bone mineral density (BMD) and bone volume over total volume (BV/TV) in rat femur on average by 25% and 41% respectively. Moreover, mineral apposition rate and bone biomarkers of bone turnover in the treatment group were at similar levels with those of the Sham group.

Due to the function of NMP as a low affinity bromodomain inhibitor and its mechanism of action involving osteoblasts/osteoclasts balance and inhibitory effect on inflammatory cytokines, NMP is a promising therapeutic compound for the prevention of osteoporosis.

Introduction

Osteoporosis is a skeletal disorder characterized by compromised bone strength that predisposes to increased risk of fracture. It is most often caused by an increase in bone resorption that is not sufficiently compensated for by a corresponding increase in bone formation [1].

Osteoporosis poses a significant public health issue. It is estimated that over 200 million people are affected by osteoporosis worldwide. The number of women with osteoporosis and subsequent fractures is bound to rise as the elderly population increases [2]. Advanced age and early menopause are the best predictors of osteoporosis, but other factors such as; low body weight, diseases, treatments, family history of osteoporosis and inactive lifestyle increase susceptibility to fractures [3]. The cost of osteoporosis-related fractures to the economy is enormous, predicted to escalate to $131.5 billion by 2050 [4]. In Europe alone 22 million women and 5.5 million men are estimated to have osteoporosis [5]. Because of the vast medical and socioeconomic challenges that osteoporosis present worldwide, the need for new treatments is both imperative and pressing.

At the moment, some of the most used therapies for osteoporosis include, but are not limited to bisphosphonates, parathyroid hormone (PTH), and selective estrogen receptor modulators (SERMs). Over the last decade, as bisphosphonates became an established treatment for osteoporosis, potential side effects of patients under this therapy have been reported. The most common include atypical subtrochanteric or femoral shaft fractures and osteonecrosis of the jaw, however, at very low frequency [6]. Also, severe suppression of bone turnover is caused by bisphosphonate usage [7]. Instead of preventing bone resorption, the opposite strategy for treating osteoporosis is the application of anabolic substances such as PTH (1–34) applied in intermittent regime [8]. SERMs have been developed and evaluated for osteoporosis treatment and prevention, including bazedoxifene, lasofoxifene, droloxifene, idoxifene, ormeloxifene, ospemifene, and arzoxifene [9]. Nevertheless, the most crucial feature to define the clinical efficacy of a SERM is generally considered to be endometrial safety [10].

A more recent trend is epigenetic drug discovery showing great potential for new therapies [11]. Epigenetics refers to transmissible

* Corresponding author. Fax: +41 44 634 3140.
E-mail address: franz.weber@zzm.uzh.ch (F.E. Weber).

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changes in gene expression that do not involve changes to the underlying DNA sequence. At least three systems including DNA methylation, histone modification and non-coding RNA (ncRNA)-associated gene silencing are currently considered to initiate and sustain epigenetic change [12]. BET (bromodomain and extraterminal domain) proteins are a group of epigenetic regulators. They were shown to serve as scaffolds for molecular complexes at recognized acetylated histone sites to regulate chromatin accessibility to transcription factors and RNA polymerase [13]. They are considered potential therapeutic targets in many distinct diseases. The role of epigenetics in osteoporosis has just started to be studied. However, it is gradually being postulated as a key concept, as epigenetic mechanisms are involved in interactions between the genome and the environment. The exact relationship between epigenotypes and disease phenotypes is still to be elucidated; it is known that epigenetic marks change during aging, including a global decrease in the abundance of 5-methylcytosines and some histone modification [14]. Since osteoporosis is an age-related disease, it could be speculated that those age-related changes in epigenetic marks participate in the pathophysiology of the disease [15]. Recent studies demonstrated JQ1 (a bromodomain inhibitor developed by the *Structural Genomic Consortium*) to suppress inflammation by a reduction of the inflammatory cytokine release, bone destruction by the inhibition of osteoclast maturation and bone formation by the inhibition of osteoblast maturation [15,16]. The latter activity makes its use for treatment of osteoporosis questionable.

Recently, it was discovered that NMP also exhibits some affinity to bromodomains [17]. Over the last years we showed that N-methyl pyrrolidone (NMP), a small water soluble molecule used as a constituent in FDA-approved medical devices, plays a significant role in the osteoblast and osteoclast differentiation [18,19]. Indeed, NMP enhances BMP-2-induced osteoblast differentiation and bone regeneration and disrupts osteoclast differentiation and bone resorption. Together these results suggest that NMP might act as clinically applicable bromodomain inhibitor and could be used for the prevention or treatment of osteoporosis.

To that end, we tested NMP for its ability to inhibit bromodomain binding for a variety of BET proteins and for the prevention of osteoporosis in ovariectomized (OVX) animals, a well-established animal model mimicking menopause in women and simulating osteoporosis.

Materials and methods

AlphaScreening assay

AlphaScreening assay was performed using recombinant bromodomains and bromodomain ligands or recombinant BET bromodomains and BET ligands from BPS Bioscience (San Diego, USA). The AlphaScreening signal from the assay is correlated with the amount of bromodomain/BET ligand binding to the bromodomain. AlphaScreening signal was measured using EnSpire Alpha 2390 Multilabel reader (Perkin Elmer).

Binding experiments were performed in duplicate. AlphaScreening data were analyzed using the computer software, Graphpad Prism. In the absence of the compound, the AlphaScreening signal (A_t) in each data set was defined as 100% activity. In the absence of the bromodomain/BET ligand, the AlphaScreening signal (A_b) in each data set was defined as 0% activity. The percent activity in the presence of each compound was calculated according to the following equation: % activity $= [(A - A_b) / (A_t - A_b)] \times 100$, where A = AlphaScreening signal in the presence of the compound, A_b = AlphaScreening signal in the absence of the bromodomain/BET Ligand, and A_t = AlphaScreening signal in the absence of the compound. The percent inhibition was calculated according to the following equation: % inhibition = 100 − % activity. Values of % activity versus a series of compound concentrations were then plotted using non-linear regression analysis of Sigmoidal dose–response curve generated with the equation $Y = B+(T - B) / 1 + 10^{((\mathrm{LogEC50} - X) \times \mathrm{Hill\ Slope})}$, where Y = percent activity, B = minimum percent activity, T = maximum percent activity, X = logarithm of compound and Hill Slope = slope factor or Hill coefficient. The IC50 value was determined by the concentration causing a half-maximal percent activity.

Assay of ALP activity and ALP staining

Alkaline phosphatase activity was measured as a marker of osteoblastic differentiation. C2C12 cells were purchased from American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics (100 U/ml penicillin G and 100 mg/ml streptomycin). For the experiments, the cells were plated one day before treatment and treated with BMP2 in the presence or absence of different agents. C2C12 cells were seeded at a density of 5×10^4 cells/cm^2 in 24-well plates ($n = 3$ per group) for ALP staining or in 96-well plates ($n = 4$ per group) for ALP activity. One day later, cells were treated as indicated in the figure, and incubation was continued for 5 more days. After 5 days of incubation, medium was removed, and cells were washed with PBS and then scraped in buffer A (0.56 M 2-amino-2-methyl-1-propanol). The pellets were then homogenized for 10 s. After centrifugation, supernatant was collected and used for ALP assay using p-nitrophenylphosphate as a substrate. The protein content of the lysates was measured using Bradford protein assay reagent (Bio-Rad). Experiments were performed independently in triplicate. To examine alkaline phosphatase activity histochemically, cells were fixed for 10 min with 3.7% formaldehyde at room temperature. After washing with PBS, the cells were stained as described in Ref. [20]. Images of stained cells were captured with a CCD camera.

Osteoporosis rat model and treatments

15 week old healthy female Sprague–Dawley (SD) rats (wt. 230 ± 10 g) were obtained from Charles River laboratories. The rats were adapted to laboratory environment for 2 weeks before the experiment. In 3 independent experiments a total of 30 animals were used. The acclimatized rats underwent either bilateral laparotomy (Sham Veh, $N_{total} = 10$) or bilateral ovariectomy (OVX, $N_{total} = 20$). One week after recovering from surgery, the OVX rats were divided into 2 groups: OVX with vehicle (OVX Veh, $N_{total} = 10$) and OVX with NMP (OVX NMP, 1/3 of LD50 = 105 μl/100 g/week, equals an overall concentration of 10.5 mM, $N_{total} = 10$). Treatment via intraperitoneal injection was initiated 1 week after OVX and lasted for 15 weeks. The body mass of each rat was monitored weekly, and the administered dose was adjusted accordingly. All animal procedures were approved by the Animal Ethics Committee of the local authorities (Canton Zurich, 40/2012). Whole blood sample was collected via abdominal aorta puncture immediately following sacrifice by CO_2 asphyxiation. Then, a serum specimen was harvested after centrifugation (2000 min). Samples were stored at −80 °C until further testing and analysis. Femurs were dissected and the adherent tissue removed before placing the samples in 70% ethanol and later used for bone mineral density (BMD) measurement and trabecular microarchitecture analysis. Liver tissues were also removed and fixed and embedded in paraffin (Sophistolab AG, Muttenz, Switzerland) stained with H&E and analyzed for pathological changes (toxicity).

Microcomputed tomography (μCT) analysis

The rat femur samples were measured with a cone-beam microCT (μCT 100, SCANCO MEDICAL AG, Brüttisellen, Switzerland) at a resolution of 14.8 μm. The reference point was used to define the region of interests (ROI) and the bone was automatically segmented, based on its gray scale value in the CT slices. 200 slices were evaluated for every sample (volume of interests: VOI). The three-dimensional images were reconstructed with the purpose of visualization and display.