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Bisphosphonate-Functionalized Hyaluronic Acid Show Selective Affinity For Osteoclasts As A Potential Treatment For Osteoporosis

Sujit Kootala, MS¹ Dmitri Ossipov, Phd¹, Jeroen JJP van den Beucken, Phd², Sander Leeuwenburgh, Phd², Jöns Hilborn, Phd¹

Current treatments for osteoporosis involve administration of high doses of bisphosphonates (BPs) over a number of years. However, the efficiency of absorption of these drugs and specificity towards target osteoclastic cells is still suboptimal. In this study, we have exploited the natural affinity of high- (H) and low- (L) molecular-weight hyaluronic acid (HA) towards cluster of differentiation 44 (CD44) receptors on osteoclasts to use it as a biodegradable targeting vehicle. We covalently bound BP to functionalised HA (HA-BP) and found that HA-BP conjugates were highly specific to osteoclastic cells and reduced mature osteoclast numbers significantly more than free BP. To study the uptake of HA-BP, we fluorescently derivatised the polymer-drug with fluorescein B isothiocyanate (FITC) and found that L-HA-BP could seamlessly enter osteoclastic cells. Alternatively, we tested polyvinyl alcohol (PVA) as a synthetic polymer delivery vehicle using similar chemistry to link BP and found that osteoclast numbers did not reduce in the same way. These findings could pave the way for biodegradable polymers to be used as vehicles for targeted delivery of anti-osteoporotic drugs.
A. Introduction

Osteoporosis involves major physiological changes in bone, rendering it porous and brittle. As such, osteoporosis is a major health concern in developed countries and affects 20% of males and 30% of females worldwide, particularly at the postmenopausal age.(1, 2) Approximately 9 million fractures occur each year worldwide on account of osteoporosis and 30% of these occur among the EU.(3) It is projected that by 2025, in the US alone, the economic burden of treating osteoporosis-related fractures will exceed $25 billion.(4) Hence, improved treatments would be of considerable societal and economical value.

Bone can be referred to as either a tissue or an organ; it is composed of a complex three-dimensional multacellular structure. Healthy bone entails a fine balance between osteoblasts and osteoclasts, which are the three major cell types required for the continuous turnover of bone.(5-7) Osteoblasts are responsible for the deposition of new hydroxyapatite bone mineral and form osteocytes after becoming embedded in the deposit extracellular matrix.(8) These osteoblasts express alkaline phosphatase (ALP), type 1 collagen, RUNX2 and osteocalcin that are used as major indicators of mature functioning osteoblasts.(9, 10)

Osteoclasts are responsible for bone resorption and appear as large multinucleated cells with ruffled borders that attach to the mineralised extracellular matrix, degrade it and release free calcium into the blood in a process that allows continuous remodelling of bone.(10) These osteoclasts arise from the hematopoietic lineage (i.e. monocytes/macrophages) and express tartrate-resistant acid phosphatase (TRAP), cathepsin K and vitronectin receptor markers indicating maturity.(11-13)

Osteoporosis is associated with insufficient bone formation and highly active population of osteoclasts, thereby causing excessive calcium resorption that cannot be naturally compensated, mineralising osteoblasts. Osteoblasts exert control over osteoclasts through the secretion of receptor activator of nuclear factor kappa B ligand (RANKL), which along with macrophage colony-stimulating factor (M-CSF) as key factors responsible for the differentiation of monocytes into osteoclasts.(16) Osteoblasts control the number of osteoclasts through secretion of osteoprotegerin (OPG), a member of the tumour necrosis factor (TNF) receptor superfamily.(17) Together, these signals molecules maintain a healthy balance of the overall numbers of osteoclasts and osteoblasts. Current clinical strategies for the treatment of osteoporosis focus on restoring the balance between mineralisation and resorption by administering drugs to reduce osteoclast numbers, thereby reducing the overall rate of resorption.(18, 19) The most common family of anti-osteoporosis drugs are bisphosphonates (BP’s) that primarily act to reduce osteoclast numbers. Alternatively, glucocorticoid-based therapies such as parathyroid hormone or oestrogen (with or without progesterone) that stimulate osteoblast activity are used.

The counterbalance increased bone resorption,(3, 20, 21) BP’s, are analogues of inorganic pyrophosphates and comprise phosphonate groups bridged through a common carbon atom. Similar to inorganic phosphates, phosphonate groups account for the chelation of calcium ions.(22) This effect renders bone the most potent BP’s.(23) Zoledronate has shown superior potency in terms of anti-osteoclastic behaviour, mainly due to the involvement of the amino group located on one of its two side groups.(22) Another parallel strategy advocates that modifying the second side group of BP’s with an amine likewise results in enhanced action against osteoclasts. The essential condition for the pharmacological activity of BP’s is the acidic microenvironment in the resorption lacunae that is created by mature functioning osteoclasts.(24) Since BP’s bind to the mineral in bone, acidic conditions result in the protonation of the phosphonate groups to trigger release of the BP moiety from the matrix. The osteoclasts absorb the released BP through the endocytic pathway and release it into the cellular compartment as they encounter the acidic environment of the vesicles. Once released intracellularly, amino BP’s inhibit farnesyl diphasphate synthase, a key enzyme involved in the synthesis of cholesterol.(25) This inhibition is known to affect the prenylation of GTPase signalling proteins, which affects the ability of the osteoclasts to anchor themselves to the surrounding matrix. The entire process results in major disruptions in the functioning of mature osteoclasts involving attachment and presentation of the ruffled membrane borders necessary for resorption.(26) Eventually, because of the inability of osteoclasts to anchor and resorb, they undergo apoptosis, leading to a reduction in their overall numbers. In vivo, BP’s may be administered either orally or intravenously. Oral administration has the disadvantage of diminished bioavailability over time, necessitating multiple sessions of administration.(27) Comparatively, intravenous administration of BP’s has resulted in stronger and longer lasting anti-resorptive effects. However, because of issues such as poor compliance, persistence of continued therapy, dosage issues and continued use of BP’s, the dropout ratio of patients is as high as 50%, resulting in poor patient recovery outcomes.(28) The main reason for discontinuing oral administration is gastrointestinal complications driving patients to discontinue their treatment midway.(29) Another issue with BP’s relates to osteonecrosis of the jawbone, which has been shown to become apparent upon cancer treatment with (high doses of) BP’s.(30, 32) Therefore, strategies to increase the therapeutic and targeting efficiency of BP’s towards osteoclasts to reduce the dosage of BP’s would be highly beneficial for the treatment of osteoporosis.

Particularly, BP’s adsorb onto the surface of bone to provide release of BP’s as the HAP is resorbed along with the adsorbed BP’s, allowing osteoclast inhibition over an extended period.(35) To change the pharmacokinetic properties of BP’s, they have also been linked to polyethylene glycol (PEG) that is known to escape protein interactions. PEG-alendronate conjugates in osteoporotic rats have been shown to effectively inhibit the decrease in the width of the growth plate in bone to a level achieved with intrapulmonary administration of alendronate alone in an effort to reduce the side effects of alendronate on the mucosal and gastric lining, however not necessarily increasing the efficiency of the drug.(36) In another study, alendronate was linked to poly(D,L-lactide-co-glycolide) (PLGA) to determine if the drug retained its anti-osteoclastic properties after the conjugation [ref]. However, the experiments showed that the PLGA-alendronate conjugate had no better anti-osteoclastic properties than PLGA alone.(32) Hence, methods to enhance the targeting of BP’s to osteoclastic cells to improve BP efficacy and reduce BP side effects are currently not available.

Hyaluronic acid (HA) is a non-sulphated glycosaminoglycan that comprises repetitive disaccharide units consisting of monomers of N-acetyl-D-glucosamine and D-glucuronic acid and forms a linear polysaccharide chain.(33) It is an essential developmental molecule.
found in many parts of the human body, such as the syn...43
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cavities, eyes, umbilical cord and connective tissues. HA...46
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synthesised and degraded in vivo, assisted by hyaluronan synth...48
and hyaluronidas, respectively. (34) Amongst others, the cluste...49
differentiation 44 (CD44) receptor-mediated pathway is the m...50
route for entry of HA into the cells and is recognised by spe...51
tubulins, hyaluronidas, for extracellular and intracellular...52
10%.

132. Synthesis of HA-thiol (HA-SH) derivatives. HA (400 mg, averag...132
133. MW 8000 Da, 1 mmol of disaccharide units) was dissolved in 40...133
134. of deionised water. Dihydrazide linker 1 (35.7 mg, 0.15 mmol) ...134
135. added to the HA solution. N-hydroxysuccinimide (NHS, 153 mg...135
136. mmol) was separately dissolved in 6 mL of acetonitrile and 1...136
137. mixture (v/v = 1:1) and added to the HA solution. The pH of ...137
138. resultant solution was adjusted to 4.7, after which the coupl...138
139. reaction was initiated by addition of solid 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC, 96 mg, 0.5 mmol) to ...139
140. reaction mixture. The mixture was stirred overnight and 15...140
141. basified to 8.5 with 1 M NaOH. DL-dithiothreitol (DTT, 116 mg, 0...141
142. mmol) was added to the solution. The mixture was stirred 16...142
143. overnight, after which the solution was transferred to a dial...143
144. tube (MW cutoff = 3500). After exhaustive dialysis against dilute HCl (pH 3.5) twice. The solution was neutralised to pH 7.4 and lyophilised. The resulting polymers were analysed by 1H NMR and 31P NMR and elemental analysis (colorimetric spectrophotometric method by OEA Labs). Specifically, 1H NMR peaks corresponding to the native HA protons (such as acetamide protons at 1.9 ppm; 2′, 3′, 4′, 5′ and 6′-protons of the HA disaccharide unit between 3.2–4.0 ppm as well as anomic 1′-protons at 4.4 ppm) were compared with peaks corresponding to the methylene protons 2 and 3 of the grafted side chains. The peak at 2.2 ppm corresponds to two methylene protons –CH₂COH(PO₃H₂) that are adjacent to a bridging carbon of the BP group.

145. Synthesis of polyvinyl alcohol-alcohol-BP (PVA-BP) derivatives. Thiolated PVA was first prepared according to a previously established protocol. (38) The degree of thiolation in PVA-SH was 4.5% (i.e. on an average, 16.2 out of 360 monomer units were thiolated in PVA of average MW 16000 Da). PVA-SH (35.3 mg, 32.4 µmol of –SH groups) was dissolved in 7 mL of degassed distilled water and acrylated BP 2 (9.4 mg, 32.4 µmol) was added to the solution. Subsequently, 4 mg of Irgacure 2959 was added and the mixture was stirred for 10 min under ultraviolet light (36 W UV timer lamp, CNC international BV, Netherlands). Thereafter, the mixture was dialysed against distilled water three times. The dialysed solution was lyophilised to give 32 mg of PVA-BP (72% yield). The resulting polymer was analysed by 1H NMR and 31P NMR. 1H NMR showed that one BP group was linked to each thiol group (Figure S1).

146. Fluorescent labelling of HA derivatives. Fluorescein B isothiocyanate (FITC) was linked to hydrazide-functionalised HA derivatives of L-HA and H-HA (L-HA-hy and H-HA-hy, respectively). Fluorescent labelling of bisphosphonated HA was performed analogously starting with HA derivatives dually functionalised with hydrazide and BP groups (L-HA-hy-BP and H-HA-hy-BP). Initial modification of either native HA or HA-BP with hydrazide groups was first accomplished according to our previously published protocol. (39) First, 20 mg each of each HA derivative (L-HA-hy and H-HA-hy; L-HA-hy-BP and H-HA-hy-BP) (Scheme S2) was dissolved in deionised water at concentrations of 12 mg/mL and 4 mg/mL, respectively. The pH of the obtained solutions was adjusted to 8 with 1 M NaOH. Then, 0.8 mg of FITC (40) was dissolved in 100 µL of dry methanol and added to the basified solution of HA-hydrazide derivative, corresponding to 0.03 molar equivalents of FITC with respect to the number of HA disaccharide units. The reaction mixture was stirred overnight in dark at room temperature (RT) and then dialysed against acidic water (pH 3.5) containing 0.1 M NaCl, followed by dialysis against acidified water twice. Dialysis tubes with a molecular weight cut-off of 3500 Da were used for H-HA-hy and H-HA-hy-BP, whereas dialysis tubes with a molecular weight cut-off of 1000 Da were used for L-HA-hy and L-HA-hy-BP. After freeze-drying the dialysed solutions, the following FITC-labelled derivatives were obtained: 10.7 mg (51.4% yield) of L-HA-hy, 15.3 mg (73.6% yield) of H-HA-hy, 11.5 mg (57.5% yield) of L-HA-hy-BP and 20 mg (100% yield) of H-HA-hy-BP. 

147. Cell culture. Cells from the murine monocyte cell line RAW 264.7 were differentiated into osteoclast-like cells by addition of murine RANKL. In brief, the cells were grown in α-minimum essential medium (α-MEM, Gibco) supplemented with 10% foetal calf serum (FCS) (Gibco) with 50 µg/mL gentamycin at 37°C in a humidified atmosphere of 5% CO₂. In order to initiate the process of osteoclast formation, the cells were seeded (0.8 mL) in 8-well Lab-Tek chamber slides (BD Biosciences, Sweden) at a density of 2 × 10⁴ cells.
Confocal microscopy. Immunos匡ning was performed 24 h after incubating with the samples from Table 1 labelled with FITC. In brief, the cells were washed with PBS, fixed with 4% parafomaldehyde and permeabilised in 0.01% Triton-PBS. Nonspecific binding sites were blocked with the addition of 10% goat serum for 1 h. To examine the role of the CD44 receptor in the uptake process, the cells were treated with a monoclonal antibody specific for the mouse CD44 receptor and counter stained with a rhodamine-linked secondary antibody for visualisation. L-HA-hy-BP and H-HA-hy-BP were labelled with FITC as described above to follow the polymer linked-BP. DAPI was used to stain the multiple nuclei. The cells were incubated for 60 min at RT with monoclonal rat anti-mouse anti-CD44 antibody (50 μL/well, 1:100 diluted 1% donkey serum with PBS) and subsequently incubated with secondary fluorescent antibody (50 μL/well, 1:200 diluted donkey anti-rat IgG conjugated with rhodamine; Molecular Probes) at RT for 30 min in darkness. Cell nuclei were stained using DAPI for 30 min at RT in dark. The cells were washed with PBS and then mounted in Vectashield mounting fluid (Vector). Fluorescence images were acquired using a Zeiss confocal laser-scanning microscope. Images were quantified using Image J (Free source software from NIH).

Statistical analysis. All quantitative experiments were performed in triplicate and the data are shown as the mean ± standard deviation of one representative experiment. The results are expressed as mean ± SD. Statistical significance was calculated with Student’s t-test for unpaired samples using the SPSS statistical analysis software. p Values less than 0.05 were considered significant.

C. Results

BP groups were linked to HA to form HA-BP. HA of MW 8 and 150 kD (designated as L-HA and H-HA, respectively) was functionalised with BP groups in two steps according to the reported procedure. (37) In brief, native HAs were first modified with thiol groups using linker 1, which facilitated synthesis of L-HA-SH and H-HA-SH, respectively (Scheme 1). In both derivatives, the degree of substitution with thiol groups was approximately 10%. Subsequently, photo-initiated thiol-ene addition of thiol groups to pamidronate acylamide 2 resulted in the bisphosphonated analogues L-HA-BP and H-HA-BP. On average, 2.5 and 3 molecules of acrylated BP molecules were added per thiol group in L-HA-BP and H-HA-BP, respectively, as was judged from 1H NMR analysis (Figure 1). HA functionalisation with BP groups was confirmed by appearance of methylene protons 1 adjacent to the bridging carbon of the BP group (see structure in Figure 1A for designation). The degree of substitution with BP’s (DS_{BP}) was found to be 25% for L-HA-BP according to the comparative integration of this peak and the singlet of HA acetamide protons at 1.9 ppm. Moreover, a characteristic singlet phosphorus peak at 18.7 ppm was observed for the attached BP’s in 31P NMR spectra (Figure 1B). 1H NMR and 31P NMR spectra of H-HA-BP were similar to those of the L-HA-BP analogue, indicating that the conjugation efficiency did not depend on the MW of HA. Elemental analysis of HA-BP derivatives showed 3.2% phosphorous and 0.7% sulphur content indicating that approximately 2.3 bisphosphonate groups were linked to each thiol group.
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In addition, we examined whether linking of the BP moiety to a synthetic polymer, devoid of cell surface receptor recognition molecules such as PVA of similar MW, would have any effect that mimics the effect of L-HA-BP on osteoclast-like cells. (SI. Table 1) L-HA-BP at a concentration of 100 µM exhibited the highest toxicity by reducing over 90% of the murine osteoclast-like cells as well as human osteoclasts, whereas H-HA-BP exhibited approximately 60% toxicity. In contrast, L-HA, H-HA and unmodified BP demonstrated only limited (approximately 35%) reduction in osteoclast numbers compared with the untreated control within 24 h. Hydrazide-linked HA of molecular weights without BP’s were treated with BPs for 24 h. Statistical analysis was performed on the different groups and quantified as represented in the graph. Statistical significance was calculated using the Student's t-test. P value < 0.01 when compared across the groups.

Figure 1. (A) Synthesis scheme for BP-functionalised HA. (B) H NMR and (C) 31P NMR spectra of the L-HA-BP derivative in D2O.

BP-linked HA exhibits anti-proliferative effects on murine osteoclast-like cells. The results in Figure 2 show that RAW 264.7 cells induced with 20 ng/mL RANKL for 5 days formed osteoclasts. TPMCs mimicking osteoclasts. We quantified the effect of H-HA and L-HA as well as H-HA-BP and L-HA-BP in comparison with paraffin oil. In addition, we examined whether linking of the BP moiety to a synthetic polymer, devoid of cell surface receptor recognition molecules such as PVA of similar MW, would have any effect that mimics the effect of L-HA-BP on osteoclast-like cells. (SI. Table 1) L-HA-BP at a concentration of 100 µM exhibited the highest toxicity by reducing over 90% of the murine osteoclast-like cells as well as human osteoclasts, whereas H-HA-BP exhibited approximately 60% toxicity. In contrast, L-HA, H-HA and unmodified BP demonstrated only limited (approximately 35%) reduction in osteoclast numbers compared with the untreated control within 24 h. Hydrazide-linked HA of molecular weights without BP’s were treated with BPs for 24 h. Statistical analysis was performed on the different groups and quantified as represented in the graph. Statistical significance was calculated using the Student's t-test. P value < 0.01 when compared across the groups.

Figure 2. (A) Effect of BPs on murine osteoclast-induced TRAP activity. RAW 264.7 cells were induced with (20 ng/mL) RANKL for 30 days. Subsequently they were treated with BPs for 24 h. Activity was measured as described in ‘Materials and Methods’.

Figure 3. (A) Effect of BPs on human osteoclast-induced TRAP activity. PBMC’s were induced with 40 ng/mL RANKL and 40 ng/mL M-CSF for 4 weeks. Subsequently they were treated with BPs for 24 h. TRAP activity was measured as described in ‘Materials and Methods’. The images show the formation of giant multi-nucleated osteoclasts in the presence/absence of osteoclast-like cells. A. H-HA (avg. MW= 150 kD); B. L-HA (avg. MW= 7.5 kD); C. Control; D. H-HA-BP (avg. MW= 150kD); E. L-HA-BP (avg. MW= 7.5 kD); F. Free BP.

Student's t-test. P value < 0.01 when compared across the groups.

Figure 4. Effects of HA-linked bisphosphonates (0, 1, 10, 50 or 100 µM) on osteoclast differentiation. Human osteoclasts were treated with different concentrations of bisphosphonates (0, 1, 10, 50 or 100 µM) for 24 h and counted both the number of osteoclasts and large osteoclasts after 4 weeks of culture. Results are shown as means ± SD for four independent experiments. P>0.05 and represents significant difference from control osteoclasts (≥2 nuclei).

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cells were exposed to BP-containing and BP-free compounds for 12 h and evaluated for cytotoxicity using the AlamarBlue® assay described above. Data are expressed as the mean ± S.D. of triplicate cultures. The experiment was performed three times, with similar results obtained in each experiment. Statistical significance was calculated with Student’s t-test. P value < 0.01 when compared across the groups.

The metabolic activity of the cells was assessed using AlamarBlue® assay and plotted as cell survival compared to untreated control. Differentiated osteoclasts demonstrate variable responses when incubated with AlamarBlue® and therefore TRAP assay showed the most reliable method to testing osteoclasts whereas the undifferentiated cells show measurable response towards AlamarBlue®. In a similar experiment, native L-HA and HA exhibited approximately 50% toxicity towards both osteoblasts and undifferentiated murine macrophages in 24 h. The experiment was performed three times, with similar results. Data are expressed as the mean ± S.D. of triplicate cultures. The experiment was performed three times, with similar results. Data are expressed as the mean ± S.D. of triplicate cultures.

Whether the potency and specificity of the drug against osteoclasts requires the development of smart delivery strategies that can enhance cellular uptake in osteoclasts. We noted that the hydrazide-modified compounds did elicit some toxicity of their own and can be attributed to the presentation of unreacted hydrazide groups as an irritant. Significantly, the polymer-linked drugs did not significantly affect undifferentiated monocytes. This corroborates with improved uptake of L-HA HA by endocytosis.(44). It has been previously demonstrated that osteoblasts show low expression levels of CD44, whereas osteocytes and osteoclasts show higher expression levels of CD44 receptors.(36) BP-based drugs are known to inhibit mature osteoclasts from attaching to the bone surface by altering the cytoskeleton and subsequent loss of the ruffled borders, thereby slowing down resorption.(45) In order to verify if the effect is replicable with a drug delivery vehicle that does not present suitable ligands for receptor-mediated uptake, we used bispophonated PVA. Indeed, this adduct had no detectable effect on the survival of osteoclasts. Another important requirement for new-generation drugs is non-toxicity towards unrelated cell types. Here we observed that the drug-linked polymer does not affect the growth or proliferation of osteoblasts, which possess the inherent mechanisms to reminealise and heal defects in bone. This makes the polymer-linked drug a multimodal vehicle, where BP targets bone and HA targets specific cell surface receptors present on osteoclasts. Further, we investigated the transport of the drug-linked polymer L-HA-BP into mature osteoclasts to rule out any involvement of toxicity arising through the interaction of L-HA-BP merely with the cell surface. We equipped the polymer with a fluorescent probe to locate its movement or attachment and performed the experiment under the same conditions as described before. Although only a few mature osteoclasts survived incubation with the drug for 12 h, the ones that survived were fixed and stained with DAPI to locate the nuclear body. We could distinctly locate pockets inside the multi-nucleated osteoclasts with the fluorescent polymer of L-AH. In the case of monocytes, we demonstrated that fluorescence appeared only at the cell surface and not inside the cell, reiterating our observations that the drug-linked polymer shows high specificity towards multinuclear mature osteoclasts only. This demonstrates the preference of the polymer-drug conjugate towards mature osteoclasts and not towards other cell types. We speculate that this could be possibly mediated through the interaction of the complex with CD44 receptors or other HA receptors, allowing higher possibility for the drug to be taken into the osteoclast cell body. It has been suggested that efficient treatment of skeletal disorders such as osteoporosis ideally requires the development of smart delivery strategies that can target osteoclasts without disturbing other functioning cell types.

We explored the idea of linking the BP drug to HA in order to increase the probability of the drug-linked polymer to be internalised through processes such as receptor-mediated endocytosis through CD44 (receptor for hyaluronan), clathrin-mediated pits and pinocytosis.(43) It has been suggested in previous work that low molecular weight HA can penetrate the cell with greater efficiency using receptor mediated endocytosis compared to high molecular weight HA.(44) We made an important observation that when the drug was linked to HA, the toxicity of the drug towards osteoclasts increased, which could be attributed to enhanced uptake. The polymer-drug conjugate also demonstrated concentration dependent toxicity, implying that dosage could be controlled and even concentrations as low as 10 μM could elicit the desired pharmacological response in vitro. The free drug and HA polymer lacking the BP moiety itself did not impose any significant toxic effect on the osteoclasts. We noted that the hydrazide-modified compounds did elicit some toxicity of their own and can be attributed to the presentation of unreacted hydrazide groups as an irritant. Significantly, the polymer-linked drugs did not significantly affect undifferentiated monocytes. This corroborates with improved uptake of L-HA HA by endocytosis.(44). It has been previously demonstrated that osteoblasts show low expression levels of CD44, whereas osteocytes and osteoclasts show higher expression levels of CD44 receptors.(36) BP-based drugs are known to inhibit mature osteoclasts from attaching to the bone surface by altering the cytoskeleton and subsequent loss of the ruffled borders, thereby slowing down resorption.(45) In order to verify if the effect is replicable with a drug delivery vehicle that does not present suitable ligands for receptor-mediated uptake, we used bisphophonated PVA. Indeed, this adduct had no detectable effect on the survival of osteoclasts. Another important requirement for new-generation drugs is non-toxicity towards unrelated cell types. Here we observed that the drug-linked polymer does not affect the growth or proliferation of osteoblasts, which possess the inherent mechanisms to reminealise and heal defects in bone. This makes the polymer-linked drug a multimodal vehicle, where BP targets bone and HA targets specific cell surface receptors present on osteoclasts. Further, we investigated the transport of the drug-linked polymer L-HA-BP into mature osteoclasts to rule out any involvement of toxicity arising through the interaction of L-HA-BP merely with the cell surface. We equipped the polymer with a fluorescent probe to locate its movement or attachment and performed the experiment under the same conditions as described before. Although only a few mature osteoclasts survived incubation with the drug for 12 h, the ones that survived were fixed and stained with DAPI to locate the nuclear body. We could distinctly locate pockets inside the multi-nucleated osteoclasts with the fluorescent polymer of L-AH. In the case of monocytes, we demonstrated that fluorescence appeared only at the cell surface and not inside the cell, reiterating our observations that the drug-linked polymer shows high specificity towards multinuclear mature osteoclasts only. This demonstrates the preference of the polymer-drug conjugate towards mature osteoclasts and not towards other cell types. We speculate that this could be possibly mediated through the interaction of the complex with CD44 receptors or other HA receptors, allowing higher possibility for the drug to be taken into the osteoclast cell body. It has been suggested that efficient treatment of skeletal disorders such as osteoporosis ideally requires the development of smart delivery strategies that can target osteoclasts without disturbing other functioning cell types.
The in vivo scenario related to bone renewal is complex. Cell-cell talk, cellular cross-talk, and built-in mechanisms have been developmentally incorporated that allow the cells to survive high levels of stress. Extreme stress changes the way cells orchestrate bone turnover, which cannot be rebalanced without intervention. The drugs available at present are capable of treating bone turnover, which cannot be rebalanced without drug developmentally incorporated that allow the cells to survive higher compliance rates. Future work should explore in detail the mechanisms involved in signalling of such complexes in cells targeting vehicles. We expect that this type of targeted delivery system can be extended to other classes of drugs in the future.

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D. Conclusion
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