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A novel hybrid compound LLP2A-Ale both prevented and rescued the osteoporotic phenotype in a mouse model of glucocorticoid-induced osteoporosis

Geetha Mohan¹, Evan Yu-An Lay¹, Haley Berka¹, Lorna Ringwood¹, Alexander Kot¹, Haiyan Chen¹, Wei Yao¹, and Nancy E. Lane¹

¹Center for Musculoskeletal Health, University of California at Davis School of Medicine, Sacramento, CA 95817, USA

Abstract

Prolonged glucocorticoid (GC) administration causes secondary osteoporosis (GIOP) and non-traumatic osteonecrosis. LLP2A-Ale is a novel bone seeking compound that recruits mesenchymal stem cells to the bone surface, stimulates bone formation, and increases bone mass. The purpose of this study was to determine if treatment with LLP2A-Ale alone or in combination with parathyroid hormone (PTH) could prevent or treat GIOP in a mouse model. Four month old male Swiss-Webster mice were randomized to a prevention study with placebo, GC (day 1 – 28), and GC + LLP2A-Ale (IV, day 1) or a treatment study with placebo, GC (day 1 – 56), GC + LLP2A-Ale (IV, day 28), GC + PTH, and GC + LLP2A-Ale + PTH (day 28 – 56). Mice were sacrificed on day 28 (prevention study) or on day 56 (treatment study). The study endpoints included bone mass, bone strength, serum markers of bone turnover (PINP and CTX-I) and angiogenesis (VEGF-A), surface-based bone turnover, and blood vessel density. LLP2A-Ale prevented GC-induced bone loss and increased mechanical strength in the vertebral body (day 28 and 56) and femur (day 56). LLP2A-Ale, PTH, and LLP2A-Ale + PTH treatment significantly increased the mineralizing surface, bone formation rate, mineral apposition rate, double labeled surface and serum PINP level on day 56. LLP2A-Ale and PTH treatment increased femoral blood vessel density and LLP2A-Ale increased serum VEGF-A on day 28. Therefore, LLP2A-Ale monotherapy could be a potential option to both prevent and treat GC-induced osteoporosis and bone fragility.

Keywords

Glucocorticoid; osteoporosis; bone loss; LLP2A-Ale; angiogenesis

Correspondence: Dr Nancy Lane, Center for Musculoskeletal Health, University of California at Davis Medical Center, 4625 2nd Avenue, Suite 2006, Sacramento, CA 95817, USA. Tel: +1-916-734-0758, Fax: +1-916-734-4773, nelane@ucdavis.edu.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Compliance with Ethical Standards

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Introduction

Glucocorticoids (GC) are potent anti-inflammatory compounds that are used in the management of rheumatoid arthritis, asthma, organ transplantation, dermatitis, and lymphoid malignancies. However, prolonged GC administration is the most common cause of secondary osteoporosis and non-traumatic osteonecrosis. An estimated 30 – 50% of patients receiving long-term GC therapy sustain osteoporotic fractures and 9 – 40% develop osteonecrosis [1]. In GC-induced osteoporosis (GIOP) there is a significant and rapid reduction in bone mineral density (BMD) of 6 – 12% measured by quantitative computed tomography, or 1 – 3% by DEXA and continued bone loss with additional years of treatment, resulting in increased fracture risk [2]. The prevalence of GIOP increases with age: about 48% of patients aged above 70 years and 30% of those aged below 60 years have had at least one vertebral fracture while on GC therapy [3]. Moreover, the risk of fractures increases with the duration and dose of GC treatment. A cohort study of patients aged 18 to 64 years which received continuous treatment with 10 mg/day of prednisone for more than 90 days reported a 7-fold increase in hip fractures and a 17-fold increase in vertebral fractures [4].

In bone, GC receptors are expressed on the osteoblasts, osteocytes, growth plate chondrocytes, and in bone marrow cells [5]. GCs reduce osteogenesis from mesenchymal stem cells (MSCs) and direct their differentiation into adipocytes, decrease terminal differentiation to osteoblasts, and reduce osteoblast function and lifespan that eventually leads to bone loss [6,7]. Recent evidence suggests that GCs have a profound effect on skeletal vasculature [8]. Endochondral ossification is important for normal longitudinal bone growth and hypertrophic chondrocytes in the growth plate secrete vascular endothelial growth factor (VEGF) which facilitates penetration of blood vessels from metaphysis. These hypertrophic chondrocytes undergo apoptosis and their space is invaded by osteoblasts that lay down bone matrix which undergoes mineralization to form bone or directly differentiate into osteoblasts/osteocytes [9,10]. GCs have been shown to have detrimental effects on endochondral ossification and longitudinal bone growth by suppressing VEGF expression in hypertrophic chondrocytes. Animal studies have shown that both dexamethasone and prednisolone suppress VEGF expression and reduce the penetration of vessels into the hypertrophic zone of growth plate [11]. GCs have also been shown to have vasoconstrictive effects mediated by endothelin-1 which causes reduced blood flow, resulting in necrosis [12].

Current treatment for GIOP includes the use of bisphosphonates such as alendronate, risedronate, and zoledronic acid, and teriparatide (recombinant human parathyroid hormone, hPTH 1-34) each of which has been approved for this indication by the US Food and Drug Administration. In patients with osteonecrosis, bisphosphonate treatment has been shown to reduce pain, increase mobility, and delay bone collapse [13]. Bisphosphonates inhibit osteoclast activity and prevent bone resorption. However, long-term bisphosphonate use could contribute to an over suppression of bone turnover, which may lead to osteonecrosis of the jaw and atypical femoral fractures [14]. Anabolic agents such as teriparatide might counteract the negative effects of GCs on osteoblasts by increasing the number of osteoblast precursors, promoting differentiation into mature cells, and increasing survival leading to

increased bone formation [15]. PTH has been shown to improve BMD in both mice and patients receiving GCs [16,17]. While injections of hPTH (1-34) can stimulate new bone formation, there are limitations to its use in subjects that have had radiation to the skeleton, Paget disease, or open epiphyses [18]. More recently sclerostin antibody has been demonstrated as a potential treatment for GIOP. Anti-sclerostin antibodies are bone-forming agents which block endogenous sclerostin and promote osteoblast maturation and function. The sclerostin antibody, Romosozumab has been shown to increase serum P1NP (bone formation marker), decrease CTX-1 (bone resorption marker) and improve lumbar spine and hip BMD in post-menopausal women with low bone mass [19,20]. Animal studies have shown that sclerostin antibody prevented GC-induced reduction in bone mass, bone strength, preserved osteoblast activity, and osteocyte viability [21,22].

LLP2A is a peptidomimetic ligand that has high affinity and specificity for integrin $\alpha 4\beta 1$, which is highly expressed on the MSC surface [23]. Recently, our research group synthesized a hybrid compound, where LLP2A is attached to a linker chemical which is then attached to alendronate, a bisphosphonate with high affinity for bone. We have shown in our earlier studies that this compound, LLP2A-Ale, can direct MSCs to the surface of bone [24]. LLP2A-Ale was found to induce $\alpha 4\beta 1$ signal transduction which enhanced MSC migration, proliferation, osteogenic differentiation, and binding to hydroxyapatite, the main component of bone [25]. The effects of LLP2A-Ale on MSC migration appeared to be mainly chemotactic as increased chemokine levels were observed, including monocyte chemotactic protein-1 and macrophage-inflammatory protein-1 α [26]. Moreover, the migration capacity of bone marrow MSCs is under the control of a large numbers of receptor tyrosine kinases (RTKs) in response to both growth factors and chemokines. In a previous study, our research group has shown that LLP2A-Ale regulated RTK activation in MSCs and their migration in the presence of hydroxyapatite [27].

In addition, we have performed experiments that demonstrate that this compound LLP2A-Ale can prevent bone loss from estrogen deficiency, and can prevent bone loss in aged mice [27]. Based on the observations that GC-induced bone loss is partially caused by a reduction in osteoblasts, and LLP2A-Ale can direct MSCs to the bone surface and stimulate osteogenic differentiation, the purpose of this study was to determine if LLP2A-Ale could prevent and restore GC-induced bone loss, compared to PTH and in combination with PTH.

Materials and Methods

Animals and experimental procedures

Four month old male Swiss-Webster mice were housed in a room that was maintained at 20°C with a 12-hour light/dark cycle. The mice were maintained on commercial rodent chow (22/5 Rodent Diet; Teklad, Madison, WI) with 0.95% calcium and 0.67% phosphate available ad libitum. Table 1 shows the different treatment groups and the number of animals in each group. The animals were randomized to either a prevention study or a treatment study as follows:

Prevention Study—The prevention study had the following groups: the baseline group in which the mice were sacrificed on day 1, the placebo group implanted with a placebo pellet

(day 1 – 28); the GC group implanted with a slow release prednisolone pellet (2.8 mg/kg/d, Innovative Research of American, Sarasota, FL) for 28 days, the GC + LLP2A-Ale group implanted with a slow release GC pellet for 28 days and LLP2A-Ale (200µg/kg) was administered by intravenous (IV) injection on day 1 of the study (Table 1).

Treatment Study—The treatment study included the placebo group implanted with a placebo pellet (day 1–56), and the GC group implanted with a slow release prednisolone pellet (2.8mg/kg/d, day 1 – 56). On day 28, the GC treated animals were then randomized into 3 different treatment groups, the GC + LLP2A-Ale group (200µg/kg IV on day 28 and day 42), the GC + PTH group (20µg/kg/day, 5x/week, day 28 – 56, hPTH (1-34), Bachem Inc., Torrance, CA), or GC + LLP2A-Ale + PTH (Table 1).

Alizarin Red (20mg/kg) was given subcutaneously (SC) to all the mice on day 0 and Calcein (20 mg/kg) was injected SC to all mice at seven and two days before euthanization. Mice were sacrificed on day 28 (prevention study) or on day 56 (treatment study). All animals were treated according to the USDA animal care guidelines with the approval of the UC Davis Committee on Animal Research.

Biochemical Markers of bone turnover and angiogenesis

Serum samples were collected at the time of sacrifice and stored at –80°C. Serum levels of Pro-collagen type 1 N terminal propeptide (P1NP) were measured using the Rat/Mouse P1NP EIA kit (Immunodiagnostic Systems, Fountain Hills, AZ, USA) and serum C-terminal telopeptide of type I collagen (CTX-1) levels were measured using RatLaps (CTX-1) EIA kit (Immunodiagnostic Systems Inc., Gaithersburg, MD, USA). The MILLIPLEX MAP mouse angiogenesis magnetic bead panel – multiplex assay was used to measure serum VEGF-A levels (MAGPMAG-24K, EMD Millipore, Billerica, MA, USA). All the assays were performed as recommended by the manufacturers and all the samples were assayed in duplicate. A standard curve was generated from each kit, and the absolute concentrations were extrapolated from the standard curves. The coefficients of variations for inter-assay and intra-assay measurements were < 10% for all assays and are similar to the manufacturers' references [28,29].

Micro-CT

Immediately after sacrifice, the femurs (left and right) and the 5th lumbar vertebral body (LVB 5) from each animal were isolated and fixed in 10 % neutral buffered formalin. The right distal femur, the LVB 5, and the mid-shaft of left femur were scanned using micro-CT (VivaCT 40; Scanco Medical, Bassersdorf, Switzerland); with an isotropic resolution of 10.5 µm. Bone samples were scanned with an X-ray source voltage of 70 kVp and 145 µA current. For the distal femurs, 200 slices were evaluated starting about 0.2 mm from the distal end of the growth plate including a total metaphysis tissue volume of 2 mm³ for each specimen. For the mid-shaft of the left femurs, 200 slices were evaluated starting about 2.2 mm from the distal end of the growth plate that included only the femoral cortical bone. The scans for LVB 5 were initiated in the sagittal plane of the vertebral body, covering the entire cortical and trabecular bone of the vertebral body. The sagittal plane was chosen to compensate for the irregularity of the growth plate. We evaluated 200 slices per specimen for

the LVB consistently including only the secondary spongiosa and excluding the primary spongiosa from analysis. Mineralized bone was separated from the bone marrow, using a matching cube 3-D segmentation algorithm. Bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and trabecular number (Tb.N) of the right distal femur and LVB 5 and the cortical bone volume (Ct. BV) of left femoral mid-shaft was determined as described previously [30,31].

Bone histomorphometry

The right distal femurs were dehydrated in 30% sucrose and embedded in Optimal Cutting Temperature compound (O.C.T.) and sectioned longitudinally with a Leica/Cryostat microtome at 8 μ m thick sections. Bone histomorphometry was performed on unstained sections using a semiautomatic image analysis Bioquant system (Bioquant Image Analysis Corp., Nashville, TN, USA) linked to a microscope equipped with transmitted and fluorescence light [31]. A counting window allowing measurement of the entire trabecular bone and bone marrow within the secondary spongiosa and mid-femoral cortex was created for the histomorphometric analysis. Measurements such as single and double labeled surface (sLS, dLS), percentage of osteoclast surface (Oc.S/BS), mineralizing surface (MS/BS), mineral apposition rate (MAR), and surface-based bone formation rate (BFR/BS) was calculated according to Parfitt & Dempster et al. [32,33].

Biomechanical testing

The endplates of the 6th lumbar vertebrae (LVB 6) were polished using an 800-grit silicon carbide paper to create two parallel planar surfaces. Before testing, caudal and cranial diameter measurements were taken at the top, middle, and bottom of the vertebral body to obtain six measurements which were averaged as the diameter. The height along the long axis was recorded and the vertebrae were modeled as a cylinder. Each lumbar vertebra was then loaded to failure under unconfined compression along its long axis using a MTS 831 electro-servo-hydraulic testing system (MTS Systems Corp., Eden Prairie, MN) at a displacement rate of 0.01 mm/s with 1 kN load cell. The tests were performed in 37 °C HBSS and sample loads and displacements were continuously recorded throughout each test. Values for the maximum load were determined as previously described [29]. To analyze the biomechanical properties of the femurs, the samples were subjected to three-point bending tests using a MTS 831 electro-servo-hydraulic testing system (MTS Systems Corp., Eden Prairie, MN, USA) such that the posterior surface was in tension and the anterior surface was in compression [29]. The major loading span was 14.5 mm. Each femur was loaded to failure in 37 °C HBSS at a displacement rate of 0.01 mm/s while its corresponding load and displacement were measured using a calibrated 1 kN load cell. Two diameter measurements were taken at the fracture location, and averaged to model the femur as a cylinder. Values for the maximum load were then determined [27,34].

Measurement of osteocyte apoptosis in un-decalcified bone sections

As an exploratory analysis we looked at osteocyte apoptosis in the placebo, GC and GC + LLP2A-Ale group from the prevention study (day 28). The right central femur was used for osteocyte apoptosis assay. Decalcified bone sections were mounted, deplasticized, and incubated in 20 μ g/ml of proteinase K in PBS for 15 minutes at room temperature. Apoptotic

cells were detected using TUNEL technology by using an in situ cell detection kit, Peroxidase (POD; Roche Applied Science, Indianapolis, IN, USA). Negative controls were generated by omitting the TdT from the labeling mix. [35]. Only osteocytes within the trabeculae of central femoral cortex were assessed for this analysis. The percentage of apoptotic osteocytes was expressed as the number of apoptotic osteocytes divided by the total number of osteocytes present per bone section [31].

Animal perfusion for quantification of vascularization

An exploratory perfusion study was performed to determine the vascular density of femur from each group (n=4 for each group) in the treatment study. On day 28, the animals were anesthetized using isoflurane and after deep sedation, each mouse was secured on its back to a fixation board. Using fine scissors the rib cage was cut open along its lateral aspect and the diaphragm was severed peripherally to expose the thoracic cavity. The heart was cannulated at the apex of the left ventricle and the right atrium was punctured to provide outflow. The blood vessels were flushed with heparinized normal saline (1000 U/ml) and pressure fixed with 10% formalin. Using a syringe, 5 mL of freshly mixed Microfil (MV-120, Flow Tech, Inc., Carver, Massachusetts) was infused into the aorta at 0.5–1 mL/min. After perfusion, the animals were stored at 4°C overnight to allow polymerization. The following day, the femurs were dissected, fixed in 10% formalin, decalcified thoroughly and scanned using a micro-CT (VivaCT 40; Scanco Medical, Bassersdorf, Switzerland), with an isotropic resolution of 10.5 µm. The entire femur was used in the analysis for blood vessel density (VV). An algorithm of thresholding 150–200, sigma 1 and support 2 were used for the quantification of the vascularization. Vascular density was presented as vessel volume per tissue volume (VV/TV).

Statistical Analysis

The group means and standard deviations were calculated for all outcome variables. Nonparametric Kruskal-Wallis tests were used to identify significant differences between the groups and if detected, non-parametric post-hoc tests were used to determine pairwise comparisons between the treatment groups. Differences were considered significant at $p < 0.05$ (IBM SPSS Statistics 20; SPSS Inc., Chicago, IL, USA).

Results

Micro-CT

Prevention Study—The baseline group (sacrificed on day 1), and the placebo group (sacrificed on day 28) had similar BV/TV values in the distal femur, mid-femur shaft (Fig. 1), and LVB 5 (supplementary table 2). On day 28 there were no significant differences in BV/TV and Ct.BV between the placebo, GC, and GC + LLP2A-Ale group in the femur. However, the LVB 5 BV/TV was significantly higher in the GC + LLP2A-Ale group compared to GC group ($p < 0.05$, supplementary table 2). We did not observe any significant differences in Tb.N, Tb.Th, and Tb.Sp between the groups in either the distal femur and LVB 5.

Treatment Study—On day 56, the GC group had significantly lower BV/TV in the distal femur and Ct.BV compared to the placebo ($p<0.05$, Fig. 1). Treatment with LLP2A-Ale, PTH, and LLP2A-Ale + PTH significantly prevented this decrease in BV/TV in both the distal femur and LVB 5 in the GC treated mice ($p<0.05$). Treatment with LLP2A-Ale, PTH, and LLP2A-Ale + PTH significantly restored Ct.BV in the GC treated mice ($p<0.05$). There were no statistically significant differences in Tb.N, Tb.Th, and Tb.Sp between the groups in both the distal femur and LVB 5. However, there was clear trend towards increased Tb.Th in the GC + LLP2A-Ale, GC + PTH, and GC + LLP2A-Ale + PTH groups compared to GC only group in both the distal femur and LVB 5.

Bone histomorphometry

Prevention Study—The baseline and the placebo group had similar MS/BS, MAR, BFR/BS, sLS/BS, dLS/BS and Oc.S/BS. On day 28, the GC group had significantly lower MS/BS and BFR/BS in the distal femur compared to placebo group ($p<0.05$, Fig. 2, supplementary table 3). The dLS/BS was significantly higher in the GC + LLP2A-Ale group compared to GC only group ($p<0.05$, Fig. 2). We did not observe any significant differences between the groups for the other parameters.

Treatment Study—We observed significantly lower MS/BS, MAR, and BFR/BS in the GC group compared to the placebo group (Fig. 2, supplementary table 3). There were no significant differences in sLS/BS, dLS/BS, and Oc.S/BS between the GC and placebo groups. On day 56, the MS/BS, MAR, BFR/BS, and dLS/BS of GC + LLP2A-Ale, GC + PTH, and GC + LLP2A-Ale + PTH groups were all significantly higher compared to GC group ($p<0.05$). There were no significant differences in dLS/BS and Oc.S/BS between the groups.

Biochemical markers of bone turnover and angiogenesis

Prevention study—On day 28, no significant differences in the serum PINP, CTX-I or VEGF-A levels were observed between the baseline and the placebo group. The serum PINP levels were significantly lower in the GC group compared to the placebo group and GC + LLP2A-Ale group ($p<0.05$, Fig. 3). On day 28, no significant differences in the serum CTX-1 levels were observed between the groups. The serum VEGF-A levels were significantly lower in the GC group compared to placebo and GC + LLP2A-Ale group ($p<0.05$, Fig. 3). The VEGF-A levels were also significantly higher in the GC + LLP2A-Ale group compared to placebo and baseline ($p<0.05$, Fig. 3).

Treatment study—On day 56, no significant differences in the serum PINP levels were observed between the placebo and GC group. However, the serum PINP levels of the GC group were significantly lower compared to GC + LLP2A-Ale, GC + PTH, and GC + LLP2A-Ale + PTH treatment groups ($p<0.05$, Fig. 3). On day 56, there were no significant differences in the serum levels of CTX-1 in any of the treatment groups. Serum VEGF-A levels in the GC group, GC + LLP2A-Ale, GC + PTH, and GC + LLP2A-Ale + PTH treatment groups were significantly lower than the placebo group.

Bone strength

Prevention study—The maximum load of femur and LVB 6 was not different between the baseline and the placebo group. The maximum load of LVB 6 measured by compression test showed significantly decreased bone strength in the GC group compared to the placebo group and the GC + LLP2A-Ale treatment group ($p<0.05$, Fig. 4). In the femur, the maximum load of the GC group was significantly lower compared to the GC + LLP2A-Ale treatment group ($p<0.05$, Fig. 4).

Treatment study—The maximum load of LVB 6 was significantly reduced in the GC group compared to placebo, GC + LLP2A-Ale, GC + PTH, and GC + LLP2A-Ale + PTH treatment groups ($p<0.05$, Fig. 4). The maximum load of femur was significantly reduced in the GC group compared to placebo, GC + PTH, and GC + LLP2A-Ale + PTH treatment groups ($p<0.05$, Fig. 4).

Osteocyte apoptosis

On day 28 an increased number of apoptotic osteocytes were observed in the GC group compared to placebo and GC + LLP2A-Ale treatment group. The mean percentage of apoptotic osteocytes for each treatment group was: 5.8 ± 0.4 for placebo, 13.6 ± 0.7 for GC group and 4.3 ± 0.6 for GC + LLP2A-Ale group.

Vascular density

Vascular density was assessed in the right femur of mice by micro-CT after vascular perfusion on day 28. GC treatment reduced vascular density compared to the placebo, GC + LLP2A-Ale, and GC + PTH treatment groups ($p<0.05$). Interestingly, GC + LLP2A-Ale and GC + PTH treatment increased vascularization more than the placebo group by 46% and 74% respectively ($p<0.05$, Fig. 5).

Discussion

In the present study, we evaluated the effect of LLP2A-Ale, a hybrid compound that directs MSCs to the bone surface, and its ability to prevent or treat GC-induced bone loss. We observed that GC treated mice had decreased bone volume, bone strength in the distal femur and vertebra, reduced bone formation, and evidence of reduced mineralizing surface and surface based bone formation [17,30,36]. Yao et al. reported that GC-induced bone loss is caused by early upregulation of genes involved in osteoclast activation, function, and adipogenesis, followed by a delayed but extended reduction in expression of genes associated with osteoblast activation and maturation [28]. Jilka et al. reported decreased osteoblasts and an increased prevalence of osteoblast and osteocyte apoptosis in GC-induced osteoporosis in both patients and mice. They also reported that GCs directly prolonged osteoclast life span [37]. In the present study we observed increased osteocyte apoptosis in the GC group compared to placebo and GC + LLP2A-Ale group. We also observed an early increase in bone resorption followed by a reduction in bone formation that was present 56 days after the initiation of GC administration. There was an increase in bone turnover with reduced bone formation in mice receiving GC. Treatment with LLP2A-Ale, PTH, and LLP2A + PTH prevented GC-induced bone loss and reductions in bone strength.

In a randomized, double-blind, placebo-controlled study, Saag et al. reported that in patients receiving GCs, alendronate treatment significantly increased spinal BMD and maintained femoral neck BMD [38]. An extension study by Adachi et al. showed significant reduction in vertebral fractures in these patients [39]. A prevention study by Cohen et al. and a treatment study by Reid et al. found that risedronate prevented bone loss at the lumbar spine, femoral neck, and femoral trochanter in patients receiving GCs [40,41]. Reid et al. also reported that a single 5 mg intravenous infusion of zoledronic acid significantly improved lumbar spinal BMD and was more effective than risedronate for prevention and treatment of GIOP.

In the current study using a mouse model of GIOP, we evaluated the effect of LLP2A-Ale treatment on day 1 (preventive study) and treatment on day 28 and 42 (treatment study). Our research group has previously reported that one injection of LLP2A-Ale directed MSCs to the bone surface and increased both their retention and osteoblast differentiation of the transplanted MSCs at the bone surface, using a xenotransplantation model [24]. We also determined that 2–3 injections of LLP2A-Ale two weeks apart was required to obtain increases in bone mass and strength similar to those reported with hPTH (1-34) treatment in ovariectomized mice [27]. Therefore, in this study we administered one injection of LLP2A-Ale on day 1 for prevention study and two injections of LLP2A-Ale, on day 28 and 42 for treatment study. In the prevention study, LLP2A-Ale treatment only had a trend to improve bone volume and bone strength in the distal femur of GC treated mice. It significantly improved bone volume and bone strength in the vertebral body. In the treatment study, LLP2A-Ale significantly improved bone volume and bone strength in the distal femur and vertebral body of GC treated mice. This difference in LLP2A-Ale efficacy between prevention study and treatment study could be due to the fact that bone turnover is high during the first few weeks as indicated by increased CTX-1 values in the GC and GC + LLP2A-Ale groups compared to the baseline and placebo groups. Therefore, the treatment period for prevention study might not have been long enough to observe significant effect of a single dose LLP2A-Ale in this animal model.

Previous literature shows that PTH treatment increases BV/TV, improves BMD, mineral apposition rate, and bone formation rate in mice receiving GCs [17,30]. In a randomized, double-blind, controlled trial, hPTH has been shown to increase spinal and hip BMD and reduce the incidence of new vertebral fractures in men and women with GIOP [16]. In the present study we observed that PTH treatment by itself or in combination with LLP2A-Ale improved bone volume and bone strength in both the femur and vertebra. Treatment with PTH and LLP2A-Ale + PTH also increased serum P1NP levels, and surface based measures of bone formation.

Water constitutes about 20 – 25% of bone's wet weight and a loss of more than 9% of the water has been reported to reduce bone strength [42]. GC excess has been shown to reduce bone vascularity, bone blood flow, and the volume of water present in the skeleton thus compromising bone strength [8]. In the present study, we observed a reduction in maximum load of both femur and vertebrae at day 56 in GC group mice. Treatment with LLP2A-Ale, PTH, and LLP2A-Ale + PTH increased maximum load compared to GC group. Measurement of vascular density of the whole femur showed that GC treatment reduced the

vascular density compared to the placebo mice. Increased vascular density was observed in the LLP2A-Ale and PTH treatment group compared to GC group. It is possible that LLP2A-Ale reverses vascular damage caused by GC thereby increasing femoral and vertebral bone strength.

Apart from hydration, a crucial protein called vascular endothelial growth factor (VEGF) also affects angiogenesis in bone. Wang et al. reported significantly lower VEGF protein and mRNA levels and decreased femoral head blood supply in rabbits after 6 weeks of GC treatment [43]. Jiang et al. reported decreased vessel volume, vessel surface, percentage of vessel volume, and vessel thickness in the femoral heads of rats treated with GC for 6 weeks. They also reported decreased serum VEGF and downregulation of VEGF mRNA in the femoral head of rats treated with GC compared to control rats [44]. In our study, we observed decreased vascular density in the femur of GC treated mice and significantly reduced serum VEGF-A level in the GC group. Whereas, GC + LLP2A-Ale and GC + PTH group mice showed increased vascular density in the femur and increased serum VEGF-A level on day 28. It has been shown that MSCs have angiogenic properties and contribute to vascular formation, function, and aid to regenerate blood supply to injured tissues [45]. MSCs secrete trophic agents and pro-angiogenic factors such as angiopoietin (Ang)-1, VEGF, and fibroblast growth factor-2 that recruit resident MSCs to the site of injury and promote angiogenesis [46]. We speculate that LLP2A-Ale recruits MSCs to the surface of bone and not only prevent bone loss, but also promote angiogenesis when the treatment is initiated at an early stage. On day 56, treatment with LLP2A-Ale, PTH, or LLP2A-Ale + PTH did not restore serum VEGF-A level to normal. This could be due to the fact that the treatment was started 28 days after GC administration during which the disease may have progressed to see any treatment effects on serum VEGF-A levels. Therefore, more experiments are required to study the mechanism by which LLP2A-Ale promotes angiogenesis in the steroid-induced bone loss model in mice.

Our study has a number of strengths including a validated pre-clinical model of GC-induced bone loss and the utilization of a number of outcome measures including: vascular density, both cortical and trabecular bone strength, and surface based bone turnover. However, our study also has some limitations. For example, we did not have a GC + PTH group in the prevention study which could have provided additional information regarding treatment initiation time points. However, PTH treatment is not used for patients on GCs to prevent bone loss – it is approved and used in the United States for the treatment of osteoporosis or bone loss due to GC administration. Also, due to multiple study endpoints, we did not have sufficient number of bone samples to process for surface-based bone histomorphometry which reduced the power of the study. While the study determined a change in vascular density due to GC treatment, and a difference with combination GC + LLP2A or GC + PTH, we observed very low frequency of osteonecrosis changes (n=2, on day 56 in the GC only group) in this study. This did not permit us to study the association between blood vessel density and osteonecrosis development. We did not have sufficient samples for RNA to evaluate tissue expression of vascular agonists such as VEGF. Additional experiments that can evaluate the tissue expression of mRNA levels of VEGF would have been useful to further explore the association with vascular density.

In summary, treatment with LLP2A-Ale by itself or in combination with PTH preserved bone mass and bone strength in GC treated mice. Although the combined treatment with LLP2A-Ale + PTH prevented GC-induced bone loss and restored bone strength compared to GC alone, this observation was not statistically significant from either treatment alone. Therefore, LLP2A-Ale monotherapy may be a potential treatment option for GC-induced osteoporosis and bone fragility. Additional studies are needed to determine if these treatments might be effective in other diseases in which vascularity is compromised, such as GC-induced osteonecrosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Effect of GC, LLP2A-Ale, PTH, and LLP2A-Ale + PTH combination treatment on trabecular bone of right distal femur and cortical bone of left mid-shaft femur assessed by micro-CT. GC administration decreased BV/TV and Ct.BV on day 56 whereas; treatment with LLP2A-Ale, PTH, and LLP2A-Ale + PTH prevented the decreased BV/TV and Ct.BV. BV/TV: bone volume fraction, Tb.N: Trabecular number, Tb.Th: Trabecular thickness, Tb.Sp: Trabecular separation, Ct.BV: Cortical bone volume. Data presented as mean ± SD, * $p < 0.05$

Figure 2.

Representative unstained bone histomorphometric images for each group from prevention study (D-28) and treatment study (D-56) are shown. GC administration induced thinner trabeculae with less mineralizing surface (decreased fluorescent labeling) compared to the placebo group on both day 28 and day 56. LLP2A-Ale, PTH, or LLP2A-Ale + PTH treatments showed thicker trabeculae, higher double-labeled surface (arrow) than placebo and GC group on day 28 and day 56. Note that the preservation of alizarin red in groups treated with LLP2A-Ale, suggesting modeling-dependent bone formation.

Figure 3.

Effect of GC, LLP2A-Ale, PTH, and LLP2A-Ale + PTH combination treatment on biochemical markers of bone turnover and angiogenesis. Treatment with LLP2A-Ale, PTH, and LLP2A-Ale + PTH increased the serum P1NP levels compared to GC group. GC treatment reduced serum VEGF-A levels compared to placebo in both the prevention (day 28) and the treatment (day 56) study. However, LLP2A-Ale treatment significantly increased serum VEGF-A levels compared to GC alone and placebo on day 28. P1NP: procollagen type 1 N-terminal propeptide, CTX-1: C-terminal telopeptides of type I collagen, VEGF-A: vascular endothelial growth factor-A. Data presented as mean $\pm$ SD. * $p < 0.05$

Figure 4.

Mechanical testing data of vertebral body (measured by compression test) and femur (measured by three-point bend testing) is shown. GC treatment significantly decreased the maximum load of vertebral body compared to placebo and GC + LLP2A-Ale on day 28. In the femur, GC treatment significantly decreased the maximum load compared to GC + LLP2A-Ale group. On day 56, the maximum load of vertebral body was significantly decreased in the GC group compared to all other groups. The maximum load of femur was significantly decreased in the GC group compared to placebo, GC + PTH, and GC + LLP2A-Ale + PTH group on day 56. Data are presented as mean $\pm$ SD, * $p < 0.05$

Figure 5.

The top panel shows the blood vessel density of mouse femurs of placebo, GC, GC + LLP2A-Ale, and GC + PTH group on day 28. GC administration significantly reduced femoral vascularity compared to placebo. Treatment with LLP2A-Ale or PTH not only prevented decreased vascularity due to GC administration but it also increased vascularity compared to placebo group. Data are presented as mean $\pm$ SD, * $p < 0.05$. The lower panel shows representative micro-CT reconstructed 3D microangiography of femur from placebo, GC, GC + LLP2A-Ale, and GC + PTH treatment groups with colored thickness maps.

Table 1

Number of animals used in different groups in prevention study and treatment study

No. of Mice	Group	Dose and treatment
Prevention study (D-28)		
13	Baseline	0mg/kg/d GC
12	Placebo	0mg/kg/d GC
13	GC	2.8mg/kg/d GC
13	GC + LLP2A-Ale	2.8mg/kg/d GC + 200µg/kg LLP2A-Ale (day 1)
Treatment study (D-56)		
13	Placebo	0mg/kg/d GC
13	GC	2.8mg/kg/d GC
13	GC + LLP2A-Ale	2.8mg/kg/d GC + 200µg/kg LLP2A-Ale (day 28 and day 42)
13	GC + PTH	2.8mg/kg/d GC + 20µg/kg PTH (20µg/kg/d, 5x/week from day 28 – 56)
13	GC + LLP2A-Ale + PTH	2.8 mg/kg/d GC + 200µg/kg LLP2A-Ale (day 28 and day 42) + 20µg/kg PTH (20µg/kg/d, 5x/week from day 28 – 56)