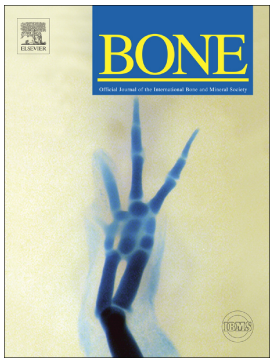

Skeletal restoration by phosphodiesterase 5 inhibitors in osteopenic mice: Evidence of osteoanabolic and osteoangiogenic effects of the drugs

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Skeletal restoration by phosphodiesterase 5 inhibitors in osteopenic mice: evidence of osteoanabolic and osteoangiogenic effects of the drugs

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Abstract

Phosphodiesterases (PDEs) hydrolyze cyclic nucleotides and thereby regulate diverse cellular functions. The reports on the skeletal effects of PDE inhibitors are conflicting. Here, we screened 17 clinically used non-xanthine PDE inhibitors (selective and non-selective) using mouse calvarial osteoblasts (MCO) where the readout was osteoblast differentiation. From this screen, we identified sildenafil and vardenafil (both PDE5 inhibitors) having the least osteogenic EC_{50}. Both drugs significantly increased vascular endothelial growth factor (VEGF) and VEGF receptor 2 (VEGFR2) expressions in MCO and the nitric oxide synthase inhibitor L-NAME completely blocked VEGF expression induced by these drugs. Sunitinib, a tyrosine receptor kinase inhibitor that also blocks VEGFR2 blocked sildenafil-/vardenafil-induced osteoblast differentiation. At half of their human equivalent doses, i.e. 6.0mg/kg sildenafil and 2.5 mg/kg vardenafil, the maximum bone marrow level of sildenafil was 32% and vardenafil was 21% of their blood levels. At these doses, both drugs enhanced bone regeneration at the femur osteotomy site and completely restored bone mass, microarchitecture, and strength in OVX mice. Furthermore, both drugs increased surface referent bone formation and serum bone formation marker (P1NP) without affecting the resorption marker (CTX-1). Both drugs increased the expression of VEGF and VEGFR2 in bones and osteoblasts and increased skeletal vascularity. Sunitinib completely blocked the bone restorative and vascular effects of sildenafil and vardenafil in OVX mice. Taken together, our study suggested that sildenafil and vardenafil at half of their adult human doses completely reversed osteopenia in OVX mice by an osteogenic mechanism that was associated with enhanced skeletal vascularity.

1. Introduction

Phosphodiesterase (PDEs) are enzymes that degrade the phosphodiester bond in the second messenger molecules including cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) to produce 5′-AMP and 5′-GMP, respectively. 11 PDEs are having differential tissue distribution and selectivity of these PDEs could be cAMP-specific, cGMP-specific or both [1]. Besides, PDE1 uses Ca^{2+}/calmodulin as a substrate and represents the third functional variety [2].

PDE-2, -3, -4 and -5 are abundantly expressed in bone cells and thus likely to regulate skeletal function [3, 4]. However, there are varied reports of the effects of PDE inhibition in the bone. For example, among the xanthine class of non-selective PDE inhibitors, theophylline caused bone loss but pentoxifylline stimulated bone formation in preclinical studies [5-7]. This class of PDE inhibitors stabilizes cAMP resulting in the rise of their levels in the cells and depending on their strength and duration of the rise cytotoxicity or differentiation of osteoblasts occurs. Regarding isozyme-specific PDE inhibitors, rolipram, a PDE4 inhibitor has been reported to prevent OVX-induced bone loss by enhancing trabecular and periosteal bone formation with concomitant suppression of bone turnover [8]. There are contrasting reports on the skeletal effects of PDE5 inhibition. For example, in mice, tadalafil stimulates bone loss [4] whereas, in OVX rats vardenafil, udenafil and tadalafil protect against bone loss [9]. However, in the case of the studies with mice, the doses of tadalafil were 20-30 fold more than the human equivalent dose which could have resulted in bone loss. In the case of the rat study that assessed the skeletal effects of PDE5 inhibitors under estrogen deficiency, OVX failed to display unequivocal features of estrogen deficiency viz. bodyweight of OVX rats was less than ovary intact groups after 8 months, and whole-body BMD and serum resorption markers showed a rather marginal decrease compared to that reported in the literature.

PDE inhibitors in general and PDE5 inhibitors, in particular, have vascular/hemodynamic effects [10]. A dense network of blood vessels supplies O_2 and nutrients to bone tissue. The generation of new blood vessels is essential for bone formation during development or repair. A study on 2401 women who were 65 or older concluded decreased vascular support associated with increased bone loss [11]. Both cAMP and cGMP PDE inhibitors are reported to increase nitric oxide (NO) production [12], the key signal molecule for vasodilation (to enhance tissue perfusion) and several endothelial cell function as well as angiogenesis. Among the various angiogenic factors, vascular endothelial growth factor (VEGF) has emerged as the major mediator of endothelial function which involves stimulation of NO production [13]. Moreover, VEGF has been reported to have a positive influence on bone development and growth [14-17] and fracture repair [18] by likely coupling angiogenesis to osteogenesis [19]. PTH, the osteoanabolic drug has been reported to have vascular regulatory function causing vasodilation and hypotension [20]. Moreover, PTH promotes peak bone accrual in growing rats which could be blocked by anti-VEGF antibody treatment, thus suggesting the mediatory role of VEGF in the osteoanabolic effect of PTH [21]. We recently reported that a non-selective methylxanthine drug, pentoxifylline restored osteopenia in ovariectomized (OVX) rats through likely osteogenic and osteo-angiogenic mechanisms [6]. From these reports, it appears that angiogenesis and vascular supply are critical for skeletal health.

Through a screen of non-selective PDE inhibitors belonging to xanthine class, we showed the bone restorative effect of pentoxifylline in osteopenic rabbits and rats [6, 7]. In rats, pentoxifylline displayed both osteogenic and osteo-angiogenic effects [6]. Pursuing with the effort to identify clinically used drugs having the dual role of osteogenesis and osteo-angiogenesis, we here screened an FDA approved library of non-xanthine PDE inhibitors to first obtain drugs with osteogenic effect in vitro. Our ultimate goal is to identify PDE

inhibitors with isozyme selectivity. Out of 17 drugs, sildenafil and vardenafil showed the least osteogenic EC_{50} and hence we tested the skeletal effects of these two drugs given through oral route to osteopenic mice by studying bone mass, microarchitecture, bone formation, bone turnover and bone strength in comparison to subcutaneously dosed teriparatide. We then studied the effect of sildenafil and vardenafil in skeletal vasculature and associated molecular mechanisms using in vitro cultures of osteoblasts.

2. Materials and Methods

2.1. Reagents and chemicals

Cell culture medium, collagenase, and all fine chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) and cell culture supplements viz., FBS and dispase were purchased from Invitrogen (Carlsbad, CA, USA). Sildenafil and vardenafil were purchased from Selleckchem (Houston, Texas, USA). Teriparatide (TPTD) was purchased from Calbiochem (USA).

2.2. In vitro studies

2.2.1. Animals and experimental procedure: All animal experimental procedures were prior approved (Institutional Animal Ethics Committee approval no. CDRI/IAEC/2016/115) and conducted as per the guidelines laid by the Committee for Control and Supervision of Experiments on Animals. All animals used for the study were obtained from National Laboratory Animal Center, CDRI and were subjected to 12 h dark-light cycle under controlled temperature (23– 25 °C) and humidity (50–60 %).

2.2.2. Primary cultures of osteoblast progenitor cells: Following our previously published protocol of sequential digestion [22], we isolated calvarial cells from neonatal mice (MCO) that are known to be mostly progenitor cells committed to the osteoblast lineage [23-25]. Briefly, calvariae from ten to twelve 1–2-day old mouse pups were harvested, cleaned, and subjected to five sequential enzymatic digestions (0.1% dispase and 0.1% collagenase I) of 10–15 min each. Cells from second to fifth digestion were collected, centrifuged, resuspended and cultured in α-MEM containing 10% FBS and 1% penicillin/streptomycin (complete growth medium).

2.2.3. ALP assay: MCO was seeded in 24-well plates and cells were treated with various drugs (10^{-11}M – 10^{-6}M) for 48 h in differentiation medium (α-MEM supplemented

with 10mM β-glycerophosphate and 50µg/ml ascorbic acid). 1,25-$(OH)_2$ vitamin D_3 (10nM) was used as a positive control for this experiment. 2mg/ml para-Nitrophenylphosphate (pNPP) in diethanolamine buffer was used to measure ALP activity calorimetrically at 405nm [22, 26]. Recombinant protein VEGF (#293-VE, R&D system), adenylate cyclase inhibitor SQ22536 (ab120642, Abcam), NO synthase inhibitor L-NAME (ab120136, Abcam) and sunitinib (3768, Tocris) were used to study the signaling mechanisms of the drugs. Sunitinib was used at 1µM, and at this concentration, it had no effect on osteoblast viability (data not shown). Osteoblasts were pre-incubated with inhibitors for 1 h before compound treatment.

2.2.4. Mineralization assay: Bone marrow cells (1×10^6) from the femur of adult mice were seeded in a 6-well plate in differentiating media (α-MEM with 10mM β-glycerophosphate, 5mg/ml ascorbic acid, and 100nM dexamethasone) with or without the drugs. Mineralized nodules were visualized by staining the fixed cultures with Alizarin red-S stain, and the stain was extracted with 10% cetylpyridinium chloride to quantify mineralization colorimetrically (OD at 595nm) [27].

2.2.5. 3′, 5′- Cyclic guanosine monophosphate (cGMP) and 3´, 5´-Cyclic adenosine monophosphate (cAMP) ELISA: MCO were cultured in 6-well plates and upon reaching 80-90% confluence, complete growth medium was replaced by the fresh medium without FBS and preincubated for 1 h. TPTD (100 nM), sildenafil (100nM) or vardenafil (100nM) was added to the cultures, and incubated for various time points. The medium was removed, and cAMP and cGMP levels in the lysates were determined by an ELISA (Cayman Co., Ann Arbor, MI, USA) following the manufacturer's protocol. Total protein in each well was determined by MicroBCA (Pierce, Rockford, IL, USA) to normalize cAMP and cGMP data [6].

2.2.6. Osteogenic gene expression study: MCO was cultured in differentiation media and drug treatments were given for 48 h. RNA was isolated using TRIzol reagent. Changes in the expression of osteogenic genes including runt-related transcription factor 2 (RunX2) [F-TTGACCTTTGTCCCAATGC; R- AGGTTGGAGGCACACATAGG], alkaline phosphatase (ALP) [F-CAGCTCCCCTCCTTTTGTG; R-CCTGGACCTCT CCCTTGAGT], type I collagen (Col I) [F- CCGCTGGTCAAGATGGTC; R- ACCCTTAGGTCCAGGGAATC] were quantified by real-time PCR (qPCR). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [F-AGGGATGCTGCCCTTACC; R-TCTACGGGACGAGGAAACAC] was used as the housekeeping gene for this study. The relative mRNA expression was calculated using the equation, relative quantity = $2^{-\Delta\Delta \text{Cycle threshold}}$ method [28].

2.2.7. Western blotting: 50µg of total protein from MCO or femur of the treated animals were resolved by 8%-12% SDS PAGE, transferred to PVDF membranes (0.22µm pore size) and detected by chemiluminescent HRP substrate-based detection system ((#WBKLS0500, Millipore) in Imager Quant LAS 4010 Chemidoc (GE Healthcare). Antibodies and dilutions for immunoblotting were as follows: VEGF (#CST12556; 1:1000), VEGFR2 (#CST9197; 1:1000) and HRP tagged β-actin (#A3854, 1:50000, Sigma Aldrich). Secondary anti-rabbit (#A0545, 1:25000) was purchased from Sigma Aldrich.

2.3. In vivo studies

2.3.1. Femur osteotomy in mice: A drill bit with a diameter of 0.4 mm was inserted in the anterior portion of the diaphysis of the bilateral femurs, 2cm above the knee joint of adult female mice. One day after surgery, mice were divided into five groups (n=10/group). One group received water (vehicle) and other groups received different doses of sildenafil or vardenafil. Human equivalent doses of sildenafil and vardenafil in mice are respectively 12mg/kg and 5mg/kg [29, 30]. For both drugs, we used the human equivalent dose and its

half dose. Daily oral dosing was performed for 12 days. Calcein labeling to measure bone formation at the osteotomy site was done as described before [31]. Sections (60 µm) through the fracture callus were made using Isomet-Slow Speed Bone Cutter (Buehler, Lake Bluff, IL) and photographed using a confocal microscope (LSM 510 Meta, Carl Zeiss, Inc., Jena, Germany) with appropriate filters. The intensity of calcein binding was calculated using Carl Zeiss AM 4.2 image-analysis software [32].

2.3.2. Pharmacokinetic (PK) study: Oral PK study of sildenafil and vardenafil was performed in adult female mice (n=6). The animals fasted 12-14 h with free access to water. Sildenafil (6mg/kg) or vardenafil (2.5mg/kg) was administered in a suspension form (Tween 80 and 0.25 % CMC). Blood and bone marrow samples were collected at 0.25, 0.5, 1, 3, 5, 7, 9, 12- and 24-hour post-dosing. Serum was harvested by centrifuging the blood at 10,000 rpm for 10 min. Bone marrow harvested from each mouse was homogenized with physiological saline using a tissue homogenizer. The bone marrow and serum samples were stored at -80^{0}C until analysis.

The single-step liquid-liquid extraction method was used for processing of serum or bone marrow sample. An aliquot of serum or bone marrow sample was mixed with ter-butyl methyl ether (TBME) to extract sildenafil, vardenafil and internal standard (I.S.). Briefly, 100 µL of serum/bone marrow sample was transferred to polypropylene tube and 2 ml TBME was added, vortexed and centrifuged. The supernatant was separated and dried under N_2 in TurboVap. The dried residue was reconstituted using the mobile phase and this solution was injected into the LC-MS/MS system for analysis.

The concentration of sildenafil and vardenafil were estimated in serum and bone marrow. Data acquisition and quantitation were performed using AnalystTM (version 1.6.3 software; SCIEX, Toronto, Ontario, Canada). The concentration-time data were subjected to non-

compartmental analysis using Phoenix WinNonlin (version 8.0; Certara Inc., Princeton, NJ, USA) to calculate different PK parameters. The observed concentration at which drug reached to systemic circulation maximum (T_{max}) and maximum bone marrow concentration (C_{max}) were acquired by visual inspection of the experimental data. The area under the curve (AUC) from 0 to T_{last} (AUC_{0-t}) was calculated using the linear trapezoidal rule.

2.3.3. Osteopenic mice by OVX: 5-months old (22±4 g) Balb/c mice were OVX bilaterally and left 6 weeks untreated to develop osteopenia. Osteopenia was confirmed by μCT. At this point, after the confirmation of osteopenia, mice were divided into 5 groups: Sham+veh, OVX+veh, OVX+sildenafil (6mg/kg, oral), OVX+vardenafil (2.5mg/kg, oral) and OVX+TPTD (40μg/kg, s.c.) (n=10/group). After 6 weeks of various treatments, calcein (20mg/kg, s.c.) was injected twice before sacrificing at 7days interval [22].

2.3.4. μCT analysis: We followed our previously published protocols to measure various bone parameters [22].

2.3.5. Measurement of bone-turnover markers: From serum, cross-linked C-telopeptide of type I collagen (CTX-I) and procollagen type I N-terminal propeptide (PINP) levels were determined by ELISA (MyBioSource, USA.), following the manufacturer's protocols.

2.3.6. Vertebral compression test: Bone mechanical strength of L5 vertebra was assessed by compression test using bone strength tester TK 252C (Muromachi Kikai Co. Ltd, Tokyo, Japan) according to a previously published protocol [6].

2.3.7. Histomorphometry of bone: For dynamic histomorphometric measurements, double calcein labeling was done with an interval of 7 days between two calcein injections. Mineralizing surface/bone surface (MS/BS), mineral apposition rate (MAR) and bone formation rate/total bone surface (BFR/ BS), were calculated following previously published

protocols [33]. Briefly we measured periosteal perimeters, single-labeled surface (sLS), double-labeled surface (dLS), and interlabeled thickness (IrLTh) and these data were used to calculate mineralizing surface/bone surface (MS/BS), mineral apposition rate (MAR), and bone formation rate (BFR) as follows: MS/BS=(1/2 sLS + dLS) / BS (%); MAR=IrLTh/7 days (μm/day); BFR/BS=MAR × MS/BS (μm/day) [6, 34].

2.3.8. Bone vasculature measurement: We measured blood vasculature using an in vivo imaging system (IVIS Spectrum In-Vivo Imaging System, Perkin Elmer). Fluorescein Isothiocyanate-Dextran (FITC dextran) [excitation 490/ emission 520] dye was injected via the tail vein (20μl/animal; 50,000beads/20 μl) for assessing vascular bed in long bones. 4 h after injection all mice were sacrificed, and femurs and tibias were cleaned of muscle for imaging. As fluorescent intensity is directly proportional to blood vasculature, total fluorescent intensity was calculated for each group to quantify blood vasculature normalized with body weight [6, 35].

2.3.9. Statistics: Data are expressed as mean ± SEM unless otherwise indicated. The data obtained in experiments with multiple treatments were subjected to one-way ANOVA followed by post hoc Dunnett's multiple comparison test of significance using GraphPad prism 5.

3. Results

3.1. Effect of PDE inhibitors on osteoblast differentiation

Using MCO (consisting of osteoblast progenitors), we screened 17 non-xanthine PDE inhibitors by ALP stimulation assay as the readout for osteoblast differentiation, and data are shown in table 1. Five of these drugs stimulated ALP production out of which sildenafil and vardenafil had lower EC_{50} than others (table 1). Out of the remaining drugs, five inhibited osteoblast differentiation out of which nafronyl oxalate had the least IC_{50} and seven had no effect (table 1).

Although sildenafil, vardenafil, rolipram, and zaprinast have PDE5 inhibitory effect however the potent osteogenic effect of sildenafil and vardenafil could be due to the presence of 1-methyl-4-(phenylsulfonyl)piperazine moiety in these two drugs. There was not much similarity among chemical structures of inactive drugs which prevents drawing a structure-activity relationship. Similarly, the drugs that suppressed osteoblast differentiation share no chemical resemblance and thus a structure-activity relationship cannot be drawn. Based on this screening, we continued our studies with sildenafil and vardenafil.

Sildenafil and vardenafil showed a concentration-dependent increase in osteoblast differentiation between 10- and 100nM, and at 100nM the effect was comparable to the positive control, 1,25-$(OH)_2$ vitamin D_3 (Fig 1A).

3.2. The osteogenic effect of sildenafil and vardenafil is mediated by a cGMP-NO-VEGF pathway

We next studied the effect of sildenafil and vardenafil on mineralization induction in vitro. Bone marrow stromal cells from adult mice, when treated with sildenafil or vardenafil (both 100nM) significantly increased mineralized nodule formation but the effect of sildenafil was more than vardenafil (Fig 1B). Furthermore, both drugs significantly increased the expression

of osteogenic genes including Runx2, col1, and ALP. Sildenafil increased Runx2 mRNA levels more than vardenafil however the mRNA levels of col1 and ALP were comparable between the two drug treated groups (Fig 1C).

We next studied the effect of these two drugs on the cAMP and cGMP levels in MCO. Sildenafil and vardenafil did not affect the intracellular cAMP levels (Fig 1D) but robustly increased the cGMP levels (Fig 1E). We then pretreated MCO with adenylyl cyclase inhibitor (SQ22536) and NO-cGMP inhibitor (L-NAME) and found that whereas SQ22536 had no effect on the induction of osteoblast differentiation, L-NAME completely abrogated the ALP inducing effect (Fig 1F).

As cGMP-PKG signaling is linked with angiogenesis [36] and both drugs significantly increased intracellular cGMP level, so we evaluated their involvement in the stimulation of pro-angiogenic factors in MCO. Both drugs significantly increased VEGF and VEGFR2 expression, although sildenafil induced VEGF more rapidly than vardenafil (24 h versus 72 h) (Fig 1G, H). Sunitinib, the broad-spectrum receptor TRK inhibitor that also blocks the VEGF receptors, abrogated osteoblast differentiation induced by sildenafil and vardenafil (Fig 1I). Taken together, these data suggested that both drugs stimulate osteoblast differentiation via the cGMP-PKG-VEFG receptor signaling.

3.3. Osteogenic dose determination of sildenafil and vardenafil

We tested the osteogenic efficacy of sildenafil and vardenafil in a femur osteotomy model at the human equivalent and half of the human equivalent doses for both drugs. Calcein intensity measurement at the osteotomy site showed that both drugs increased calcein deposition equally over the control (Fig 2A). μCT-based determination of callus bone volume showed a similar result (Figure 2B). Given that sildenafil at 6mg/kg dose and

vardenafil at 2.5mg/kg dose displayed significant new bone regenerative effect, these doses were selected as the minimum effective doses and used for all subsequent studies.

Single-dose pharmacokinetic studies of sildenafil and vardenafil were performed in adult female mice. The pharmacokinetics data are shown in table 2. Both the drugs were found to be rapidly absorbed and achieved maximum serum concentrations (C_{max}) at ~0.25 h. C_{max} for sildenafil was 671.69±131.31ng/mL (~1μM) and vardenafil was 291.6±47.47ng/mL (~0.5μM). Both drugs cleared rapidly from systemic circulation (sildenafil, 15.01±0.82 L/h/kg and vardenafil, 23.07±1.35 L/h/kg) and as their systemic clearance values were higher than the total mice hepatic blood flow (4.32 L/h/kg) [37], high hepatic elimination appeared to have contributed in the process. In bone marrow, vardenafil showed higher clearance (23.07±1.35 L/h/Kg) than sildenafil (15.01±0.82 L/h/Kg). The maximum bone marrow concentrations (C_{max} achieved at 1 h) of sildenafil was 219±20.38ng/mL (~461 nM) and vardenafil was 61.67±4.86ng/mL (~124nM). From the C_{max} values of serum and bone marrow it appeared that at bone marrow, sildenafil and vardenafil respectively attained 46% and 21% of their serum levels. Thus, bone marrow levels of both drugs at their selected doses are greater than their in vitro osteogenic EC_{50} (~100nM).

The volume of distribution of both the drugs in serum (sildenafil, 7.91±1.05 L/kg and vardenafil, 8.31±1.47 L/kg) and bone marrow (sildenafil, 71.002±3.69 L/kg and vardenafil, 45.44±4.03 L/kg) were higher than the total body water volume (0.58 L/kg in mice) [37] which suggested a significant extravascular distribution and tissue penetration. Average elimination terminal half-life of sildenafil and vardenafil in serum were 0.66±0.05 h and 0.72±0.07 h, respectively, and that in bone marrow were 3.28±0.11 and 1.35±0.04h respectively, which further indicated their rapid metabolism and/or excretion. The overall per-oral systemic exposure denoted by the area under the concentration-time profile curve

($AUC_{0-\infty}$) of sildenafil was 403.68.±21.63 h*ng/mL for bone marrow and 720.03±42.55 h*ng/ml for serum and that of vardenafil was 109.68±6.93 h*ng/mL for bone marrow and 317.19±55.91 h*ng/mL for serum.

3.4. Trabecular restoration by sildenafil and vardenafil involve an osteoanabolic mechanism

Sildenafil (6 mg/kg) and vardenafil (2.5 mg/kg) were administered for 6 weeks to OVX osteopenic mice. At the endpoint, serum P1NP (osteogenic marker) was reduced in the OVX+veh group compared to sham. Sildenafil or vardenafil treatment to OVX mice restored PINP to the sham level (Fig 3A). TPTD increased serum P1NP level over sham. By contrast, the OVX-induced rise in serum CTX-1 (resorption marker) levels was unchanged by sildenafil, vardenafil or TPTD (Fig 3A).

At the tissue level, the osteogenic effect of sildenafil and vardenafil were confirmed by double calcein labeling. Both drugs and TPTD increased MS/BS, MAR and BFR/BS over the OVX group (Fig 3B).

The mRNA levels of osteogenic genes including Runx2 and Col1 in the bones were down-regulated in OVX+veh compared to sham. Sildenafil, vardenafil, and TPTD increased the mRNA levels of osteogenic genes more than the sham. Notably, sildenafil increased the expression of the osteogenic transcription factor, Runx2 more than TPTD (Fig 3C). By contrast, the OVX-induced rise in the bone mRNA levels of TRAP and RANK was unaffected by sildenafil, vardenafil, and TPTD. These data corroborated the serum biochemical marker data showing the osteoanabolic effect of sildenafil and vardenafil without the anti-resorptive effect, similar to TPTD (Fig 3C).

3.5. Sildenafil and vardenafil restored trabecular bones in osteopenic mice

Since, both drugs showed an osteoanabolic effect, as shown in the preceding section, we investigated whether they could restore the OVX-induced osteopenia. In vehicle-treated OVX mice BMD, BV/TV, Tb.Th, and Tb.N were lower than sham whereas Tb.Sp was higher (Fig 4). Sildenafil and vardenafil completely restored all the parameters to the levels of sham or TPTD. Since our in vitro data showed that both drugs upregulated VEGF and VEGFR2 expressions and that sunitinib blocked the drug-induced differentiation of MCO, we investigated whether sunitinib blocked the bone restorative effect of these two drugs. Indeed, sunitinib completely blocked the restorative of both drugs at femur metaphysis (Fig 4).

Besides, at L5, sildenafil, and vardenafil restored all trabecular parameters to the levels of sham and TPTD, and sunitinib blocked the effect (Fig 5A).

In OVX mice, the load-bearing capacity of L5 vertebra was significantly decreased. Sildenafil and vardenafil treatments completely restored all vertebral strength parameters to the levels of sham and TPTD (Fig 5B).

3.6. Sildenafil and vardenafil increase bone vascularity

As both drugs increased VEGF and VEGFR2 expression in cultured osteoblasts, we evaluated their expressions in vivo. OVX bones expressed significantly lower levels of VEGF and VEGFR2 compared with sham, and vardenafil and TPTD completely restored these proteins to the sham levels. Sildenafil not only restored these two proteins to the sham levels but also increased the expression of VEGF more than the sham (Fig 6A).

We next assessed vasculature at femur and tibia and observed a significant reduction in fluorescent signals indicative of decreased vasculature in the OVX rats compared with sham, and sildenafil and vardenafil increased the fluorescent signal more than the sham group whereas TPTD restored it to the sham levels (Fig 6B).

4. Discussion

cGMP elevating agents including 2,29-(hydroxy nitroso hydrazino)bis-ethanamine and atrial natriuretic peptide have been shown to stimulate osteoblast function in vitro [38, 39]. Cinaciguat, a soluble guanylate cyclase activator when administered by the parenteral route, mimics some of the bone anabolic effects of E2 in OVX mice [40]. Cinaciguat stimulated bone formation but did not affect osteoclast number and function. cGMP via the activation of cGMP-dependent protein kinase promotes bone marrow vasculogenesis, the de novo formation of blood vessels from endothelial progenitor cells [41]. VEGF is a critical regulator of vasculogenesis and angiogenesis and the regulation of osteogenesis and bone angiogenesis by this growth factor has been extensively studied [42]. In the present study, through a screen of 17 PDE inhibitors belonging to the non-xanthine class, we found that sildenafil and vardenafil have osteogenic and osteo-angiogenic effects likely mediated by VEGF, but did not alter the OVX-induced bone resorption.

Osteoblast precursor cells express high levels of VEGF [43, 44] and VEGF stimulates osteoblast differentiation [45]. VEGF also favors osteoblastic differentiation over adipocytic differentiation of bone marrow-derived mesenchymal stem cells (MSC) [46]. Moreover, with age, VEGF is remarkably reduced in MSCs that give rise to osteoblasts [47]. Our data showed that sildenafil and vardenafil stimulate osteogenic differentiation of MCO (osteoblast progenitors) and upregulate osteogenic genes in vitro and in vivo (in bones of osteopenic mice). Both drugs also stimulated VEGF and VEGFR2 expression in MCO and bones of osteopenic mice. The osteogenic differentiation induced by both drugs was suppressed by sunitinib, an RTK inhibitor that also inhibits VEGF receptor signaling [48]. Although sunitinib inhibits platelet-derived growth factor receptors and c-Kit in addition to VEGFR, however, in the present study, since sunitinib completely blocked the differentiation induced

not only by sildenafil and vardenafil but also by VEGF, it is reasonable to assume that the two PDE5 inhibitors acted via the VEGF receptor to accomplish their osteoanabolic effect. Complete blockade of the bone restorative effect of sildenafil and vardenafil by sunitinib further supported the mediatory role of VEGF signaling in achieving the skeletal effects of these drugs. Given that the activation of NO-cGMP-PKGII pathway stimulated osteoblast function via the Erk and wnt signaling [49, 50], a similar mechanism may also be operative in the case of sildenafil and vardenafil. Moreover, since PDE5 inhibition by taladafil promotes survival of bone marrow-derived MSC under oxidant stress [51] and stimulates neoangiogenesis in endothelial progenitor cells [52], sildenafil and vardenafil could have similar effects in the bone marrow giving rise to the beneficial outcomes in OVX mice as observed in this study.

A copious supply of blood through the generation of new blood vessels is essential for bone formation during development or repair [53]. A study on postmenopausal women suggested that decreased vascular support was associated with increased bone loss [11]. We observed that in mice, osteopenia was associated with reduced bone vascular volume indicative of reduced perfusion/circulation, and sildenafil and vardenafil restored both bone mass as well as vascular volume. Indeed the later effect of the drugs was better than the sham and TPTD treated groups, thus suggesting a robust skeletal vascular support being afforded to OVX mice by these two PDE5 inhibitors. To the best of our knowledge, this is the first report to quantitatively determine the impact of PDE5 inhibitors in the skeletal vascular bed. Recently we have shown that a non-selective PDE inhibitor, pentoxifylline (a xanthine class of drug) had positive effects on bone mass and vascularity [6]. Restoration of bone vascular volume by PTX was comparable to sham whereas vascular volume in response to sildenafil and vardenafil treatments were significantly higher than sham which suggested to us that selective PDE5 inhibition had a superior vascular impact in osteopenic bones than pentoxifylline.

Expression of PDE4 and PDE5 in bone cells and high affinities of sildenafil and vardenafil for PDE5 (higher than that of cGMP itself) [54] appear to explain such robust skeletal vascular effect shown by these drugs. Because PDE5 inhibition causes systemic vasodilation [55, 56], we surmise a vasodilatory effect in the skeletal vascular bed causing increased tissue perfusion resulting in the positive skeletal outcomes.

The skeletal effects of sildenafil and vardenafil were achieved at half of their respective human equivalent doses. The maximum bone marrow levels of vardenafil were equal to and sildenafil was greater than their osteogenic effect in vitro that was determined from ALP assay. Therefore, it is possible that a lower dose of sildenafil than that used here could restore bones in OVX mice. N-desmethyl sildenafil and M1 (desethylation at the piperazine moiety of vardenafil) respectively are the predominant circulating metabolites of sildenafil and vardenafil. Both metabolites have a similar PDE selectivity to the parent drugs and have significant in vitro inhibitory potency for PDE5 [57-59]. Therefore, the osteogenic and osteo-angiogenic (induction of VEGF and VEGFR2 expression in osteoblasts) effects of these two drugs in vivo could be a combination of the parent molecules and their major metabolites.

In men, sildenafil and vardenafil are prescribed for erectile dysfunction [60]. Female sexual arousal dysfunction (FSAD) is common. Currently, there is no regulatory approval for any drug to treat FSAD in postmenopausal women. However, randomized clinical trials reported moderate effectiveness of sildenafil in postmenopausal women with FSAD with good safety profile and have been approved for enhancing sexual function in women [61, 62]. Therefore, a positive skeletal effect at half the doses of the drugs in addition to their indicated use is sufficiently appealing to test their efficacy in postmenopausal osteoporosis.

Our study has several limitations. 1) Taladafil, another specific PDE5 inhibitor was not included in our list of drugs. Among sildenafil, vardenafil, and tadalafil, the least IC_{50} (highest affinity) for PDE5 has been reported for vardenafil, thereby suggesting that the most

potent PDE5 inhibitor has been included in our study. 2) The predominant metabolites- N-desmethyl sildenafil for sildenafil and M1 for vardenafil were not estimated in the PK studies. 3) The efficacy of irsogladine maleate, a PDE4 inhibitor showed osteogenic EC_{50} close to vardenafil was not assessed in OVX mice due to difficulties arising from managing too many experimental groups. Future studies would assess the skeletal impact of irsogladine maleate in a preclinical model of post-menopausal osteopenia. 4) We have not studied the time taken for osteopenia to develop following the withdrawal of these drugs which would have enabled us to predict the drug-free period that could be allowed for sildenafil and vardenafil. 5) Fracture healing is compromised in the osteopenic condition and we have not studied this issue here. 6) Although, the surface referent bone formation at the cancellous site was comparable between the PDE5 inhibitors and TPTD however the same was not assessed at the cortical site. 7) We have not assessed whether increased angiogenesis and vascularity mediated the osteogenic response of these two drugs in OVX mice.

Based on our screening of non-xanthine classes of PDE inhibitory drugs we identified sildenafil and vardenafil having an osteoanabolic effect comparable to TPTD that resulted in complete restoration of bone mass and strength. The underlying mechanism involved the NO-cGMP-VEGF-dependent increase in osteoblastic differentiation. The same pathway likely promoted skeletal vascularity to further support the osteoanabolic impact of these drugs. Our data support carrying out a clinical trial of orally dosed sildenafil/vardenafil at half of their respective recommended doses in postmenopausal women with osteoporosis with TPTD as the comparator.

Figure legend

Figure 1: The osteogenic effect of sildenafil and vardenafil is mediated by a cGMP-NO-VEGF pathway. (A) In MCO, sildenafil and Vardenafil both stimulate osteoblast differentiation as evident from measuring ALP activity. 1,25-dihydroxyvitamin D3 (Vit D - 10nM) was used as a positive control. (B) Sildenafil and vardenafil (both 100nM) increased mineralized nodule formation by bone marrow stromal cells. (C) The expression of different osteogenic genes was increased by sildenafil and vardenafil in MCO. (D) cAMP profile and (E) cGMP profile of sildenafil (100nM) and Vardenafil (100nM) were compared with TPTD (10nM) in MCO. (F) In MCO, cGMP-NO signaling inhibitor L-NAME (3 μM) inhibited sildenafil- or vardenafil-mediated osteoblast differentiation, but adenylate cyclase inhibitor SQ 22536 (1 μM) did not. (G) Sildenafil (100nM) and (H) vardenafil (100nM) increased VEGF and VEGFR2 expression in MCO. (I) VEGFR inhibitor sunitinib (1 μM) completely blocked sildenafil- or vardenafil-mediated osteoblastic differentiation in MCO. VEGF (100ng/ml) was used as a positive control. Data represented as mean ± SEM of three experiments. *p<0.05, **p<0.01, ***p<0.001 compared to vehicle.

Figure 2: Sildenafil and vardenafil promoted new bone formation in mice femur osteotomy model. (A) Representative calcein images (20X) at the osteotomy site and quantification are shown in the indicated treatment groups. (B) Representative 3D-μCT images at the osteotomy site and quantification of callus volume (BV/TV) are shown in the indicated treatment groups. Data represented as mean ± SEM, n=10. *p<0.05, **p<0.01, ***p<0.001 compared to vehicle.

Figure 3: Trabecular restoration by sildenafil and vardenafil involves an osteoanabolic mechanism. (A) Serum PINP and CTX-1 were measured in different treatment groups to asses bone turnover. (B) Mineralized bone surface (MS/BS), mineral apposition rate (MAR), and bone formation rate (BFR) were measured in different treatment groups by dynamic

histomorphometry. (C) Expression of osteogenic (RunX2 and Col1) and osteoclastogenic (TRAP and RANK) genes were measured in femur metaphysis of indicated treatment groups. Data represented as mean ± SEM, n=6. *p<0.05, **p<0.01, ***p<0.001 compared to sham.

Figure 4: Sildenafil and vardenafil completely restored femur trabecular parameters in OVX mice. Upper panels showing representative 3-D μCT images of femur metaphysis of different treatment groups. Lower panel showing quantified data of various parameters including bone mineral density (BMD), bone volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular separation (Tb.Sp). Data represented as mean ± SEM, n=10. *p<0.05, **p<0.01, ***p<0.001 compared to sham.

Figure 5: Sildenafil and vardenafil completely restored L5 parameters in OVX mice. (A) Upper panel showing representative 3-D μCT images of different treatment groups and indicated parameters quantified below. (B) L5 compression strength parameters. Data represented as mean ± SEM, n=6-10. *p<0.05, **p<0.01, ***p<0.001 compared to sham.

Figure 6: Sildenafil and vardenafil increased vascularity in osteopenic bone. (A) Protein extracted from proximal femur was subjected to Western blotting and VEGF and VEGFR2 expression were assessed. (B) Bone vascularity was determined by measuring FITC dextran intensity in the isolated bone of different treatment groups. Left panel showing images of In-Vivo Imaging System of bone vasculature in the different treatment groups. FITC dextran intensity was normalized by the bodyweight of each mouse and data were expressed as fold change. Data represented as mean ± SEM, n=3-6. *p<0.05, **p<0.01 compared to the sham group.

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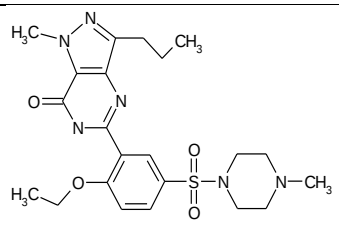
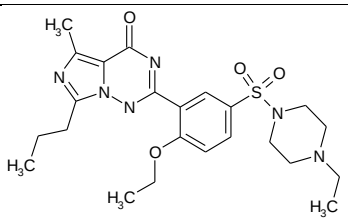
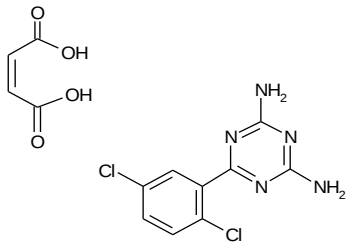
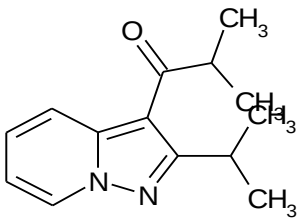
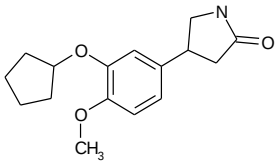
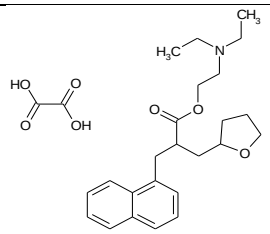
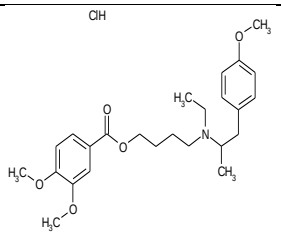
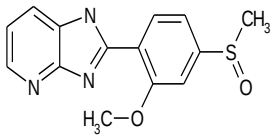
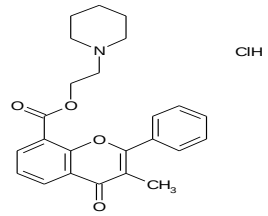
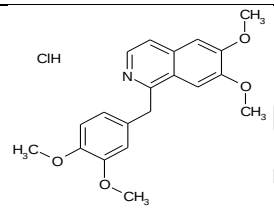
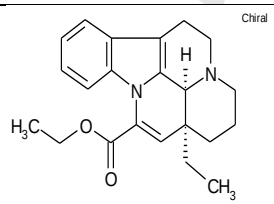
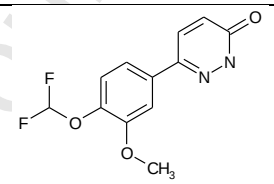
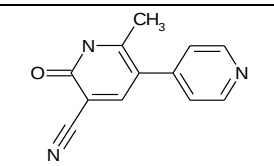
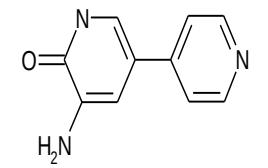
Credit Author Statement:

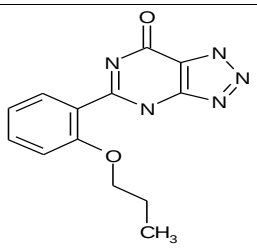
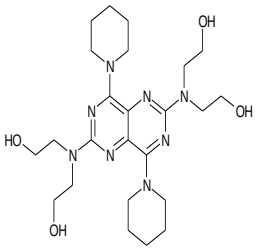
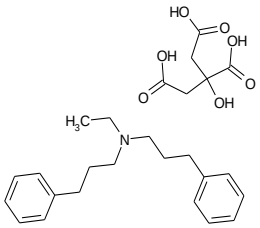
Subhashis Pal: Performed experiments and wrote manuscript; **Konica Porwal & Priya Singh**: Performed experiments; **Mamunur Rashid, Sandeep Kumar Singh & Riyazuddin Mohamed:** Pharmacokinetic study; **Jiaur R Gayen & Muhammad Wahajuddin:** supervised the pharmacokinetic experiments; **Naibedya Chattopadhyay:** conceptualized and supervised the experiments.

All authors analysed data and contributed to discussion.

Table 1: PDE inhibitors and their effects on osteoblast differentiation as determined by ALP assay

Compound	Structure	PDE specificity	EC_{50}	IC_{50}
1. Sildenafil (C22H30N6O4S)		PDE5 inhibitor	93.37±8.81 nM	-
2. Vardenafil (C23H32N6O4S)		PDE5 inhibitor	112.13±10.5 nM	-
3. Irsogladine maleate (C13H11Cl2N5O4)		selective PDE4 inhibitor	132.82±26.42 nM	-
4. Ibudilast (C14H18N2O)		Nonspecific PDE inhibitor	179.25±26.69 nM	-
5. Rolipram (C16H21NO3)		PDE 4,5	2961.15±1083.02 nM	-
6. Nafronyl oxalate (C26H35NO7)		Non-specific PDE inhibitor	-	28.17±9.13

7. Mebeverine hydrochloride (C25H36ClNO5)		Non-specific PDE inhibitor	-	407.27±65. nM
8. Sulmazole (C14H13N3O2S)		PDE 3 inhibitor	-	575.15±64. nM
9. Flavoxate hydrochloride (C24H26ClNO4)		Non-specific PDE inhibitor	-	907.33±52. nM
10. Papaverine hydrochloride (C20H22ClNO4)		Non-specific PDE inhibitor	-	1176.25±179 nM
11. Vinpocetine (C22H26N2O2)		Nonspecific PDE inhibitor	-	-
12. Zardaverine (C12H10F2N2O3)		PDE 3,4	-	-
13. Milrinone (C12H9N3O)		Nonspecific PDE inhibitor	-	-
14. Amrinone (C10H9N3O)		PDE 3 inhibitor	-	-

15. Zaprinast (C13H13N5O2)		PDE 5 inhibitor	-	-
16. Dipyridamole (C24H40N8O4)		Non-specific PDE inhibitor	-	-
17. Alverine citrate salt (C26H35NO7)		Non-specific PDE inhibitor	-	-

Data presented in the order of most potent to least potent for EC_{50}/IC_{50}

Table 2: Pharmacokinetic parameters of sildenafil and vardenafil in adult Balb/c mice serum.

Parameters	Sildenafil (6 mg/kg)		Vardenafil (2.5 mg/kg)	
	Serum	**Bone Marrow**	**Serum**	**Bone Marrow**
Maximum concentration [C_{max}] (ng/ml)]	671.69±131.31	219±20.38	291.6±47.47	61.67±4.86
C_{max} achieving time [T_{max} (h)]	0.25	1	0.25	1
Half-life [$T_{½}$ (h)]	0.66±0.05	3.28±0.11	0.72±0.07	1.35±0.04
Total Systemic exposure [AUC_{0-24} (ng h/ml)]	719.69±42.69	402.35±21.51	317.19±55.94	109.38±6.97
Total Systemic exposure $AUC_{0-\infty}$ (ng h/ml)	720.03±42.55	403.68±21.63	317.19±55.91	109.68±6.93
Volume of distribution [V_d (L/kg)]	7.91±1.05	71.002±3.69	8.31±1.47	45.44±4.03
Systemic clearance [Cl (L/h/kg)]	8.34±0.47	15.01±0.82	8.06±1.42	23.07±1.35

Highlights

- Among 17 non-xanthine class of PDE inhibitors, sildenafil and vardenafil showed the most potent osteogenic effect in vitro
- The osteogenic effect of the two drugs is mediated by a cGMP-NO-VEGF pathway
- At half of their respective adult human doses, these two drugs completely restored trabecular bones in osteopenic mice
- Both drugs increased bone strength in osteopenic mice
- Both drugs increased bone vascularity in osteopenic mice