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ORIGINAL ARTICLE

In vivo effects of two novel ALN-EP4a conjugate drugs on bone in the ovariectomized rat model for reversing postmenopausal bone loss

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Abstract
Summary Two alendronate-EP4 agonist (ALN-EP4a) conjugate drugs, C1 and C2, which differ in structure by a short linker molecule, were evaluated in ovariectomized (OVX) rats for their anabolic effects. We showed that C1 led to significant anabolic effects on cortical and trabecular bone while anabolic effects associated with C2 were minimal.
Introduction EP4as were covalently linked to ALN to create ALN-EP4a conjugate anabolic bone drugs, C1 and C2, which differ in structure by a short linker molecule in C1. When administered systemically, C1 and C2 are delivered to bone through targeted binding of ALN, where local hydrolytic enzymes liberate EP4a from ALN to exert anabolic effects. Here, we compare effects of C1 to C2 in a curative in vivo study.
Methods Three-month-old female Sprague Dawley rats were OVX or sham operated and allowed to lose bone for 3 months. Animals were then treated via tail vein injections for 3 months and sacrificed. Treatment groups were as follows: C1L (5 mg/kg biweekly), C1H (5 mg/kg weekly), C2L (15 mg/kg monthly), C2H (15 mg/kg biweekly), OVX and sham control (phosphate-buffered saline (PBS) biweekly), and ALN/EP4a-unconjugated mixture (0.75 mg/kg each biweekly).
Results MicroCT analysis showed that C1H treatment significantly increased vertebral bone mineral density (vBMD) and trabecular bone volume versus OVX controls while C2 treatments did not. Biomechanical testing showed that C1H treatment but not C2 treatments led to significant improvement in the load bearing abilities of the vertebrae compared to OVX controls. C1 stimulated endocortical bone formation and increased load bearing in femurs, while C2 did not.
Conclusions We showed that C1 led to significant anabolic effects on cortical and trabecular bone while anabolic effects associated with C2 were minimal. These results led us to hypothesize a mode of action by which presence of a linker is crucial in facilitating the anabolic effects of EP4a when dosed as a prodrug with ALN.

Keywords Anabolic drug · Biomechanical testing · Bisphosphonates · Bone histomorphometry · Conjugate drug · Osteoporosis

Introduction

Current intervention strategies for postmenopausal osteoporosis are primarily based around antiresorptive drug therapies such as bisphosphonates (BPs), which have the ability to bind selectively to bone mineral and decrease bone resorption by inducing osteoclastic apoptosis [1, 2]. Alendronate (ALN) is a nitrogen-containing BP which has been shown in previous randomized trials to significantly increase bone mineral density (BMD) and decrease spinal fracture risks in women [3–5]. However, emerging evidence suggests that chronic use of BPs not only suppresses bone formation but also leads to severe reduction in the overall bone turnover over the long term [6, 7].

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Since osteoporosis is often diagnosed after significant bone loss has occurred, optimal treatment of osteoporosis would involve not only inhibiting bone resorption but also increasing bone formation through bone anabolic agents to reverse bone loss in osteoporotic patients. Currently, the only clinically approved anabolic therapy for the treatment of osteoporosis is teriparatide (Forteo™), which is a recombinant form of parathyroid hormone (PTH). Past studies have shown that intermittent administration of PTH has significant bone anabolic effects on rats and humans [8–10]. Unfortunately, treatments with Forteo™ require daily injection and long-term use of PTH has been associated with osteogenic sarcoma in rats [11–13], and thus, its use may be limited due to safety concerns. Prostaglandin E_2 (PGE_2) is an arachidonic acid derivative widely produced within the body and an in vivo bone anabolic agent currently under extensive investigation. Past studies have shown that PGE_2 is a potent multifunctional regulator of bone turnover in vitro. It can stimulate osteoclastogenesis and bone resorption [14, 15] as well as increase bone formation by stimulating osteoblast differentiation in vitro [16, 17]. Furthermore, PGE_2 has been shown to have an overall anabolic effect in vivo, including effects on increasing rat cortical and trabecular bone mass [18, 19], preventing ovariectomy-induced cancellous bone loss [20], and induction of periosteal and endosteal bone formation [21, 22]. Unfortunately, systemic administration of exogenous PGE_2 has been associated with a number of adverse effects including headache, gastrointestinal problems, lethargy, flushing, and uterine contraction. Furthermore, PGE_2 requires daily administration as well as a fairly high dose of around 1 to 3 mg in order to achieve robust anabolic bone effects on rats [23, 24]. As a result, its therapeutic use in humans may be limited.

Past studies using PGE_2 receptor knockout mice have shown that the G protein-coupled receptor EP4 is strongly implicated in mediating PGE_2's stimulatory effects on bone resorption and bone formation [25, 26]. Furthermore, a number of synthetic selective EP4 agonists (EP4as) have been shown to strongly mimic PGE_2's effects on bone, including (1) stimulation of bone resorption in mouse calvarial cultures [25], (2) induction of woven bone formation by local infusion in mice [26], and (3) restoring trabecular and cortical bone mass and bone mechanical strength due to ovariectomy in rats [27]. Unfortunately, despite the overall anabolic effects of EP4 agonists on bone, systemic administration of EP4a in rodents also produces a number of unwanted side effects including thickening of intestinal epithelium, hypotension, and diarrhea [26], thus limiting the clinical application of these agents in systemic anabolic therapy for osteoporosis.

To minimize adverse effects associated with systematic administration of EP4a, the current study adopts a conjugate bone-targeting approach where a synthetic, stable EP4 agonist is covalently linked to the bisphosphonate ALN [28]. The rationale is that when administered systemically, the conjugate drugs should selectively bind to bone surface through ALN's ability to bind to bone mineral [1], where local hydrolytic enzymes liberate the EP4a components in a sustained release manner to exert anabolic effects on bone while ALN remains attached to bone to exert antiresorptive effects.

The present study aims to investigate the in vivo effects of the conjugate drugs as an anabolic therapy for reversing postmenopausal bone loss. Specifically, two conjugate drugs—C1 and C2—were tested in the study [28], whose structures differ by a 4-hydroxyphenylacetic acid linker (LK) moiety found in the C1 conjugate but not in the C2 conjugate (Fig. 1). 4-Hydroxyphenylacetic acid is a natural compound commonly found in foods such as olive oil and is not expected to have pharmacological effects if liberated in vivo [29]. Note that in both the C1 and C2 conjugates, ALN's terminal amine group and EP4a's C-15 hydroxyl group are implicated in the drug conjugation. Since these chemical groups have been shown to be required for the biological functions of ALN [1] and EP4a [30], respectively, the conjugate drugs are not expected to exert drug effects until they are cleaved to yield the ALN and EP4a components. Past in vivo experiments conducted by our laboratory with C1 have shown efficacy for bone growth stimulation in rats, and that cleavage of the LK is required for ALN to fully exert its antiresorptive effects on bone [31].

As a result of their differential structures, C1 and C2 exhibit distinct stabilities in vivo. Past radiolabeling study using EP4a-^{3}H-labeled conjugates showed that upon systemic administration of the conjugates, 5.9 % of total administrated C1 and 9.4 % of C2 were taken up by bone [28] while the majority was excreted in the feces and urine. For the C1 conjugate, radiolabeling studies further showed that the EP4a component was released with a halftime of about 5 days via the cleavage of the ester bond [28] while the ALN component was liberated with a much longer halftime of about 22 days presumably due to the slower cleavage of the carbamate bond between the ALN and LK components [32]. For the C2 conjugate, the EP4a and ALN are obligatorily released simultaneously from the conjugate with a halftime of 28 days through cleavage of the direct carbamate linkage [28].

The current study adopts the ovariectomized (OVX) rat model of postmenopausal bone loss to investigate and compare the in vivo anabolic effects of the C1 and C2 conjugates on bone. Past studies have shown that ovariectomy-induced bone loss in rats shares many similar characteristics with postmenopausal bone loss in humans, making it a suitable animal model for the current study [33]. Previously, our laboratory conducted a 6-week long treatment study examining the effects of the C1 conjugate on OVX rats, which was shown to exert robust anabolic effects on the lumbar vertebrae and femurs [31]. The current study aims to confirm and further characterize the effects of C1 conjugate on bone in a longer, 3-month drug treatment study and to investigate the anabolic effects of the more stable C2 conjugate in comparison to C1 on reversing bone loss

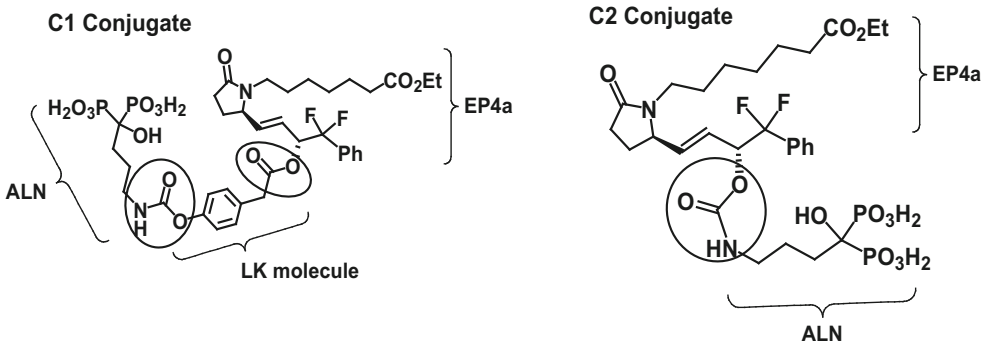


Fig. 1 C1 and C2 conjugate structures. A 4-hydroxyphenylacetic acid linker molecule (*LK*) is present in the C1 conjugate but absent in the C2 conjugate. In the C1 conjugate, ALN is covalently linked to the LK molecule through a carbamate bond, while the other end of the LK is covalently linked to EP4a through an ester bond. In the C2 conjugate, the ALN and EP4a components are directly linked through a carbamate bond. Carbamate and ester linkages are outlined in *circles*

due to ovariectomy. We hypothesized that systemic administration of C1 and C2 will lead to significant bone anabolic effects on treated rats over the course of the treatment compared to the OVX vehicle control (OX) and the unconjugated drug mixture (C2M) control. It is very likely two conjugates may exert unequal levels of anabolic effects on bone due to their differential conjugate structures and in vivo stabilities. Furthermore, we also hypothesized that the slower cleavage of drug components from the C2 conjugate could allow a less frequent dosing (every 2 or 4 weeks) than the C1 conjugate.

Materials and methods

Animal husbandry

Seventy 3-month-old female virgin Sprague Dawley rats were obtained from Charles Rivers Laboratories (Quebec, Canada). Of these, 60 were subjected to bilateral ovariectomy while 10 were sham operated. Prior to drug treatments, animals were allowed to lose bone for 3 months to achieve established osteopenia [34]. All animal procedures were reviewed and approved by the University of Toronto Animal Care Committee (UTACC).

Treatments

After the 3-month bone loss period, animals received tail vein injections of various drug treatments (Table 1) for a period of 3 months. Treatment groups ($n=10$) consisted of high and low dosages for C1 and C2 conjugates (C1H, C1L, C2H, and C2L, respectively), vehicle-treated ovariectomy-negative controls (OX), vehicle-treated sham healthy controls (SV), and an unconjugated mixture of ALN and EP4a in identical concentration to control for the effect of conjugation between the components (C2M). Sterile phosphate-buffered saline (PBS) at pH 7.2 was used for vehicle treatments.

C1H was dosed at 5 mg/kg weekly, while C1L was dosed at 5 mg/kg every 2 weeks. The appropriate dosage was determined based on the results from our earlier study [31] as well as the pharmacokinetic parameters of the C1 conjugate as determined by radiolabeling studies, which allows the calculation that a 5 mg/kg weekly dose of C1H should provide a sustained release of EP4a at approximately 14 μg/kg/day. This is comparable to the rate of PGE_2 release in a previous conjugate study shown to be effective in reversing bone loss in OVX rats [35]. In contrast, the C2 conjugate has an initial uptake rate of 9.4 % and a slower EP4a release halftime of approximately 28 versus 7 days in the C1 conjugate [28]. As a result, a calculated C2H dosage of at least 15 mg/kg every 2 weeks is needed to maintain a similar rate of EP4a release at, on average, 14 μg/kg/day in the bone. The dosage of the C2M control group, in which the ALN and EP4a were dosed in an unconjugated mixture, was chosen with the purpose of matching the highest possible dosage of ALN and EP4a in the C1 and C2 conjugates. Since C2H is dosed at 15 mg/kg every 2 weeks with approximately 10 % uptake rate into bones, only about 1.5 mg/kg of the conjugate would be taken up by bone. This calculates to a maximum possible dosage of approximately 0.75 mg/kg every 2 weeks for each of the two components, which is the dosage we used for ALN and EP4a in the C2M group. We are aware that this calculation is based on the assumption that all of the C2 components are being rapidly cleaved within the 2-week window once it is taken up by bone. However, since the release halftime of EP4a and ALN from C2 is about 28 days, the dosage for the C2M group is likely about two to four times greater than the possible maximum dose released by C2. This was done to avoid any possibility of underdosing the C2M control.

Histomorphometry

Rats were injected with calcein green (10 mg/kg; Sigma-Aldrich) subcutaneously at 12 and 2 days before sacrifice.

Table 1 Study treatment groups (n=10/group, seven groups)

Group	Number	Animals	Treatment	Dosage (mg/kg)	Molar dosage (μmol/kg)	Frequency	Total dosage (mg/kg)
1 Sham control (SV)	10	Sham	Vehicle (PBS)	–	–	Every 2 weeks	–
2 OVX control (OX)	10	OVX	Vehicle (PBS)	–	–	Every 2 weeks	–
3 C1 high dose (C1H)	10	OVX	C1	5	5.69	Weekly	60
4 C1 low dose (C1L)	10	OVX	C1	5	5.69	Every 2 weeks	30
5 C2 high dose (C2H)	10	OVX	C2	15	20.20	Every 2 weeks	90
6 C2 low dose (C2L)	10	OVX	C2	15	20.20	Monthly	45
7 Unconjugated C2 mixture (C2M)	10	OVX	ALN+EP4a	EP4a, 0.75; ALN, 0.75	EP4a, 1.79; ALN, 2.54	Every 2 weeks	EP4a, 4.5; ALN, 4.5

SV and OX are healthy and negative controls, respectively. C1 and C2 both have high- and low-dose treatment groups. C2M is a mixture of unconjugated ALN and EP4a to examine the effects of conjugation. All solutions were administered at 1 mL/kg via IV tail vein injections

Following sacrifice, tibiae were processed and stained for dynamic and static histomorphometry analysis according to procedures described in our previous study [31]. All static and dynamic histomorphometric analyses were performed using the Bioquant Osteo 11.2.6 MIR software (Bioquant Image Analysis Corporation). The region of interest was defined as the tibial proximal metaphysis beginning 1 mm from the distal end of the growth plate and extending 2 mm into the metaphysis. Histomorphometric parameters were measured following the guidelines of the American Society of Bone and Mineral Research for Bone Histomorphometry [36].

X-ray micro-computed tomography

The sixth lumbar vertebrae and left femurs were scanned using the SkyScan 1174 Compact Desktop Micro-CT machine (Bruker microCT, Belgium) to determine volumetric bone mineral density (vBMD) and bone microarchitecture based on the setup described previously [31]. All scans were performed using the following settings: X-ray voltage=5 kV, X-ray current=800 μA, and frame averaging=2, with a 0.25-mm aluminum filter to remove image noise and an isotropic voxel size of 11.6 μm. Images were reconstructed using the SkyScan NRecon software and analyzed using the SkyScan CT-Analyzer software (version 1.5.0). The region of interest was defined to be trabecular secondary spongiosa of the lumbar vertebrae, excluding the primary spongiosa near the cranial and caudal vertebral growth plates as well as a 1-mm-thick volume in the femur mid-diaphysis.

Biomechanical testing

All biomechanical tests were performed using an Instron 4465 testing machine (Instron, Canton, MA, USA) based on the experimental setup described previously [31]. For three-point bending test, a span of 15 mm was used. All time points and load data were recorded with the LabView Acquisition software (LabView v5.0; National Instruments, TX, USA). Force-displacement curves were generated from the data collected and normalized to the geometry of the specimen to construct stress-strain curves.

Statistical analysis

All statistical analyses were performed using the SPSS statistical software (version 21; IBM). The results were compared using one-way analysis of variance (ANOVA) and Bonferroni post hoc tests. Kruskal-Wallis non-parametric test was performed on the samples with non-normal distribution. Significance was defined as $p<0.05$ for two-tailed probability at 95 % confidence interval. Data are presented as mean±SD.

Results

Conjugate effects on bone turnover

The results from histomorphometric analysis of the proximal tibial metaphyseal trabecular bone are shown in Table 2. In the proximal tibia, ovariectomy led to as much as 64 % ($p<0.001$) loss of trabecular bone volume (BV/TV) in the OX group compared to the SV control. In comparison to OX, only C1H and C1L conjugate treatments led to significant increase in trabecular BV/TV (237 and 104 %, respectively; $p<0.001$) while the other drug treatments including C2 and C2M did not significantly reverse ovariectomy-induced bone loss. Furthermore, ovariectomy led to elevated levels of tissue-level bone turnover, as evidenced by significantly increased levels of dynamic bone parameters, including mineralizing surface (MS/BS), mineral apposition rate (MAR), bone formation rate (BFR/BS), as well as increased percent osteoid volume (OV/BV) and percent osteoclast surface (Oc.S/BS) in the OX group compared to SV.

Levels of bone dynamic parameters of C1H were comparable to those of OX control with the exception of BFR/BV, which showed a 28 % reduction compared to OX ($p<0.05$). In

Table 2 Histomorphometric analysis of tissue-level bone turnover in the proximal tibial metaphysis

Treatment group	Sham	OVX					
	SV (vehicle) Mean±SD	OX (vehicle) Mean±SD	C1H Mean±SD	C1L Mean±SD	C2H Mean±SD	C2L Mean±SD	C2M Mean±SD
BV/TV (%)	42.410±3.151	15.397±4.014*	51.869±11.600$	31.318±9.153*,$,#	18.019±4.603*,#,&	21.312±1.649*,#	21.825±4.105*,#
MS/BS (%)	5.289±1.006	20.285±5.719*	19.036±6.081*	12.977±2.922*,$,#	8.234±1.338$,#	7.797±2.031$,#	3.658±1.176$,#,&
MAR (μm/day)	0.974±0.118	1.321±0.025*	1.395±0.141*	1.373±0.146*	1.106±0.182$,#,&	1.098±0.062$,#,&	0.949±0.153$,#,&
BFR/BS (μm/day/mm)	0.054±0.005	0.287±0.050*	0.266±0.087*	0.171±0.041*,$,#	0.082±0.020$,#,&	0.098±0.017$,#,&	0.033±0.012$,#,&
BFR/BV (μm/day/mm^2)	1.150±0.103	5.407±1.661*	3.890±1.172*,$	2.995±0.626*,$	1.787±0.425$,#	2.160±0.549$,#	0.699±0.187$,#,&,Δ
OS/BS (%)	0.457±0.089	3.997±0.765*	1.189±0.248*,$	0.931±0.131$	0.623±0.116$,#	0.645±0.121$,#	0.576±0.120$,#
OV/BV (%)	0.025±0.015	0.210±0.067*	0.061±0.004$	0.054±0.011$	0.034±0.005$	0.034±0.011$	0.028±0.010$
N.Oc/BS (1/mm)	2.394±0.140	3.735±0.446*	2.297±0.359$	1.858±0.329*,$,#	1.100±0.217*,$,#,&	0.994±0.191*,$,#,&	0.783±0.125*,$,#,&
Oc.S/BS (%)	4.922±0.953	7.86±0.888*	4.862±0.609$	3.716±0.698*,$,#	2.038±0.437*,$,#,&	1.955±0.528*,$,#,&	1.545±0.198*,$,#,&

BV/TV trabecular bone volume, *MS/BS* mineralizing surface, *MAR* mineral apposition rate, *BFR/BS* bone formation rate (surface referent), *BFR/BV* bone formation rate normalized over bone volume, *OV/BV* percent osteoid volume, *OS/BS* percent osteoid surface, *Oc.S/BS* percent osteoclast surface. *N.Oc/BS* osteoclast density

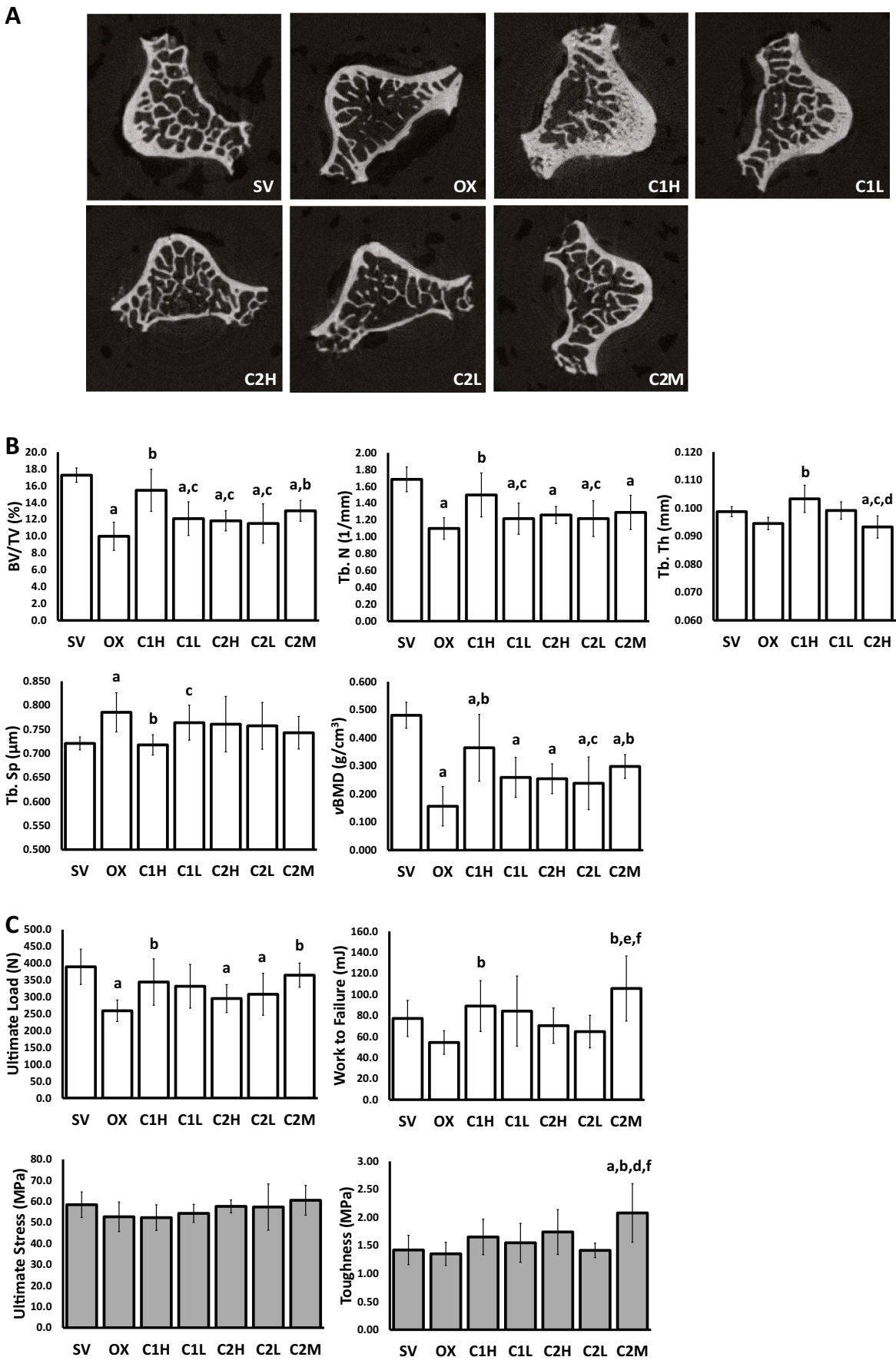
*$p<0.05$, compared to SV; $ $p<0.05$, compared to OX; # $p<0.05$, compared to C1H; & $p<0.05$, compared to C1L; Δ $p<0.05$, compared to C2H

contrast, levels of all bone dynamic parameters of C1L with the exception of MAR were significantly decreased compared to those of OX, while levels of all bone dynamic parameters of the C2H, C2L, and C2M treatment groups were significantly decreased compared to those of OX. These results suggest that all drug treatments with the exception of C1H led to significant reduction of tissue-level bone formation compared to OX control. Furthermore, osteoid formation parameters OS/BS and OV/BV were decreased in all drug-treated groups (including C1H) compared to OX, indicating suppression of osteoid formation by these treatments. In addition, Oc.S/BS and osteoclast density (N.Oc/BS) were also decreased in all drug-treated groups in comparison to OX control, indicating suppression of tissue-level bone resorption by all of the drug treatments.

Effects on trabecular microarchitecture and vertebral mechanical properties

Micro-computed tomography (μCT) analysis showed that ovariectomy led to significant trabecular bone loss and compromised trabecular structural indices in the sixth lumbar vertebrae, as shown by a 42 % ($p<0.001$) reduction in BV/TV, 67 % reduction in vBMD ($p<0.001$), 35 % ($p<0.001$) reduction in trabecular number (Tb.N), and 9 % ($p<0.05$) increase in trabecular separation (Tb.Sp) in the OX group compared to SV control (Fig. 2b). In comparison to the OX control group, C2M treatment led to a 30 % ($p<0.05$) increase in BV/TV and 90 % increase in vBMD ($p<0.01$). Also compared to OX, C1H treatment led to a 55 % ($p<0.001$) increase in BV/TV, 36 % ($p<0.001$) increase in Tb.N, 9 % ($p<0.001$) increase in trabecular thickness (Tb.Th), as well as 133 % increase in vBMD ($p<0.001$) of the vertebral trabecular bone, while a 9 % ($p<0.01$) reduction in Tb.Sp was observed. Furthermore, all of these trabecular structural indexes with the exception of vBMD were comparable between C1H-treated vertebrae and that of the SV control vertebrae. In contrast, C1L, C2H, and C2L conjugate treatments did not lead to significant anabolic effects on the tested vertebrae, as evidenced by comparable trabecular structural indices and vBMD in these groups to OX controls.

Vertebral compression tests of the L6 vertebrae showed that ovariectomy significantly compromised the load bearing ability of the vertebrae as shown by a 33 % reduction ($p<0.001$) in ultimate load in the OX group compared to SV control. Compared to OX, C2M treatment led to a 41 % recovery ($p=0.001$) of ultimate load (Fig. 2c). C1H but not C1L treatment significantly improved the ultimate load and work to failure of the vertebrae compared to OX, by 33 % ($p<0.05$) and 64 % ($p<0.05$), respectively. Furthermore, the ultimate load and work to failure of the C1H group vertebrae were also comparable to those of the SV group vertebrae. However, after normalizing for vertebral geometry, we found


A
SV
OX
C1H
C1L
C2H
C2L
C2M
B
BV/TV (%)
Tb. N (1/mm)
Tb. Th (mm)
Tb. Sp (μm)
vBMD (g/cm³)
C
Ultimate Load (N)
Work to Failure (mJ)
Ultimate Stress (MPa)
Toughness (MPa)

◀ **Fig. 2** Treatment effects on the microarchitecture and mechanical properties of the sixth lumbar vertebrae. **a** Representative μCT cross-sectional images of the L6 vertebrae. **b** Trabecular bone structural indices measured by μCT analysis. *BV/TV* percent bone volume, *Tb.N* trabecular number, *Tb.Th* trabecular thickness, *Tb.Sp* trabecular separation, *vBMD* volumetric bone mineral density. **c** Mechanical properties of the vertebrae determined by vertebral compression testing. *White bars* represent external properties; *gray bars* represent material properties after normalizing for sample geometry. [a]$p<0.05$, compared to SV; [b]$p<0.05$, compared OX; [c]$p<0.05$, compared to C1H; [d]$p<0.05$, compared to C1L; [e]$p<0.05$, compared to C2H; [f]$p<0.05$, compared to C2L. Mean±SD

that the mechanical properties of vertebrae were comparable across all groups, indicating that the improvement seen in the C1-treated vertebrae in comparison to OX was due to the improvement in the trabecular microarchitecture but not due to changes in the intrinsic material properties of the bone. In contrast, C2 conjugate treatment did not lead to improvement in either the extrinsic or intrinsic mechanical properties of the vertebrae.

Conjugate effects on cortical bone

μCT analysis of femur cortical bone showed that C1 treatment stimulated endocortical bone formation in the femoral midshaft, with C1H treatment showing a more pronounced effect than C1L (Fig. 3a). Also, the amount of endocortical bone formation varied largely between animals within the same treatment group, indicating differential animal response to the drug treatment. In contrast, no endocortical bone formation was observed in any C2- or C2M-treated femurs. Furthermore, no visible periosteal bone formation was observed in the femurs of any treated groups possibly due to the low resolution of the current μCT machine used.

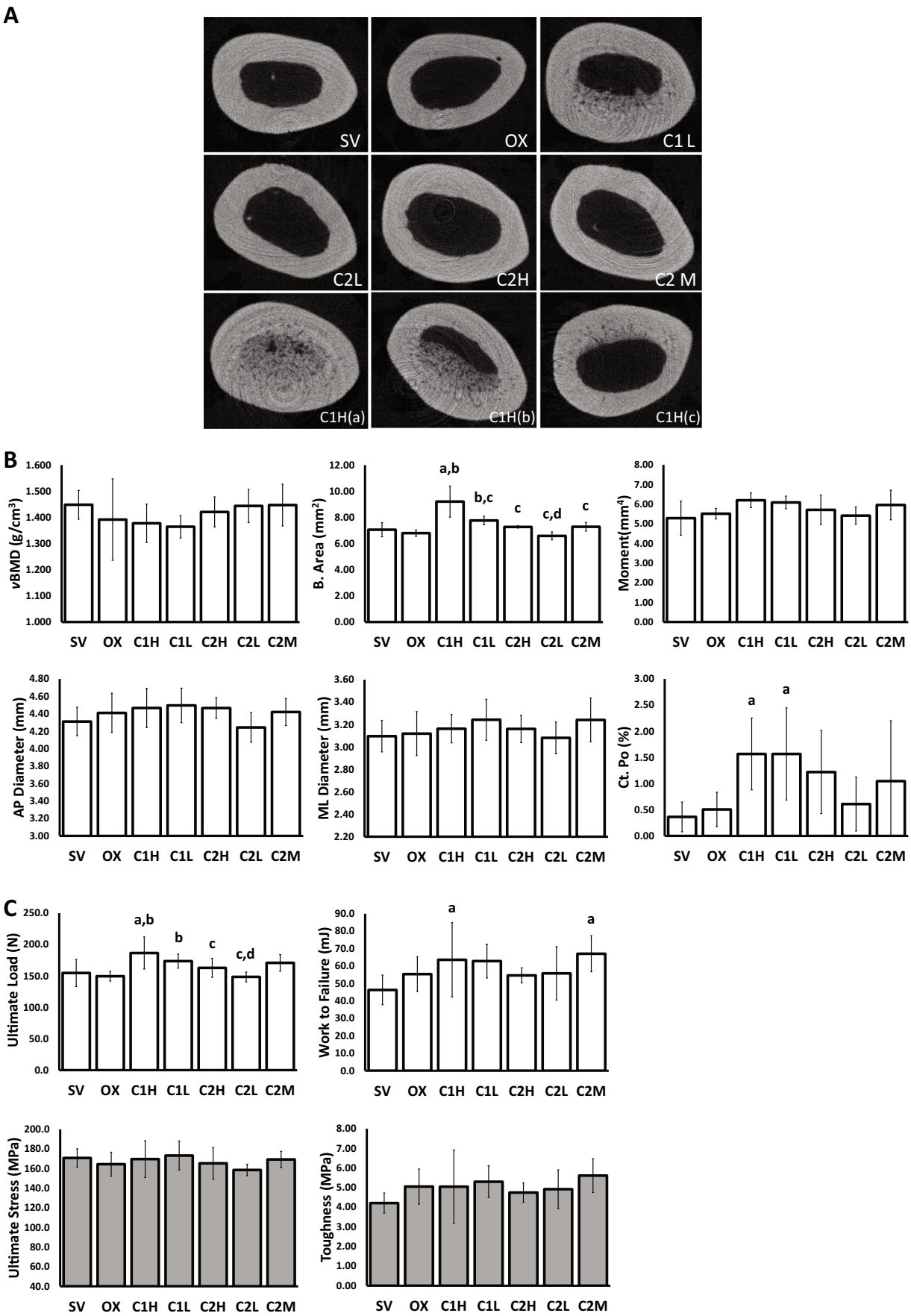
Quantitative μCT analysis showed that the vBMD of femur cortical bone was comparable across all treatment groups (Fig. 3b). A 35 % ($p<0.001$) and 14 % ($p<0.05$) increase in the cross-sectional femoral bone area was observed in C1H and C1L treatments, respectively, most likely attributed to the femoral endocortical bone formation seen in these groups. Such increase in the cross-sectional femoral bone area was not observed in C2H, C2L, or C2M treatment groups. Furthermore, we found no difference in the femurs' anterior-posterior diameter, medial-lateral diameter, and minimum principal moment of inertial (MMI_{min}) between groups, indicating that the external geometry of the femurs was not affected by drug treatments. In addition, we found significant increase in the cortical porosity of C1H- and C1L-treated femurs compared to SV ($p<0.05$), but not compared to OX.

Three-point bending tests of the femurs showed that C1H and C1L treatments significantly improved the ultimate load of the femurs by 25 % ($p<0.001$) and 16 % ($p<0.05$), respectively, compared to OX, while the ultimate load of the C1H-treated femurs were also significantly improved compared to SV group femurs. This was not observed in C2H-, C2L-, or C2M-treated femurs (Fig. 3c). Furthermore, significant increase in work to failure was observed in C1H- and C2M-treated groups by 37 % ($p<0.05$) and 45 % ($p<0.01$), respectively, compared to SV, indicating improved abilities of the femurs to absorb energy before failure. C1L-, C2H-, and C2L-treated groups did not show this improvement. After normalizing for specimen geometry, calculated parameters of bone material properties including ultimate stress and toughness were comparable across all groups, indicating that the intrinsic material properties of femurs were not improved by any of the drug treatments.

Discussion

The goal of the present study was to compare the effects of two novel EP4a-ALN conjugate drugs C1 and C2 in an in vivo rat model of postmenopausal osteoporosis. The current study employed the OVX rat model as the animal model for postmenopausal bone loss [33]. At the study endpoint, we saw that ovariectomy led to significant trabecular bone loss in the lumbar vertebrae leading to decreased vBMD, decreased vertebral mechanical strength, as well as increased bone turnover in the proximal tibial metaphysis compared to SV. These changes are consistent with previous reports of ovariectomy-induced bone loss in rats, thus confirming the validity of the OVX model in our study. The unconjugated drug mixture (C2M) control group led to significant increase in trabecular bone volume in the rat vertebrae compared to OX but did not induce endocortical bone formation in the femurs. Given the lack of conjugation between ALN and EP4a in C2M, as well as the short in vivo half-life of EP4a of around 1–2 h [28], the EP4a component in the C2M treatment likely only has a very short window to exert anabolic effects on bone before being quickly metabolized after each biweekly injection. As a result, the biweekly dosing regiment of C2M was most likely insufficient for EP4a to produce robust anabolic effects on the femurs and the observed increase in trabecular bone volume in C2M-treated vertebrae is mostly due to effects of ALN alone to prevent ovariectomy-induced bone loss.

In contrast to C2M, the conjugate drugs—C1 and C2—were designed to provide a slow but continuous release of EP4a locally in the bone as the cleavage of the conjugates occurs. As such, we hypothesized that the systemic administration of C1 and C2 will lead to significantly greater bone anabolic effects on treated bones over the course of the treatment compared to the OX control and the unconjugated C2M control. Consistent with our hypothesis, the results of the current study showed that the C1H treatment led to significant increase in trabecular bone volume, improved trabecular structural indices, as well as improved mechanical properties of the vertebrae compared to OX control. Most strikingly, we


A
SV
OX
C1 L
C2L
C2H
C2 M
C1H(a)
C1H(b)
C1H(c)
B
vBMD (g/cm³)
B. Area (mm²)
Moment(mm⁴)
AP Diameter (mm)
ML Diameter (mm)
Ct. Po (%)
a,b
b,c
c
c,d
c
a
a
SV OX C1H C1L C2H C2L C2M
C
Ultimate Load (N)
Work to Failure (mJ)
Ultimate Stress (MPa)
Toughness (MPa)
a,b
b
c
c,d
a
a
SV OX C1H C1L C2H C2L C2M

◀ **Fig. 3** Treatment effects in cortical bone. **a** Representative cross-sectional μCT images of the left femora at mid-diaphysis. *Bottom row* represents high (6/10 animals), medium (3/10 animals), and low level (1/10 animals) of endocortical bone formation found in the C1H-treated femora (10 animals in total). Note that high is defined as >50 % marrow occlusion by endocortical one formation, medium as 10–50 % marrow space occlusion, and low as <10 % marrow space occlusion. **b** Structural indices of femur mid-diaphysis measured by μCT analysis. *vBMD* volumetric bone mineral density, *B.Area* cross-sectional bone area, *Moment* minimum principle moment of inertia, *Ct.Po* cortical porosity. **c** Femur mechanical properties determined by three-point bending tests. *White bars* represent external properties; *gray bars* represent material properties after normalizing for femur sample geometry. [a]$p<0.05$, compared to SV; [b]$p<0.05$, compared OX; [c]$p<0.05$, compared to C1H; [d]$p<0.05$, compared to C1L; [e]$p<0.05$, compared to C2H. Mean±SD

saw obvious endocortical bone formation in the femur mid-diaphysis of the C1-treated groups as well as increase in cortical porosity, which was not observed in the C2M-treated femurs. Past studies have shown that endocortical bone formation is a downstream anabolic effect of PGE_2 on rats [37, 38, 22], while increase in cortical porosity is also consistent with the effect of PGE_2 in stimulating intracortical remodeling leading to a porous cortex [22, 37]. Since EP4a mimics the bone effects of PGE_2, the EP4a component is, thus, most likely responsible for the observed anabolic effects on the C1H-treated femurs and possibly on the vertebrae. Given that the dosage of C2M was chosen to match the highest dosage of ALN and EP4a that is possibly released by the conjugate drugs in vivo, these results suggest that the C1H treatment led to the overall greater anabolic effects than the C2M treatment. As such, the conjugation between ALN and EP4a in the C1 conjugate is, therefore, beneficial in terms of facilitating anabolic effects of EP4a through target delivery of the drug to bone by ALN, leading to the slow release of the EP4a in bone. This is superior to the C2M treatment, which delivers a bi-weekly bolus dose of EP4a in bone that is eliminated quickly due to local metabolism. In comparison to C1H treatment, the C1L dose treatment led to lesser amounts of endocortical bone formation in treated femurs and did not have significant anabolic effects on the vertebrae. These results suggest that the C1L treatment dose was likely too low to provide sufficient amount of continuous EP4a release.

In the current study, we hypothesized that the C2 conjugate treatments should lead to apparent anabolic effects on treated bones, although their effectiveness may be unequal to the C1 conjugate treatments due to their distinct conjugate structure and pharmacokinetics in vivo [28]. Furthermore, we had hoped that the slower cleavage of C2 conjugate may allow less frequent dosing of C2 than the C1 conjugate. To our surprise, we saw no apparent anabolic effects on C2H- or C2L-treated femurs, which had no apparent endocortical bone formation or increase in cortical porosity. Furthermore, the treated vertebrae exhibited significant ovariectomy-induced trabecular bone loss comparable to that of OX control. These results suggest that the antiresorptive effects of ALN as well as the anabolic effects of EP4a in C2H and C2L treatments were insufficient to prevent and/or recover lost bone due to ovariectomy. This was surprising since the C2H conjugate treatment was dosed to provide an average EP4a release rate of 14 μg/kg/day [28] into the bone, which is similar to the EP4a release rate of the C1H treatment. Given that the C1 and C2 conjugates contained the same drug components but slightly different conjugate structures, such results suggest that their differing conjugate structures may be implicated in their distinct pharmacodynamics.

Finally, in the analysis of tissue-level turnover by histomorphometry study of tibial metaphysical trabecular bone, we saw that all drug treatments including C1L, C2H, C2L, and C2M with the exception of C1H led to significant reduction in the levels of bone resorption and bone formation parameters compared to OX. As past studies have shown that ALN can lead to reduction in overall bone turnover [39, 7], these results indicate that the ALN components in all of these drug treatments have exerted some levels of pharmacological effect on bone. In the C1H-treated bones, a significant reduction in tissue-level resorption was observed while levels of dynamic bone formation parameters were comparable to those of OX. However, analysis of osteoid seams did further show a decrease in osteoid formation in the C1H group, which may predict a greater reduction in overall bone formation in response to a longer C1H treatment term.

To summarize, the results of the current study suggest that the EP4a-related anabolic effects on bone are better facilitated by the C1 conjugates than C2. Furthermore, ALN-related effects on reducing bone turnover are present in all of the drug treatments, though not sufficient to prevent ovariectomy-induced bone loss in some of the treatment groups. To better understand the mechanisms underlying the distinct in vivo effects of C1 and C2, their specific conjugate structures should be considered (Fig. 1). As mentioned previously, C1 and C2 differ structurally with the presence or absence of the LK molecule, resulting in their distinct in vivo stabilities [28]. In the C2 conjugate, the ALN is directly linked to EP4a through a carbamate bond, which has a cleavage halftime of about 28 days. As a result, the cleavage of the conjugate link in the C2 conjugate leads to the release of the EP4a and ALN drug components simultaneously in a 1:1 ratio, which means that the concentration of newly freed EP4a and ALN in bone is the same at any given time. Given the previously reported effects of ALN on reducing osteoclastic bone resorption as well as suppressing bone formation [7, 39], it is likely that these effects may be antagonistic to the anabolic effects of PGE_2 on promoting osteoblastogenesis and new bone formation [16, 17, 22, 40] in vivo. This is supported by the results of the current study where we saw the lack of anabolic effects in the C2H-treated vertebrae and femurs, despite a daily release rate of EP4a that has been previously shown to be sufficient in

reversing ovariectomy-induced bone loss in rats [35]. In addition, past studies have shown that though the effects of PGE_2 are predominantly anabolic in vivo [22], PGE_2 and EP4 receptor agonists are also strong stimulators of osteoclastic differentiation and bone resorption in vitro [41, 25, 42–44]. Therefore, it is possible that EP4a's stimulatory effects on osteoclastogenesis and bone resorption may have antagonized ALN's inhibitory effects on osteoclastic resorption in the C2-treated bones, leading to blunting of ALN-related antiresorptive effects. This is demonstrated in the current study where we saw significant trabecular bone loss in the C2H-treated vertebrae due to ovariectomy despite a sufficient and relatively high-ALN dose provided by the C2H treatment [45].

Contrary to what was observed for the C2 conjugate treatments, EP4a-related anabolic effects did not appear to be significantly blunted by ALN in the C1-treated bones. As mentioned previously, C1 conjugate's structure differs from that of C2 by a LK molecule (Fig. 1), which allows the differential release of the EP4a and ALN components from the conjugate at halftimes of approximately 5 days [28] and 22 days [32], respectively. This means that for every one ALN released from the C1 conjugate, four EP4a molecules are released locally in bone. At a 4:1 ratio, the results in the vertebrae and femurs suggest that EP4a's anabolic effects are not significantly blunted by ALN and are able to induce new bone formation in these skeletal sites. As such, we propose a possible pharmacodynamic antagonistic relationship between EP4a and ALN, specifically when dosed at a low ratio of around 1:1, where the two components can significantly oppose and antagonize their respective anabolic and antiresorptive actions in vivo. However, at a greater release ratio such as 4:1 in the C1 conjugate, this antagonistic relationship most likely becomes insignificant.

Unfortunately, with the exception of the present study, there are currently no combination drug studies that examine the potential antagonistic effects between EP4a and ALN when specifically dosed at a low (1:1) ratio. For example, in Lauritzen et al.'s study which reported that ALN did not prevent PGE_2's stimulation of bone formation in rats [24], the daily dosage of PGE_2 used in the study was over 3000 times greater than the daily dosage of ALN (3 mg/kg/day for PGE_2 and 0.8 μg/kg/day for ALN). As such, the currently available literature on ALN and EP4a/PGE_2 combination effects in vivo does not actually reject the possibility of such an antagonistic relationship when dosed at a 1:1 ratio. Future studies are therefore needed to confirm this possibility.

A limitation of the current study is the different dosing schedules used for the C1 and C2 conjugate treatments, which vary between the weekly dosing versus the biweekly dosing for the C1H and C2H treatments, respectively, and the biweekly and monthly dosing for the C1L and C2L treatments,

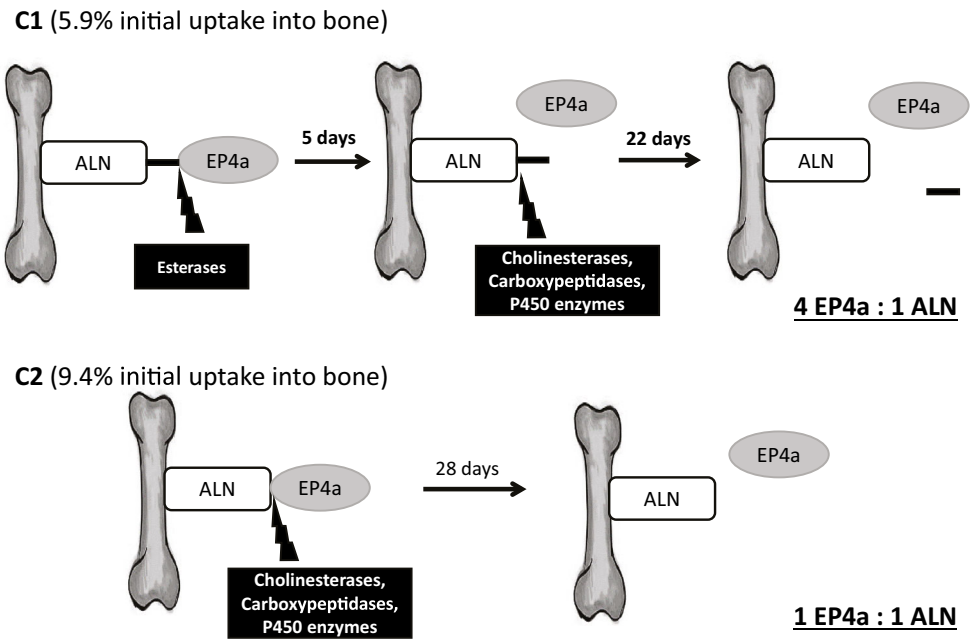


Fig. 4 Proposed mode of action of the C1 and C2 conjugates. In the C1 conjugate, esterases cleave the bond between LK and EP4a quickly to liberate the EP4a component (release halftime of 5 days), leaving ALN attached to the LK. Enzymes such as carboxypeptidases then cleave the carbamate bond to release ALN from the LK molecule (release halftime of 22 days). *Black bar* represents the LK molecule. The difference in the release halftime between the two components leads to the release of EP4a and ALN in a 4:1 ratio, which gives EP4a an opportunity to exert its anabolic effects on bones without significant antagonistic effects from ALN. In the C2 conjugate, enzymes cleave the carbamate bond to release EP4 and ALN molecules from the conjugate simultaneous in a 1:1 ratio (release halftime of 28 days), where ALN significantly suppresses the anabolic effects of EP4a on bone

respectively. This was done to match more closely the EP4a release halftime from each conjugate drug as well as to test our hypothesis regarding whether the slower release rate of EP4a in the C2 conjugate can allow less frequent dosing than the C1 conjugate in animals. We acknowledge that such dosing pattern may cause difficulties in the interpretation of the results in terms of comparing the in vivo effects of the two drug conjugates. However, it should be noted that the C1H and C1L treatments were in fact dosed to provide very similar daily release rates of EP4a into bone as the corresponding C2 treatments [28]. Thus, although the dosing schedules of these treatments vary, their relative in vivo anabolic effects relating to EP4a may still be sufficiently evaluated and compared.

In conclusion, the results of the current study suggest that the C1 conjugate has greater anabolic effects in vivo compared to the C2 conjugate, likely owing to their differential conjugate structures and pharmacokinetics in vivo. Based on the results of the current study, we propose a model of action for the C1 conjugate (Fig. 4), in which the functional EP4a and ALN components are released in a roughly 4:1 ratio, giving EP4a a "window of opportunity" to overcome the inhibition of ALN to exert robust anabolic effects on bone. In the C2 conjugate, the ALN and EP4a components are released simultaneously at a 1:1 ratio, where the antagonistic effects of ALN and EP4a in vivo can significantly reduce their respective antiresorptive and anabolic effects on bone. To our knowledge, this is the first study which compares the effectiveness of two ALN and EP4a conjugates in an in vivo model and the first report on the potential antagonistic effects between EP4a/PGE_2 and ALN at a 1:1 ratio. Although this hypothesis needs to be further elucidated, the results of the current study suggest that the C1 conjugate is a more promising future therapy than C2 for reversing bone loss due to postmenopausal osteoporosis.

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Conflicts of interest None.

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