Repurposing Erectile Dysfunction Drugs Tadalafil and Vardenafil To Increase Bone Mass

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ABSTRACT

We report that two widely–utilized drugs for erectile dysfunction, tadalafil and vardenafil, trigger bone gain in mice through a combination of anabolic and anti–resorptive actions on the skeleton. Both drugs enhanced osteoblastic bone formation *in vivo* using a unique gene footprint, and inhibited osteoclast formation. The target enzyme, phosphodiesterase 5A (PDE5A) was found to be expressed in mouse and human bone, as well as in specific brain regions, namely the *locus coeruleus*, *raphe pallidus*, and the paraventricular nucleus of the hypothalamus. Localization of PDE5A in sympathetic neurons was confirmed by co–immunolabeling with dopamine β–hydroxylase, as well as by retrograde bone–brain tracing using a sympathetic–nerve–specific pseudorabies virus, PRV152. Both drugs elicited an anti–anabolic sympathetic imprint in osteoblasts, but with net bone gain. Unlike in people where vardenafil is more potent than tadalafil, the relative potencies were reversed with respect to their osteoprotective actions in mice. Structural modeling revealed a higher binding energy of tadalafil to mouse PDE5A than vardenafil. This was due to steric clashes of vardenafil with a single methionine residue at position 806 in mouse PDE5A. Collectively, our findings suggest that a balance between peripheral and central actions of PDE5A inhibitors on bone formation, together with their anti–resorptive actions, specify the osteoprotective action of PDE5A blockade.

SIGNIFICANCE STATEMENT

Tadalafil and vardenafil are among the most widely used drugs for erectile dysfunction that affects nearly 19% of men over 20 years of age. Older men also suffer with age–related bone loss resulting in crippling fractures. We show that, in mice, both agents act on bone cells resulting in the formation of new bone and reduced removal of old bone. Due to net gain in bone mass, we posit that tadalafil and vardenafil could be utilized for co–treating erectile dysfunction and osteoporosis in men of advancing ages, as well as for treating osteoporosis in post–menopausal women. We recommend future clinical studies to establish the ability of the drugs to increase bone density and reduce fracture risk in people.

/body

INTRODUCTION

Since the initial description of the effects of nitric oxide (NO) on bone cells (1), physiological studies over two decades have confirmed its critical role in skeletal homeostasis. Notably, NO and its donors suppress the activity of the osteoclast, the cell that resorbs bone, and additionally, stimulate bone formation by osteoblasts (1-7). Osteoclasts also generate NO in the local resorptive microenvironment (7) and mice lacking NO synthase display an osteoporotic phenotype (8). The therapeutic potential for long–term NO donor therapy, such as nitroglycerin, sodium nitroprusside and nitrosyl-cobinamide for osteoporosis has also been explored (4, 9-13). Individuals receiving NO donor therapy display higher hip bone mineral density (BMD) and a reduced risk of fracture (14, 15). Nonetheless, chronic NO–based therapy is currently restricted for use in conditions of vascular dysfunction, such as recurrent angina and pulmonary hypertension (16, 17).

The downstream target of NO is soluble guanylate cyclase (sGC)–cyclic guanosine monophosphate (cGMP)–dependent protein kinase G (PKG). PKG is a serine–threonine protein kinase that is inactivated by family of specific cGMP–degrading phosphodiesterases (PDEs). Gain– and loss–of–function of PKG in murine models, namely in *Prkg2*^{R242Q} and *Prkg2*$^{-/-}$ mice, results in increases and decreases in bone mass, respectively (18, 19). Likewise, soluble guanylate cyclase has also been targeted for bone gain (20, 21). In all, the results to date establish a primary role for the NO–cGMP–PKG axis in skeletal regulation, and suggest that the inhibition of PDEs could offer osteoprotection by activating PKG.

Of the eleven PDEs in mammalian tissues (22), PDE5A is the inhibitory target for the three widely utilized drugs for erectile dysfunction, namely sildenafil, tadalafil, and vardenafil. Pharmacologic studies using recombinant PDE5A show that vardenafil is 10–fold more potent

than tadalafil in inhibiting the human enzyme (22). Of note is that 70% of men older than 70 years who have experienced erectile dysfunction are potential candidates for PDE5A inhibitor therapy (23). In fact, following the release of the first PDE5 inhibitor, sildenafil, in 1998, the use of PDE5A inhibitors in the Veterans Health Administration grew to 105 patients per 1,000 male veterans (24). With the availability of generic forms of the drugs, their usage is likely to accelerate in an increasingly aged male population.

The relatively ubiquitous expression of PDEs has nonetheless prompted a careful examination of the extra–genital actions of PDE5A inhibition. For example, tadalafil and vardenafil have been used for pulmonary hypertension (25). Likewise, PDE5A is expressed in the brain, and its inhibition affects neurogenesis, memory, and stroke progression (26-28). PDE5A was also expressed in chondrocytes, but inhibiting PDE5A in 1–month–old rats for three weeks did not affect long bone growth or bone modeling (29). Other studies on putative skeletal effects of PDE5A inhibition in animal models have yielded inconsistent results, including hyperresorption and low bone density (30), positive effects on bone in ovariectomized and glucocorticoid–treated mice (31, 32), and accelerated fracture healing (33).

Here, we report a comprehensive analysis of the effects of PDE5A inhibition on bone formation, bone resorption and bone mass. We also evaluate the contribution of central actions mediated *via* PDE5A–containing neurons in the brain. We find that tadalafil and vardenafil increase bone mass through combined actions on osteoblasts and osteoclasts, as well as on hippocampal neurons. This net positive effect on bone mass could be therapeutically meaningful to protect against bone loss in men over 50 years, 47% of whom have osteopenia (34).

RESULTS

We first carried out unbiased Taqman–based expression profiling of 20 murine PDE isoforms using whole bone RNA (Fig. 1A). Three PDEs, namely *Pde2a*, *5a* and *4d*, were expressed at high levels (>15 copies *per* cell) compared even with *Tnfrsf11a* and *Tnfsf11* that encode for RANK and RANKL, respectively (Fig. 1A). Of note is that *Pde5a* expression in 40–week–old mice was significantly greater than that in young mice, suggesting that PDE5A could be targeted in older individuals to prevent bone loss. Furthermore, other molecular components of the NO–cGMP–PKG axis, including soluble guanylate cyclase (*Gucy1a2* and *Gucy1a3)* and protein kinase G (*Prkg1* and *Prkg2*) isoforms, were also expressed in bone. Prior studies with bovine tissue have documented high *Pde2a* expression in the adrenal gland, kidney, heart and hippocampus (35). PDE2A binds cGMP at physiological concentrations to induce a conformational change that enhances its affinity for 3',5'–cyclic adenosine monophosphate (cAMP). Although at higher concentrations, it competes to hydrolyze cGMP preferentially, PDE2A is not a target for tadalafil or vardenafil, which exhibit IC_{50}s that are >10,000 fold higher than for PDE5A (22). Unlike PDE2A, PDE4D hydrolyzes cAMP, but not cGMP, and is again not a known target for tadalafil or vardenafil.

Fig. 1B shows Affymetrix microarray data using cultured human $CD14^+$ hematopoietic stem cell precursors, osteoclasts, and osteoblasts. Notably, genes encoding for the corresponding human PDE isoforms, namely *PDE5A* and *PDE6D*, were expressed in osteoblasts (Fig. 1B). In addition, *PDE6D* was expressed in $CD14^+$ cells as well as osteoclasts (Fig. 1B). However, PDE6D is a non–catalytic PDE subunit, and hence, is not a target for tadalafil or vardenafil. *PDE6A* and *PDE9A* were not expressed in human bone cells, consistent with their very low expression in mouse bone (Fig. 1A and 1B). The osteoclast–specific genes, namely *ATP6V0D2* and *ACP5*, as well as osteoblast–specific genes, namely *COL1A1* and *ALPL*, were expressed in the two cell types, respectively, confirming cellular identity. Fig. 1C

shows the co–localization (purple) of PDE5A immunoreactivity (blue) with RUNX2 (brown) in murine bone marrow stromal cells; this confirms the localization of PDE5A protein in osteoblast precursors. No such co–staining of PDE5A– and CSF1R–positive cells was noted, suggesting the absence of the enzyme in osteoclasts.

We therefore examined the effects of tadalafil and vardenafil on the formation of mineralizing osteoblasts from these precursors. To study osteoblastogenesis *in vitro*, we cultured murine bone marrow stromal cells in differentiating medium in the presence of tadalafil or vardenafil for 21 days. A significant increase in colony formation, colony–forming–units–osteoblastoid (Cfu–ob), was noted with tadalafil and vardenafil (Fig. 1D). In parallel, both drugs reduced tartrate–resistant acid phosphatase– (ACP5–) positive osteoclasts formed when hematopoietic stem cells were cultured for 5 days in the presence of RANK-L and macrophage colony-stimulating factor (M-CSF) (Fig. 1E). These studies together document both pro–osteoblastic and anti–osteoclastic actions of the two agents.

Increases in bone mass can arise from cell–autonomous actions of a molecule on bone cells – osteoblasts, best illustrated by the anabolic actions of parathyroid hormone (PTH) (36), or osteoclasts, as with calcitonin (37). Central neural circuits converging on sympathetic and parasympathetic relay at the level of the osteoblast also affect bone mass (38-40). Sympathetic relay, in particular, exerts an anti–anabolic action by reducing osteoblast proliferation, and drugs, such as propranolol, show positive actions on bone mass and reductions in fracture risk (41, 42). Thus, overall effects on bone mass are a composite of anabolic and anti–anabolic actions on osteoblasts, as well as the modulation of osteoclastic bone resorption.

Given that PDE5A is expressed in the brain with multiple predicted neural actions (26-28), we sought to explore whether PDE5A inhibition has a role in bone mass regulation *via* the well–characterized sympathetic innervation of bone (39). Confocal immunofluorescence

imaging of brain tissue from wild type C57BL/6 mice showed PDE5A labeling in the *locus coeruleus*, *raphe pallidus*, and paraventricular nucleus of the hypothalamus (Figs. 2A, S1A and S1B). A sub–population of PDE5A–positive neurons exhibited immunoreactivity for dopamine β–hydroxylase (DBH), suggesting that multiple distributed nodes of the sympathetic nervous system could be modulated by PDE5A (Fig. 2A).

To further examine if these PDE5A/DBH double–labeled neurons project into bone, we utilized a trans–synaptic tracing technique using a pseudorabies virus strain, PRV152. PRV152 expresses enhanced green fluorescent protein (EGFP) under the control of the human cytomegalovirus immediate early promoter. When injected into peripheral tissues, the virus travels exclusively in a retrograde manner and localizes to central neurons (Fig. 2B). It has therefore been utilized widely for tracing tissue–neuronal connections (43). Injection of PRV152 under the bone periosteum or into metaphyseal bone, areas of abundant sympathetic innervation, resulted in detection of EGFP in the PDE5A–rich areas noted above, 6 days following injection in anaesthetized mice (Fig. 2B). Figs. S1C and 1D show no EGFP signal when PRV152 was placed on the bone surface, rather than injected under the periosteum or into metaphyseal bone. Collectively, the data establish a direct anatomical connection between PDE5A–containing neurons in specific brain areas and bone, raising the possibility of a contribution of this central axis to the bone–forming actions of PDE5A inhibitors.

To evaluate the effect of PDE5 inhibition on bone mass and microarchitecture *in vivo*, we used 14–week–old female C57BL/6 mice. Areal bone mineral density (BMD) was evaluated by dual energy X–ray absorptiometry (DXA, *Piximus*) at baseline and then every two weeks following oral gavage with vehicle, tadalafil (2 mg/kg/day), or vardenafil (10 mg/kg/day). In vehicle–treated mice, areal BMD increased over 6 weeks, while tadalafil– and vardenafil–treated mice displayed greater increases in BMD, with the effect of tadalafil reaching statistical

significance compared with vehicle (Fig. 3A). There were also variations in BMD gain at the different sites (Fig. 3A).

To replicate the areal BMD data, we sacrificed the mice at the end of 6 weeks of treatment. The vertebral column was dissected and processed for micro-computed tomography (μ–CT) to assess structural parameters. Consistent with representative images (shown), volumetric BMD (vBMD) and fractional bone volume (BV/TV) were increased significantly or showed trends–to–significance with tadalafil and vardenafil, respectively. The somewhat larger response magnitude with tadalafil was consistent with the areal BMD data (*c.f.* Figs. 3B and 3A). Trabecular number (Tb.N) tended to be higher with corresponding decrements in trabecular spacing (Tb.Sp), while there was no effect on trabecular thickness (Tb.Th). From the collective DXA and μ–CT data, tadalafil displayed more robust increases in bone mass compared with vardenafil.

To achieve further granularity of the bone mass effect, we examined the effects of the two drugs on bone formation and resorption. For bone formation parameters, dynamic histomorphometry was performed on the vertebral column after 6 weeks of drug treatment and following the injection of calcein (15 mg/kg) at days –7 and –2 before sacrifice (Fig. 4A). Shown are fluorescent labels indicative of new bone formation with magnification of selected areas together with quantitative analysis of sections from mice for each group (Figs. 4A and 4B). Both tadalafil and vardenafil increased mineralizing surface (MS), mineral apposition rate (MAR) and bone formation rate (BFR) significantly (Fig. 4B). To explore effects of PDE5A inhibition on bone resorption, we examined the number of osteoclasts labeled for ACP5 (tartrate–resistant acid phosphatase), shown in the photomicrographs (Fig. 4A). Both tadalafil and vardenafil reduced ACP5–positive osteoclast number expressed *per* bone surface or bone volume (Fig. 4C); this is consistent with an action of both drugs on osteoclast formation noted *in vitro* (*c.f.* Fig. 1E).

For the PDE5A–inhibitor–induced anabolic actions, we sought to dissect direct actions of the respective drugs on osteoblasts *versus* potential indirect effects exerted *via* sympathetic relay. Tadalafil and vardenafil yielded matched gene signatures in terms of up–regulated and down–regulated osteoblastogenic genes in bone marrow stromal cells following *in vivo* exposure (Figs. 4D and 4E). Namely, *Ogn* and *Bsp* were up–regulated by both drugs, consistent with an anabolic action, whereas *Bmp2* was down–regulated (Figs. 4D and 4E). The expression of *Alp, Runx2, Tnfsf11* and *Col1a1* remained relatively unchanged with both drugs. We also examined the expression of protein kinase G isoforms, of which PKG2 is a downstream target of PDE5A. *Prkg2* was up–regulated significantly in osteoblast precursors derived from tadalafil–treated mice, with a trend–to–significance (p=0.08) in vardenafil–exposed cells (Fig. 4F). The expression of *Prkg1*, which is not a PDE5A–target, was unaffected by either drug.

Because centrally located PDE5A–positive sympathetic neurons were found to innervate bone (Fig. 2), we also studied the well–characterized 'sympathetic–bone' gene signature (39, 44), consisting mainly of clock genes, in osteoblast precursors from tadalafil– and vardenafil–treated mice. This latter footprint is known to modulate precursor proliferation, rather than differentiation (39, 44). Both tadalafil and vardenafil suppressed this gene signature with the down–regulation of *Per1 Per2* and *Bmal1*, as well as *Myc* and *Ccnd* (Fig. 4G). Such a reduction in genes known to be regulated by sympathetic discharge, prominently cyclin D, would be consistent with reduced proliferation of osteoblast precursors. In parallel, the direct anabolic actions of PDE5A inhibition on this pool of precursors would induce their differentiation into mature mineralizing osteoblasts (*c.f.* Fig. 1D). The data together highlights the intricate control of osteoblast homeostasis by PDE5A, with a net skeletal anabolic advantage of PDE5A inhibition exerted through enhanced mineralization and new bone formation (Figs. 3, 4A and 4B).

DISCUSSION

We report that the two widely–utilized drugs for erectile dysfunction, tadalafil and vardenafil, increase bone mass by coordinated peripheral and central actions. Notably, despite yielding an anti–proliferative gene signature consistent with sympathetic relay, which can be traced upstream to specific brain neurons, the two drugs also displayed a unique peripheral gene signature with demonstrable increases in osteoblastogenesis *in vitro* and bone formation *in vivo*. In parallel, there was a reduction in osteoclastic bone resorption *in vivo* arising from the inhibition of osteoclastogenesis. In all, an anabolic action plus reduced bone removal combined to provide the robust increase in bone mass noted on DXA and replicated through μ–CT–based microstructural analyses.

Interestingly, unlike most other known osteoblast stimuli, such as parathyroid hormone or oxytocin, that trigger the upregulation of the so–called 'osteoblastogenesis gene program' (45), PDE5A inhibitors modulated select genes, with *Ogn* and *Bsp* being up–regulated and *Bmp2* being suppressed. These effects could be time– or dose–dependent or both, noting that gene expression can oscillate in the sustained presence of a stimulus (46). However, despite this observed pattern in which *Runx2*, *Col1a1* and *Alp* also remained unaltered, the net response was anabolic, suggesting that the 'osteoblastogenesis gene program' on its own cannot be used as a sole surrogate for bone formation.

Equally intriguing is our finding that PDE5A immunoreactivity was co–localized with DBH in three brain regions, namely *locus coeruleus*, *raphe pallidus* and paraventricular nucleus of the hypothalamus. Specifically, using the pseudorabies virus, PRV152, we could trace sympathetic innervation from PDE5A–DBH dual–stained neurons directly to bone. PDE5A has been shown to be present in brain regions with potential functions in neurogenesis, memory, and stroke

progression (26-28). Questions remain whether PDE5A is present in the human brain, and if so, what might be the consequences of any central actions of PDE5A inhibition in people.

Erectile dysfunction is the most prevalent sexual dysfunction, which affects ~19% of men >20 years of age, with a prevalence that increases with aging (23). Furthermore, ~47% of men above the age of 50 years have osteopenia (34). There is also a strong association between erectile dysfunction, reduced BMD and an increased risk of hip fracture (47, 48). Our data therefore has considerable promise for the co–therapy of erectile dysfunction and bone loss in aging men. The significantly increased *Pde5a* expression in bone in older compared with young mice (Fig. 1A) supports this premise. Additionally, when used for erectile dysfunction in androgen–deficient, older men, oftentimes together with testosterone, PDE5A inhibition may, through an anabolic action, protect against the osteoporosis that is driven primarily by reduced bone formation. It is noteworthy that when administered to mildly hypogonadal men, testosterone does not itself affect bone mass or muscle strength (49).

We also posit that, when used for treating erectile dysfunction in androgen–ablated prostate cancer patients, PDE5 inhibitors may prevent the acute, rapid and severe bone loss that is poorly amenable to most other therapies (50). The drugs are also used for penile rehabilitation as a standard–of–care after prostatectomy (51), and this use could be extended to bone protection. In addition, and in view of the extensive safety record of PDE5A inhibitors as a class, the possibility of their repurposing for a sole use in post–menopausal women with a high fracture risk should indeed be considered. Towards this, and based on our data, we recommend safety and efficacy trials towards the utilization of all four FDA–approved agents, namely vardenafil, tadalafil, sildenafil, and the more recently approved avanafil, in women.

Interestingly, we found a notable discrepancy between the effective doses of vardenafil and tadalafil in mice *versus* humans. Vardenafil is known to have a 10–fold higher affinity (0.89

nM) compared with tadalafil (9.4 nM) with respect to human recombinant PDE5A (22). However, in the mouse, tadalafil, used at a 5–fold lower dose, displayed an almost similar if not better efficacy than vardenafil. To explore this difference, we modeled mouse PDE5A using the available crystal structures of the catalytic domain of human PDE5A in combination with the respective drugs (PDB id 1UHO for vardenafil and 1UDU for tadalafil). This allowed us to delineate subtle variations in binding modes of the respective drugs with mouse PDE5A *in silico.*

With a chemical structure similar to the cGMP, vardenafil binds the catalytic pocket of PDE5A and competes with cGMP binding (22, 52). In contrast, tadalafil is not related structurally to cGMP, but has a similar mechanism of therapeutic action (53). Our model shows that both drugs bind mouse PDE5A and make H–bond interactions with Q807 (Fig. 5). However, the more robust actions of tadalafil on mouse bone (Figs. 3 and 4) can be explained by its greater binding energy to PDE5A (-19.4 kJ/mol) compared with vardenafil (-16.6 KJ/mol). This difference in *in silico* binding energy is consistent with a greater number of interactions made by tadalafil than by vardenafil (Fig. 5C). More importantly, the M806 residue in mouse PDE5A displays steric clashes with bound vardenafil (Fig. 5C), likely reducing its binding energy, and hence, its potency. On a broader note, this analysis highlights the critical importance of what might appear as relatively minimal structural variations in binding modes, but that could underpin significant potency differences of small molecules across species.

LEGENDS TO FIGURES

Figure 1: **Expression and *In Vitro* Actions of PDE5A Inhibitors Tadalafil and Vardenafil.** (**A**) Taqman–based PCR using bone RNA showing the expression of 20 murine phosphodiesterase (PDE) isoforms, soluble guanylate cyclase isoforms (*Gucy1a2* and *Gucy1a3*), protein kinase G isoforms (*Prkg1* and *Prkg2*), as well as *Tnfrsf11a* and *Tnfsf11* (controls). SYBR–Green–based PCR using bone RNA from 10– and 40–week–old mice showing the expression of *Pde5a*. Data is expressed as copy number *per* cell (normalized to *Tubulin*, *Rps11*, *Actb* and/or *Gapdh*). Mean ± sem; N=3 biological replicates for Taqman; N=4 biological replicated for SYBR Green. (**B**) Data obtained from Human Genome U133 2.0 GeneChip Arrays (Affymetrix, Santa Clara, CA). Presence of transcripts was determined from the signal of perfect-match and mismatch probe pairs in each probe set, with statistical confidence (*P* value) indicated. Characteristic highly expressed osteoclastic and osteoblastic transcripts are also included, as controls. (**C**) Photomicrographs showing immune–labeling of PDE5A (blue, solid arrowhead) and RUNX2 (left panel) or CSF1R (middle panel) (brown, hollow arrowhead) in mouse bone marrow (co–localization is shown in purple, solid arrow), together with a control image without primary antibody (right panel) (See 'Methods' for details). (**D**) Effect of tadalafil and vardenafil on colony forming units–osteoblastoid (Cfu-ob) in 21–day cultures of bone marrow stromal cells isolated from marrow of 10–month–old male mice in differentiating medium, shown as representative alizarin–red–positive Cfu-obs and mean absorbance of extracted dye *per* well ± sem (in triplicate). (**E**) Effect of tadalafil and vardenafil on tartrate–resistant–acid–phosphatase–positive (ACP5+) osteoclasts 5 days following the incubation of bone marrow hematopoietic stem cells with RANKL and M-CSF, shown as representative plates and mean ACP5+ osteoclast number *per* well ± sem (in triplicate). Statistics for D and E: unpaired 2–tailed Students t-test; **P*<0.05 and ***P*<0.01 or as shown.

Figure 2: Localization of PDE5A in Sympathetic Neurons in Three Brain Regions. (**A**) Representative confocal photomicrographs showing the co–localization (yellow) of PDE5A (green) and dopamine β–hydroxylase (DBH, red) immunolabeling in a number of neurons of the *locus coeruleus* (LC), *raphe pallidus* (RPa), and paraventricular nucleus of the hypothalamus (PVH). Also shown is the map of brain areas. (**B**) Retrograde trans–synaptic virus–mediated tract tracing using a pseudorabies virus strain, PRV152, which expresses enhanced green fluorescent protein under the control of the human cytomegalovirus immediate early promoter. PRV152 was injected into the metaphysis or subperiosteally (shown as schematic) in live anesthetized mice 6 days prior to sacrifice. Brain regions were dissected and processed for PDE5A (green) and EGFP (red) immunohistochemistry. The virus traversed from bone *via* the sympathetic nervous system to the three brain regions LC, RPa and PVH, where it co–localized with PDE5A (yellow). Please also refer to Fig. S1.

Figure 3: **PDE5A Inhibitors Tadalafil and Vardenafil Increase Bone Mass**. (**A**) Areal bone mineral density of the total skeleton and of L4-L6 vertebrae and right femur and tibia in female mice treated with vehicle (grey), tadalafil (green) or vardenafil (blue) measured at 0, 2, 4, and 6 weeks by a *Piximus* small animal densitometer (see 'Methods' for details and doses). (**B**) Representative images of 3–dimensional reconstructions of L5 vertebral trabecular bone 6 weeks following vehicle or drug treatment. Static quantitative morphometry from micro-computed tomography, providing measures of volumetric bone mineral density (vBMD), bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular spacing (Tb.Sp), and connectivity density (Conn.D). Shown as mean percent change ± sem, (*versus* vehicle). Statistics: Two–tailed Student's t-test; **P*<0.05 and ***P*<0.01 or as shown *versus* vehicle; *N*=4-5 mice *per* group.

Figure 4: PDE5A Inhibitors Tadalafil and Vardenafil Stimulate Bone Formation and Reduce Bone Resorption. (**A**) Representative low power photomicrographs (750 µm field) of calcein double labels with magnified view (bottom), as well as ACP5–stained surfaces in sections of vertebral trabecular bone 6 weeks following vehicle, tadalafil, or vardenafil treatment. Histomorphometric analysis showing measured and calculated parameters of bone formation, namely mineralizing surface (MS), mineral apposition rate (MAR), and bone formation rate (BFR) (**B**) (5 to 24 sections from 2 to 3 mice), as well as number of osteoclasts (N.Oc) per bone surface (BS) or volume (BV) (**C**) (9 to 20 sections from 3 to 5 mice) (see 'Methods' for details and doses). Blinded measurements were made. Statistics: Two–tailed Student's t-test; **P*<0.05 and ***P*<0.01 or as shown *versus* vehicle. Osteoblastic gene signature (SYBR Green qPCR) consisting of a set of differentially regulated differentiation genes, namely *Ogn*, *Bsp*, *Alp*, *Runx2*, *Tnfsf11*, *Col1a1* and *Bmp2* (**D**, **E**), protein kinase G isoforms (*Prkg1* and *Prkg2*) (**F**), and the unique sympathetic relay signature (39, 44), comprising *Fos*, *Jun*, *Clock*, *Per1*, *Per2, Bmal1, Ccnd* and *Myc* (**G**), in differentiating osteoblast precursors from mice treated with tadalafil or vardenafil. (**H**) Schematic representation of the predicted roles of central and peripheral PDE5A inhibition and of reduced osteoclastic resorption in bone gain.

Figure 5: Structural Modeling of Tadalafil and Vardenafil with Mouse PDE5A. (**A**) Structures of tadalafil (blue) and vardenafil (orange) positioned in the mouse PDE5A catalytic domain. The positions of Zn^{2+} (grey) and Mg^{2+} (magenta) are highlighted in the pocket. Note that catalytic activity occurs *via* direct interactions between cGMP and the divalent cations Zn^{2+} or Mg^{2+} (52). (**B**) Close–up of interactions made by tadalafil (Ta) and vardenafil (Va) in the catalytic pocket. (**C**) 2D plot of interactions of tadalafil and vardenafil in the binding site. Note the steric clash that vardenafil makes with the side chain of M806. Please also refer to Figure S2 for validation details.

LEGENDS TO SUPPLEMENTARY FIGURES

Figure S1: Hippocampus sections without primary anti–PDE5A (**A**) or anti–DBH antibodies (**B**). Sections of the paraventricular hypothalamic nucleus (**C**) or *raphe pallidus* (**D**) following inoculation of PRV152 on the bone surface.

Figure S2: (**A**) Sequence alignment of mouse PDE5A (UniProt id Q8CG03) with the human sequence extracted from 1UDU and 1UHO structures. In these two structures, atomistic coordinates are not available for the disordered region at residues 665–675. Conserved residues are in dark (>80% conservation) and light (>50% conservation) green. Conservative replacements are in yellow. (**B**) Ramachandran plot highlighting the Phi and Psi angles of residues. (**C**) Z–score plot representing how close the quality of the MODELLER–program–built model is to that of the proteins of the same length generated experimentally. (**D**) Energy plot highlighting the local energies against residue positions.

METHODS

Syngeneic, 14–week–old C57BL/6 mice, back–crossed for >10 generations, were maintained on a 12–hour light/12–hour dark cycle, and fed on normal chow. Groups of 5 mice were given DMSO (vehicle), tadalafil (2 mg/kg), or vardenafil (10 mg/kg) by oral gavage every day for 6 weeks. Areal bone mineral density was measured every 2 weeks on anaesthetized mice using a small animal densitometer (*Piximus,* Lunar-GE). Before sacrifice at week 6, the mice were given two intraperitoneal injections of calcein (15 mg/kg) 5 days apart, and were then sacrificed. Micro-computed tomography (μ-CT) with 3-D reconstructions (μCT 40, SCANCO) and dynamic histomorphometry (Zeiss Axio Observer Z1) were performed, as before (54). For histomorphometry, images of individual sections were analyzed blinded for single and double labeled surface area, interlabel distance, mineralizing surface (MS), and derived bone formation calculations. Resorption surfaces, quantified blindly by TRAP staining (Sigma, Cat. #387A-1KT), were conducted as before (54). All experimental protocols were approved by the Institutional Animal Care and Use Committee of Icahn School of Medicine at Mount Sinai.

Human $CD14^+$ cells were isolated and osteoclasts were produced *in vitro* using RANKL, whereas mineralizing osteoblasts were differentiated from human mesenchymal stem cells, all as described in detail (55, 56). Gene screening was performed as described, using isolated RNA to make double-stranded cDNA, from which biotin-labeled cRNA was made and hybridized to the oligonucleotide DNA array on glass (57).

For immunohistochemistry, frozen marrow sections were stained with antibodies against PDE5A (rabbit polyclonal, Invitrogen, Cat. #PA5-79796), and RUNX2 (rabbit monoclonal, Abcam, Cat. #ab192256) or CSF1R (rabbit monoclonal, Invitrogen, Cat. #MA5-15151). Microwave treatment was applied after first staining to eliminate cross–reactivity *per* Toth *et al.* (58). Secondary antibodies included ImmPRESS-AP horse anti-rabbit IgG polymer kit (Vector

Laboratories, Cat. #MP-5401) for PDE5A, and anti-rabbit poly-HRP-IgG (Leica Biosystems, Cat. #RE7161) for RUNX2 and CSF1R. For immunolabeling frozen brain sections, we used chicken anti-GFP (ThermoFisher, Cat. #A10262) and sheep anti-DBH (Abcam, Cat. #ab19353) as primary antibodies.

For the analysis of osteoblastogenesis, bone marrow stromal cells were grown in differentiation medium for 21 days with vehicle (DMSO), tadalafil or vardenafil. Cells were washed, fixed and stained with alizarin red-S (30 min) and photographed, before the alizarin stain was extracted using 10% (v/v) acetic acid (30 min) and processed for spectrophotometry at 405 nm *per* Gregory et al. (2004) (59). Quantitative PCR assays were carried out using premade Taqman probes (Applied Biosystems) or SYBR Green I dye for the respective genes. The data was normalized against a mixture of *Tubulin, Rps11*, *Hprt1*, *Gapdh* and *Actb*. Data comparing groups have been expressed as mean ± sem. Comparisons used two–tailed Student's t-tests, with *P* values less than 0.05 considered significant.

For molecular modeling, we first extracted the amino acid sequence for mouse PDE5A (UniProt accession no. Q8CG03). There are two human crystal structures of the catalytic domain PDE5A in complex with vardenafil (PDB id 1UHO) and tadalafil (PDB id 1UDU) (60), with a sequence homology of 96% over 313 amino acids with the mouse sequence (Fig. S2A). Residues 525-850 from the mouse were modeled based on the human template. The spatial positions of the tadalafil and vardenafil were retained from the human structure into the mouse model. The models were built using MODELLER (https://salilab.org/modeller/), and evaluated based on internal score with stereochemical check *via* a Ramachandran Plot (Fig. S2B). Five lowest Z–score models were selected and were evaluated using ProSA (61, 62), where a Z–score plot and a local model quality graph were generated (Fig. S2C). The Z–score plot represents how close the quality of the MODELLER–program–built model is to that of the proteins of the same length generated experimentally. The energy plot shows the local energies

against residue positions (Fig. S2D). Since a positive value indicates errors in the modeled structure, an appropriate model should have most negative scores. Combining the results from both plots, the ‘best’ model was then selected and was minimized to relieve any steric clashes. Figures were generated using ICM-Molsoft (63) and ezCADD (64).

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