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Neuropeptide Y Y1 receptor antagonism increases bone mass in mice

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ABSTRACT

The neuropeptide Y system has emerged as one of the major neural signalling pathways regulating bone homeostasis. Absence of Y1 receptor signalling from bone forming osteoblasts is responsible for an enhancement on bone mass in mice, suggesting that pharmacological blockade of Y1 receptors may offer a novel anabolic treatment option for improving bone mass. Here we show that oral administration of the selective Y1 receptor antagonist BIBO3304 for 8 weeks dose-dependently increases bone mass in mice. Histomorphometric analysis revealed a significant 1.5-fold increase in cancellous bone volume in the femora of mice treated with BIBO3304. Furthermore, bone microarchitecture was improved, with greater trabecular number and trabecular thickness. This increase in bone mass was associated with a significant increase in bone anabolic activity of osteoblasts and, interestingly, was evident despite a coincident increase in bone resorption, as evidenced by an increase in the number of the osteolytic osteoclasts. Changes were also evident in cortical bone, with a significant increase in periosteal mineral apposition rate. Importantly, no adverse extra-skeletal side effects were observed through Y1 receptor antagonism over the 8-week treatment period, with no effects of even the higher BIBO3304 dose on body weight, adiposity, energy metabolism or circulating corticosterone levels. Taken together, this work describes the first NPY-based anabolic treatment for improving bone mass, and highlights the therapeutic potential of blocking Y1 receptor signalling for the prevention of, or recovery from, degenerative skeletal diseases.

Introduction

Skeletal diseases are a leading cause of debilitation and reduced quality of life. The gradual reduction of bone mass with increasing age remains one of the most common features characterizing a range of degenerative skeletal disorders. Due to increased longevity, altered lifestyle and hormonal imbalance, we have witnessed a dramatic increase of osteopenic-driven skeletal diseases such as osteoporosis [1]. Osteoporosis is an extremely widespread disease, representing a major economic burden on health care systems worldwide, with direct costs in the US of $19 billion in 2005 [2] and indirect costs being far greater. So widespread is this condition that it is estimated that osteoporotic fracture will occur in one in two women and one in three men over the age of 60 [3].

The development of new agents for the treatment of osteoporosis is an area of intense effort in drug discovery. The majority of pharmacologic therapies developed to date involve anti-resorptive agents. Antiresorptive drugs, such as bisphosphonates, estrogens selective receptor modulators or calcitonin, specifically inhibit the bone-resorbing osteoclastic system. However, while such drugs may halt the progression of bone loss, they do not lead to net accrual of bone in the skeleton. For that reason, potent anabolic drugs, such as parathyroid hormone analogues and strontium salts, have proven to be more therapeutically effective, since they actually improve bone structure and increase bone size [1,4]. However, the cost of treatment imposes some limitations on their use, and the discontinuation of an anabolic therapy leads to rapid bone loss [4,5]. Thus, the development of new anabolic therapeutic agents has proven difficult, and useful cost-effective anabolic strategies are still a therapeutic challenge.

Advances in understanding of the molecular and cellular biology of bone remodelling have identified the nervous system as a critical modulator of the complex regulatory machinery controlling bone metabolism [6], uncovering potential new targets for bone remodelling

Abbreviations: BAT, brown adipose tissue; BMC, bone mineral content; BMD, bone mineral density; DXA, dual-energy X-ray absorptiometry; MAR, mineral apposition rate; micro-CT, micro-computed tomography; NPY, Neuropeptide Y; RER, respiratory exchange ratio; WAT, white adipose tissue.

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therapy. Neuropeptide Y (NPY) is a recently identified osteo-neuromodulator shown to regulate bone cell function [7,8]. NPY is a neurotransmitter widely expressed through the peripheral and central nervous system, and its actions are mediated through the activation of G-protein coupled receptors, namely Y1, Y2, Y4, Y5 and y6 receptors [9]. Through specific activation of the Y1 receptor, NPY has been shown to play a critical role in a number of centrally-regulated physiological functions, such as energy homeostasis [10], anxiety [11], cardiovascular function [12] and neurogenesis [13]. More recently, NPY has been shown to regulate bone homeostasis via actions in peripheral tissues. Insights from Y1 receptor knockout (KO) models have revealed that the absence of peripheral Y1 receptors led to pronounced anabolic effects on bone [14–16]. As a result of these anabolic effects, germline Y1 receptor KO mice displayed an increase in bone mass, with consistent changes in femoral, tibial, and vertebral bones. Importantly, despite strong expression of Y1 receptors in the hypothalamus, specific deletion of these central Y1 receptors did not alter bone mass, indicating the likelihood of peripheral pathways being involved [14]. This finding opens the exciting possibility that peripherally-acting Y1 antagonism may be a suitable anabolic avenue for pharmacological intervention in osteoporosis/osteopenia. This potential was further heightened by the discovery that the Y1 receptor is the only Y receptor proven to be robustly expressed in osteoblasts [7,17] and in bone marrow stromal cells [16], suggesting a direct role of Y1 receptors on bone remodelling. Indeed, we have recently shown that specific deletion of Y1 receptors from the osteoblasts of mice results in a marked increase in osteoblast activity and bone mass, confirming the importance of osteoblast-specific Y1 receptors in the control of bone anabolism [8]. These studies strongly suggest that pharmacologically blocking peripheral Y1 receptor signalling may represent an anabolic therapeutic strategy to prevent or reverse bone loss occurring in common skeletal disorders such as osteoporosis.

The potential therapeutic application of NPY receptor ligands is a promising area of research. In fact, the Y1 receptor has been identified as a novel therapeutic target for the treatment of certain disorders, namely obesity and cancer [18,19]. In recent years, a number of effective Y1 receptor antagonists have been made commercially available. These agents represent powerful tools to determine the pharmacological potential of specifically targeting Y1 receptors for the benefit of disease treatment. Among these compounds, BIBO3304 remains one of the most potent and selective Y1 receptor antagonist described [20], with the added advantage that it can be administered orally. While, the therapeutic potential of antagonizing central Y1 receptors using centrally-administered BIBO3304 has been studied in vivo [20–22], particularly with regard to feeding behaviour, this is the first study to explore the potential utility of orally-administered BIBO3304 as a therapeutic tool to improve bone mass.

In order to explore the therapeutic potential of this anti-Y1 receptor strategy, we have investigated the peripheral effects of the highly selective non-peptide Y1 receptor antagonist BIBO3304 on bone metabolism. In order to avoid stress induced by gavage or transcutaneous delivery, BIBO3304 was incorporated into an artificially flavoured jelly [23] and given to mice that had been trained to eat it on a daily basis for 8 weeks. Due to the wide distribution of Y1 receptors in peripheral tissues [24] and the Y1 receptor-mediated actions in other physiological systems such as energy homeostasis [10], metabolic changes were also monitored throughout the treatment period.

Materials and methods

Animals

All research and animal care procedures were approved by the Garvan Institute/St. Vincent's Hospital Animal Ethics Committee and were conducted in accordance with relevant guidelines and regulations. Male wild type C57/BL6 mice were housed under conditions of controlled temperature (22 °C) and illumination (12 h light cycle, lights on at 07:00 h). All mice were fed a normal chow diet *ad libitum* (8% calories from fat, 21% calories from protein, 71% calories from carbohydrate, 2.6 kcal.g^{-1}; Gordon's Speciality Stock Feeds, Yanderra, NSW, Australia). Water was available *ad libitum* throughout the experiments.

Voluntary oral administration

To avoid stress induced by gavage or daily injections, BIBO3304 N-[(1R)-1-[[[[4-[[(Aminocarbonyl)amino]methyl]phenyl]methyl]amino]carbonyl]-4-[(aminoimino-methyl)amino]butyl]-α-phenyl-benzeneacetamide ditrifluoroacetate (Tocris Bioscience, Bristol, UK) was delivered orally to mice via a novel method previously described [23]. Briefly, BIBO3304 was incorporated into an artificially flavoured and sweetened jelly and given to mice trained to eat the jelly once daily for 8 weeks. Jelly without BIBO3304 was used as vehicle. In general, mice trained in this way consume the entire quantity of jelly in less than 1 min. Wild type mice were treated orally with a sub-therapeutic dose of 500 nmol/mouse/day (an estimated dose of 0.02 mg/kg), based on concentrations tested *in vitro* [8]. Considering potential hepatic clearance after oral administration, a ten-fold higher dose was also tested (i.e. 5 μmol/mouse/day, an estimated dose of 0.2 mg/kg).

Experimental procedure

9-week-old male wild type mice were housed individually and allowed to acclimatize before being trained to eat the jelly [23]. Prior to treatment commencement, body weight and body composition were measured by DXA. Subsequently, mice were separated into groups of equal average body weight and body composition. From 10-weeks of age, mice received BIBO3304 jelly once per day at 09:00 h for 8 weeks, while control mice received vehicle jelly. Animals maintained a high avidity for jelly throughout the study period. Given the wide peripheral distribution of Y1 receptor in rodents [24], we checked for possible side effects of BIBO3304 by monitoring body weight twice a week at the same time of the day. Body composition changes were also examined using DXA analysis at the fourth and eighth week of treatment. Additionally, spontaneous food intake was measured following 1 or 5 weeks of jelly treatment, as described below. These time points are referred to as 'acute' or 'chronic' in data presentation. During the fourth week of the treatment period, mice were put in metabolic chambers to determine metabolic rate, respiratory exchange ratio (RER) and physical activity as described below. Rectal temperature was also measured at the sixth week. During the seventh week of treatment, mice were submitted to a glucose tolerance test. To this end, mice were fasted for 24 h before intraperitoneal injection of a 10% D-glucose solution (1.0 g/kg). Blood samples were obtained from the tail tip at 0, 20 and 60 min after injection and glucose levels were measured using an AccuCheckII glucometer (Roche, NSW, Castle Hill, Australia). All mice were injected with the fluorophore calcein (15 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) 3 and 10 days prior to tissue collection to enable subsequent calculation of bone formation rate.

Tissue collection

At the end of the 8-week treatment period, mice were culled between 13:00 and 16:00 h by cervical dislocation followed by decapitation for collection of trunk blood. Serum was separated, immediately frozen and stored at −20 °C for subsequent analysis of corticosterone using a radioimmunoassay kit (ICN Biomedicals, Irvine, CA, USA) and analysis of serum insulin like growth factor (IGF)-1 using a radioimmunoassay detection kit from Bioclone (Sydney, NSW, Australia). The white adipose tissue (WAT) depots (right inguinal, right retroperitoneal, right epididymal and mesenteric), and white muscle were removed,

weighed, frozen on dry ice and stored at −80 °C. Femurs, tibias and caudal vertebrae were excised, fixed overnight in 4% paraformaldehyde in phosphate buffered saline at 4 °C and then stored in 70% ethanol at 4 °C before undergoing processing.

Food intake

Spontaneous daily food intake was measured over 3 consecutive days in individually housed mice. Actual food intake was calculated as the weight of food taken from the hopper minus the weight of food spilled in the cage. The weight of spilled food per day was determined as the 24 h increase in weight of the cage bedding, after removing all faeces and drying for 24 h at 22 °C to eliminate weight changes due to urine and water bottle drips. The average was used for statistical analysis.

Indirect calorimetry

Metabolic rate was measured by indirect calorimetry using an eight-chamber open-circuit calorimeter (Oxymax Series; Columbus Instruments, Columbus, OH, USA) as described previously [10]. Briefly, pre-weighed mice were housed individually in specially built Plexiglas cages (20.1 × 10.1 × 12.7 cm). Temperature was maintained at 22 °C with an airflow of 0.6 l/min. Food and water were available *ad libitum*. Mice were transferred into Plexiglas cages and were acclimatized to the new cages for 24 h before recordings commenced. Mice were subsequently monitored in the system for 24 h. Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured every 27 min. The respiratory exchange ratio (RER) was calculated as the quotient of VCO_2/VO_2, with 100% carbohydrate oxidation resulting in a value of 1 and 100% fat oxidation resulting in a value of 0.7. Energy expenditure (kcal heat produced) was calculated as Calorific Value (CV) x VO_2, where CV is $3.815 + 1.232 \times$ RER. Data for the 24 h monitoring period was averaged for 1-h intervals for energy expenditure and RER. Ambulatory activity of individually-housed mice was evaluated within the metabolic chambers using an OPTO-M3 sensor system (Columbus Instruments, Columbus, OH, USA), whereby ambulatory counts were a record of consecutive adjacent photo-beam breaks. Cumulative ambulatory counts of X and Y directions were recorded every minute and summed for 1 h intervals.

Rectal temperature measurements

Body temperature was measured at 09:00 h with a thermometer connected to a rectal probe (Physitemp Instruments Inc, Clifton, NJ, USA). Temperature readings were taken within 10 s of removing the mouse from its cage. Repeat readings were taken from each mouse on 3 consecutive days, and the average of the three readings was used for statistical analysis.

Body composition densitometry

Whole body bone mineral content (BMC), bone mineral density (BMD), lean mass and fat mass were measured on mice ventral side down by dual energy X-ray absorptiometry (DXA), using a mouse PIXImus densitometer (Lunar Piximus II, GE Medical Systems, Madison, WI, USA). The head and the tail were excluded from the analysis.

Bone histomorphometry

Bone histomorphometry was carried out on 5 μm sagittal sections of the distal half of the right femur as previously described [25]. Briefly, sections were stained for mineralized bone (using a von Kossa technique), and cancellous bone volume, trabecular thickness and trabecular number were calculated. Bone formation indices, namely mineral apposition rate, mineralising surface and bone formation rate were calculated, whilst osteoclast surface and osteoclast number were estimated on tartrate-resistant acid phosphatase (TRAP)-stained sections, using LeicaQWin analysis software (Leica Microsystems, Heerbrugg, Switzerland). Cortical mineral apposition rate was measured in an endosteal and a periosteal region both extending 1000 μm proximal from the posterior aspect of the growth plate, as previously described [25].

Bone micro-computed tomography (micro-CT)

Following fixation, left femora were cleaned of muscle and analyses of the cortical bone were carried out using micro-CT with a Skyscan 1174 scanner and associated analysis software (Skyscan, Aartselaar, Belgium) as previously described [23]. Briefly, analyses of the cortical bone were carried out in 150 slices at 1.07 mm thick with CTAnalyser software (version 1.10.10). These analysed slices were 750 slices (5.37 mm) proximal from the distal growth plate. The following parameters were calculated: total tissue area, bone area, marrow area, endosteal perimeter, periosteal perimeter, cortical thickness, and polar moment of inertia (an index of strength). In addition, three-dimensional (3D) images were generated using CTVol Realistic Visualisation software (version 2.1.0).

Statistical analysis

All data are expressed as means ± SEM (standard error of the mean). Differences between treated and untreated mice were assessed by two-tailed Student's *t*-test. Statistical analyses were performed with SPSS for Mac OS X, version 19 (SPSS Inc., Chicago, IL, USA). For all statistical analyses, $p < 0.05$ was accepted as being statistically significant and $p \leq 0.1$ was accepted as showing a trend of change.

Results

Peripheral Y1 receptor antagonism enhances bone formation in a dose-dependent manner

In order to investigate the potential for Y1 receptor blockade to improve bone mass, wild type mice were initially treated, based on *in vitro* pharmacological information [8], with a minimal dose of 500 nmol/mouse/day (~0.02 mg/kg/day) of BIBO3304 for 8 weeks, referred to herein as 'low dose'. Histomorphometric analysis of distal femurs isolated from these mice is shown in Figs. 1A–B. When compared to vehicle-treated mice, 8-weeks of treatment with this low dose resulted in a trend to increased cancellous bone volume (Table 1), corresponding to a 1.15-fold increase, however this did not reach statistical significance ($p = 0.15$). The bone formation indice of mineralising surface (MS, the surface extent of active bone formation) was slightly increased ($p = 0.10$), however, mineral apposition rate (MAR, the speed of mineralized tissue production) and bone formation rate remained unaltered. Treatment with this 500 nmol/mouse/day BIBO3304 dose also did not significantly alter any bone resorption indices, with osteoclast surface being unchanged (Table 1).

In order to examine the safety and off-target effects of low-dose BIBO3304 treatment, a number of specific indices were examined (Table 2). Low dose BIBO3304 did not alter body weight, food intake, body composition (assessed by DXA, dual energy X-ray absorptiometry), white or brown adipose tissue mass, energy expenditure (assessed by indirect calorimetry) or fasting insulin or glucose levels.

Encouraged by the positive trends seen on bone mass with this initial treatment paradigm, as well as the lack of any indication of adverse effects on metabolic parameters, we next treated mice with a ten-fold higher dose of BIBO3304 (5 μmol/mouse/day, ~0.2 mg/kg/day). DXA analysis performed over the 8-weeks of treatment revealed

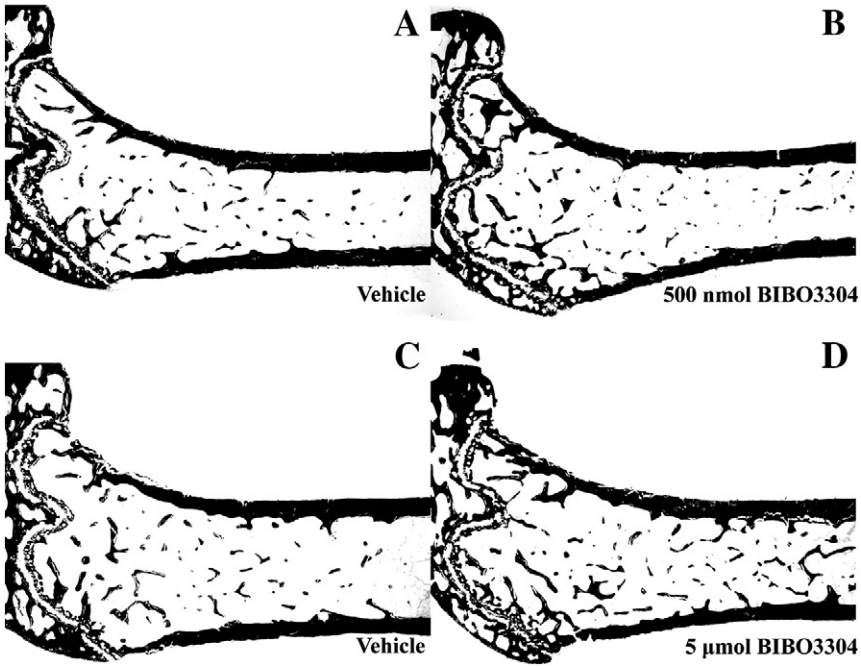

Fig. 1. Representative saggital micrographs of the distal femoral metaphysis of BIBO3304-treated mice at a low (500 nmol/mouse/day) and high dose (5 μmol/mouse/day) (B, D) after 8 weeks of daily oral treatment, compared to their correspondent vehicle-treated littermates (A, C). Figures show mineralized bone tissue and are representative of the respective groups.

that when compared to baseline levels (before initiating treatment), 5 μmol BIBO3304-treated mice showed a significant increase in whole body BMC after 4- and 8-weeks of treatment ($p<0.05$), and this effect was not observed in vehicle-treated mice, with a 2-fold greater increase in BMC in BIBO3304-treated mice over the period compared to control (Table 3). These increases in whole body BMC corresponded to increments of 7.3 and 10.9% at 4 and 8 weeks of treatment, respectively. In contrast, vehicle-treated mice showed only non-significant increments in whole body BMC of 4.9 and 5.0% at 4 and 8 weeks, respectively.

Interestingly, when the skeletal response to BIBO3304-treatment was examined in detail by histomorphometric analysis, 5 μmol/mouse/day BIBO304 resulted in a significant 1.5-fold increase in cancellous bone volume, as indicated by the representative sections shown in Figs. 1C–D. The increase in cancellous bone volume following 8-weeks of treatment with the 5 μmol dose (Fig. 2A; $p=0.001$), also resulted in a significant improvement in microarchitecture, with a concomitant increase in trabecular number (Fig. 2B; $p<0.01$) and thickness (Fig. 2C; $p<0.05$). Moreover, mineral apposition rate, the marker of osteoblastic anabolic activity, was significantly higher in BIBO3304-treated mice (Fig. 2D; $p<0.01$), although there were no significant effects on either mineralising surface (Fig. 2E) or bone formation rate (Fig. 2F). Bone resorption rate was also greater in 5 μmol BIBO3304-treated mice. Indeed, both osteoclast number (Fig. 2G) and osteoclast surface (Fig. 2H) were significantly elevated ($p<0.05$) in the treated mice compared to vehicle-administered controls. Together, these data recapitulate the bone phenotype previously observed for germline and osteoblast-specific Y1 receptor deficient mice [14,15], confirming the importance of Y1 receptor signalling for controlling bone mass and revealing the potential of Y1 targeting as a novel therapeutic intervention for modulating bone mass.

Peripheral Y1 receptor antagonism maintains cortical bone strength

Both germline and osteoblast-specific Y1 receptor deletion contribute to a marked anabolic effect on cortical bone [14,15]. Consistent with these genetic models, 5 μmol BIBO3304 stimulated a significant increase in osteoblast activity on the external (periosteal) surface (Fig. 3A), with a trend for a similar effect on the internal (endocortical) surface ($p=0.10$) (Fig. 3B). Therefore, cortical bone changes were further assessed by micro-CT analysis. When compared to vehicle-treated mice, 5 μmol BIBO3304-treated mice exhibited a

Table 1
Cancellous bone parameters in the distal femoral metaphysis, following 500 nmol BIBO3304 treatment compared to vehicle-treated mice as determined by histomorphometric analysis.

Parameters	Vehicle	500 nmol BIBO3304
Cancellous bone volume (%)	11.3 ± 1.3	13.0 ± 0.68
Trabecular thickness (μm)	28.5 ± 1.5	30.1 ± 1.1
Mineral apposition rate (μm/day)	1.6 ± 0.1	2.0 ± 1.0
Mineralising surface (%)	21.0 ± 1.0	24.7 ± 2.5[a]
Osteoclast surface (%)	7.4 ± 0.7	6.0 ± 0.6

Values are means ± SEM of 6–10 mice per group.
[a] $p=0.10$ versus vehicle-treated mice.

Table 2
Effects of peripheral Y1 receptor blockade on body weight, food intake, body composition, white and brown adipose tissue mass, energy expenditure, fasting serum insulin and glucose levels.

Parameters	Vehicle	500 nmol BIBO3304
Body weight (g)	33.1 ± 1.1	32.4 ± 0.9
24-hour food intake (g/BW)	0.126 ± 0.004	0.125 ± 0.004
Whole body lean mass (g)	23.6 ± 0.8	24.7 ± 0.7
Whole body fat mass (g)	5.4 ± 0.4	4.9 ± 0.2
White adipose tissue mass (g)	0.88 ± 0.05	0.87 ± 0.06
Brown adipose tissue mass (g)	0.11 ± 0.01	0.08 ± 0.01
Energy expenditure (kcal per hour)		
24 h	0.498 ± 0.013	0.508 ± 0.014
Dark phase	0.520 ± 0.013	0.522 ± 0.015
Light phase	0.475 ± 0.010	0.493 ± 0.012
Fasting serum insulin (pM)	161 ± 34	244 ± 44
Fasting serum glucose (mM)	10.4 ± 0.4	10.0 ± 0.4

Values are means ± SEM of 6–10 mice per group. No statistical differences were detected between vehicle- and BIBO3304-treated mice at this dose.

Table 3
Effects of peripheral Y1 receptor signalling blockade on bone parameters throughout the 8 weeks of treatment period, as determined by dual energy X-ray absorptiometry (DXA).

Weeks of treatment	Whole body BMD (g/cm^2)		Whole body BMC (g)	
	Vehicle	5 μmol BIBO3304	Vehicle	5 μmol BIBO3304
0	0.058 ± 0.001	0.057 ± 0.001	0.475 ± 0.019	0.458 ± 0.013
4	0.059 ± 0.002	0.059 ± 0.001	0.497 ± 0.015	0.491 ± 0.014[a]
8	0.057 ± 0.002	0.058 ± 0.001	0.498 ± 0.015	0.507 ± 0.013*

Abbreviations: BMD, bone mineral density; BMC, bone mineral content. Values are means ± SEM of 6 mice per group.
* $p<0.05$ versus basal levels (0 weeks).
[a] $p=0.10$.

significant reduction in bone marrow volume (Fig. 3C; $p<0.05$) and a trend towards reduced total bone volume (Fig. 3D; $p=0.09$). In fact, histomorphometric analysis of the isolated distal femoral diaphysis also showed a decrease in bone marrow area in 5 μmol BIBO3304-treated compared to vehicle-treated mice (4.35 ± 1.15 and 4.68 ± 1.20 mm^2, respectively, data are means ± SEM of 6 mice per group; $p=0.08$). However, no significant changes were observed in either cortical volume (Fig. 3E) or cortical thickness (Fig. 3F) as determined by micro-CT analysis. Likewise, no changes were detected in the polar moment of inertia (Fig. 3G). These findings are illustrated by the representative cross-sectional micro-CT images of cortical bone (Fig. 3H). These results, which showed more pronounced effects of BIBO3304 treatment on cancellous than on cortical bone, are not entirely unexpected. The far greater surface area of cancellous bone results in more rapid and identifiable structural changes than upon cortical bone [5].

High dose BIBO3304 treatment has no significant effect on food intake, body weight, energy metabolism, adiposity or serum corticosterone levels

The Y1 receptor has been commonly implicated in NPY-mediated hyperphagia. The selective Y1 antagonist BIBO3304 was proven to significantly attenuate hyperphagia induced by intracerebroventricular NPY injection or following fasting [20,21]. In order to rule out potential side effects induced by the higher dose of BIBO3304, we evaluated the effects of 5 μmol BIBO3304 on spontaneous food intake after 1- and 5-weeks of treatment (termed 'acute' and 'chronic' treatment, respectively). As shown in Fig. 4A, acute treatment with BIBO3304 had no significant effect on daily food intake, however, there was a trend to decreased food intake with chronic treatment ($p=0.06$). This reduction in food intake, however, did not result in significant differences in body weight compared to vehicle-treated mice over this 8-week period (Fig. 4B).

Both germline and peripheral-specific Y1 receptor deletion has been shown to strongly influence energy homeostasis, thermogenesis and fat accretion [10,26]. After 4-weeks of treatment, no significant differences were observed between vehicle- and higher dose BIBO3304-treated mice with respect to metabolic parameters analysed by indirect calorimetry (Table 4). Namely, BIBO3304-treatment had no effects on physical activity, energy expenditure and respiratory exchange ratio, during either the light or the dark phase (Table 4). In addition, BIBO3304-treated mice showed no significant difference from vehicle-treated controls in rectal temperature, suggesting no major effects of the drug on thermogenesis (Table 4).

In order to investigate possible effects of Y1 receptor antagonism on adiposity, we examined whole body fat mass by DXA and by weighing individual dissected white adipose tissue (WAT) depots

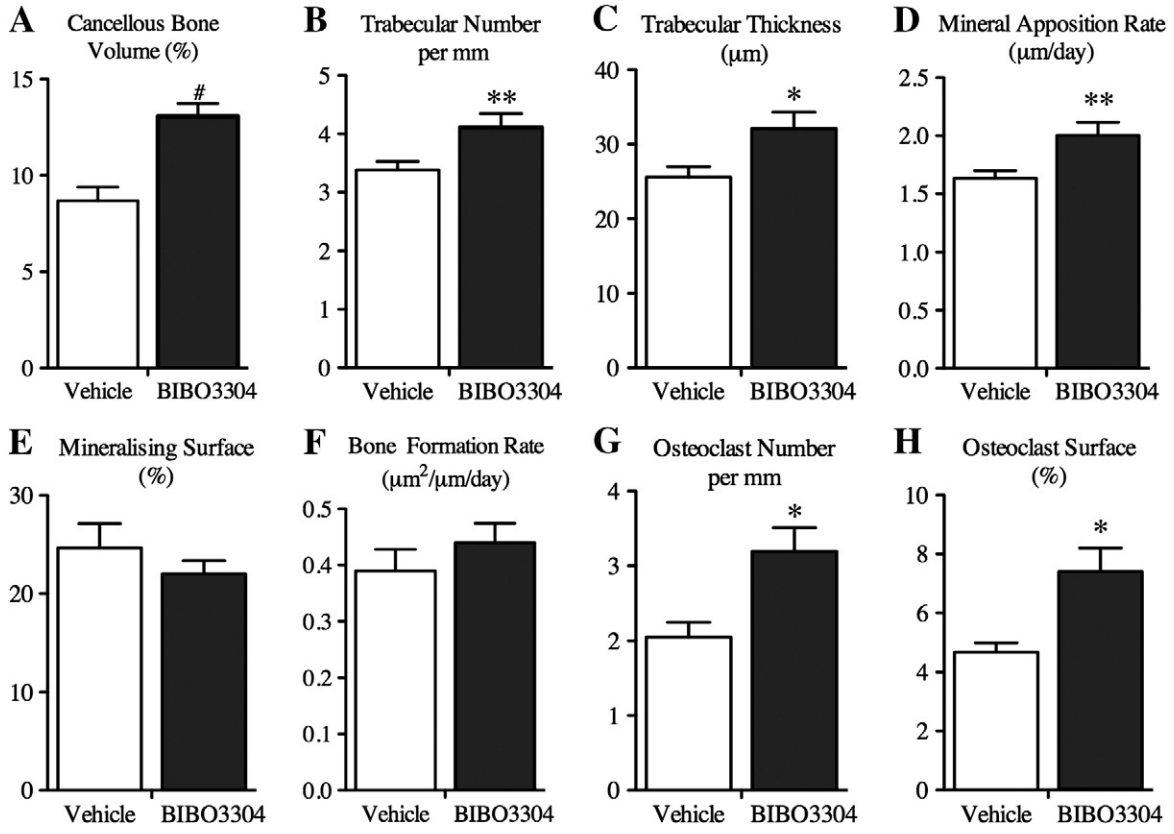

Fig. 2. Peripheral blockade of Y1 receptor signalling increases cancellous bone, as determined by histomorphometry analysis. 8-weeks of treatment with a high dose of BIBO3304 (5 μmol/mouse/day) resulted in a significant increase in cancellous bone volume (A), trabecular number (B) and trabecular thickness (C), which was associated with an increase in mineral apposition rate (D), albeit with no changes in mineralising surface (E) and bone formation rate (F). Bone resorption rate was also elevated as shown by an increased in osteoclast number (G) and osteoclast surface (H). Values are means ± SEM of 6 mice per group. *$p<0.05$, **$p<0.01$, #$p<0.001$ versus vehicle-treated mice.

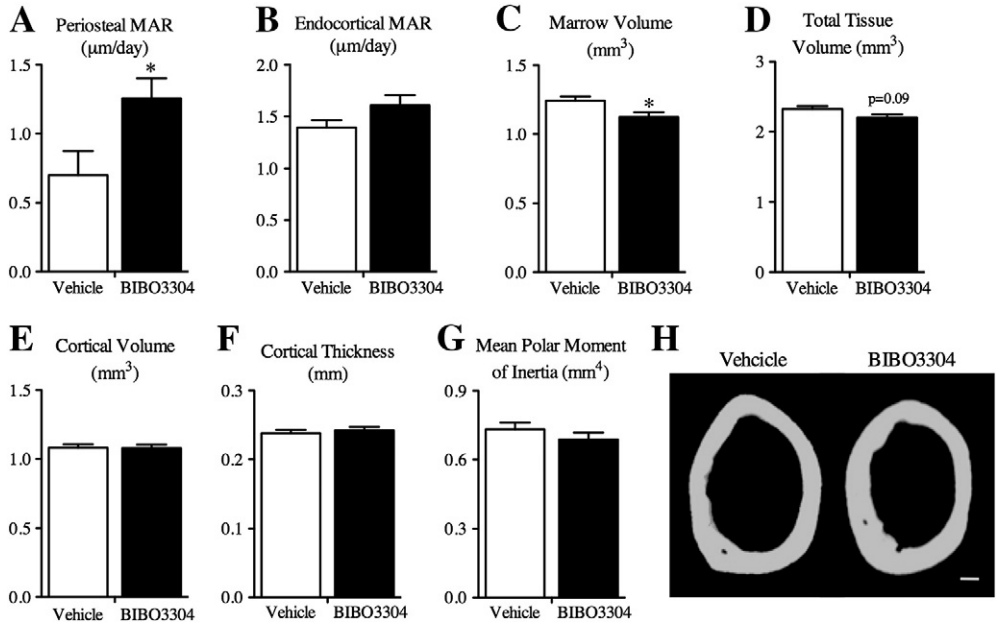

Fig. 3. Peripheral blockade of Y1 receptor signalling produces minor changes in cortical bone parameters. 8-weeks of treatment with a high dose of BIBO3304 (5 μmol/mouse/day) resulted in increased MAR on the periosteal (A) and endocortical (B) surfaces. Micro-CT analysis reveals a reduction on marrow volume (C) and a trend towards reduced total tissue volume (D), although no significant changes were observed on cortical volume (E), cortical thickness (F), and mean polar moment of inertia (G), as illustrated by representative cross-sectional micro-CT images of cortical bone (H). Values are means ± SEM of 6 mice per group. *$p<0.05$ versus vehicle-treated mice. Scale bar represents 0.20 mm.

(namely the inguinal, epididymal, mesenteric and retroperioneal depots as well as the summed total weights of these depots) and of dissected brown adipose tissue (BAT; Figs. 4C–E; expressed as percentage of body weight). The 5 μmol BIBO3304 treatment induced a trend to reduced WAT (Figs. 4C, D), and BAT mass ($p=0.10$) (Fig. 4E), albeit these differences were not statistically significant.

Y1 receptors in the adrenal cortex are known to inhibit corticosterone production, and Y1 antagonism reverses this action [27].

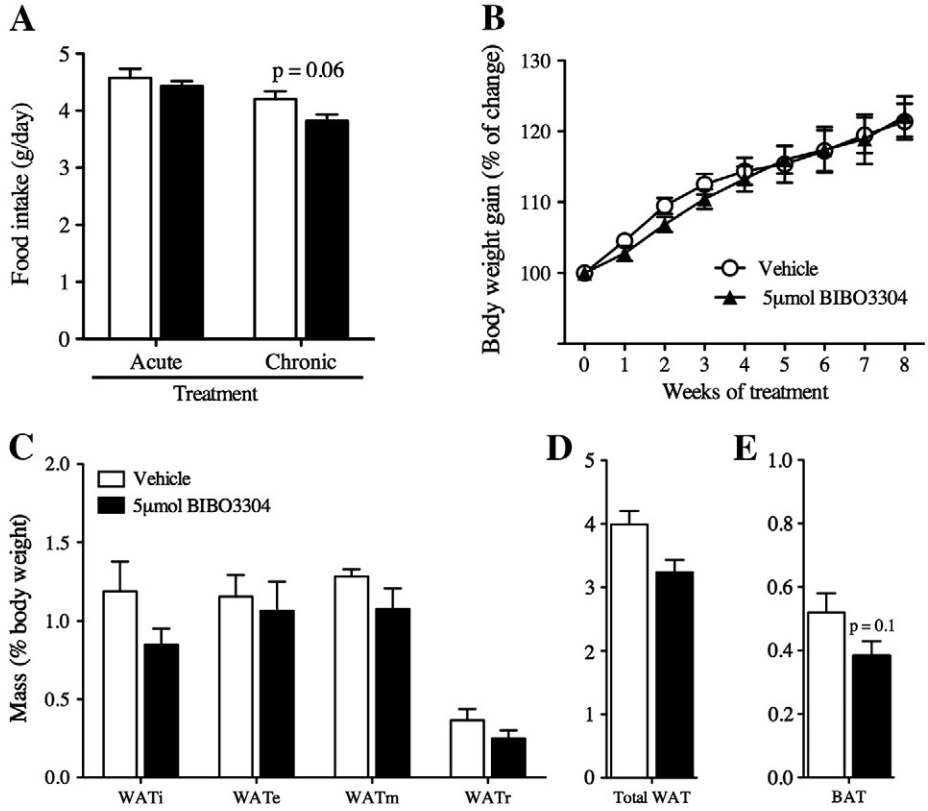

Fig. 4. Effects of peripheral Y1 receptor signalling blockade on energy metabolism. (A) Spontaneous food intake after 1 week (acute) or 5 weeks (chronic) of treatment with BIBO3304 (5 μmol/mouse/day) or vehicle. (B) Body weight gain, expressed as percentage of change of initial value. (C) Single dissected white adipose tissue (WAT) depots weight (B), total weight of dissected WAT depots (D) and brown adipose tissue (BAT) weight (E), expressed as percentage of body weight. Values are means ± SEM of 6 mice per group. Abbreviations: WATi, inguinal WAT; WATe, epididymial WAT; WATm, mesenteric WAT; WATr, retroperitoneal WAT.

Table 4
Effects of peripheral Y1 receptor signalling blockade on body weight, body composition, rectal temperature, physical activity, respiratory exchange ratio and energy expenditure as determined during indirect calorimetry.

Parameters	Vehicle	5 μmol BIBO3304
Body weight (g)	33.1 ± 1.1	32.4 ± 0.9
Whole body lean mass (g)	24.8 ± 0.5	25.0 ± 0.6
Whole body fat mass (g)	6.5 ± 1.1	4.9 ± 0.7
Rectal temperature (°C)	36.1 ± 0.22	36.3 ± 0.1
Physical activity (ambulatory counts per hour)		
24 h	351.8 ± 71.3	405.8 ± 80.9
Dark phase	404.9 ± 84.1	517.3 ± 93.7
Light phase	298.6 ± 56.7	294.3 ± 61.4
Respiratory exchange ratio (RER)		
24 h	0.950 ± 0.013	0.968 ± 0.011
Dark phase	0.975 ± 0.012	0.993 ± 0.010
Light phase	0.926 ± 0.013	0.943 ± 0.010
Energy expenditure (kcal per hour)		
24 h	0.551 ± 0.011	0.554 ± 0.012
Dark phase	0.578 ± 0.010	0.579 ± 0.013
Light phase	0.524 ± 0.009	0.529 ± 0.009

Values are means ± SEM of 6 mice per group. No statistical differences were detected between vehicle- and 5 μmol BIBO3304-treated mice.

Importantly, at the end of treatment, serum corticosterone levels were not statistically different between vehicle-treated and 5 μmol BIBO3304-treated groups (322 ± 62 and 263 ± 58 ng/mL, respectively; data are means ± SEM of 6 mice per group; $p = 0.51$).

Y1 receptor antagonism does not affect glucose homeostasis

Mice deficient in Y1 receptors are characterized by altered fuel storage and hyperinsulinaemia, suggesting dysfunctional regulation of glucose metabolism [28]. Therefore, at week 7 of the treatment period, the effects of chronic BIBO3304 administration on whole body glucose metabolism were evaluated in 24 h fasted mice. Animals were challenged with an intraperitoneal glucose tolerance test. Compared with vehicle-treated mice, 5 μmol BIBO3304-treated mice showed no statistically significant differences in serum glucose levels, either in the fasted state or in response to intraperitoneal glucose injection, albeit the area under the glucose tolerance curve tended to be higher in the BIBO3304-treated when compared to the vehicle-treated animals (Table 5).

Taken together, these data indicate that Y1 receptor antagonism at the higher dose tested had clear effects on bone but did not induce any marked metabolic imbalance that would be expected to compromise oral treatment.

Discussion

In the present study we show that oral administration of a non-peptide Y1 receptor antagonist, BIBO3304, exerted consistent anabolic effects on cancellous and cortical bone. Importantly, the bone changes observed in BIBO3304-treated mice occurred without any deleterious effects on body weight, food intake, energy metabolism, adiposity or glucose metabolism compared to vehicle-treated mice. Taken together, our data highlight the potential therapeutic effectiveness of inhibiting Y1 receptor signalling for the treatment of low bone mass.

Table 5
Effects of peripheral Y1 receptor signalling blockade on glucose metabolism.

Parameters	Vehicle	5 μmol BIBO3304
Daily water intake (g/day)	5.75 ± 0.38	5.93 ± 0.32
Fasting serum glucose (mM)	6.8 ± 0.3	6.7 ± 0.5
Area under glucose tolerance curve (mM × 60)	593 ± 17	627 ± 31

Values are means ± SEM of 6 mice per group. No statistical differences were detected between vehicle- and 5 μmol BIBO3304-treated mice.

Our results, from an 8-week oral treatment with a specific Y1 receptor antagonist at a dose of 5 μmol, demonstrate an increase in osteoblast activity in cortical (weight bearing) and cancellous (metabolic) bone, resulting in enhanced cancellous bone volume and microarchitectural indices and a 2-fold increase in whole body BMC accrual during the treatment period. This improvement in bone mass occurred in association with an increase in the rate of mineralized tissue production (mineral apposition rate). These data extend our previous observations of effects of germline or osteoblast-specific Y1 receptor ablation [14,15] by demonstrating the clinically relevant finding that Y1 receptor ablation can be manipulated pharmacologically to stimulate bone growth.

Although, we had observed a decreased of marrow volume in BIBO3304-treated mice, cortical bone thickness and strength indices were maintained. The exact reasons for the lack of pronounced anabolic effects of BIBO3304 treatment on cortical bone are not clear. One likely explanation would be the rather short treatment period, with far larger cortical bone responding more slowly to cellular changes than cancellous bone, due to the fact that cancellous bone has a larger surface area per unit volume [5,29]. Keeping this in mind, Y1 receptor antagonism remains a potential anabolic agent, which might be useful for the systemic therapeutic prevention of bone loss disorders.

The changes evident in bone formation parameters display a similar pattern to those evident for both germline and osteoblast -specific Y1 receptor null mice, with an increase in osteoblast activity (mineral apposition rate), but no change in surface extent of bone formation (mineralizing surface) [14,15]. In terms of magnitude, the changes in anabolic activity and cancellous microarchitecture are comparable with those from osteoblast-specific Y1 receptor null mice, however despite the increase in mineral apposition rate, bone formation rate was not significantly elevated. Importantly, the increase in bone mass following 5 μmol BIBO3304 treatment was evident despite a coincident increase in bone resorption indices, as indicated by an increase in osteoclast number and surface. This is consistent with the increase on osteoclast activity observed after the global deletion of Y1 receptor [14]. In this manner, Y1 receptor blockade would act to elevate bone mass, as required of an anabolic agent, but would also ensure adequate bone turnover, thereby avoiding any possible issue with hypocalcaemia, which can lead to tetany, cardiac arrhythmias and ECG changes [30]. In keeping with this, there is a well described requirement for the development of therapeutic drugs that regulate the activity of both osteoclasts and osteoblasts functions [4].

The increase in osteoclast number suggests that the absence of Y1 receptor signalling alters osteoclastogenesis, which may result from indirect actions via osteoblasts, or potentially by direct actions on the osteoclast. Osteoblastic lineage cells stimulate osteoclast formation through production of receptor activator of nuclear factor (NF-κB) ligand (RANKL). Previous *in vitro* studies have shown that NPY can alter osteoclastogenesis by inhibiting RANKL activation in bone marrow stromal cells [31] and osteoblasts [7]. Thus, it is possible that such alterations might occur as a result of the lack of Y1 receptors in osteoblasts. However, the deletion of Y1 receptors solely in mature osteoblasts did not affect either osteoclast indices or RANKL expression, suggesting that the elevated activity of the osteoclast lineage observed here and in germline Y1 receptor KO mice is not due to the lack of Y1 receptor on mature osteoblasts [15]. Indeed, besides osteoblast-like cells, Y1 receptor is also expressed on peripheral blood mononuclear cells (PBMCs), the osteoclast precursor, and also on macrophages [32], which share the same progenitor lineage with osteoclasts. Therefore, Y1 receptor antagonism might influence osteoclast recruitment and activity by directly controlling osteoclast progenitor cells. However, the cellular and molecular effects of Y1 receptor antagonism on osteoclast activity remain to be elucidated. Furthermore, the immune system is also known to regulate osteoclastogenesis. Facilitated by their close proximity in the bone marrow, immune cells produce cytokines, which directly or indirectly regulate

osteoclastogenesis and osteoclast formation [33]. Importantly, NPY has been shown to regulate immune cell functions, namely T and B lymphocytes, natural killer cells and macrophages [34]. For this reason, we cannot exclude a possible regulation of Y1 receptor signalling blockade on osteoclastogenesis via interaction with immune cells expressing Y1 receptors. Overall, however, it is important to note that although the BIBO3304 treatment had altered bone resorption rate, it was not sufficient to counteract the strong anabolic effects induced by Y1 receptor antagonism.

In general, use of anti-osteoporotic agents is only initiated in those patients who have already sustained an osteoporosis-related fracture [5]. However, by the time osteoporosis is diagnosed, usually after a first fracture, extensive bone loss has already occurred. Most common antiresorptive treatments can only prevent further bone loss and for that reason are of limited use. On the other hand, the Y1 receptor-mediated anabolic mechanism, identified herein, would provide a potential means for increasing bone mass even in osteopenic patients. This conclusion is consistent with previously published data showing that the global Y1 receptor deficiency protects against gonadectomy-induced bone loss in female mice [35]. Such an anabolic treatment would be of marked clinical utility, because current anabolic treatments (i.e. parathyroid hormone and strontium ranelate) are limited by dosage efficiency, delivery issues, appropriated timing of treatment and forthcoming side effects. For instance, the use of parathyroid hormone analogues, which require daily transcutaneous injection, has been questioned based upon hypercalcaemic activity [36] and increases the risk of osteosarcoma [37]. Clearly new avenues of anabolic treatment for osteoporosis are required.

The Y1 receptor mediates remarkable effects in an extensive variety of important physiologic functions, namely in the regulation of feeding behaviour [20,21], energy balance [26] and adiposity [10,14], consistent with its wide distribution in central and peripheral tissues [24]. Therefore, an oral delivery of Y1 receptor antagonist might have been expected to induce some additional effects on other physiological systems besides bone. Indeed, the global deletion of the Y1 receptor significantly influences adiposity and circulating insulin levels [14,28]. Importantly, our findings show that 8 weeks of oral treatment with a Y1 receptor antagonist BIBO3304 does not produce significant extra-skeletal side effects, particularly with regard to food intake, body weight, energy metabolism, adiposity or glucose homeostasis, that could contraindicate its use for bone therapy. Overall, with the exception of the anabolic effects noticed on skeletal homeostasis, the blockade of Y1 receptor by oral BIBO3304 treatment prompted no adverse effects on the other peripheral or brain functions investigated.

In conclusion, this study has shown that Y1 receptor antagonism achieved by oral administration of BIBO3304 at a dose of 5 μmol enhances osteoblast activity resulting in increased mineral apposition rate both in cortical and cancellous bone of mice. This resulted in increased bone mass, while maintaining bone turnover through a coincident increase in bone resorption. This was achieved after only 8 weeks of daily treatment at a dose of 5 μmol/mouse/day. These findings suggest that BIBO3304 or other similarly potent Y1 receptor antagonists might represent new anabolic agents that could be used as a therapeutic tool to prevent or reverse bone loss and reduce fracture in such common conditions as osteoporosis. Further studies are warranted to more closely examine the therapeutic role of BIBO3304 as an oral anabolic agent in challenged mice, for instance in osteoporotic mice and following fracture.

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