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Neuropeptide Y Y2 Antagonist Treated Ovariectomized Mice Exhibit Greater Bone Mineral Density

KL Seldeen^{2,*}, PG Halley^{1,*}, CH Volmar¹, MA Rodríguez³, M Hernandez³, M Pang², SK Carlsson¹, LJ Suva⁴, C Wahlestedt¹, BR Troen², and SP Brothers^{1,**}

¹Center for Therapeutic Innovation and Department of Psychiatry and Behavioral Sciences, University of Miami Miller School of Medicine, Miami, FL USA

²Division of Geriatrics and Palliative Medicine, University at Buffalo and Research Service, Veterans Affairs Western New York Healthcare System, Buffalo, NY USA

³Bruce W. Carter VA Geriatric Research Education and Clinical Center (GRECC), Miami, FL, USA; University of Miami Miller School of Medicine, Miami, FL USA

⁴Department of Orthopaedic Surgery, Centre for Orthopaedic Research, University of Arkansas Medical School, Little Rock, AR USA

Abstract

Osteoporosis, a disease characterized by progressive bone loss and increased risk of fracture, often results from menopausal loss of estrogen in women. Neuropeptide Y has been shown to negatively regulate bone formation, with amygdala specific deletion of the Y2 receptor resulting in increased bone mass in mice. In this study, ovariectomized (OVX) mice were injected once daily with JNJ-31020028, a brain penetrant Y2 receptor small molecule antagonist to determine the effects on bone formation. Antagonist treated mice had reduced weight and showed increased whole-body bone mineral density compared to vehicle-injected mice. Micro computerized tomography (micro-CT) demonstrated increased vertebral trabecular bone volume, connectivity density and trabecular thickness. Femoral micro-CT analysis revealed increased bone volume within trabecular regions and greater trabecular number, without significant difference in other parameters or within cortical regions. A decrease was seen in serum P1NP, a measure used to confirm positive treatment outcomes in bisphosphonate treated patients. C-terminal telopeptide 1 (CTX-1), a blood biomarker of bone resorption, was decreased in treated animals. The higher bone mineral density observed following Y2 antagonist treatment, as determined by whole-body DEXA scanning, is indicative of either enhanced mineralization or reduced bone loss. Additionally, our findings that *ex vivo* treatment of bone marrow cells with the Y2 antagonist did not affect osteoblast and osteoclast formation suggests the inhibitor is not affecting these cells directly, and suggests a central role for

^{**}To Whom Correspondence Should Be Addressed: Shaun P. Brothers, Ph.D., Center for Therapeutic Innovation (CTI), Dept. of Psychiatry and Behavioral Sciences, University of Miami Miller School of Medicine, 1501 NW 10th Ave, BRB #416 (M860), Miami, FL 33136, 305-243-6113 (office), 772-480-0554 (cell), 305-243-2523 (fax), sbrothers@med.miami.edu.

^{*}These authors contributed equally to this work.

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compound action in this system. Our results support the involvement of Y2R signalling in bone metabolism and give credence to the hypothesis that selective pharmacological manipulation of Y2R may provide anabolic benefits for treating osteoporosis.

Keywords

Neuropeptide Y; Y2 receptor; bone; menopause; JNJ-31020028

Introduction

In adults, bone remodelling is necessary to maintain the shape, structure and strength of bone in response to mechanical stress. This homeostatic process occurs when bone tissue is broken down by resorptive osteoclasts and is replaced by bone-forming osteoblasts (Aguila and Rowe, 2005; Raggatt and Partridge, 2010). However, a greater rate of bone resorption and/or a decreased rate of bone formation result in osteoporosis, a disease characterized by progressive bone loss and increased risk of fracture (Duque et al., 2009; Raisz, 1999, 2005; Troen, 2003). Osteoporosis is most prevalent in post-menopausal women, and estrogen has been shown to prevent bone resorption by inhibiting osteoclast activity (Jilka et al., 1992; Kameda et al., 1997; Troen, 2003).

NPY is processed from a 94–95-amino acid prohormone and has been classified into the same family as peptide YY and pancreatic polypeptide (Balasubramaniam, 1997; Tatamoto et al., 1982). Since its original isolation, it has been linked to numerous processes such as inhibiting anxiety and depression (Heilig, 2004), regulating learning-associated synaptic plasticity (Redrobe et al., 2004; Thorsell et al., 2000), and modulating circadian rhythm (Medanic and Gillette, 1993; Yannielli and Harrington, 2001), vascular smooth muscle contraction (Glenn and Duckles, 1994; Pernow and Lundberg, 1988) and pain transmission (Naveilhan et al., 2001; Solway et al., 2011). In humans, NPY mediates its effects by interacting with a diverse family of G-protein coupled receptors (Y1, Y2, Y4, Y5), which are highly expressed across a number of different brain regions and throughout the sympathetic nervous system (Blomqvist and Herzog, 1997; Brothers and Wahlestedt, 2010; Fetissov et al., 2004b; Jacques et al., 1997; Nozdrachev and Masliukov, 2011; Redrobe et al., 2002). While Y1, Y4 and Y5 receptors are generally considered to be post-synaptically expressed, Y2 receptors (Y2R) are primary located pre-synaptically and function as autoreceptors to inhibit NPY release, along with other neurotransmitters such as noradrenaline, glutamate and GABA (Chen and van den Pol, 1996; Chen et al., 1997; King et al., 1999; Martire et al., 1995; Silva et al., 2007; Wahlestedt et al., 1986). Importantly, Y2 receptors are highly abundant in the hypothalamus, where they regulate body weight, feeding, and energy homeostasis (Baldock et al., 2007; Fetissov et al., 2004a; Sainsbury et al., 2002; Sainsbury and Zhang, 2010).

Over the last decade, there has been an increasing body of experimental evidence to suggest that bone remodelling is under central, hypothalamic and amygdalar regulation (for review see (Driessler and Baldock, 2010)). Results from a number of studies, using transgenic mouse models, now contradict the traditional view that bone formation is primarily

controlled by autocrine or paracrine mechanisms (Shi and Baldock, 2012). Altering the signalling of neuropeptide Y (NPY), a 36-amino acid peptide that primarily acts as a neurotransmitter in the brain and autonomic nervous system, can also independently affect bone physiology (Gu et al., 2016; Liu et al., 2016; Xiao et al., 2016) (Baldock et al., 2006; Baldock et al., 2005; Baldock et al., 2002; Dumont et al., 1992; Heilig and Widerlov, 1995). A role for NPY Y2 receptors in bone metabolism was first reported by Baldock and colleagues, who demonstrated increased trabecular bone volume, trabecular number and trabecular thickness in germ line Y2R knockout mice compared to wild-type control animals (Baldock et al., 2002). Y2 receptors are not apparently present in either wild-type mouse bone tissue including osteoblasts, thus excluding the possibility that these changes in bone structure were the result of direct Y2R-mediated effects on bone cells. A central mechanism was confirmed, as selective deletion of hypothalamic Y2 receptors in adult mice also led to an identical two-fold increase in bone volume, relative to experimental control animals (Baldock et al., 2002). Indeed, similar enhancement of bone mass and bone formation has been observed in subsequent studies, thus positioning the Y2 receptor as a potential drug target for enhancing bone formation (Baldock et al., 2006; Lundberg et al., 2007). However, it has also been shown that germ line NPY Y1 receptor knockout mice display a comparable increase in cancellous bone volume, along with greater trabecular number and thickness (Baldock et al., 2007). In contrast, these effects were not centrally mediated, as selective knockout of hypothalamic Y1 receptors did not recapitulate this phenotype (Baldock et al., 2007). Instead the increase in bone volume appeared to be the result of altered direct NPY activity on Y1 receptors expressed on osteoblasts (Baldock et al., 2007). Reduced Y1 receptor expression has also been seen in bone stromal cells isolated from Y2R knockout mice (Lundberg et al., 2007), possibly an adaptive response to an increase in NPY due a complete lack of Y2R-mediated feedback. More recently, a novel inducible Y2 receptor system was used to show central disruption of Y2 signalling in the brain decreases bone mineral content (Qi et al., 2016).

Experimental approaches using selective Y2 receptor antagonists may help to further elucidate this process by minimizing possible complications associated with chronic gene deletion, such as compensatory signalling responses. One peptidomimetic Y2 receptor antagonist, BIIE0246, has been used in this capacity in a limited fashion in the past. However, with the emerging evidence of several off-target impacts, lack of brain penetration and the fact that BIIE0246 is an irreversible inhibitor, such studies have had mixed results (Brothers et al., 2010). Traditionally, NPY receptor selective small molecule drugs were difficult to develop due to lack of good screening systems and functional Y2-assays, although, in recent years, an increasing number of small molecule antagonists have been characterized (Bonaventure et al., 2004; Brothers et al., 2010; Doods et al., 1999; Grouzmann et al., 1997). Of these, JNJ-31020028 is a highly selective Y2-antagonist being sufficiently brain penetrant to achieve very high central receptor inhibition (Cippitelli et al., 2011; Shoblock et al., 2010; Swanson et al., 2011). To assess whether central Y2 receptor inhibition represents a potential therapeutic approach for the treatment of osteoporosis, we examined the effects of systemic Y2 receptor antagonism on bone mass in adult female ovariectomized (OVX) mice, a model of post-menopause characterized by osteopenia and osteoporosis phenotypes.

Materials and methods

Measurement of JNJ-31020028 Activity in Cell Lines

The cAMP biosensor assay was performed as previously described (Brothers et al., 2010; Saldanha et al., 2010). Briefly human embryonic kidney (HEK) 293 cells stably expressing a cyclic nucleotide-gated (CNG) channel and either Y2R or Y1R were cultured in T-175-cm² flasks at 37°C and 95% relative humidity. Cells were plated and maintained in growth medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 25 mM HEPES, 5 mM L-glutamine, 250 µg/ml G418 (Geneticin), 1 µg/ml puromycin, and 1% antibiotic mix containing penicillin, streptomycin, and neomycin. A 10-point dose-response curve with a 1:3 dilution series was used, with compounds being tested in quadruplicate for each point. For JNJ-31020028 and BIIE0246 (Y2R) or BIBP3226 (Y1R), cells were simultaneously treated with NPY peptide at a concentration of 100 nM for all points (antagonist mode).

Animals

All animal work was carried out according to protocols reviewed and approved by University of Miami Institutional Animal Care and Use Committee. Ovariectomized female C57BL/6J mice, 10 weeks of age, were purchased from Jackson Laboratories (Stock 000664; JAX Mice and Services, Bar Harbor, Maine, USA). The mice were housed in groups of five animals per standard open-top mouse cage, and maintained on a regular chow diet and tap water. Both food and water were available *ad libitum*.

JNJ-31020028 was synthesized as described in (Shoblock et al., 2010; Swanson et al., 2011) by Reagent 4 Research, LLC (Hangzhou, China) and suspended in a 10% (v/v) vehicle (10% DMSO in saline). All mice received a daily intraperitoneal injection of either JNJ-31020028 (10 mg/kg) or vehicle control, in a final volume of 100 µl, for eight weeks (n=10 per treatment group). 10 mg/kg is a dose that is expected to have >90% receptor occupancy in rodent brains (Shoblock et al., 2010). During this treatment period, weights and food intake were recorded every three days. 24 hours after the last injection the mice were anesthetised and whole blood was collected (6% EDTA) by cardiac puncture, immediately following this the mice were culled for follow up experiments. Blood plasma was isolated by centrifugation at 1000 × g for 10 minutes. Percent weight was calculated on a per animal basis for each day. The initial weights were set at 100% and each subsequent weighing was calculated as a percentage of the baseline weight. Percent weight change was calculated by subtracting 100% from the calculated % weight on each treatment day. A negative number indicates weight loss and a positive number indicates weight gain.

Dual-energy X-Ray Absorptiometry

Following sacrifice mice were positioned onto sample trays for analysis. Total body BMD, total body bone mineral content (BMC), body fat % and lean mass were determined using a Lunar PIXImus II (GE Healthcare) using a single scan and excluding the head (Perrien et al., 2007).

Micro computerized tomography

Assessment of bone morphology and microarchitecture was performed using a μ CT40 (Scanco Medical, Brüttisellen, Switzerland) consistent with current guidelines for the assessment of bone microstructure in rodents using micro-computed tomography (Bouxsein et al., 2010). Briefly vertebrae and femurs were harvested and fixed in 10% neutral buffered formalin. Specimens were imaged, and three-dimensional reconstructions were created by stacking the regions of interest from each two-dimensional slice and then applying a gray-scale threshold and Gaussian noise filter with all data acquired at 55 kVp, 114 mA, and 200-ms integration time (Fowler et al., 2012). MicroCT slices were segmented into bone and marrow regions by applying a visually chosen fixed threshold for all samples after smoothing the image with a three-dimensional Gaussian low-pass filter ($\sigma = 0.8$, support = 1.0) to remove noise. The trabecular analysis was performed in a cancellous bone region that spanned 240 μ m to 2040 μ m distal of the growth plate in the femoral metaphysis region. For cancellous bone regions assessment included bone volume fraction (BV/TV; %), trabecular thickness (Tb.Th.; mm), trabecular separation (Tb.Sp.; mm), trabecular number (Tb.N.; 1/mm), connectivity density (ConnD.; 1/mm³) and structure model index (SMI).

For cortical analysis, transverse CT slices were acquired in a 1-mm-long region centered at the femoral midshaft. The outer contour of the bone was found automatically using the builtin manufacturer's contouring tool. Total area was calculated by counting all voxels within the contour, bone area by counting all voxels that were segmented as bone, and marrow area was calculated as total area - bone area. This calculation was performed on all 25 slices (1 slice = $\sim 12.5 \mu$ m), using the average for the final calculation. The outer and inner perimeter of the cortical midshaft was determined by a three-dimensional triangulation of the bone surface (BS) of the 25 slices, and cortical thickness, total cross-sectional area (CSA, mm²), cortical bone area (BA, mm²) and medullary area (MA, mm²), endosteal and periosteal perimeter (EP and PP, mm) as well as diameter of the midshaft (mm) determined as described (Fowler et al., 2012).

Analysis of osteoclast and osteoblast formation

Bone marrow was harvested from tibia and femur bones of mice during both our *ex vivo* analysis of Y2 antagonist treated mice as well as from a three 4-month-old female C57BL6 mice, for the analysis of Y2 antagonist impacts *in vitro*. Cells were then centrifuged, or pooled from the three mice then centrifuged for our *in vitro* analysis, at 1500 RPM for 5 minutes at room temperature. Cells were suspended in 5 ml Ammonium-Chloride-Potassium lysing buffer (Life Technologies Inc., Carlsbad, United States) and after 5 minutes minimal media (α MEM media containing 100 U/ml penicillin and 100 μ g/ml streptomycin) was added and then centrifuged at 1500 RPM for 5 minutes. Cells were suspended in culture media (minimal media (Invitrogen, Carlsbad, United States) containing 10% Fetal Bovine Serum (Gibco, Gaithersburg, United States)). For the osteoclast formation assay, mouse M-CSF (R&D systems, Minneapolis, United States) was added to 20 ng/ml and cells incubated overnight at 5% CO₂ at 37°C. Non-adherent cells were then plated 500,000/well in a 24-well plate and incubated with 20 ng/ml M-CSF and 40 ng/ml RANK ligand (Santa Cruz Biotechnology, Dallas, United States) for 5 days with a media change at 3 days. Cells were then stained for tartrate resistant acid phosphatase (TRAP) expression using a leukocyte acid

phosphatase kit (Sigma, St. Louis, United States) to allow visualization of osteoclast. Osteoclast were then identified by an investigator who was blind to the identify of the samples, and then osteoclast number and area coverage (total osteoclast area/total well area) were calculated using ImageJ software (NIH, Bethesda, United States) as the mean of 3–5 wells per mouse. For the osteoblast formation assay, cells were plated at 10^6 /well in a 24-well plate in osteoblast culture medium (minimal media, 10% Fetal Bovine Serum, 10 mM β -glycerolphosphate (Sigma, St. Louis, United States) and 50 μ M ascorbic acid (Sigma, St. Louis, United States). Media was changed every 2–3 days and cells were stained for alkaline phosphatase (Sigma, St. Louis, United States) after 14 days. Alkaline phosphatase stain intensity was then measured with ImageJ software as the mean of 3 wells per mouse. Values were obtained by converting scanned images of equal sized wells to grayscale, applying consistent threshold limits, and applying ImageJ integrated pixel density measuring tools. Osteoblast cells were stained using alizarin red (Sigma, St. Louis, United States). Alizarin red stain intensity was measured with ImageJ software as the mean of 3 wells per mouse in a similar manner as for alkaline phosphatase stain intensity.

ELISA analyses

Levels of plasma Procollagen Type 1 N-Terminal Propeptide (PINP) and C-telopeptide of Type 1 Collagen (CTX-1) were measured via ELISA assays according to manufacturer instructions (MBS703389 and MBS818051, respectively; MyBiosource Inc, San Diego, United States).

Data Analysis

Graphpad Prism software was used for graph preparation and statistical analysis. Data outcomes were compared between groups using a one-way or two-way analysis of variance (ANOVA) as indicated. When a significant value was obtained, comparisons were carried out using Bonferroni post hoc analysis. Data are mean $\pm$ SEM, and the level of significance was set to 0.05.

Results

JNJ-31020028 is a NPY Y2 receptor antagonist

HEK cells over-expressing either the Y2 or Y1 receptors were treated with increasing concentrations of JNJ-31020028 in comparison to known selective antagonists BIIE0246 (Y2) and BIBP3226 (Y1). Note that NPY receptors are Gi coupled receptors and thus stimulation of the receptor is expected to decrease intracellular cyclic AMP levels. JNJ-31020028 is a selective Y2 receptor antagonist and is ineffective in the cells expressing the Y1 receptor (Figure 1).

Effect of Y2R antagonism on total body weight

Conditional knockout of hypothalamic NPY Y2 receptors has been shown to significantly reduce body weight in mice (Sainsbury et al., 2002). In the current study, the effects of treatment with the selective Y2R antagonist JNJ-31020028 on overall body weight were examined in these OVX mice. Baseline body weight was recorded 24 hours prior to beginning daily injections and was similar between groups (vehicle: 24.7 ± 0.5 g, Y2

antagonist: 25.0 ± 0.4 g). During the first three weeks of injections, both treatment and control groups had reduced body weight due to the inherent stress of daily injections, however the body weights of the mice in the vehicle group gradually returned to baseline (Figure 2A). In contrast the Y2 antagonist treated mice demonstrated significantly lower body weights relative to mice in the control group for the duration of the treatment period, and did not return to baseline (treatment x time interaction; 2-way ANOVA; $p = 0.0005$). This discrepancy was not apparently due to reduced feeding behavior, as the average daily food intake was identical for both groups (Figure 2B). Furthermore, dual energy x-ray absorptiometry (DEXA) body composition analyses did not detect any significant treatment-related differences in body fat percentage or lean body mass between groups (Figures 2C, D and Table 1).

Effects of Y2R inhibition on bone structure

To explore skeletal impacts of JNJ-31020028 treatment, bone mineral density was assessed in OVX mice using dual energy X-ray absorptiometry (DEXA). Representative whole-body scans from JNJ-31020028-treated and vehicle mice and the regions of interest are shown in Figure 3A. The largely soft tissue containing areas highlighted in green were excluded from analyses. Bone mineral density (BMD) was significantly higher in Y2 antagonist-treated mice compared to mice receiving the vehicle control ($p=0.045$; 2-tailed t-test) (Figure 3B and Table 1). The BMD values observed for vehicle- and JNJ-31020028-treated OVX mice were within normal range (Delahunty et al., 2009).

To validate the DEXA measurements, the femoral and vertebral bones were next assessed using micro-computerized tomography (micro-CT). Consistent with DEXA, quantitative 3-D imaging of isolated vertebral samples revealed a significant increase in bone volume (BV/TV) (Figure 4 and Table 2). Correspondingly connectivity density (ConnD) and trabecular thickness (TB.Th) were also significantly greater in Y2 antagonist-treated mice compared to mice in the vehicle control group. Furthermore, treated mice exhibited lower structural model index (SMI), indicative of greater plate-like, as opposed to rod-like, composition within trabecular bone (Table 2). No statistically significant differences were detected for trabecular number (TB.N) or separation (TB.Sp).

Analysis of femurs showed greater BV/TV within trabecular regions of Y2 antagonist-treated mice, which was associated with an increase in trabecular number and a reduction in trabecular separation (Figure 4, Table 2). However, unlike vertebrae, there were no statistically significant differences in trabecular thickness, connective density or SMI within these regions. Furthermore, quantitative imaging of cortical regions of the femur did not detect differences in microarchitecture between Y2 antagonist-treated and vehicle control groups (Table 2).

Effects of Y2R inhibition on bone metabolic markers

To investigate whether the observed differences in bone morphology could be attributed to altered metabolic activity, the effect of pharmacological Y2R inhibition on bone metabolism was examined. Procollagen Type 1 N-Terminal Propeptide (P1NP) and C-terminal telopeptide 1 (CTX1) serve as biomarkers for bone formation and resorption, respectively

(Hale et al., 2007; Rosen et al., 2000). ELISA analysis of plasma samples from antagonist-treated mice revealed a significant reduction in both PINP1 ($p=0.034$; 2-tailed t-test) and CTX1 ($p=0.004$; 2-tailed t-test) levels compared to mice in the vehicle control group (Figure 5).

Primary bone cell cultures were isolated from the tibiae of both Y2 antagonist- and vehicle-treated mice, and osteoblast and osteoclast formation were assessed *in vitro*. Osteoblast differentiation was stimulated with 10 mM β -glycerolphosphate and 50 μ M ascorbic acid, and formation was determined by staining for alkaline phosphatase (ALP) activity (Leung et al., 1993). The intensity of ALP staining was significantly greater in cultured cells isolated from the Y2 antagonist-treated tibiae compared to those isolated from tibiae of mice in the vehicle control group ($p=0.0094$; 2-tailed t-test) (Figure 6). Osteoclast differentiation was induced with 20 ng/ml M-CSF and 40 ng/ml receptor activator of nuclear factor κ -B ligand (RANKL), and formation was assessed by number of osteoclasts and total areal coverage of multinucleated osteoclasts. Somewhat surprisingly, bone marrow cultures obtained from the Y2 antagonist-treated mice exhibited significantly more osteoclasts and greater areal coverage with *in vitro* induction ($p=0.0254$ and 0.0382 respectively; 2-tailed t-test) (Figure 7). Furthermore, primary bone cell cultures were isolated from the tibiae of wild type female mice, and similarly treated to form osteoblast (Figure 8A) and osteoclast (Figure 8B) in the presence of increasing concentration of Y2 antagonist. Neither the intensity of the alkaline phosphatase staining, nor the number of osteoclasts formed or total osteoclast area were affected by Y2 antagonist treatment ($p > 0.05$ for all comparisons). To further examine differences in osteoblast activity, we assessed calcium deposition by staining with alizarin red (Figure 9), and found cells isolated from Y2 antagonist-treated tibia also exhibited greater stain intensity ($p=0.024$; 2-tailed t-test).

Discussion

Conditional deletion of hypothalamic NPY Y2 receptors (Y2R) leads to increased bone volume in adult mice (Baldock et al., 2006; Baldock et al., 2002), suggesting that targeted strategies to inhibit the Y2 receptor may have therapeutic potential for the treatment of bone loss disorders, such as osteoporosis. Here we provide evidence that female ovariectomized mice treated with a selective Y2 antagonist showed higher trabecular bone volume. Indeed, there are key similarities between the pharmacological effects observed in this study and the reported phenotype of conditional hypothalamic Y2R knockout mice. In the current study, increased bone volume was observed in femoral and vertebral trabecular bone, and was associated with enhanced trabecular thickness in vertebral bone. Consistent with this, per Baldock *et al.* (2002), hypothalamic knockout effects also appear to be specific to trabecular bone, as cortical bone was unchanged. In contrast, the same authors later reported an increase in cortical density along the entire femur bone using quantitative computed tomography (QCT), suggesting that similar drug-mediated effects on cortical bone might be apparent using more sensitive analyses (Baldock et al., 2006). Y2 antagonist treatment also led to a reduction in trabecular separation in femurs, which is otherwise found to be significantly higher in osteoporosis patients than in those without bone loss (Croucher et al., 1994; Schnitzler et al., 2005).

Furthermore, the higher bone mineral density following Y2 antagonist treatment, observed using whole-body DEXA, is due to either enhanced mineralization and/or reduced bone loss. Lundberg and colleagues showed mineralization was an important factor leading to greater bone mineral density in cultured bone marrow stromal cells isolated from germ line Y2R knockouts (Lundberg et al., 2007). This coincided with an increase in the number of multi-potential mesenchymal progenitor cells in the bones of these mice compared to wild-type controls. The greater bone volume in the Y2R knockout mice may be due to an enhanced ability of these mesenchymal cells to differentiate into bone-forming osteoblasts (Lundberg et al., 2007). Germ line Y2R knockout has also been shown to induce greater osteoblast activity and enhance bone mineralization *in vivo* (Baldock et al., 2006). In line with this, both alkaline phosphatase and alizarin red staining was significantly higher in primary bone marrow cell cultures isolated from Y2 antagonist-treated mice compared to those obtained from the vehicle control animals, which indicates greater osteoblast formation potential (Leung et al., 1993).

Given that current treatment with bisphosphonates reduces bone loss by interfering with osteoclast formation and activity (Drake et al., 2008), Y2 antagonists may act synergistically with such an agent to promote bone formation in people with advanced osteoporosis and therefore represents a potential strategy for the treatment of bone loss disorders, though this hypothesis remains to be tested. In support of this notion, the levels of procollagen type 1 N-terminal propeptide (P1NP) were also reduced in Y2 antagonist treated mice. A decrease in P1NP levels is used as a desirable clinical biomarker to assess efficacy in individuals receiving bisphosphonate therapies (Gallagher et al., 2006; Hale et al., 2007; Saarto et al., 1998). However, we really do not know enough about the time course of the reduction in the P1NP in our study. A study on the longitudinal changes in both P1NP and CTX biomarkers will likely yield a better understanding of the *in vivo* dynamic/coupling between bone resorption and formation in the mice treated with the NPY antagonist.

Plasma CTX1 levels from Y2 antagonist-treated mice were decreased suggesting that there is an overall lower degree of bone resorption. Therefore, it was interesting to note that cultured bone marrow cells from these mice also displayed higher levels of osteoclast differentiation during the *ex vivo* growth assays. Since bone resorption and bone formation are finely balanced (Aguila and Rowe, 2005), it may not be surprising to have found a corresponding increase in osteoclast formation in response to enhanced osteoblast formation following Y2R inhibition. Indeed, similar examples of such counterintuitive increases in both osteoclast and osteoblast activities have been observed following experimental manipulation of *in vivo* osteoblast or osteoclast formation (Bennett et al., 2007; Eleftheriou et al., 2006). Enhanced osteoclast formation in the *ex vivo* analysis suggests that Y2 antagonism enhances the potential for osteoclastogenesis. However, as inferred by the biomarkers, Y2 antagonist treatment reduced bone turnover in these OVX mice concurrent with increases in bone mineral density and improvements in microarchitecture. In contrast, the *ex vivo* data suggested greater potential for osteoblast and osteoclast formation. It should be noted that the formation observed in *ex vivo* assays is due to factors added to induce differentiation, and these assays may also reflect how bone marrow stem cells respond to stimulus. Taken together, these data may indicate the Y2 antagonist slows bone metabolism *in vivo*, while also potentiating the system to respond vigorously to bone differentiating

stimuli. Although this notion warrants further investigation, others have reported similar differences between observed turnover *in vivo* and *ex vivo* (Cho et al., 2014). Additionally, our findings that *ex vivo* treatment of bone marrow cells with the Y2 antagonist did not affect osteoblast and osteoclast formation suggests the inhibitor is not affecting these cells directly, and is likely working through endocrine signaling mechanisms.

In addition to its effects on bone, treatment with the Y2R antagonist also led to a sustained reduction in body weight in the ovariectomized mice. No differences in food intake, percentage fat or lean body mass were observed, suggesting a decrease in both fat and lean mass together, which may indicate an overall increased metabolic rate, though this remains to be tested. Therefore, inhibition of the Y2R signaling pathway could be useful as an approach to decrease body weight as has been previously suggested (Sainsbury et al., 2002). While in this study, we aimed only to assess the effects on mice that have been ovariectomized to start to address questions over the potential effects of Y2 receptor antagonism in post-menopausal women, the question remains whether the compound is having an anabolic effect, or whether the effect is preventative of loss. Either way, the compound still may hold value as a potential treatment, especially in combination with currently used therapies.

However, the study has several limitations that should be considered. Although we report the Y2R antagonist increases BMD in OVX mice, the lack of sham mice does not allow confirmation that bone density in the OVX mice was indeed decreased, and further, whether treatment was able to completely mitigate the impacts of OVX induced bone loss. A sham cohort would have also provided greater insight in our interpretation of microCT, serum bone markers, and *ex vivo* osteoblast and osteoclast formation assays. Interestingly, here we also report our OVX mice did not appreciably gain body weight as is often reported in the literature (Beranger et al., 2014; Chung et al., 2014). One possibility for this observation is potential stress from daily intraperitoneal injections of Tx or placebo, and a sham cohort would have been useful to verify if this treatment protocol indeed provokes stress in the animals. Finally, the addition of bone histology would provide greater understanding of our *in vivo* and *ex vivo* bone metabolism data.

Our results support the involvement of Y2R signalling in bone metabolism, suggest that selective pharmacological manipulation of Y2R may provide therapeutic benefits in the setting of osteoporosis, and warrant further studies to expand this work and elucidate underlying mechanisms.

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Highlights

- Treatment of female ovariectomized mice, a model for human menopause, with a selective Y2 antagonist showed higher trabecular bone volume and bone mineral density compared to controls.
- NPY Y2 antagonist treated mice displayed a decrease in serum P1NP, a measure used to confirm positive treatment outcomes in bisphosphonate treated patients.
- NPY Y2 receptor antagonist treatment in mice resulted in higher osteoclast and osteoblast activity which, taken together with increased bone mineral density, suggests a higher overall rate of bone remodelling.
- Osteoclast and osteoblast cells taken from untreated mice then exposed to antagonist *in vitro* did not respond to the Y2 receptor antagonist, suggesting a role for central NPY Y2 receptors.
- These studies suggest some potential for Y2 receptor antagonists to be useful in addressing bone diseases in menopause.

Figure 1. Structure and activity of JNJ-31020028

A. JNJ-31020028 is shown with known pharmacokinetic parameters. JNJ-31020028 tested in Y2R expressing HEK cells (B) or Y1R expressing HEK cells (C), compared to controls.

Figure 2. Selective Y2 antagonist treatment reduces total body weight in ovariectomized mice
 The treated group exhibited significantly lower body weights relative to vehicle control for the duration of the treatment period. *** represents $p < 0.001$, ($n = 10$; treatment x time interaction; 2-way ANOVA;). No effects of treatment were seen for (B) average daily food intake, (C) percentage body fat or (D) lean body mass. Data are shown as mean values $\pm$ standard error mean.

Figure 3. Selective Y2 antagonist treatment increases bone mineral density in ovariectomized mice

DEXA measurements revealed that bone mineral density (BMD) was significantly higher in drug-treated mice compared to those receiving the vehicle control. Data are shown as mean values $\pm$ standard error mean. * represents $p < 0.05$ ($n = 10$; 2-tailed t-test)

Figure 4. Selective Y2 antagonist treatment increases vertebral and femoral trabecular bone volume in ovariectomized mice

(A) Representative microCT 3D-renderings of vertebrae samples isolated from JNJ-31020028- and vehicle-treated mice. (B) Representative microCT 3D-renderings of trabecular regions of femurs isolated from JNJ-31020028- and vehicle- treated mice. Quantification of these renderings is shown in Table 2.

Figure 5. Selective Y2 antagonist treatment reduces plasma CTX1 and P1NP levels in ovariectomized mice

ELISA analysis of murine plasma revealed a significant reduction in both (A) CTX1 and P1NP levels compared to vehicle control. Data are shown as mean values $\pm$ standard error mean. ** and * represent $p < 0.05$ and $p < 0.01$ respectively (n=10 2-tailed t-test).

Figure 6. Selective Y2 antagonist treatment of ovariectomized mice enhances *ex vivo* osteoblast formation

(A) Representative images of alkaline phosphatase staining in primary bone marrow cell cultures derived from the tibiae of Y2 antagonist- and vehicle-treated mice (B) Intensity of ALP staining, using ImageJ (NIH), was significantly greater in cultures of cells isolated from the drug-treated group to those in vehicle control group. Data are shown as mean values from 3 wells for each mouse $\pm$ standard error mean. * represents $p < 0.05$ ($n = 5$; 2-tailed t-test).

Figure 7. Selective Y2 antagonist treatment of ovariectomized mice increases *ex vivo* osteoclast formation and areal coverage

(A) Representative images of TRAP+ osteoclast formation in primary bone marrow cell cultures derived from the tibiae of Y2 antagonist- and vehicle-treated mice. The number (B) and areal coverage (C) of multi-nucleated osteoclasts was significantly greater in cultures of cells isolated from the drug-treated group to those in vehicle control group. Data are shown as mean of 3–5 wells for each mouse values $\pm$ standard error mean. * represents $p < 0.05$ (n=5; 2-tailed t-test).

Figure 8. Ex vivo osteoblasts (A) and osteoclasts (B) taken from untreated mice treated with indicated concentrations of Y2 receptor antagonist
 Untreated marrow was extracted from tibia and femur bones (harvested and pooled from three 4-month-old female C57BL/6 mice) then cultured as described in Methods. For the osteoclast formation assay cells were treated with CSF/RANKL then stained for tartrate resistant acid phosphatase (TRAP) expression. For the osteoblast formation assay, cells were stained for alkaline phosphatase after 14 days. Data were quantified using ImageJ from 3–5 wells per treatment for osteoclast formation (number and total area) and 3 wells per treatment for integrated alkaline phosphatase intensity. Intensity of the alkaline phosphatase

staining, the number of osteoclasts formed and total osteoclast area were not affected by Y2 antagonist treatment ($p > 0.05$ for all comparisons).

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Figure 9. Ex vivo osteoblasts taken from vehicle or treated mice stained for calcium with Alizarin Osteoblasts from vehicle (A) or compound treated mice were stained with Alizarin red as described in methods. Data were quantified using ImageJ for stain intensity. Intensity of the Alizarin red staining was significantly higher in osteoblasts taken from JNJ-31020028 treated animals. (* represents $p = 0.024$; 2-tailed t-test).

Table 1

Dual X-ray absorptiometry bone and body composition analysis, (*) indicates p<0.05 from vehicle).

Group	Bone mineral density (g/cm ²)	Bone mineral content (g)	Absolute lean mass (g)	Absolute fat mass (g)
Vehicle	0.0478 ± 0.0014	0.401 ± 0.011	19.4 ± 1.8	3.6 ± 0.7
JNJ-31020028	0.0493 ± 0.0017*	0.413 ± 0.028	18.9 ± 1.1	3.7 ± 0.5

Table 2

Effect of JNJ-31020028 treatment on trabecular and cortical bone microstructure

	Femur		Vertebrae	
	Vehicle	JNJ-31020028	Vehicle	JNJ-31020028
Trabecular				
BV/TV	0.039 ± 0.010	0.054 ± 0.017 *	0.198 ± 0.050	0.245 ± 0.021 *
ConnD (mm ⁻³)	11.2 ± 5.7	21.7 ± 15.9	110.7 ± 31.3	146.4 ± 29.4 *
SMI	2.75 ± 0.22	2.65 ± 0.33	0.83 ± 0.36	0.47 ± 0.20 *
Tb.N (mm ⁻¹)	2.19 ± 0.27	2.55 ± 0.39 *	6.34 ± 0.52	6.42 ± 0.43
Tb.Th (mm)	0.049 ± 0.003	0.051 ± 0.003	0.046 ± 0.003	0.049 ± 0.002 *
Tb.Sp (mm)	0.463 ± 0.056	0.402 ± 0.053 *	0.178 ± 0.018	0.169 ± 0.015
DA	1.44 ± 0.11	1.40 ± 0.12	1.71 ± 0.18	1.64 ± 0.14
Cortical				
Cort. CSA (mm ²)	0.569 ± 0.029	0.584 ± 0.032		
Cort.TH (mm)	0.186 ± 0.006	0.189 ± 0.012		
Med.area (mm ²)	0.704 ± 0.032	0.709 ± 0.066		
Endo.peri (mm)	2.563 ± 0.100	2.641 ± 0.134		
Med.dia (mm)	0.542 ± 0.021	0.528 ± 0.025		
Total CSA (mm ²)	1.334 ± 0.061	1.356 ± 0.066		
Perio.Peri (mm)	3.517 ± 0.126	3.565 ± 0.108		
Dia.Mid (mm)	0.746 ± 0.019	0.750 ± 0.026		

BV/TV = bone volume fraction; ConnD = connective density; SMI = structure model index; Tb.N. = trabecular number; Tb.Th. = trabecular thickness; Tb.Sp. = trabecular separation; DA = degree of anisotropy; Cort. CSA = cortical cross sectional area; Cort.TH = cortical thickness; Med.area = medullary area; Endo.peri = endosteal perimeter; Med.dia = medullary diameter; Total CSA = total cross sectional area; Perio.peri = periosteal perimeter; Dia.mid = diameter of midshaft.

* indicates $p < 0.05$, $n=10$.