

## Improved Bone Delivery of Osteoprotegerin by Bisphosphonate Conjugation in a Rat Model of Osteoarthritis

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**Abstract:** This study investigated the delivery of a model therapeutic protein, namely, osteoprotegerin (OPG), to bone sites in an animal model of osteoarthritis. The OPG was chemically conjugated to a “bone seeking” thiol-bisphosphonate (thiolBP) via a disulfide linkage. The BP conjugates of OPG were shown to display a higher hydroxyapatite affinity in vitro as compared to unmodified OPG. After intravenous injection, the bone uptake of OPG–thiolBP conjugate was increased 2-fold over that of control OPG under conditions of normal bone turnover. Furthermore, the retention of the OPG–thiolBP conjugate was significantly higher after 72 h. When administered to osteoarthritic rats undergoing active bone remodeling, the delivery of OPG–thiolBP conjugate to bone was increased more than 4-fold over that of control OPG after 24 h. These results suggest a significant advantage of BP conjugation as a drug delivery strategy for therapeutic cytokines in osteopenic bone diseases.

**Keywords:** Osteoprotegerin; bisphosphonate; bone remodeling; osteoarthritis; anterior cruciate ligament; bone targeting

### Introduction

The microarchitectural adaptation of bone mass and bone mineral density due to osteoclast-mediated resorption is of great clinical concern for many bone diseases, including osteoporosis and osteoarthritis.<sup>1,2</sup> During bone resorption, trabecular bone microstructure is altered, often influencing

functional strength and the integrity of cartilage support. Osteoclast formation (osteoclastogenesis) is induced in the presence of “macrophage colony stimulating factor” (M-CSF) when the membrane-bound factor “receptor activator of NF- $\kappa$ B ligand” (RANKL) binds its receptor RANK. This process of osteoclastogenesis can be perturbed by local factors that interrupt RANKL–RANK intercellular signaling. One such factor is osteoprotegerin (OPG), a soluble, circulating glycoprotein from the tumor necrosis factor (TNF)-receptor family.<sup>3</sup> OPG found in the extracellular milieu can inhibit osteoclastogenesis by binding with RANKL and blocking

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its interaction with RANK on osteoclast precursors. Thus, administration of recombinant OPG protein can inhibit osteoclastogenesis at bone sites, leading to conservation of bone mass and bone mineral density (BMD) in vivo.<sup>4</sup> However, high quantities of recombinant OPG (e.g., >10 mg/kg) need to be administered systemically to attain sufficiently high concentrations in the local bone microenvironment to elicit a therapeutic effect on bone mass.<sup>4,5</sup> Such supraphysiological doses pose the risk of unwanted side effects, particularly in non-bone related loci of RANK/RANKL/OPG regulation, such as the immune and cardiovascular systems.<sup>6,7</sup> Targeting recombinant cytokine drugs, such as OPG, to bone will maximize the drug concentration in local bone microenvironment actively undergoing remodeling, and potentially provide a therapeutic effect using a significantly reduced quantity of drug.

One approach to improve therapeutic protein delivery to bone tissue is to conjugate the proteins to bone-seeking bisphosphonates (BPs).<sup>8</sup> BPs are nonhydrolyzable analogues of naturally occurring inorganic pyrophosphate, and they rapidly adsorb to bone surfaces after systemic administration. These bone-seeking properties of BPs are utilized clinically in bone scintigraphy, in which the  $\gamma$ -emitting technetium (<sup>99m</sup>Tc) is delivered to bones with BP carriers.<sup>8</sup> Proteins have been recently targeted to bone after BP conjugation, but this work has typically employed nonactive model proteins.<sup>9</sup> Proteins such as albumin and IgG were employed in exploring the feasibility of bone targeting in normal animals (i.e., nondisease models). The BP conjugation and bone targeting of therapeutically important cytokines, such as OPG, had not been previously attempted. Therefore, this

study was conducted to investigate if bone-seeking BPs are able to improve delivery of OPG to subchondral bone in a clinically relevant disease model. We chose to employ a surgically induced model of osteoarthritis in rats, where periarticular bone undergoes sequelae of initial bone resorption, followed by bony sclerosis of the subchondral bone plate. Using a thiol-based coupling approach, we elected to construct a cleavable disulfide conjugate of OPG and a thiolBP. The protein's amine groups were used to link to the thiol group of the thiolBP. In this communication, we demonstrate that such BP-conjugates are able to target to disease sites after intravenous injection and result in ~4-fold increased deposition of the proteins as a result of BP conjugation.

## Materials and Methods

**BP Conjugation to OPG.** The OPG obtained from Peptrotech Inc. (Rocky Hill, NJ) was dissolved at 1 mg/mL using ddH<sub>2</sub>O, and dialyzed against 0.1 M phosphate buffered saline (PBS, pH 7.0) to remove amines in the original formulation buffer. After confirming the obtained OPG concentration with reverse phase high pressure liquid chromatography (RP-HPLC), 40  $\mu$ g of OPG was reacted with 5 mM *N*-ethylmaleimide in 160  $\mu$ L of 0.1 M phosphate buffer (pH 7.0) for 30 min to quench the free thiol group of OPG. The capping of the thiol group was deemed necessary to minimize chances of protein–protein cross-linking during the conjugation reaction while OPG is exposed to the cross-linker. A 10 mM thiolBP (2-(3-mercaptopropylsulfanyl)-ethyl-1,1-bisphosphonic acid; synthesized as described in ref 10) solution was reacted with 5 mM *N*-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP; Molecular Biosciences, Boulder, CO) for 30 min, after which it was combined with the OPG solution to give a final concentration of 1 mM SPDP. After 3 h at room temperature, the solution volume was brought to 600  $\mu$ L, and the solution was dialyzed extensively against 0.1 M PBS (pH 7.0). The OPG–thiolBP conjugates were stored at 4 °C and used in experimentation within the week, or otherwise lyophilized for storage.

**Conjugate Characterization.** RP-HPLC was used for OPG analysis by using a Vydac 214TP C4 column (Grace-Vydac, Hesperia, CA), flow rate of 1 mL/min, and mobile phases of 0.1% TFA in H<sub>2</sub>O (A) and 0.1% TFA in 90/10% acetonitrile/H<sub>2</sub>O (B). The gradient was 0.0–1.0 min of 0% B, a linear increase to 70% B between 1.0 and 4.0 min, a linear increase to 80% B between 4.0 and 6.0 min, 80% B between 6.0 and 9.5 min, and a linear decrease to 0% B at 14.0 min. Native OPG was used for calibration, based on spectrophotometric detection at 280 nm. To determine the extent of thiolBP conjugation, OPG conjugates were collected after column purification and ashed to determine the amount of thiolBP as described.<sup>11</sup> The thiolBP concentration

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of a sample (mM) was divided by its protein concentration (mM) to determine the average number of thiolBP conjugated per OPG.

SDS-PAGE was used to determine whether inadvertent protein-protein cross-linking occurred during BP conjugation. Typically, 10  $\mu\text{g}$  of proteins were run on 4–20% Tris-HCl gels (Bio-Rad), which was stained with 0.1% Coomassie Blue R-250 in 10:10:80 methanol/acetic acid/deionized water. Native OPG and different molecular weight proteins were used as the reference standards.

**In Vitro Hydroxyapatite (HA) Binding.** The HA affinity of protein samples was determined by using in-house prepared HA and  $^{125}\text{I}$ -labeled proteins.<sup>11</sup> To obtain iodinated proteins, the microfuge tubes were coated overnight with 200  $\mu\text{L}$  of 20  $\mu\text{g}/\text{mL}$  1,3,4,6-tetrachloro-3 $\alpha$ ,6 $\alpha$ -diphenylglycouril (SIGMA; St Louis, MO) in chloroform. Approximately 10  $\mu\text{g}$  of protein in phosphate buffer was added to the tubes, along with 0.02 mCi of  $^{125}\text{I}$ -Na (in 0.1 M NaOH). After 30 min, free  $^{125}\text{I}$  was separated from radiolabeled protein using a NAP-5 column. Five microliters of iodinated protein was then added to 300  $\mu\text{L}$  of 5 mg HA suspension in different solutions, including 0, 50 and 100 mM phosphate buffer (pH = 7.0), and rat serum, all containing 50 mg/mL BSA as the protein background. The samples were incubated for 3 h at room temperature, after which they were centrifuged at 10,000 rpm. The supernatant was decanted into counting tubes, along with the supernatant from two subsequent rinses of HA pellets. The HA pellet was then counted in a  $\gamma$ -counter (Wizard 1470; Wallac Inc., Turku, Finland) and %HA binding was calculated as:  $100\% \times [(\text{HA pellet cpm}) / (\text{supernatant cpm} + \text{HA pellet cpm})]$ .

**Pharmacokinetics Study.** The animal studies were conducted with either normal 6 week-old Sprague-Dawley rats (Biosciences, University of Alberta) or rats that had undergone surgical induction of knee osteoarthritis (see below). Radiolabeled OPG proteins were diluted with sterile injectable saline to obtain a concentration of 0.1  $\mu\text{g}$  protein/ $\mu\text{L}$  and radioactive counts of  $\sim 10^6/300$   $\mu\text{L}$ . Rats were placed in restraining cylinders, and a precounted 300  $\mu\text{L}$  of protein formulation was injected as a bolus into the tail vein. Three rats were euthanized at indicated time points ( $n = 3/\text{time point}$  for each study group), and specific tissues (see figures) were dissected and directly counted using the  $\gamma$ -counter. The amount of protein retention in harvested tissues was calculated at each time point and expressed as a percentage of the injected dose.

**Anterior Cruciate Ligament Transection (ACLX).** All surgical procedures were conducted using ethically approved standard operating procedures and according to an established protocol.<sup>12</sup> For all ACLX animals, surgery was performed on the right knee alone, with the intact contralateral knee serving as an internal control. Briefly, rats were anaesthetized

using isoflurane inhalation (2%/L O<sub>2</sub>). Using a scalpel, the knee underwent a medial capsulotomy, with retraction of the skin followed by sharp dissection of the capsule. The medial collateral ligament was transected to expose the medial meniscus, which was resected at both tibial insertions. The exposed ACL was entirely transected in the ligament midsubstance using Vannas spring-scissors. The capsular tissues were reapposed and closed using resorbable 5–0 Vicryl suture to provide joint stability. The wound was subsequently closed with 5–0 Vicryl suture. The wound was swabbed with iodine, and the rats were removed from anesthesia and permitted to recover in their cages. Postsurgically, rats were monitored daily for the first week and administered analgesia for the first 2 days postsurgically. They were permitted free mobility and maintained on their regular diet for 6 weeks postsurgery, at which point they were used for pharmacokinetic analyses of radiolabeled OPG conjugates, as described previously. Histological analysis of the rats confirmed the substantial remodeling of periarticular trabecular bone, and the erosive loss of articular cartilage (Figure 1).

**Statistical Analysis.** All data are presented as mean  $\pm$  standard deviation (SD). Analysis of variance (SPSS; Chicago, IL) was conducted for the HA binding and pharmacokinetics studies to ascertain statistical significance among groups. Where indicated, “post hoc” statistical differences were conducted using the two-sided *t*-test ( $p \leq 0.05$ ).

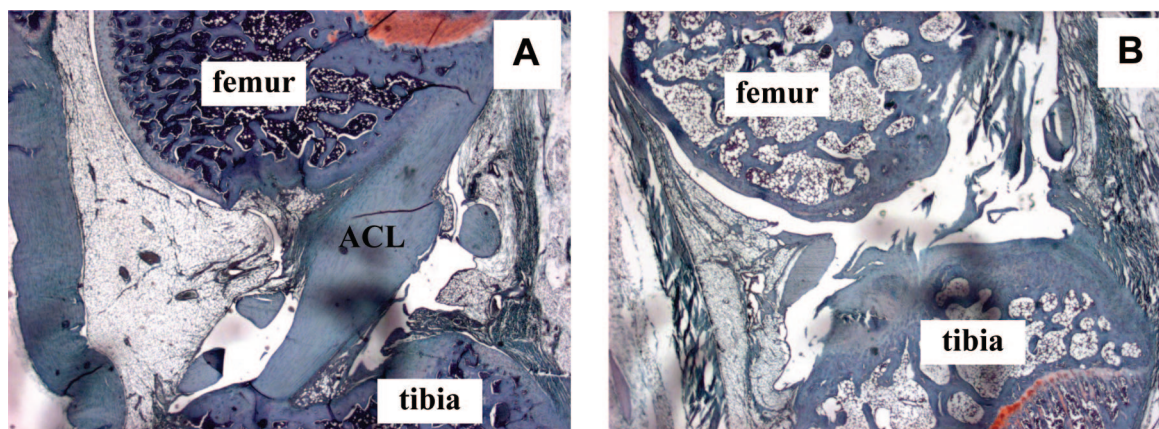
## Results

During HPLC analysis, native OPG characteristically eluted in one sharp peak from 8.5 until 9.0 min and, in contrast, OPG-thiolBP conjugates consistently eluted at longer retention times, from 8.5 until 10.5 min as a single broad peak (Figure 2). Subsequent SDS-PAGE analysis of 10  $\mu\text{g}$  of the control and thiol-BP conjugated proteins confirmed that the modified OPG-thiolBP conjugate was not degraded, and was run as a single protein band at the expected molecular weight for OPG monomer of  $\sim 20$  kDa (not shown). The extent of thiolBP substitution on OPG was between 2.4 and 3.8 thiolBP/OPG at the concentration of SPDP used (1 mM; 3 independent experiments).

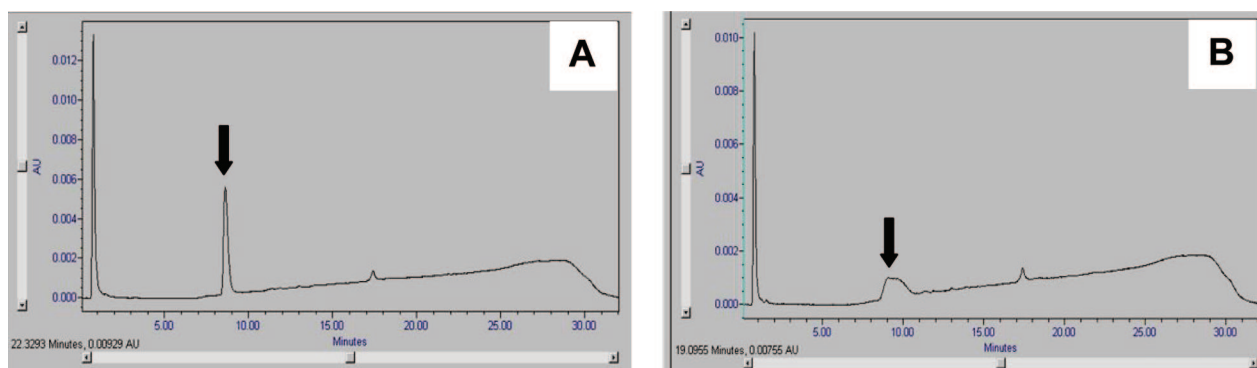
The OPG-thiolBP conjugate had a significantly greater affinity to HA, with 10-fold greater binding at both 50 and 100 mM phosphate buffers, and 4-fold greater HA binding in rat serum, as compared to the control native OPG (Figure 3). Nonphysiological solvation conditions of “water alone” resulted in a strong, but equivalent adsorption of both control OPG and OPG-thiolBP conjugate to HA. This was consistent with the strong adsorption binding of a wide range of proteins to HA in the absence of buffer salts.

The pharmacokinetic study in normal rats indicated that bone deposition of intravenously injected OPG-thiolBP was significantly increased (2-fold) over that of control OPG at 24 h (Figure 4). The OPG-thiolBP levels at the bones was significantly higher at 72 h as well, whereas control OPG was detectable to a lower extent at the bones. In general,

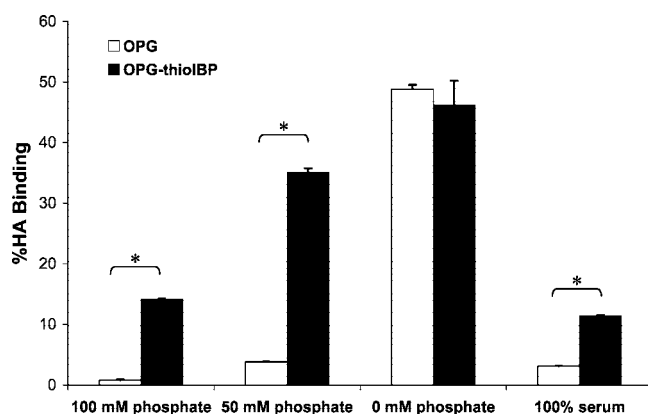
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**Figure 1.** Safranin-O trichrome stained histological sections of the knee joint. (A) Normal control rat knee joint with prominent (intact) ACL and full thickness articular cartilage. (B) ACL-transected knee joint, demonstrating the loss of both articular cartilage and periarticular trabecular bone, along with cartilage fibrillation and sclerosis of the underlying subchondral bone plate.



**Figure 2.** RP-HPLC chromatograms for native (control) OPG (A), and OPG-thiolBP conjugate (B). Whereas unmodified OPG eluted as one sharply resolved peak at 8.5–9.1 min (arrow in A), the OPG-thiolBP (arrow in B) eluted as a broad peak at 8.5–10.5 min.



**Figure 3.** In vitro HA affinity of OPG and OPG-thiolBP conjugates in 0, 50 and 100 mM of phosphate buffer (pH 7.0) and in 100% rat serum. Except for 0 mM phosphate (i.e., ddH<sub>2</sub>O), the OPG-thiolBP conjugate displayed significantly higher binding affinity in all conditions.

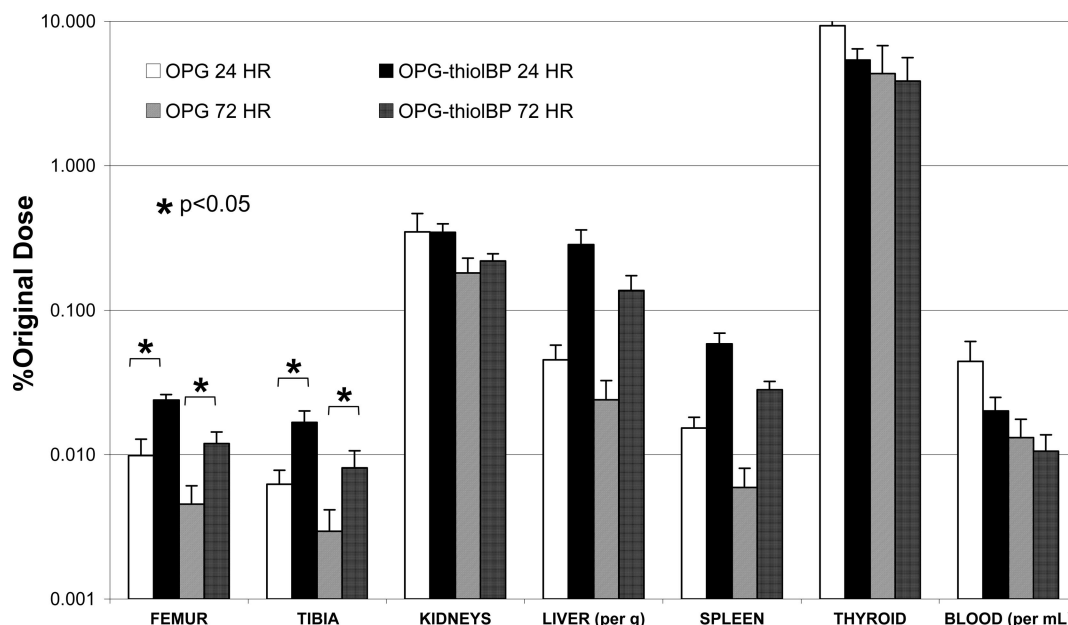
other organs demonstrated an equal distribution of control and conjugated OPG, except liver and spleen that displayed increased uptake of the OPG-thiolBP conjugate. The radioactivity at the thyroid was the highest among the

harvested organs and this is likely to represent the uptake of the liberated <sup>125</sup>I from the proteins.

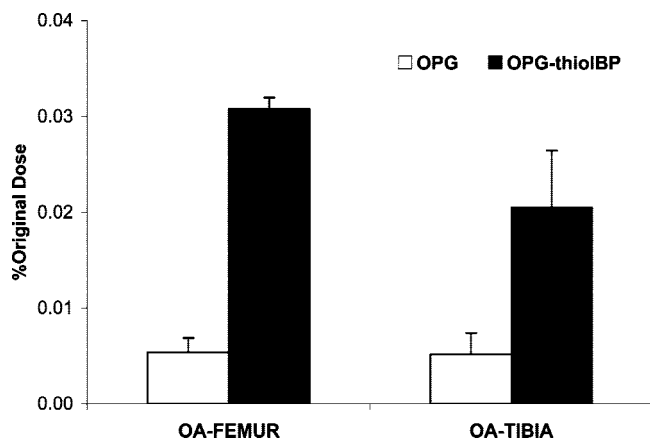
In the rat model of osteoarthritis, the OPG-thiolBP again displayed significantly increased bone targeting after intravenous injection as compared to control OPG at the 24 h time point (Figure 5). There was a 4-fold (tibia) and 6-fold (femur) increased targeting of the OPG-thiolBP conjugate to bones in the knee joint of rats, which were allowed to develop OA for 6 wk after ACLX surgery. As in normal rats, the uptake of the OPG-thiolBP conjugate was higher in the liver and spleen as compared to the control OPG (not shown).

## Discussion

Conjugation of a thiol-containing BP to OPG was successfully achieved in this study, based on a conjugation strategy originally adopted for bovine serum albumin.<sup>10</sup> The extent of conjugation obtained was typically ~3 thiolBP per OPG. This conjugation efficiency was in line with previous studies with similar size proteins (e.g., lysozyme of 14.4 kDa), but lower than the conjugation efficiency for larger proteins (e.g., >10 BPs/protein for ~69 kDa albumin).<sup>8</sup> The extent of BP conjugation was expected to be proportional to extent of mineral affinity imparted,<sup>11</sup> and this could be



**Figure 4.** Biodistribution of control OPG and OPG–thiolBP conjugates at select bone sites (femur and tibia) and other tissues in normal rats. The results are summarized as mean + SD of % recovered doses at select sites. For tibia, femur, spleen, thyroid and kidney, the results represent % recovered dose in the total organ excised, whereas for liver and blood, the % recovered doses were normalized with the weight (per g) and volume (per mL) of the recovered tissue, respectively. The significantly improved targeting of the OPG–thiolBP conjugate was evident at bones at both time points.



**Figure 5.** Targeting of control OPG and OPG–thiolBP conjugates to femur and tibia in rats with osteoarthritis (6 weeks after ACLX). More than 4-fold increase in OPG–thiolBP levels was detected in the femur and tibia after systemic administration, as compared to the control OPG.

altered by modifying reaction conditions, such as thiolBP or cross-linker concentration, or medium pH. Such detailed studies were not conducted for OPG, since the main focus of this study was to demonstrate its bone targetability in an animal model. Excessive thiolBP substitution of bioactive proteins may influence their functional activity or release characteristics in situ after delivery to bone, and as such, our investigation focused on a minimally modified protein that still attained a significantly improved bone targeting. An improved mineral affinity was observed in our well-established HA binding assay, and this observation provided

the impetus for subsequent animal studies. As with other proteins, the affinity of OPG and OPG–thiolBP to HA was inversely related to the phosphate concentration in the binding medium, but the latter retained a superior HA affinity in the presence of phosphate. Retention of a superior affinity even under serum conditions is critical since this represent a more realistic microenvironment for binding. Binding in the presence of serum was relatively less for the conjugates, possibly due to competition from serum proteins and/or cleavage of the disulfide linked conjugate with physiological thiols.

The results of this study showed that BP-conjugated OPG behaved in a similar fashion as other BP-conjugates of model proteins, giving increased deposition at bone sites. This was shown both in normal rats and also in rats developing adaptive bone and cartilage pathogenesis of osteoarthritis. A 2-fold better delivery as a result of thiolBP conjugation was not as high as the targeting efficiency obtained with other proteins; proteins such as lysozyme and albumin displayed as much as 6-fold increased delivery in normal rats after 24 h. Although we expected a better retention of OPG–thiolBP at bones at longer time points, a significant protein loss was evident for both types of proteins after 72 h postadministration, resulting in again ~2-fold increased concentration of OPG–thiolBP at bone sites. It is not known whether the cleavage of disulfide linkage in thiolBP plays a role in protein loss; however, given the relatively similar loss of proteins for both native and thiolBP-conjugated OPG, this is likely to be a minor reason for OPG loss. Rather, the passive diffusion from the site may be more important in these short study periods.

The protein targeting in the OA model was higher than the normal rats (more than 4-fold vs ~2-fold, respectively) and presumably reflects the presence of more exposed mineral sites due to increased turnover of mineralized tissues at the disease sites. The higher uptake of BPs at “exposed” HA sites is well established during bone scintigraphy. This preferential affinity is used to detect regions of bone-metastasizing cells with osteolytic activity,<sup>13</sup> sites of bone fractures<sup>14</sup> and growth plates with active sites of bone deposition.<sup>15</sup> The results from the OA model were closer to the pronounced difference seen in the in vitro HA binding assay, where the mineral surfaces are more readily available for protein binding. A significant amount of free <sup>125</sup>I was obtained in the thyroid tissue, and this is likely from the <sup>125</sup>I liberated from the proteins, resulting in some underestimation of the organ levels of the proteins. We are assuming that both the native and BP-conjugated OPG are affected by this process to the same extent, so that the relative levels of the proteins in each organ are independent of label release.

Several recent studies have tested the therapeutic utility of OPG in animal models of OA. In a murine OA model, secondary to medial meniscectomy, Kadri et al. used excessively high 10 mg/kg injections of OPG<sup>16</sup> and concluded that systemic OPG administration prevented meniscectomy-related bone loss in trabecular and subchondral regions, which resulted in the prevention of cartilage degradation. Given the involvement of OPG in other physiological processes, administering such high doses is not desirable and may preclude OPG use in a clinical situation. In a related study by Shimizu et al., a significantly reduced dose of recombinant OPG was administered intra-articularly in mice (daily injection of 100 ng in 10  $\mu$ L solution, 5 days a week, for 4 weeks).<sup>17</sup> Such an injection regimen is likely to lead to joint inflammation, tissue scarring, changes in synovial fluid composition, or even direct cartilage damage. The intravenous route employed in our study is more likely to be a feasible strategy for clinical administration of OPG. We did not evaluate the pharmacological effect of the

administered OPG–thiolBP conjugates in this study, since such a study would have required extensive analysis and was considered beyond the scope of this bone targeting study. Our future studies will focus on this aspect of the OPG conjugates and explore the feasibility of reducing bone turnover as a result of increased concentration of OPG at disease sites. Nonspecific distribution of BP conjugates to other sites needs to be also addressed in these studies, since BP conjugates are known to be deposited at nontarget sites as a result of complex formation with systemic calcium.<sup>18,19</sup>

Following systemic administration, the protein–thiolBP constructs are at a potential risk of disulfide cleavage, particularly in the presence of cysteine (the most abundant thiol in extracellular milieu), cysteinylglycine, glutathione or homocysteine.<sup>20</sup> Ultimately, the in situ release of bioactive proteins from the BP bone anchor may further diversify desired therapeutic outcomes not only by targeting pharmacologically active compounds to the bone matrix but also by effecting a timed or sustained release in the local bone microenvironment to induce cellular responses. Work from our laboratory recently detailed the cleavage of disulfide-conjugated model proteins in vitro,<sup>20</sup> and their sustained release after implantation in vivo.<sup>21</sup> In the latter study involving an ectopic implantation model, cleavage of disulfide-linked protein-BP conjugates was found to be relatively slow, if any. The present study further confirmed that such conjugates are able to retain their integrity in circulation, allowing the BP moiety to target bone and anchor the protein cargo in bones. How much disulfide cleavage occurs (either in circulation or at bone sites) in the case of OPG–thiolBP is an open question at this stage, but a control for the stability of such a linkage may allow control of in situ levels of therapeutic agents. Linkages other than disulfides, such as cathepsin K sensitive linkages, could be alternatively used to control release of bioactive agents deposited at bones.<sup>22,23</sup>

In conclusion, the current study demonstrated the ability to deliver OPG to the microenvironment of bone during OA

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pathogenesis by using a thiolBP conjugation strategy. This promising approach could be used for other proteins, and may be further tailored to achieve a steady state regimen of protein dosing.

### Abbreviations Used

ACLX, anterior cruciate ligament transection; BP, bisphosphonate; HA, hydroxyapatite; HPLC, high pressure liquid chromatography; OA, osteoarthritis; OPG, osteopro-

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tegerin; SPDP, *N*-succinimidyl-3-(2-pyridyldithiol) propionate; and thiolBP, 2-(3-mercaptopropylsulfanyl)-ethyl-1,1-bisphosphonic acid.

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