Supporting Information

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SI Materials and Methods

Formulation and Characterization of Targeted (Alendronate-PEG-PLGA) Nanoparticles. To optimize nanoparticle (NP) formulation with suitable physicochemical characteristics, with varying ratios of target ligand [alendronate (Ald)] to polyethylene glycol (PEG) density on NP surface, and to maximize the drug load, we prepared a library of NPs. The NP library was designed using different polymer molecular weights, blending different polymer ratios, using different formulation techniques such as nanoprecipitation and emulsions, and varying the conditions of formulations. We further maximized the drug load by optimizing the initial drug feed. The NPs were purified and concentrated. To formulate the NPs by blending polymers, we synthesized carboxylate-functionalized copolymer poly(D,L-lactic-co-glycolic acid) (PLGA)-*b*-PEG by amide conjugation of COOH-PEG-NH_2 to PLGA-COOH. The synthesis is described in detail elsewhere (1). We also synthesized and characterized PLGA-*b*-PEG-Ald by multistep synthesis as described in Fig. S1. Furthermore, we evaluated the release kinetics of bortezomib from the NPs (single emulsion), at 37 °C, in PBS (1×) for varying time periods as analyzed by using HPLC. At all time points, the buffer was replaced to maintain the sink conditions. The affinity of Ald-conjugated NPs [Ald-PEG-PLGA (Ald-PP)] toward bone mineral [hydroxyapatite (HA)] was investigated in comparison with nontargeted (PP) NPs. Transmission electron microscopy (TEM) was used to analyze the interaction of targeted (Ald-PP) NPs to HA in the form of NP solution (targeted vs. nontargeted). The binding of targeted NPs to biomimetic HA crystals was analyzed under high-resolution scanning electron microscopy (SEM). Specific binding of Ald-PP NPs (PLGA-$Alexa_{647}$ labeled) to ex vivo bone fragments was also investigated by imaging under fluorescence microscope. In the next set of experiments, we studied the in vivo biodistribution, where $Alexa_{647}$-labeled Ald-PP NPs were used with whole-mouse imaging. NPs were injected i.p. and imaging was done at 1- and 24-h time points. Each mouse had >30 point source selections [excitation (ex): 675 nm; emission (em): 720 nm]. Thereafter, the mouse bones were dissected, sectioned, and imaged for investigation of bone homing of labeled NPs.

In Vitro and in Vivo Efficacy of Ald-PP NPs. Fluorescent-labeled NPs (PLGA-$Alexa_{647}$, single emulsion) were incubated with peripheral blood mononuclear cells (PBMCs) or MM1S cells and analyzed with flow cytometry for NP uptake (24 h). Furthermore, we investigated the in vitro efficacy of Bort-NPs (0.1 mg/mL, 0.04–0.09 wt/wt% drug load) by measuring apoptosis via flow cytometry, where MM1S cells were exposed to ∼0.9, ∼3.6, or ∼7.3 nM effective bortezomib concentrations (24 h). Controls consisted of an equivalent mass of empty NPs, and 10 nM free bortezomib (2). The same treatments were also studied by bioluminescence assay at 24 and 48 h. The in vivo efficacy studies used female Nod/SCID beige mice in treatment or pretreatment regimes. For treatment studies, mice injected with Luc^+/GFP^+ MM1S cells were randomly divided into four groups ($n = 7$). After injecting cancer cells, on day 21 mice were treated with the following: Ald-Empty-NPs, Free Drug (bortezomib), Ald-Bort-NPs, and Nontargeted Bort-NPs, and were imaged twice a week until day 38. Mice were injected (i.p.) twice a week with 0.5 mg/kg bortezomib or with an equivalent amount of Ald-Empty-NPs. In the case of NP pretreatment regime, female Nod/SCID beige mice were randomized into three groups ($n = 10$) and injected (i.p.) thrice a week for 3 wk, with 0.3 mg/kg bortezomib or with an equivalent amount of Ald-Empty-NPs. The pretreatment groups were as follows: Ald-Bort-NPs, Free Drug, and Ald-Empty-NPs in study 1 and Ald-Bort-NPs, Ald-Empty-NPs, and Nontargeted Bort-NPs in study 2. After 3 wk, the mice were injected with Luc^+GFP^+ MM1S cell. Bioluminescent imaging (BLI) was performed weekly on these mice, and survival was assessed. Additionally, an ex vivo micro-computed tomography (micro-CT) analysis and static histomorphometry (3) of mouse bones (femur, tibia, and fibula) were performed after 3-wk pretreatment period to validate bortezomib-induced increase in osteogenesis.

Statistical Analysis. Prism 5.0 or 6.02 software was used to compute all statistical calculations. BLI and $Alexa_{647}$ signal was compared between groups using Student's *t* tests with correction for multiple comparisons made and statistical significance determined using the Holm–Sidak method with $\alpha = 0.05$. ANOVA with Dunnet's or Tukey's post hoc multiple-comparison tests were used with $P < 0.05$ considered significant for analysis of data with more than one comparison. Survival statistics were determined using the log-rank (Mantel–Cox) test or the Gehan–Breslow–Wilcoxon test. Data are plotted as means ± SEM, unless otherwise stated.

Materials. The polymer poly(D,L-lactic-co-glycolic acid) (50/50) with terminal carboxylate groups (PLGA, inherent viscosity, 0.55–0.74 dL/g in hexafluoroisopropanol; M_r, 45 kDa) was obtained from Lactel Absorbable Polymers. NH_2-PEG-COOH (M_r, 5 kDa) and *t*BOC-NH_2-PEG-COOH (M_r, 5 kDa) were purchased from Laysan Bio. All reagents were analytical grade or above and used as received, unless otherwise stated. Alexa cadaverine dyes (647 and 405) were purchased from Invitrogen. The sodium alendronate, *N*-hydroxysuccinimide (NHS), ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), HA, and any other chemicals were purchased from Sigma-Aldrich.

Cell Culture. The human multiple myeloma cell line MM1S was purchased from ATCC and engineered to express GFP and firefly luciferase (Luc^+/GFP^+ MM1S cells) as previously described (4) and cultured in 500 μg/mL geneticin (Invitrogen) for selection. PBMCs were obtained from healthy subjects by Ficoll-Hypaque density separation as recommended by the manufacturer (StemCell Technologies). Cells were cultured at 37 °C in RPMI 1640 containing 10% (vol/vol) FBS (Sigma-Aldrich), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco). Primary human BM-derived mesenchymal stromal cells (MSCs) obtained from normal healthy subjects (ND-MSCs) were isolated and cultured as previously described (5) in expansion media of DMEM (Invitrogen), 20% (vol/vol) FBS (Invitrogen), 100 U/mL penicillin, and 10 μg/mL streptomycin (Invitrogen). Clinical samples were collected from patients from Brigham and Women's Hospital; approval for these studies was obtained from the appropriate institutional review boards. Informed consent was obtained in accordance with the Declaration of Helsinki.

NP Formulation and Characterization. To optimize NP formulation with suitable physicochemical characteristics, and to vary the target ligand density on NP surface along with maximization of NP drug load, we prepared a library of NPs. The NP library was designed using different polymer molecular weight, blending different polymer ratios, using different formulation techniques such as nanoprecipitation and emulsions, and varying the conditions of formulations. We further maximized the drug load by optimizing the initial drug feed. In the solvent dispersion method

of formulation, a blend of polymers PLGA-*b*-PEG-COOH and PLGA-*b*-PEG-Ald (10 mg/mL, in acetonitrile) was mixed with bortezomib (Selleck; 100 μM, in DMSO, 0.5% by weight of polymer). The polymer–drug mixture was added dropwise to a large excess of water [1:10 organic to aqueous (vol/vol)], at a constant stirring (600 rpm, Corning MP9I), to give a final NP concentration of 1 mg/mL. NPs were stirred for another 4 h to harden the NPs and remove excess organic solvents. The NPs were filtered, concentrated, and purified from the organic solvent, unencapsulated drug, and excess polymer. The NPs were washed (PBS) three times using an Amicon centrifugation filtration membrane with a molecular mass cutoff of 100 kDa.

In the case of the single-emulsion formulation method, the polymer blend of PLGA-*b*-PEG-COOH and PLGA-*b*-PEG-Ald (5 mg/mL, in chloroform) was mixed with bortezomib (1 mM, DMSO, 3% weight of polymer), and emulsified with an aqueous phase [1:12 organic to aqueous phase (vol/vol)] using a probe sonicator. The emulsions were stabilized by adding polyvinyl alcohol (0.2%) to the aqueous phase. The sonication conditions of output power and duration significantly affected the size and drug load of NPs. We standardized the sonication conditions to using an output power of 45 W for 3 min (5-s pulse with 1-s stop time) for maximal drug load and optimal NP size. The NP emulsions were then diluted to 10 mL of water and stirred at room temperature for 6 h. The NPs were purified and concentrated as described above.

The hydrodynamic diameter and surface charge (ζ potential; in millivolts) of NPs were determined by quasi-elastic laser light scattering using a ZetaPALS dynamic light scattering (DLS) detector (Brookhaven Instruments) in 20 mM isotonic 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes) buffer saline, pH 7.4, at room temperature (RT). Experiments were performed in triplicate.

TEM images of NPs and NP interaction with calcium phosphate nanocrystals were obtained using JEOL 2011 instrument at an accelerated voltage of 80 kV. The sample was prepared by depositing 10 μL of NP suspension (0.1 mg/mL) onto a 300-mesh carbon-coated copper grid. The samples were blotted away after 5- to 10-min incubation, air-dried, and allowed it to further dry in vacuum. The grids were counterstained with 1% uranyl formate to enhance the contrast. Representative images are shown from experiments in triplicate.

Synthesis and Characterization of Polymers: PLGA-*b*-PEG-COOH, PLGA-*b*-PEG-Ald, PLGA-Alexa$_{647}$, and PLGA-Alexa$_{405}$. Carboxylate-functionalized copolymer PLGA-*b*-PEG was synthesized by amide conjugation of COOH-PEG-NH_2 to PLGA-NHS according to ref. 1. In brief, 50:50 poly(D,L-lactic-co-glycolic acid) (0.55–0.74 dL/g) (100 mg, 0.0023 mmol) was dissolved in 1 mL of dry dichloromethane (DCM); to this was added EDC (4.41 mg, 0.023 mmol) in dry DCM (0.5 mL), and the reaction was stirred for 10 min followed by addition of NHS (2.65 mg, 0.023) in dry DCM (0.5 mL). The reaction was stirred at RT for 60 min. The PLGA-NHS–activated polymer was precipitated with 20 mL of cold 1:1 MeOH/Et_2O mixture and centrifuged at 2,700 × *g* for 10 min to remove residual EDC/NHS. The washing and centrifugation steps were repeated twice, and the polymer was then dried under vacuum for 90 min to remove residual solvents. After drying, PLGA-NHS was dissolved in DCM (1 mL) followed by the addition of NH_2-PEG$_{(5kDa)}$-COOH (13.33 mg, 0.0028 mmol) dissolved in dry chloroform (0.5 mL), in dry chloroform, followed by the addition of *N*,*N*-diisopropylethylamine (DIEA) (3.62 mg, 0.028 mmol), and the reaction was stirred for 12 h at RT. The copolymer was then precipitated by dropwise addition into cold diethylether/methanol (15:35 mL) and centrifuged (3,000 × *g* for 5 min). The supernatant was decanted, and the pellet redissolved in $CHCl_3$ (1 mL), precipitated, and washed further (three times) and dried under vacuum to yield PLGA-b-PEG-COOH (yield 85%). Polymer characterization: ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.22 (m, [-OC<u>H</u>(CH_3)CONH-]), 4.82 [m, (-OC<u>H_2</u>COO-)], 3.63 [s, (-C<u>H_2</u>C<u>H_2</u>O-)], 1.58 (d, [-OCH(C<u>H_3</u>)CONH-]) ppm.

To synthesize NH_2-PEG-*b*-Ald, *t*BOC-PEG-NHS was stirred with Ald (free acid form) in DMSO, at 1:1 molar ratio, for 6 h at RT. The reaction mixture was concentrated under vacuum and added to cold diethyl ether. After 1 h, at −20 °C, the product, *t*BOC-PEG-Ald, thus obtained, was filtered and dried under vacuum. Subsequently, *t*BOC-PEG-Ald (anhydrous dichloromethane) was stirred at RT for 1 h, with tin (IV) chloride (5 mmol) under anhydrous conditions, for BOC- deprotection, and the product was dried under vacuum. NH_2-PEG-Ald was purified as described above. Polymer characterization: ^{1}H NMR (400 MHz, DMSO-d6): δ NH_2-PEG-Ald: 3.63 [s, (-O-C<u>H_2</u>C<u>H_2</u>)], 2.98 (m, Ald: -CC<u>H_2</u>C<u>H_2</u>C<u>H_2</u>NHCOC<u>H_2</u>-), 1.92 (m, Ald: -CC<u>H_2</u>C<u>H_2</u>C<u>H_2</u>NHCO-) ppm.

PLGA-*b*-PEG-Ald was synthesized by conjugation of NH_2-PEG-Ald to PLGA-COOH by using EDC and NHS to activate PLGA-COOH, as described above (yield, 70%). Polymer characterization: ^{1}H NMR (400 MHz, DMSO-d6): δ PLGA-*b*-PEG-Ald: 5.20 (m, [-OC<u>H</u>(CH_3)COO-]), 4.89 [m, (-OC<u>H_2</u>COO-)], 3.50 [s, (-C<u>H_2</u>C<u>H_2</u>O-)], 3.02 (m, Ald: -CC<u>H_2</u>C<u>H_2</u>C<u>H_2</u>NHCOC<u>H_2</u>-), 1.89 (m, Ald: -CC<u>H_2</u>C<u>H_2</u>C<u>H_2</u>NHCO-), 1.47 (-OCH(C<u>H_3</u>)CONH-) ppm.

To synthesize PLGA-Alexa$_{647}$ or PLGA-Alexa$_{405}$, 50:50 poly(DL-lactide-coglycolide) (0.55–0.74 dL/g) (100 mg, 0.0023 mmol) was dissolved in 1 mL of dry DCM; to this was added EDC (4.41 mg, 0.023 mmol) in dry DCM (0.5 mL), and the reaction was stirred for 10 min followed by addition of NHS (2.65 mg, 0.023) in dry DCM (0.5 mL). The reaction was stirred at RT for 60 min. The PLGA-NHS–activated polymer was precipitated with 20 mL of cold diethylether/methanol (1:1) mixture and centrifuged at 2,700 × *g* for 10 min to remove residual EDC/NHS. The washing and centrifugation steps were repeated twice, and the polymer was then dried under vacuum for 90 min to remove residual solvents. After drying, PLGA-NHS was dissolved in DCM (1 mL) followed by the addition of Alexa$_{647}$ cadaverine or Alexa$_{405}$ cadaverine (0.0028 mmol) dissolved in dry DCM (0.5 mL), followed by the addition of DIEA (3.62 mg, 0.028 mmol), and the reaction was stirred for 12 h at RT. The polymer was then precipitated with 20 mL of cold 1:1 MeOH/Et_2O mixture and centrifuged at 2,700 × *g* for 10 min. The washing was repeated twice more, and the product dried under vacuum (to obtain a blue polymer compound). The supernatant was decanted, and the pellet was redissolved in $CHCl_3$, precipitated, washed further (three times), and dried under vacuum to yield fluorescently labeled PLGA (yield, 90%). Polymer characterization: 1H NMR (400 MHz, $CDCl_3$): δ PLGA peaks: 5.21 (m, [-OCH(CH_3)CONH-]), 4.83 [m, (-OCH_2COO-)], 1.59 (d, [-OCH(CH_3)CONH-]) ppm.

Phosphate Assay. The ratio of Ald to polymers in the Ald-PP NPs (single emulsion) was determined using a phosphate assay. The PLGA-*b*-PEG-Ald NPs (10 μL of 10 mg/mL NPs in water) were mixed with 60 μL of concentrated H_2SO_4 and 10 μL of H_2O_2 in glass tubes, and heated for 10 min at 200 °C. Thereafter, 690 μL of $Na_2S_2O_5$ (3 mg/mL) was added to the tubes, and the samples were incubated at 100 °C for 5 min. This was followed by adding 200 μL of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (20 mg/mL) and 20 μL of ascorbic acid (100 mg/mL) to the glass tubes and incubation for another 10 min at 100 °C. The absorbance of the sample solutions was determined at 820 nm and was used to determine phosphate concentrations of the samples. The calibration curves were based on known concentrations of Na_2HPO_4 as standards. PP NPs were used as negative controls (6). Experiments were performed in triplicate.

Drug Encapsulation and Release Kinetics. To evaluate the release kinetics of bortezomib from the NPs, a NP (single-emulsion) solution (1 mg/mL, in PBS) was incubated at 37 °C, in triplicates

for varying time periods. NPs were made as described encapsulating small molecule therapeutics, such as bortezomib. In the case of the small molecular drugs, such as chemotherapeutic drugs, the NP solution (1 mg/mL, in PBS), were placed in a dialysis bag with suitable molecular weight cutoff range based on the drug used (e.g., molecular mass cutoff, 3,400 Da for bortezomib). At each time point, the NPs were collected from the dialysis bag, dissolved in organic solvent (acetonitrile), and analyzed using HPLC. At all time points, the buffer was replaced to maintain the sink conditions. Experiments were performed in triplicate.

In Vitro Bone Targeting (HA Binding and Bone Affinity). The affinity of Ald-conjugated PEG-PLGA NPs (Ald-PP, single emulsion) toward bone mineral and bone fragments (ex vivo) was investigated in comparison with nontargeted PEG-PLGA (PP, single emulsion) NPs. Fluorescent labeled NPs (PLGA-$Alexa_{647}$) stable in physiological salt concentration and temperature, at pH 7.4, were incubated with HA (HA microparticles) in microfuge tubes and stirred (Sanyo Orbital Incubator) for varying lengths of time (5, 30 min, 2, 4, 6, 12, and 24 h). At the end of incubation time, the HA microparticles and the NPs bound to them settled down, when the microfuge tubes were spun at 85 × *g* for 5 min. The binding affinity of targeted NPs (fluorescent labeled) was determined by measuring the decrease in the relative concentration (by measuring the fluorescence) of targeted Ald-PP NPs in the supernatant compared with that of nontargeted PP NPs. Experiment was performed in triplicate.

Interactions of Ald-PP NPs to bone mineral (HA NPs) was further clearly confirmed at the nanoscale by TEM imaging of HA in the form of NP rods in solution phase when incubated with polymeric NP solutions (targeted vs. nontargeted). The sample solution was loaded on TEM grids, air-dried, and counterstained with 0.5% uranyl formate, as described above in the NP characterization section. Representative images are shown from experiments in triplicate.

The binding of Ald-PP NPs to biomimetic calcium phosphate (HA) was further investigated by SEM. The biomimetic calcium phosphate crystals were generated by simulated body fluid (5× concentration) on a solid surface (1 × 0.5 cm glass). The crystals were incubated with NPs in aqueous solution (1 mL of 0.5 mg/mL concentration) for 3 min with constant stirring on an orbital shaker, washed three times with deionized water (1 mL), dried overnight under vacuum, and coated with gold to visualize under high-resolution SEM with backscattering electron detector. Representative images are shown from experiments in triplicate.

Specific binding of Ald-PP NPs (PLGA-$Alexa_{647}$ labeled) to ex vivo bone fragment (skull, mice, 2 mm × 2 mm) compared with that of PP NPs was investigated by imaging under fluorescent microscope. Bone fragments were incubated with NP solution (0.1 mg/mL) for 30 min in physiological salt, pH, and temperature conditions, with constant stirring at 100 rpm (Lab Industries Inc. Labquake Shaker CAT No. 400-110), and after incubation, the bone pieces were washed three times with PBS, air dried in dark, and imaged again. Representative images are shown from experiments in triplicate.

Alizarin Red Staining of Bortezomib-Induced Osteogenesis. Osteogenic media (OM) consisting of αMEM supplemented with 10% (vol/vol) FBS, 100 U/mL penicillin, 10 μg/mL streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen), 0.05 mM ascorbic acid, 100 nM dexamethasone, and 10 mM β-glycerophosphate, was used. MSCs were seeded into 96-well plates grown to 95% confluence, allowed to proliferate in one of the following culture media for 1 wk, and stained for mineralization using Alizarin red: (*i*) regular MSC expansion media, (*ii*) OM, (*iii*) OM plus 0.5 mM Bort, (*iv*) OM plus 1 mM Bort. Media was then removed and cells were fixed with 4% (wt/vol) paraformaldehyde for 5 min, washed with deionized water, stained with Alizarin Red solution (Sigma) [2% (wt/vol), pH 4.2] for 10 min, washed again with deionized water, and imaged. Bright-field images were taken with an Olympus CKX41 microscope and an Olympus DP72 camera and a dry 4× objective. Representative images are shown from experiments in triplicate.

Cellular Uptake. Fluorescently labeled NPs (PLGA-$Alexa_{647}$ or PLGA-$Alexa_{405}$) (single emulsion) were incubated with PBMCs or MM1S cells, and analyzed with flow cytometry on a FACsCanto II (BD) for NP uptake. Total cell populations were identified based on forward-scatter (FSC) and side-scatter (SSC) gates and, if needed, fluorescently labeling (DiO^+ PBMCs, GFP^+ MM1S cells), and the fraction of these that contained NPs were identified by $Alexa_{647}$ positivity. All gates were identical within individual experiments. Cells were seeded in 1 mL of media in 24-well plates, 0.25×10^6 cells per well, incubated with 200 μg of NP solution (PBS), and sorted in FACS buffer [PBS plus 5% (vol/vol) FBS] immediately after sampling.

Fluorescent and Two-Photon Microscopy. To image cellular uptake of NPs labeled with the fluorescent dye $Alexa_{647}$ (Invitrogen), GFP^+ MM1S cells were incubated with labeled NPs (PLGA-$Alexa_{647}$) (single emulsion) for 24 h, washed repeatedly, and imaged using GFP and Cy5.5 filter cubes in a Nikon inverted TE2000 microscope (100× Plan-Apochromat Phase, N.A. 1.4, oil-immersion objective) with a Hamamatsu Orca ER digital CCD camera. Images were collected using Leica software where the GFP signal was colored green and $Alexa_{647}$ signal was colored red. To image $Alexa_{405}$-labeled NPs, GFP^+ MM1S cells were incubated with labeled NPs for 24 h, washed repeatedly, and imaged using a two-photon Zeiss 710 confocal system Coherent Chameleon Vision II laser (wavelength range, 680–1,040 nm). Samples were excited with 800 nm, 14% power with a 63× W-Plan Apochromatic, N.A. 1.0, objective on an upright Axio Examiner Z1 stand. GFP signal from cells was detected in NDD2 using a BP filter (500–550 nm), and $Alexa_{405}$ signal was detected in NDD3 using a short-pass filter (485). Images were collected using eight line sum averages with GFP signal colored green and $Alexa_{405}$ colored red, and processed using Zeiss software (Zen 2009).

Apoptosis Flow Cytometry Assays. MM1S cells were grown in 1 mL of media in 24-well plates, 0.25×10^6 cells per well, and treated with NPs [0.1 mg/mL, 0.04–0.07% (wt/wt) drug loading, single emulsion], with an effective drug concentration of low (~0.9 nM), medium (~3.6 nM), or high (~7.3 nM) doses of bortezomib. As a control, an equivalent amount of empty (no drug) NPs, and 5 and 10 nM free bortezomib were used. At 24 h, Annexin V–FITC and propidium iodide staining were used to detect and quantify apoptosis by flow cytometry (BD FACSCanto II HTS) following manufacturer's instructions, as previously described (2).

In Vitro Bioluminescence Assays. MM1S cells were seeded at 50,000 cells per well in opaque 96-well plates in 100 μL of media. Cells were treated with NP (0.1 mg/mL concentration, single emulsion) loaded with bortezomib [0.04–0.07% (wt/wt)] in an effective drug concentration range of low (~0.9 nM), medium (~3.6 nM), or high (~7.3 nM) doses of NPs for 12, 24, and 48 h. D-Luciferin substrate (7.5 mg/mL, 5 μL per well) was added to wells 5 min before measurement on a BMG LABTech PHERAstar plate reader with PHERAstar software, version 1.60, using an open emission filter.

In Vivo Biodistribution Studies. Athymic nude mice and BALB/c at 6 wk were purchased from Charles River for biodistribution studies. $Alexa_{647}$-labeled Ald-conjugated NPs (single emulsion) were synthesized with 5% of the total polymer as PLGA-

$Alexa_{647}$, and characterized for stability before injection. Mice were fed Non-Fluorescent Diet 48 h before injection and kept on the diet for the remainder of the study. For whole-mouse imaging, 1 mg of $Alexa_{647}$-labeled NPs in PBS was injected i.p. for in vivo imaging system (IVIS) FLIT imaging (Caliper Life Sciences) in the Koch Institute Whole Animal Imaging Core, and time points examined were 1 and 24 h. Each mouse had >30 point source selections (ex: 675 nm; em: 720 nm). All quantitative imaging analysis was conducted using Living Image 4.2 software.

NP Treatment of Multiple Myeloma in Vivo. Approval for all animal studies was obtained from the Dana-Farber Cancer Institute and Massachusetts Institute of Technology Institutional Animal Care and Use Committee offices and institutional review boards. Female Nod/SCID beige mice, 6–7 wk old (Charles River Laboratories), were injected with 4×10^6 Luc^+/GFP^+ MM1S cells per mouse via the tail vein. Using $n = 7$ mice per group, mice were randomly divided into the following groups: group 1, no bortezomib Ald-PP NPs; group 2, Free Drug (bortezomib in PBS); group 3, Ald-PP NPs loaded with bortezomib; group 4, PP NPs loaded with bortezomib. Mice were injected i.p. with 0.5 mg/kg bortezomib twice a week or with an equivalent amount of no drug NPs (group 1). BLI was performed weekly until signal was detected (day 21), and the mice were then injected with the drug treatments and imaged twice a week. Specifically, mice were imaged at 0, 7, 14, 21, 24, 28, 31, 35, and 38 d. Mice were killed via CO_2 inhalation at endpoints described or, for survival studies, mice when they had hindlimb paralysis, cachexia, weight loss of >15%, or become moribund.

NP Pretreatment of Multiple Myeloma in Vivo. Female NOD/SCID beige mice, 6–7 wk old (Taconic), were randomized into three groups ($n = 7$) and pretreated for 3 wk as follows: group 1, Ald-Bort-NPs; group 2, Free Drug; group 3, Ald-Empty-NPs. Mice were injected i.p. thrice a week with 0.3 mg/kg bortezomib or with an equivalent amount of no drug NPs (group 3). Then mice were injected with 4×10^6 Luc^+GFP^+ MM1S cell per mouse via the tail vein. BLI was performed on $n = 10$ mice per group weekly until signal was detected and then imaged twice a week at days 7, 14, 21, 24, and 29 until mice began to succumb to the disease and were then observed for survival. In another study, mice were randomized into three groups ($n = 10$) and pretreated for 3 wk as follows: group 1, Ald-Bort-NPs (targeted); group 2, Bort-NPs (nontargeted); group 3, Ald-Empty-NPs. Mice were injected i.p. three times a week with 0.3 mg/kg bortezomib or with an equivalent amount of no-drug NPs (group 3) and monitored for survival.

Detection of Tumor Progression by Bioluminescence Imaging. Mice were injected with 75 mg/kg luciferin (Caliper Life Sciences), followed by whole-body real-time bioluminescence imaging (Xenogen IVIS imaging system; Caliper Life Sciences) 5 min after injection. BLI signal was collected for 15 s, 1 and 5 min using the following settings: Emission Filter Open on an IVIS 1327 camera; binning (HR), 4; field of view, 25; flux stop, 1. Total flux (photons per second) was calculated based on 4 × 8-cm regions of interest of dorsal images.

Bone Histomorphometry. Tibiae from the mice were dissected and fixed. After fixation, tibiae were dehydrated and embedded in methyl methacrylate without demineralization. Undecalcified 4-μm-thick sections were taken, and Von Kossa staining was performed. Consecutive sections were stained by tartrate-resistant acid phosphatase and toluidine blue to quantitate structure and cellular parameters by bone histomorphometry. Bone histomorphometry was performed at secondary spongiosa 200 μm below the growth plate using the Osteomeasure system (OsteoMetrics), and the results were expressed according to the standardized nomenclature (3).

Ex Vivo Micro-CT Analysis of Bones from NP Pretreatment. Micro-CT of leg bones (femur, tibia, and fibula) from pretreated mice were dissected from killed mice after the 3-wk pretreatment period to validate bortezomib-induced increase in osteogenesis. Femurs stored in 4% (wt/vol) paraformaldehyde solution were analyzed on a Scanco Medical MicroCT 35 system with an isotropic voxel size of 7 μm. Scans were conducted with an X-ray tube potential of 70 kVP, an X-ray intensity of 0.145 mA, and an integration time of 600 ms per tomographic projection.

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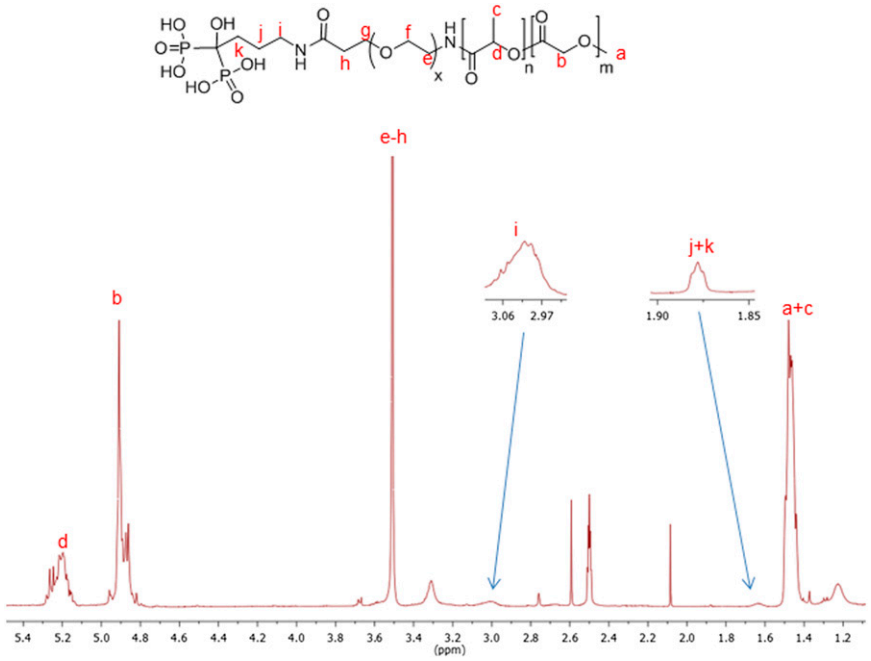

Fig. S1. Scheme of PLGA-*b*-PEG-Ald and associated 1H NMR.

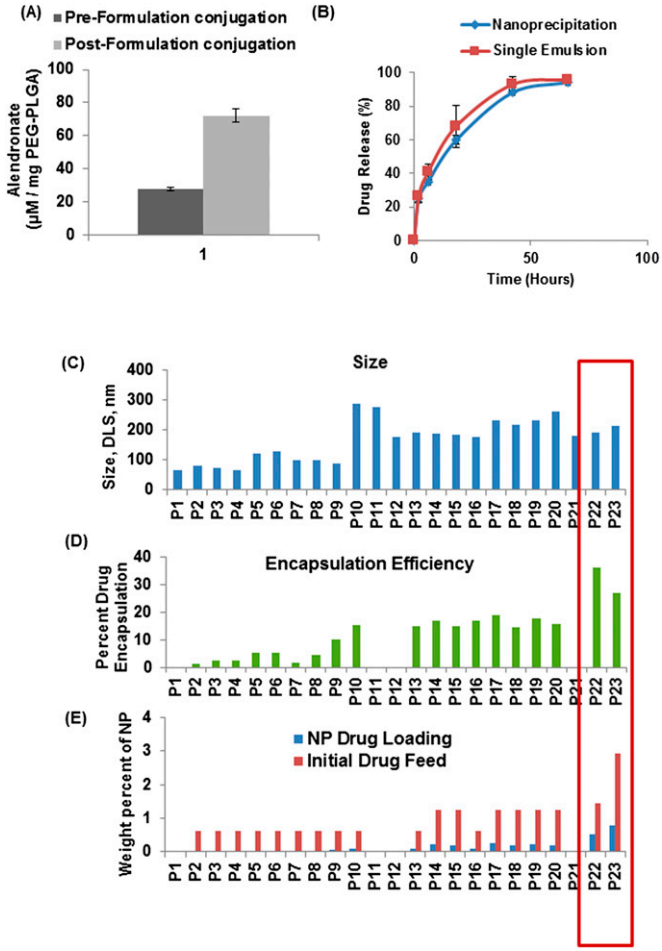

Fig. S2. (*A*) The content of Ald in Ald-PP NPs using phosphate assay. The Ald was incorporated in the NPs in two different ways: preformulation and postformulation conjugation. In preformulation conjugation, the Ald-conjugated polymer (PLGA-*b*-PEG-Ald) was blended with the PLGA-*b*-PEG polymer to formulate Ald-PEG-PLGA NPs, and in postformulation conjugation, the Ald was conjugated to the HOOC- groups on the surface of the formulated PEG-PLGA NPs made by polymer blends (PLGA-b-PEG-COOH; PLGA-b-PEG-OMe). The preformulation method of incorporating Ald to the NPs was chosen for all of the experiments performed in the study, as it gives a precise control over Ald density on NP surface with minimal batch-to-batch variability. (*B*) Release kinetics of bortezomib for NPs presented as percentage of the total drug encapsulated. (*C*) Size (hydrodynamic diameter, dynamic light scattering (DLS)], (*D*) drug encapsulation efficiency (percentage), (*E*) initial drug feed and drug loading (percentage) of a library of NPs. The NP library was synthesized using different formulation technique such as nanoprecipitation (P1–P9), double emulsion (P10–P11), and single emulsion (P12–P23). Formulation conditions such as organic to aqueous phase ratio (vol/vol), concentration of polymer in organic phase, initial feed of drug, and polymer molecular weights were varied. The optimized NPs (P22, P23) with optimal size (~190 nm), nearly neutral surface charge, maximal drug encapsulation efficiency (25–30%), and maximal drug load (0.75%) were formulated by single emulsion as described in *Materials and Methods*.

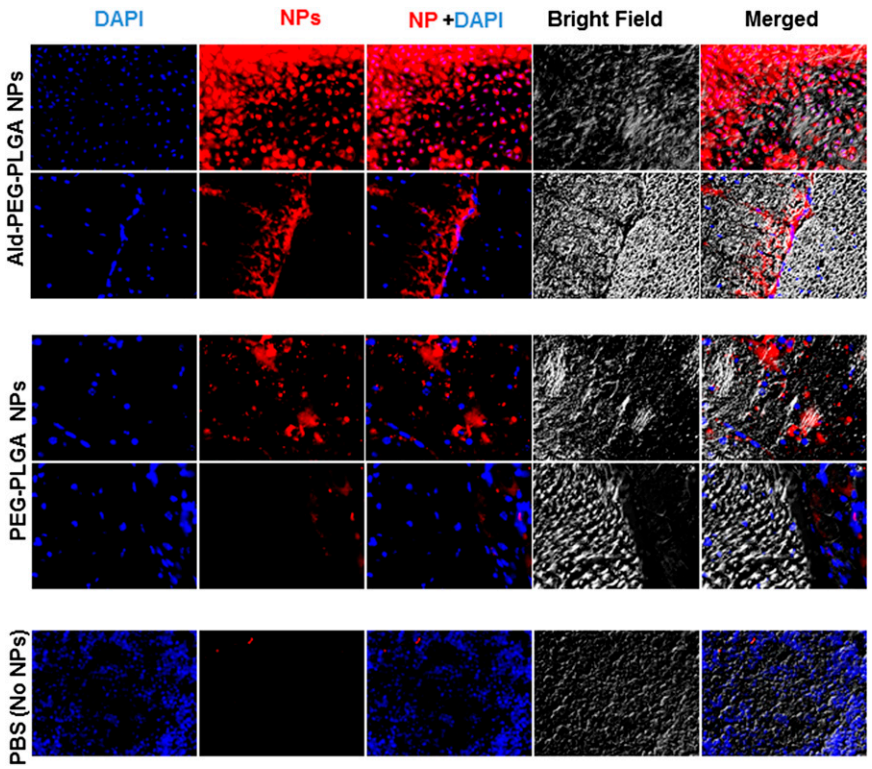

Fig. S3. Representative images of bone histology images in fluorescent channel 405 (DAPI, nuclear material), 647 (NPs, PLGA-Alexa647), bright field, and merged; for targeted (Ald-PP) NPs (*Top*), nontargeted (PP) NPs (*Middle*), and control (*Bottom*). (Scale bar: 100 μm.)

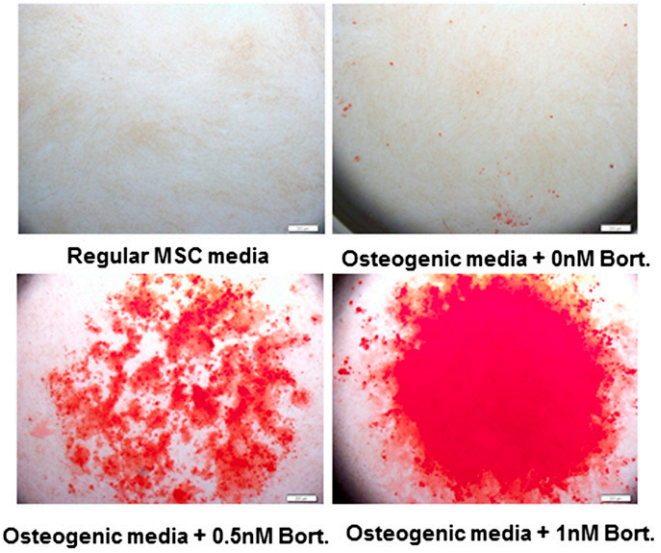

Fig. S4. Alizarin Red staining after 1 wk of differentiation demonstrate ability for bortezomib to increase osteogenic differentiation of bone marrow-derived mesenchymal stem cells.

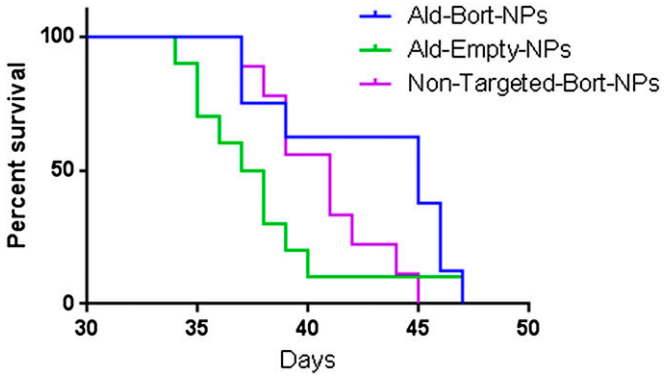

Fig. S5. Pretreatment with Ald-Bort-NPs inhibits myeloma growth. Mice were pretreated for 3 wk with Ald-Bort-NPs (targeted, blue line), Ald-Empty-NPs (without bortezomib) (green line), and Bort-NPs (nontargeted, purple line). They were then injected with $GFP^{+}Luc^{+}$ MM1S cells and monitored for survival. Survival improved in the mice pretreated with targeted-bortezomib vs. non-targeted-bortezomib NPs, and both treatments significantly increased survival for mice vs. empty nanoparticle treatment (Gehan–Breslow–Wilcoxon test, $P = 0.02$).

Table S1. Histomorphometry data of mouse bones after 3 wk of pretreatment

				Statistical analysis		
Parameters	Ald-Empty-NP ($n = 6$)	Free drug (FD) ($n = 5$)	Ald-Bort-NPs ($n = 6$)	One-way ANOVA	FD vs. Ald-Empty-NP	Ald-Bort-NPs vs. Ald-Empty-NPs
BV/TV, %	8.59 ± 3.63	14.78 ± 6.18	13.16 ± 6.48	NS	NS	NS
Tb.Th, μm	37.09 ± 8.89	46.92 ± 7.17	42.72 ± 9.78	NS	NS	NS
Tb.N, /mm	2.29 ± 0.75	3.06 ± 0.77	2.94 ± 0.89	NS	NS	NS
Tb.Sp, μm	430.5 ± 121.5	295.5 ± 85.2	324.5 ± 115.8	NS	NS	NS
N.Ob/B.Pm, /mm	6.46 ± 2.35	6.95 ± 1.59	7.23 ± 2.21	NS	NS	NS
Ob.S/B.Pm, %	9.86 ± 3.91	11.06 ± 2.97	11.14 ± 3.41	NS	NS	NS
OS/BS, %	3.35 ± 2.18	3.84 ± 1.59	4.17 ± 1.76	NS	NS	NS
O.Th, μm	3.27 ± 1.50	4.89 ± 0.78*	5.06 ± 0.63*	$P < 0.05$	$P < 0.05$	$P < 0.05$
N.Oc/B.Pm, /mm	1.22 ± 0.31	0.82 ± 0.20*	1.01 ± 0.15	$P < 0.05$	$P < 0.05$	NS
Oc.S/B.Pm, %	3.68 ± 0.67	2.81 ± 0.90	3.32 ± 0.53	NS	NS	NS
ES/BS, %	4.67 ± 0.98	3.12 ± 0.71*	4.15 ± 0.65	$P < 0.05$	$P < 0.05$	NS

Data are mean ± SD. BV/TV, bone volume/total volume; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness. Osteoblastic parameters: N.Ob/B.Pm, osteoblast number; Ob.S/B.Pm, osteoblast surface per bone perimeter; OS/BS, osteoid surface; O.Th, osteoid thickness. Osteoclastic parameters: ES/BS, eroded surface per bone surface; N.Oc/B.Pm, osteoclast number; Oc.S/B.Pm, osteoclast surface. *$P < 0.05$ compared with ET-NP, one-way ANOVA and the Dunnet's post hoc test.