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TGFβ inhibition stimulates collagen maturation to enhance bone repair and fracture resistance in a murine myeloma model

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Abstract

Multiple myeloma is a plasma cell malignancy that causes debilitating bone disease and fractures, in which TGFβ plays a central role. Current treatments do not repair existing damage and fractures remain a common occurrence. We developed a novel low tumour phase murine model mimicking the plateau phase in patients, as we hypothesized this would be an ideal time to treat with a bone anabolic. Using in vivo microCT we show substantial and rapid bone lesion repair (and prevention) driven by SD-208 (TGFβ receptor I kinase inhibitor) and chemotherapy (bortezomib and lenalidomide) in mice with human U266-GFP-luc myeloma. We discovered that lesion repair occurred via an intramembranous fracture repair-like mechanism and that SD-208 enhanced collagen matrix maturation to significantly improve fracture resistance. Lesion healing was associated with VEGFA expression in woven bone, reduced osteocyte-derived PTHrP, increased osteoblasts, decreased osteoclasts and lower serum TRACP-5b. SD-208 also completely prevented bone lesion development mice with aggressive JJN3 tumors, and was more effective than an anti-TGFβ neutralizing antibody (1D11). We also discovered that SD-208 promoted osteoblastic differentiation (and overcame the TGFβ-induced block in osteoblastogenesis) in myeloma patient bone marrow stromal cells in vitro, comparable to normal donors. The improved bone quality and fracture-resistance with SD-208 provides incentive for clinical translation to improve myeloma patient quality of life by reducing fracture risk and fatality.

Key words

Tumor-induced bone disease, TGFβ, anabolics, pre-clinical studies, collagen

Introduction

Multiple myeloma is a plasma cell neoplasm that is incurable for most patients. Myeloma is the second most common hematological malignancy and the incidence has risen by 66% since the 1970s.[1,2] Virtually all patients (~90%) develop a destructive bone disease[3], due to disruption of bone remodeling.[4,5] Sixty percent of patients experience bone fractures[6], which increases fatality[7,8] and treatment costs.[9-11] Current treatments for myeloma bone disease are limited to anti-resorptives, which can prevent further bone loss but are ineffective at repairing bone. Bone lesions (67%) and bone pain (60%) are frequently present at diagnosis[12], hence incorporation of a bone anabolic into myeloma patient care would dramatically reduce the occurrence of bone fractures and benefit patients immensely.

Transforming growth factor beta (TGFβ) is central to development and progression of myeloma bone disease. TGFβ levels are elevated due to release from myeloma cells[13,14], bone marrow stromal cells (BMSCs)[13] and from the bone matrix during resorption.[15] TGFβ regulates osteoclast and osteoblast differentiation, resulting in increased bone resorption and reduced bone formation.[15,16] Inhibition of TGFβ has both bone anabolic and anti-resorptive effects in non-tumour-bearing mice[17,18], leading to increased bone volume and improved bone quality.[19] Similarly, TGFβ inhibition prevented development of myeloma bone disease[20-22], and repaired existing bone lesions if administered with the bisphosphonate zoledronic acid.[23]

Until recently, healing of lytic lesions in myeloma patients was generally considered to only occur in rare instances. Myeloma cells induce epigenetic changes that represses the Runx2 promoter in osteoblast progenitor cells, resulting in persistent repression of osteoblast differentiation which prevents formation of new bone.[24] However, recent observations

showed 43% of patients on total therapy 4 exhibited lesion remineralization.[25] Healing was in part attributed to the bone modulating effects of the proteasome inhibitor bortezomib (btz)[26] and immunomodulatory drug lenalidomide (len). Btz has direct bone anabolic and anti-resorptive activity in vitro[27-32] and in vivo[32-34] and improves bone outcomes for myeloma patients.[25,29,35-42] Similarly, len has exhibited anti-resorptive effects[43,44] and reduced bone resorption in patients.[44] Furthermore, btz/len treatment would facilitate recovery of the bone marrow microenvironment by reducing tumour and hence normalize bone remodeling.

While it is promising that btz and len have positive effects on bone, myeloma patients continue to experience skeletal-related events despite receiving these treatments, and new therapies are needed. This must be driven by identification of bone anabolic agents (e.g. TGFβ inhibitors) that can heal and not just prevent further bone disease, in a reliable, clinically representative in vivo model. Considering osteoblast differentiation is repressed in myeloma patients, it is also integral that the ability of potential bone anabolics to overcome differentiation suppression is confirmed in patient samples. After treatment with chemotherapy, myeloma patients frequently enter a 'plateau phase' of clinical stability where tumour burden is (usually) low. We hypothesized that the plateau phase is the ideal time for anabolic treatment to repair bone.

Here, we developed a murine model of myeloma where combination chemotherapy using btz and len induces a low tumour phase that mimics the plateau phase observed clinically, and allows for repair of bone lesions. Using this model, we showed that the TGFβRI kinase inhibitor, SD-208, stimulated rapid maturation of the collagen matrix leading to enhanced intramembranous bone repair and improved fracture resistance, without the use of an anti-

resorptive. This mechanism of bone lesion repair has never before been identified in myeloma bone disease. We also provide evidence for efficacy in myeloma patients through stimulation of osteoblast differentiation of patient BMSCs with SD-208.

Methods

Study approval

All animal experiments were approved by the University of Sheffield Animal Ethics Committee and the UK Home Office (PPL 70/8799) in strict compliance with the Animal (Scientific Procedures) Act 1986. Bone marrow aspirates were obtained from myeloma patients and healthy donors with written informed patient consent and approval from the South Sheffield Research Ethics Committee (REC reference 05/Q2305/96).

In vivo myeloma studies

Female, 8-week-old NOD scid gamma (NSG, NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) mice (Charles River Laboratories) were randomized into groups and injected i.v. with PBS (naïve) or 10^6 human JJN3 cells. After 1 week, JJN3-bearing mice (n=8) were treated 5 days/week for 2 weeks with 60 mg/kg[17] SD-208 (Selleck Chemicals) by oral gavage, 20 mg/kg[23] 1D11 (anti-TGFβ antibody; Bio-Techné) by i.p. injection or controls (Fig. 1A). All mice were sacrificed after 2 weeks of treatment.

To identify the effect of chemotherapy and SD-208 on bone repair, a low tumour model was developed. Female, 9-week-old NSG mice were injected i.v. with 10^6 human U266-GFP-luc cells (Fig. 2A). Tumour burden was monitored weekly by bioluminescent imaging using an IVIS Lumina II (Caliper Life Sciences) and serum paraprotein was measured by human IgE ELISA (Thermo Fisher Scientific). At 8 weeks post-tumour inoculation (referred to as 0 weeks post-treatment), tibiae were scanned with a VivaCT80 pre-clinical μCT scanner

(Scanco) to confirm development of bone lesions and mice were treated with vehicles or chemotherapy (btz + len) ± SD208 (Selleck Chemicals), and sacrificed after 2 weeks of treatment (n=7). Vehicle mice were sacrificed after 1 week due to effects of the tumour on animal welfare and a separate group receiving chemotherapy were treated until relapse (n=5). Btz (i.p.), len (oral gavage) and SD-208 dosing schedules are described in Fig. 2A. Flow cytometry was used to measure tumour burden using a FACSCalibur (BD Biosciences) and FlowJo 10 (Treestar). All mice were group housed in individual ventilated cages with a 12-hour light/dark cycle and ad libitum access to food and water. Mouse numbers were calculated prospectively using G*Power software. Endpoint and in vivo analyses were blinded.

Micro computed tomography analyses

Bone structure and osteolytic disease was monitored in vivo at -1, 0, 1 and 2 weeks post-treatment by in vivo µCT, as previously described.[23] Briefly, mice were anaesthetized by isoflurane inhalation and tibiae were scanned at 10.4 µm isotropic voxel size, 55 kV, 145 µA, 200 ms integration time for a 360° scan with a 0.24° rotation step on a VivaCT80 (Scanco) and images were reconstructed with Scanco software. Image registration was performed in DataViewer (1.5.4.6). Ex vivo µCT was performed on the contralateral tibia, femur, calvaria and L2 and L3 vertebrae using a SkyScan 1272 µCT scanner (Bruker, Kontich, Belgium) at 4.3 µm voxel size, 0.5 mm aluminium filter, 50 kV, 200 µA for a 180° scan with a 0.7° rotation step and image reconstruction was performed using Skyscan software NRecon (1.6.9.4). Trabecular analysis was performed using DataViewer (1.5.4.6) and CTAn (1.8.1.2) following standard guidelines.[45] Tibial and femoral trabecular bone was analyzed in a 1mm region with a 0.2 mm and 0.4 mm distal offset from the growth plate, respectively. The region analyzed for vertebral (L2 and L3) trabecular bone was a 400 µm offset from the point

of 50% trabecular bone and 50% endplate on both ends of the vertebrae. Cortical osteolytic lesions of the proximal tibia to the tibial crest were measured using Osteolytica v1.9[46], using a minimum lesion size of 20,000 μm^2. 3D models were created using Drishti software (v. 1.1, ANU Vizlab, Australia) and ParaView 5.4.1 software (New York, USA).

Histomorphometry, immunohistochemistry and bone turnover markers

Tibiae were fixed in 10% formalin for 24 hours then stored in 70% ethanol. One tibia from each mouse was decalcified, embedded in paraffin and 3 µm longitudinal sections were stained for tartrate-resistant acid phosphatase (TRAP), as previously described.[47] Histomorphometric analysis of osteoclast and osteoblast numbers and surfaces on endocortical bone were measured using Osteomeasure (OsteoMetrics, Atlanta, GA, USA) using standard guidelines.[48] Immunohistochemistry was performed on paraffin-embedded tibial sections using a rabbit anti-PTHrP (R87) antibody[49] (1:5000) specific to the amino terminus of PTHrP (1-34) or rabbit anti-VEGFA antibody (ab46154 1:1000, Abcam, Cambridge, UK) as described previously.[49,50] Contralateral tibiae were embedded in LR White medium resin (TAAB Laboratories, Aldermaston, UK) and 10 µm sections were used for dynamic histomorphometry analysis of alizarin/calcein labels.[47] Serum bone turnover markers TRAP5b (Oxford Biosystems, Oxford, UK) and P1NP (Immuno Diagnostic Systems, Tyne & Wear, UK) were measured by ELISA.

Bone material characteristics

Raman spectroscopy was performed on a XploRA Plus system with a 532 nm air-cooled Ar^+ laser source (Horiba Scientific, Kyoto, Japan). Resin-embedded tibia were cut longitudinally into 10 µm sections and dried onto charged microscope slides. Spectra were obtained from 3 evenly-spaced positions within the medial cortical bone (termed endocortical, middle and periosteal) across a 400 cm^{-1} to 1800 cm^{-1} spectral range.[51] All spectra are an average of 100

consecutive spectra, with a 10 second exposure, 5 mW laser power using a 100X objective. A least squares regression algorithm was used to fit the data with a polynomial function to remove background fluorescence. Data were normalized to the primary phosphate peak and smoothed by adjacent-averaging using OriginPro 2018b b9.5.5.409 (OriginLab Corporation, Northampton, MA, USA). Band fitting for the primary phosphate ($\boldsymbol{\nu}_1PO_4$, ~960 cm^{-1}), carbonate (CO_3, ~1070 cm^{-1}) and amide I (~1610 cm^{-1} to ~1690 cm^{-1}) bands were performed by fitting Gaussian functions. Amide I sub-peak analysis was performed by fitting 4 peaks at ~1610 cm^{-1}, 1638 cm^{-1}, 1668 cm^{-1} and 1692 cm^{-1} using least squares regression, as previously identified.[52] An additional overlapping peak was included at 1601 cm^{-1} in the deconvolution. Mineral crystallinity was measured as the inverse of the full width at half maximum of the $\boldsymbol{\nu}_1PO_4$ band. Relative peak integral areas were used to calculate the mineral to matrix ratio ($\boldsymbol{\nu}_1PO_4$:amide I), carbonate to phosphate ratio (CO_3:$\boldsymbol{\nu}_1PO_4$) and amide I sub-peak ratios (1668/1610, 1668/1638, 1668/1692 cm^{-1}).

Bone mechanical testing

Femurs and vertebrae (L3 and L4) were fixed in 10% formalin for 24 hours then stored in 70% ethanol prior to 3-point bending and compression testing, respectively. Three-point bending was performed on a Bose ElectroForce 3200 test instrument with a 450 N load cell, as previously described.[53] Femurs were placed on two rounded supports 6 mm apart, with load applied anteroposteriorly to the midshaft. A 0.5 N preload was applied, followed by a 0.25 N/sec constant force until failure. The force-displacement curve was used to obtain ultimate force (N) given by the curve peak, and stiffness (n/mm) given by the gradient of the linear region of the curve.

Vertebral compression testing was performed on an Instron 5543 load frame with a 500 N load cell and Bluehills2 software (Instron, Norwood, MS, USA).[54] As the vertebral processes reduce sample stability during compression testing, processes were removed from L4 vertebrae prior to testing using a dental drill, whereas L3 vertebrae were kept intact. Vertebrae were glued to the mount with cyano-acrylate glue and compressed at a constant rate of 0.03 mm/s and a 20 Hz sample rate until 1 mm displacement. The force-displacement curve was used to obtain the ultimate force (N).

Osteogenic differentiation of patient bone marrow stromal cells

CD138- BMSCs were purified from bone marrow aspirates using CD138 MicroBeads (MACS Miltenyl Biotec). BMSCs were cultured in osteogenic media (αMEM with Glutamax supplemented with 10% (v/v) FCS, 100 µg/ml Streptomycin, 100 U/ml Penicillin, 0.01 mM dexamethasone, 200 µM ascorbate and 10 mM β-glycerophosphate)[55] supplemented with 5 ng/ml recombinant TGFβ (rTGFβ), 1 µM SD-208, rTGFβ + SD-208 or vehicles. Osteoblast formation was assessed by gene expression and alkaline phosphatase (ALP) activity.[55]

Gene expression analyses

Total RNA was isolated from human BMSCs or mouse bone marrow using a ReliaPrep RNA Cell MiniPrep System (Promega, Madison, WI, USA) and cDNA synthesized using a High-Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific). qPCR was performed using primers specifically targeting either mouse or human genes or TaqMan Gene Expression Assays (Supplemental Table I) with TaqMan Universal Master Mix II (Thermo Fisher Scientific) or SYBR Green Master Mix (Thermo Fisher Scientific) and 5 pmol of primers and detected with an ABI Prism 7900HT and SDS 2.1 software (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

All data are presented as mean±SD. Statistical analysis was performed in Prism 7 (GraphPad) using an unpaired two-tailed Student's t test, one-way ANOVA or two-way ANOVA with Tukey's multiple comparisons test.

Data sharing statement

The data generated during this study are available from the corresponding author (a.c.green@sheffield.ac.uk) upon reasonable request.

Results

SD-208 prevents myeloma bone disease

We previously reported prevention and repair of myeloma bone lesions with an anti-TGFβ antibody, 1D11, in combination with zoledronic acid but without chemotherapy.[23] Numerous small molecule inhibitors of TGFβ signaling are in clinical trials for other cancers[56] and SD-208 had a bone anabolic effect in naïve mice.[17] Thus, we investigated the efficacy of SD-208 (a TGFβRI kinase inhibitor) in treating myeloma bone disease using the aggressive JJN3 human xenograft model (Fig. 1A). Tibial trabecular bone volume and trabecular number were significantly lower in JJN3-bearing control mice compared to naïve mice (Fig. 1B-C). SD-208 or 1D11-treated mice had significantly higher trabecular bone volume and number compared to JJN3 controls (Fig. 1B-C). Trabecular thickness was comparable between all groups (Fig. 1D). The total lesion area in the proximal tibia was highest in the vehicle group (6.3±2.8%) and somewhat lower with 1D11 treatment (4.3±2.4%), whereas SD-208-treated mice had significantly lower lesion area (1.4±1%) compared to both control and 1D11-treated JJN3 mice, appearing similar to naïve mice (0.26±0.3%; Fig. 1E, H-K). This corresponded with significantly fewer lesions following SD-208 treatment compared to control and 1D11-treated mice (Fig. 1F), but no change to average lesion size (Fig. 1G). This indicates that development of myeloma bone disease was substantially impaired with SD-208 treatment, which was more effective than 1D11 treatment. Endocortical osteoblasts and osteoclasts were unaltered by SD-208 or 1D11 treatment at endpoint (Fig. 1L-O), indicating that the effects of SD-208/1D11 at the cellular level must have been occurring at earlier timepoints to reflect the improved bone architecture at endpoint.

Bortezomib and lenalidomide induce a low tumour phase in U266-GFP-luc-bearing mice

To develop a low tumour phase model that mimics the plateau phase observed clinically, Btz chemosensitivity of JJN3-GFP-luc, OPM2-GFP-luc and U266-GFP-luc cells was tested in vitro and in vivo (Supplemental Information and Supplemental Fig.1). Btz reduced the number of viable cells compared to the vehicle for all cell lines in vitro (Supplemental Fig.1). In vivo, btz treatment was the most effective at reducing tumour in the U266-GFP-luc mouse xenograft model, whereas no effect was observed in OPM2-GFP-luc-bearing mice and minimal effect was observed in JJN3-GFP-luc mice (Supplemental Fig. 1). Therefore, the U266-GFP-luc model was selected for further optimization with combined btz and len chemotherapy (Supplemental Fig. 2) to establish a low tumour phase model (Fig. 2A), mimicking the plateau phase. Btz+len administration at late-stage disease reduced tumour after 1 week by bioluminescent imaging in hindlimbs (~50 fold), vertebrae (~10 fold) and calvaria (~1000 fold) and serum paraprotein (Fig. 2B-G). This corresponded to >99% reduction bone marrow tumour burden by flow cytometry compared to vehicle (Fig. 2H). Tumour burden remained persistently low for 5-6 weeks prior to relapse (Fig. 2B-G). SD-208 did not affect tumour burden by bioluminescent imaging, serum paraprotein or flow cytometry (Supplemental Fig. 3).

SD-208 enhances bone lesion repair in combination with chemotherapy

In the low tumour model, we performed in vivo µCT of tibiae prior to treatment (week 0) and after 1 and 2 weeks of chemotherapy with or without (±) SD-208. Lesions in vehicle-treated mice were unaltered or increased in size by week 1 (+18%; Fig. 3). Mice treated with chemotherapy exhibited similar bone lesion area and number after 1 week, but after 2 weeks lesion area was reduced by 52%, indicating partial lesion repair (Fig. 3A-C). Three-

dimensional reconstructions showed thin bridging of holes in chemotherapy-treated mice (Fig. 3A-B). In comparison, after only 1 week, SD-208 + chemotherapy reduced total lesion area by 21% compared to vehicle (Fig. 3C). After 2 weeks, total lesion area was reduced by 76%, showing significant improvement over mice treated with chemotherapy alone (Fig. 3C), with repair on all surfaces of the proximal tibia (Video 1). In mice treated with btz+len±SD-208, lesion size and number were lower after 2 weeks (Fig. 3D-E). This was most pronounced with SD-208 treatment, with a 57% reduction in average lesion size and 49% reduction in lesion number, indicating lesions were smaller and nearly half were completely healed with SD-208 treatment. In vivo µCT monitoring of bone over successive timepoints identified that repair occurred from the marrow cavity out towards the bone exterior. New bone bridged perforating lesions at the endocortical surface, but lesions remained visible on the external surface as craters (Fig. 3A). Furthermore, the new bone formed between week 0 and 1 in SD-208-treated mice was generally less dense bone (red-yellow) that was then replaced by more dense bone (yellow-blue) by week 2 (Fig. 3B). Despite the presence of tumour in calvaria, bone lesions were not present (Supplemental Fig. 4A-C).

SD-208 increases trabecular bone volume in long bones

Myeloma also causes a loss of trabecular bone, thus we investigated changes in trabecular bone by µCT for in vivo scanned tibiae, and bones isolated ex vivo including the contralateral tibia, femur and lumbar vertebrae (L2 and L3). There was a significant increase in trabecular bone volume in mice treated with btz+len+SD-208 compared to btz+len after 2 weeks in vivo (Fig. 4A). This was accompanied by an increase in trabecular number, with unaltered trabecular thickness or trabecular spacing (Fig. 4B-D). Ex vivo, there was also an increase in trabecular bone volume in the contralateral tibia by 60% (Fig. 4E) and femur by 74% (Fig. 4J). Trabecular number increased and trabecular thickness was unchanged with btz+len+SD-

208 treatment compared to vehicle in the contralateral tibia (Fig. 4F-G) and femur (Fig. 4K-L). Trabecular separation was lower in mice treated with btz+len±SD-208 compared to vehicle-treated mice (Fig. 4H&M). In comparison, changes to vertebral trabecular bone were less pronounced and SD-208 provided no significant benefit beyond chemotherapy (Supplemental Fig. 4D-K). Thus, 2 weeks of SD-208 improved long bone trabecular bone architecture, but minimal benefit occurred in lumbar vertebrae.

Bone remodeling is re-established during the low tumour phase and bones heal via intramembranous repair

To identify the bone repair mechanism, we investigated changes to bone remodeling and myeloma bone disease regulators at endpoint. Histomorphometry revealed abundant TRAP+ osteoclasts on endocortical bone surfaces in vehicle mouse tibiae (Fig. 5A), which was noticeably diminished with btz+len±SD-208 treatment (Fig. 5B&C). Furthermore, btz+len±SD-208 treatment stimulated formation of woven bone along endocortical surfaces, which was particularly noticeable in regions with lytic lesions (Fig. 5B&C inset). The bridging of cortical lesions with woven bone mimics the repair mechanism reported in fracture repair models (e.g. drill-hole models[57]), suggesting myeloma bone lesions similarly heal by intramembranous bone repair. Histomorphometry also found that endocortical osteoclasts were reduced whereas osteoblasts were increased in mice treated with btz+len±SD-208 compared to vehicle (Fig. 5D-G). There was no difference between btz+len and btz+len+SD-208-treated mice, although this may have occurred at earlier timepoints (i.e. during repair) as lesions were already healed in the chemotherapy + SD-208 group at endpoint. The reduction in osteoclasts was supported by lower serum TRACP5b (Fig. 5H), indicating decreased resorption in mice administered btz+len±SD-208. Serum P1NP (bone

formation biomarker) was unchanged (Fig. 5I), indicating a change in total body bone formation was not detectable.

Myeloma cells secrete or stimulate the microenvironment to express factors that cause myeloma bone disease.[4] We investigated expression of some myeloma bone disease genes by human U266-GFP-luc myeloma cells and mouse microenvironment cells isolated from femoral bone marrow. In vehicle-treated mice there was expression of human U266-derived TGFB1, DKK1, IL6, PTHLH, MET, TNF, CCL3, and FZB but not TNFSF11 (Fig. 5J). Consistent with McDonald et al.[58], we found SOST was not expressed by myeloma cells (Fig. 5J). None of the genes (including human GAPDH) were detected in bone marrow from btz+len±SD-208-treated mice, indicating tumour reduction prevented further production of bone disease-promoting factors. Furthermore, bone marrow expression of murine Tnfsf11 (encoding RANKL) and Tnfrsf11b (encoding osteoprotegerin), commonly aberrant in myeloma, were unaltered (Supplemental Fig. 5A&B). Given osteocytes are the primary source of RANKL[59], it is still possible that RANKL signaling was altered.

TGFβ stimulates microenvironment expression of genes involved in tumorigenesis and bone disease in myeloma, including Il6, Vegfa, Mcp1[60], Pthlh (encoding PTHrP)[61] and Thbs1.[20] Thsp1, Mcp1 and Il6 expression in bone marrow was comparable between all treatment groups (Supplemental Fig. 5C-E). Vegfa expression was higher and Pthlh was lower in mice treated with chemotherapy ± SD-208 compared to vehicle mice (Fig. 6A&B).

To identify the source of PTHrP within the microenvironment we performed immunohistochemistry. In vehicle mice, U266-GFP-luc myeloma cells and 86% of bone-embedded osteocytes stained positive for PTHrP (Fig. 6C&D). In comparison, there was no

PTHrP expression in the bone marrow or osteocytes in mice treated with btz+len±SD-208 (Fig. 6E&F). This indicates that chemotherapy inhibited expression of PTHrP by osteocytes, likely by reducing tumour burden. While non-tumour bearing (naïve) mice were not included in this study, we stained sections from age- and sex-matched mice from a previous study, finding that 58.8% ± 7.1% of osteocytes were PTHrP positive. Together, this identified that tumour cells increase expression of PTHrP levels in the bone marrow, not only from tumour-derived production of PTHrP but also by stimulating expression in the microenvironment. Furthermore, considering chemotherapy ablated osteocyte PTHrP expression, this is not only reflective of reduced tumour, it also suggests bortezomib or lenalidomide may also reduce osteocytic PTHrP mechanism irrespective of tumour.

VEGFA levels would be expected to decrease in response to TGFβ inhibition[60] or chemotherapy[62], suggesting a different cause for the increase in Vegfa. VEGFA is a critical mediator of intramembranous facture repair[63,64], as such Vegfa would help facilitate lesion healing. Indeed, VEGFA immunohistochemistry (Fig. 6G-I) revealed VEGFA expression within the woven bone in mice treated with chemotherapy ± SD-208 (Fig. 6H&I). VEGFA was also expressed in the periosteum (Fig. 6G-I), tumour bone marrow (Fig. 6G) and megakaryocytes (Fig. 6H&I inset).

SD-208 enhances femoral fracture resistance and collagen maturation

Fractures are a common repercussion of myeloma bone disease that increase morbidity and fatality. To determine whether SD-208 improved fracture resistance, mechanical properties were assessed by femoral 3-point bending and vertebral compression. In femurs, the ultimate force (maximum load required to break the bone) and stiffness were increased in

btz+len+SD-208 compared to vehicle-treated mice but not chemotherapy alone (Fig. 7A&B). Btz+len significantly increased the ultimate force for L3 and L4 vertebrae, but no additional benefit was observed with SD-208 treatment (Fig. 7C&D). Prior to compression, L4 vertebrae had their processes removed whereas L3 vertebrae were kept intact; this did not affect the trend between groups, however the force to failure in the vehicle groups was higher in L3 (40.75 N ± 11.28 N) compared to L4 (31.48 N ± 6.35 N).

MicroCT revealed changes to bone structure, although as material composition and organization can also influence bone strength we performed Raman spectroscopy. MicroCT and histology analysis indicated that cortical bone lesions were healing from the endocortical surface out towards the periosteal surface. Raman spectra were collected from 3 regions in the cortical bone where lesions were undergoing repair: close to the endocortical surface, in the middle of the cortical bone and close to the periosteal surface (Fig. 7E&F). Mineralization (mineral-to-matrix ratio) and type B carbonate substitution (carbonate to phosphate ratio) were unchanged (Fig. 7H&I) and mineral crystallinity was the same for each sample (data not shown). Sub-peak analysis of the amide I band (Fig. 7G) was used to determine the effect of chemotherapy ± SD-208 on collagen cross-linking, as a surrogate measurement of matrix maturity. As bone matures, collagen fibrils undergo a series of cross-linking reactions where immature divalent cross-links form, some of which then convert to mature trivalent cross-links.[65] This conversion is associated with an increase in the ~1668 cm^{-1} to ~1692 cm^{-1} sub-peak ratio.[65] In the region close to the periosteal surface, the 1668:1692 ratio was significantly higher in mice treated with btz+len+SD-208 compared to chemotherapy alone (Fig. 7J), indicating enhanced matrix maturation with SD-208 treatment. The improvements to bone architecture and collagen cross-linking with SD-208 treatment both contribute to making stronger, more fracture-resistant bone.

SD-208 promotes myeloma patient and normal osteoblast formation in vitro

To determine the effect of TGFβ inhibition on osteoblast formation, BMSCs isolated from normal donor (N-BMSCs) and myeloma patient (MM-BMSCs) samples were cultured in osteogenic media with recombinant TGFβ (rTGFβ) and/or SD-208. N-BMSCs and MM-BMSCs treated with SD-208 had significantly higher ALP activity compared to rTGFβ-treated BMSCs at all timepoints (Fig. 8A&B). At day 7 and 10, SD-208-treated BMSCs had higher ALP activity than vehicle-treated N-BMSCs and MM-BMSCs (Fig. 8A&B). BMSCs treated with rTGFβ+SD-208 had significantly higher ALP activity at day 7 and 10 compared to rTGFβ alone, with levels comparable to vehicle-treated BMSCs (Fig. 8A&B). No significant differences were observed in osteoblastic gene expression between treatment groups (Fig. 8C-L), likely due to substantial variation between patient cells. However, there was a trend towards increased expression of the early osteoblastic gene SP7 (encoding Osterix) with rTGFβ treatment (Fig. 8D&I) and increased expression of later genes ALPL and PTH1R with SD-208 treatment (Fig. 8E&F, J&K) in both N-BMSCs and MM-BMSCs. Furthermore, BGLAP (encoding Osteocalcin) expressed by mature osteoblasts was only detectable in BMSCs treated with SD-208, and in vehicle-treated MM-BMSCs (but not N-BMSCs) (Fig. 8G&L). This indicates that SD-208 treatment blocks the inhibitory effect of rTGFβ on osteoblast differentiation, while preventing earlier progenitor cell proliferation.[15,16] The data also suggest SD-208 treatment alone can promote osteogenic differentiation of N-BMSCs and MM-BMSCs compared to vehicle without rTGFβ supplementation.

Discussion

Virtually all myeloma patients develop bone disease, which is painful, debilitating and increases fractures and mortality.[3,6-8] New bone-targeted therapies that heal bone and increase strength are needed to improve patient quality of life, especially given overall survival is increasing. We developed a novel low tumour xenograft model of myeloma to mimic the plateau phase by treating with first-line chemotherapeutics btz and len. SD-208 treatment during the low tumour phase induced rapid intramembranous healing of bone disease by modulating collagen maturation, PTHrP and Vegfa. We also identified for the first time improved fracture resistance following repair of myeloma bone disease in vivo. Furthermore, we discovered that SD-208 enhances formation of osteoblasts from MM-BMSCs, providing evidence of efficacy in patients.

SD-208 combined with chemotherapy induced rapid healing of myeloma bone disease in U266-GFP-luc-bearing mice. After only 2 weeks, all mice treated with SD-208 plus chemotherapy had substantial repair of bone lesions. Half of the lesions were completely repaired, and remaining lesions were reduced in size by nearly 60% with SD-208 treatment + chemotherapy, which was substantially more effective than chemotherapy alone. Similarly, SD-208 increased trabecular bone volume in long bones, supporting previous studies in naïve mice[17] and with 1D11 in myeloma-bearing (JJN3, U266 and 5TGM1) mice.[21,23] Chemotherapy-treated mice also exhibited lesion repair, reflecting recent observations in patients.[25,39-41] However, chemotherapy-driven repair was less effective than when combined with SD-208. Importantly, by combining SD-208 with chemotherapy we observed more effective repair within 2 weeks, even without anti-resorptive therapy and despite administration at a very late disease stage, differing from previous studies treating for longer periods or prior to tumour development.[21,23] Furthermore, we identified that chemotherapy

alleviated not only the tumour-derived pro-osteoclastic signals, but also eliminated expression of pro-osteoclastic PTHrP in osteocytes, which were previously an unidentified source of PTHrP in myeloma. Given TGFβ stimulates PTHrP expression[61] we would expect SD-208 to also inhibit PTHrP expression, but given the complete elimination of osteocytic PTHrP in mice receiving chemotherapy alone, any further benefit with SD-208 was not measurable. Thus, effective reduction of tumour can remove the osteoclastic drive from the microenvironment, suggesting conventional anti-resorptives may not always be necessary in vivo. The bone effects of SD-208 were not limited to the U266-GFP-luc model, as myeloma bone disease was also reduced with preventative treatment of JJN3-bearing mice and was more effective than 1D11 at preventing bone lesions. Thus, SD-208 effectively prevented myeloma bone disease and when administered with chemotherapy rapidly repaired bone lesions across multiple murine models, and was more effective than 1D11.[21,23]

Reports of osteolytic lesions healing are increasing, yet the mechanism of repair is unknown and it is unclear why only some patients exhibit repair. We identified that healing of myeloma bone disease occurred through deposition of 'trabecular-like' woven bone along the endocortical surface that bridged lesions, without evidence of a cartilage callus. This suggests lesion repair occurred via a process reminiscent of intramembranous fracture repair (as in drill-hole repair models[57]) rather than typical cortical remodeling or endochondral ossification. Supportive of this, we observed an increase in Vegfa, which plays a critical role in coupling of angiogenesis and osteogenesis during intramembranous repair.[63,64] In myeloma patients, healing of bone lesions has been observed in flat bones of the pelvis[25], which form via intramembranous ossification. As such, it is plausible that this could be the mechanism of repair in patients. Furthermore, it reinforces that the treatment for myeloma bone disease can be improved through pharmacological routes and that this has the potential

to make a substantial difference to patient morbidity and mortality, alleviating the reliance on anti-resorptive therapy, radiotherapy and invasive orthopedic approaches.

One area of concern in healing of myeloma bone disease relates to the quality and integrity of the new bone. In patients, μCT has provided evidence of mineralized tissue in place of previous lesions, which appears to resemble bone.[25] A novel finding from our study was that SD-208 does not affect mineralization during healing, but stimulates maturation of the collagen matrix. Changes in collagen cross-linking, without changes to mineral, in newly formed bone can determine mechanical integrity[66] and thus the increase in matrix maturity with SD-208 may contribute to the improved mechanical strength.

A major cause of morbidity in myeloma patients is pain and mobility loss resulting from fractures caused by myeloma bone disease. SD-208 treatment improved bone architecture and material properties in long bones, leading to enhanced femoral fracture resistance. Vertebral fracture resistance improved significantly in response to chemotherapy. Considering the effect on vertebral bone volume was moderate, and only significant in L3 vertebrae, it is likely that a reduction in cortical lesions may also be contributing to the improved fracture resistance. In comparison to long bones, no additional improvement in vertebrae was observed with SD-208, reflecting the less pronounced trabecular bone architectural changes. This contrasts to findings in naïve mice where SD-208 (60 mg/kg for 6 weeks) increased vertebral fracture resistance in males and increased bone formation in male (but not female) lumbar vertebrae.[17] Our studies used female mice, so it is possible that the effect of SD-208 in the vertebrae is less pronounced in females or requires a longer treatment duration. Furthermore, we observed an increase in ultimate force to failure with chemotherapy (34% in L3 and 45% in L4 vertebrae) after 2 weeks, whereas Mohammad et

al. observed a 27% increase after 6 weeks of SD-208 treatment[17], indicating that in the vertebrae the strong anabolic effect in response to chemotherapy (either attributed to direct effects of bortezomib or to the reduction in tumour load) masks any anabolic effect from SD-208. Site-specific effects similarly occur in response to PTH bone anabolic therapy in mice, with anabolic responses in lumbar vertebrae being slower (not observed until 7 weeks, compared to 3 weeks in long bones) and of smaller magnitude than in long bones.[67] Importantly, this is the first study to show improved fracture resistance with bone anabolic-driven repair (rather than prevention) of myeloma bone disease. Many patients present with myeloma bone disease at diagnosis[12], thus the ability to improve bone strength by repairing bone is vital and has the potential to vastly improve patient quality of life and reduce the need for more extreme surgical interventions.

The improved prevention of bone lesions with SD-208 over 1D11 in JJN3-bearing mice was somewhat surprising. However, this may explain why Nyman et al. did not observe improved fracture resistance when treating myeloma-bearing mice with 1D11±btz prior to lesion development[21], despite the fact that 1D11 improved fracture resistance in naïve mice.[19] TGFβ signals by binding to TGFβRII, which phosphorylates and activates TGFβRI and downstream SMAD signaling. TGFβ signaling is specific to TGFβRI[68], and would be targeted by both 1D11 and SD-208. The reason for the improved lesion prevention with SD-208 compared to 1D11, cannot conclusively be identified in our study, but may be due to the differences in administration (i.e. dose, route) or the pharmacokinetics and pharmacodynamics of each drug. For instance, there may be additional benefits to bone with SD-208 due to off-target effects, as SD-208 also has a >25% inhibition of other kinases including AKT1-3, protein kinase C (ε, η and θ isoforms), epidermal growth factor receptor, protein kinase D and mitogen-activated protein kinase-activated protein 2.[69,70]

Bone anabolics show great promise in healing myeloma bone disease. Prevention of lesion development has been observed with anti-TGFβ therapies (1D11[21,23] and now SD-208), an anti-sclerostin antibody[58,71], an anti-DKK1 antibody[72] and a soluble decoy receptor for activin A[73] in vivo. Repair of bone lesions was observed with 1D11 in combination with zoledronic acid.[23] Here we show more rapid repair with SD-208 combined with btz and len, without the use of conventional anti-resorptives, in a novel U266-GFP-luc low tumour phase model. Our findings identify the bone anabolic and anti-resorptive actions of SD-208 have the potential to treat pre-existing myeloma bone lesions as well as prevent further bone disease, significantly enhancing the bone effects of first-line chemotherapeutics. This model was developed for improved clinical representation, and optimized in NSG immunocompromised mice bearing U266-GFP-luc tumors. This was based on a number of factors, most importantly being that 1) the U266 cells induce osteolytic disease and 2) the mice exhibited a prolonged low tumor phase after chemotherapy. The lack of a fully functioning immune system in NSG mice is a potential limitation to the low tumour model, however our findings are supported by Nyman and colleagues' treatment of immunocompetent 5TGM1-bearing mice with 1D11.[21] Further, in accordance with previous studies[21,23], we did not observe any effect on the tumour with SD-208, supporting use as a bone anabolic in myeloma.

The potential for SD-208 to stimulate osteoblastic differentiation was confirmed in myeloma patient BMSCs, and comparable to the response in healthy donor BMSCs. Promisingly, SD-208 not only alleviated TGFβ-induced repression of osteoblastic differentiation, but also promoted osteogenesis of MM-BMSCs and N-BMSCs without rTGFβ supplementation. TGFβ inhibitors have been reported to overcome the impaired osteoblast differentiation

caused by TGFβ or myeloma conditioned medium in osteoblastic cell lines and primary murine BMSCs.[22,23] However, this is the first time that a TGFβ inhibitor has been shown to promote osteoblast differentiation of MM-BMSCs. Thus, the ability of SD-208 to promote osteoblast formation suggests bone anabolic efficacy in humans and substantiates our findings in murine models.

We have identified that the TGFβRI inhibitor, SD-208, enhances repair of myeloma bone disease when combined with first-line chemotherapeutics, btz and len. We found SD-208 healed bone through a process similar to intramembranous fracture repair, stimulating faster matrix maturation to rapidly improve bone architecture and increase femoral fracture resistance. We also identified that myeloma patient BMSCs are capable of forming osteoblasts, and that this is enhanced by SD-208. Inhibitors of TGFβ signaling, SD-208 and 1D11, have now been consistently shown to prevent and repair bone disease in immunocompetent syngeneic (5TGM1) and immunodeficient xenograft (JJN3, U266) murine myeloma models. TGFβ inhibition in combination with first-line chemotherapies has the potential to substantially benefit patient quality of life, survival and cost of treatment, by improving bone architecture and strength and hence reducing skeletal related events.

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Author contributions

ACG designed all research studies, conducted or directed all experiments, acquired, processed, analyzed and interpreted all data, wrote and revised the manuscript and takes responsibility for the integrity of the data. DL, KH, BW, RO, JMD, JPH and MAL assisted with some experimental work. HRE and BW assisted with some data processing. GCR provided access to equipment for mechanical tests. MAL and ADC conceived the study and assisted with manuscript revision. All authors approved the final manuscript.

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Figure 1: TGFβ inhibition with SD-208 or 1D11 prevents osteolytic lesion development and loss of trabecular bone. (A) Schematic showing in vivo treatment schedule. NSG mice were inoculated with 10^6 JJN3 cells or PBS (naïve). After 1 week, mice were treated with 60 mg/kg SD-208, 20 mg/kg 1D11 or controls 5 days/week for 2 weeks. Mice were then sacrificed for analysis of bone disease by ex vivo µCT and histomorphometry. MicroCT analysis of tibial (B) trabecular bone volume/tissue volume (BV/TV), (C) trabecular number (Tb.N), (D) trabecular thickness (Tb.Th), (E) osteolytic lesion area, (F) average lesion size and (G) lesion number. Representative reconstructed images are shown for (H) naïve, (I) JJN3 control-treated, (J) SD-208 and (K) 1D11 mice. Histomorphometry was performed on TRAP-stained, decalcified paraffin-embedded tibiae. Histomorphometric analysis of (L) osteoclast number/endocortical surface (N.Oc/Ec.S), (M) osteoclast surface/endocortical surface (Oc.S/Ec.S), (N) osteoblast number/endocortical surface (N.Ob/Ec.S) and (O) osteoblast surface/endocortical surface (Ob.S/Ec.S). Data are mean±SD (n=8 mice). # p=0.05, ## p=0.01, ### p=0.001, #### p=0.0001 (unpaired two-tailed Student's t test between naïve and JJN3 controls) and *p=0.05, **p=0.01, ***p=0.001 and ****p=0.0001 (one-way ANOVA with Tukey's multiple comparisons test between JJN3 control, SD-208 and 1D11 mice).

Figure 2: Bortezomib and lenalidomide combined chemotherapy induces a low tumour phase when administered at late stage disease. (A) Schematic showing in vivo treatment schedule. NSG mice were inoculated with 10^6 U266-GFP-luc cells. After 8 weeks (when bone lesions were present), treatment was commenced (week 0). Vehicle mice were sacrificed after 1 week (*1, n=7 mice) due to effects of tumour, btz + len mice were sacrificed after 2 weeks (*2, n=7 mice) and after relapse (*3, n=5 mice) and btz+len+SD-208 mice after 2 weeks (*4, n=7 mice). Bioluminescent imaging was performed weekly and in

vivo µCT was performed weekly from week 7. Bioluminescent quantification of tumour in the (B) hindlimbs, (C) vertebrae and (D) calvaria and the representative images show tumour burden in vehicle and btz+len treated mice imaged (E) anteriorly and (F) posteriorly. (G) Paraprotein (human IgE) was measured in mouse sera by ELISA. (H) The proportion of GFP+ tumour cells was assessed in femur-flushed bone marrow by flow cytometry. Data are mean±SD. ****p=0.0001 (unpaired two-tailed Student's t test).

Figure 3: SD-208 enhances lesion repair in combination with chemotherapy. Representative 3D reconstructed µCT images of tibia in the same mouse showing (A) osteolytic cortical bone lesions from week 0, 1 and 2 post-treatment (arrows indicate lesions that are worse (red), the same (yellow), show some repair (dark blue) or complete repair (blue) compared to week 0) and (B) transverse cross sections with density gradient coloring showing lesion repair and cortical thickening (white asterisk) in btz+len±SD-208 treated mice. Osteolytica was used to quantify (C) total lesion area, (D) average lesion size and (E) lesion number of the proximal tibia as % change from week 0. Data are mean±SD (n=7 mice). *p=0.05 (week 1; one-way ANOVA with Tukey's multiple comparisons test and week 2; unpaired two-tailed Student's t test).

Figure 4: SD-208 increases trabecular bone volume in combination with chemotherapy. Trabecular bone architecture was assessed by in vivo µCT of (A-D) one tibia (week 0, 1 and 2 post-treatment) and ex vivo µCT of the (E-I) contralateral tibia and (J-M) femur at endpoint. MicroCT analysis of tibial and femoral (A, E, J) trabecular bone volume/tissue volume (BV/TV), (B, F, J) trabecular number (Tb.N), (C, G, L) trabecular thickness (Tb.Th) and (D, I, M) trabecular separation. Representative reconstructed images of trabecular bone are

shown for ex vivo scanned tibiae. Data are mean±SD (n=7 mice). *p=0.05, **p=0.01 and ***p=0.001 (one-way ANOVA with Tukey's multiple comparisons test).

Figure 5: Chemotherapy reduces osteoclasts and bone resorption, increases osteoblasts and alters microenvironment expression of Vegfa and PTHrP. Histomorphometry of endocortical surfaces was performed on TRAP-stained, decalcified paraffin-embedded tibiae. Representative images of (A) vehicle, (B) btz+len and (C) btz+len+SD-208 treated mice show osteoclasts (red arrowheads), osteoblasts (blue arrowheads), bone marrow (BM) tumour (T)-BM) and the formation of woven bone (WB) on the endocortical surface of cortical bone (CB). Scale bars are 400 µm (proximal tibia) and 100 µm (inset). Histomorphometric analysis of (D) osteoclast number/endocortical surface (N.Oc/Ec.S), (E) osteoclast surface/endocortical surface (Oc.S/Ec.S), (F) osteoblast number/endocortical surface (N.Ob/Ec.S) and (G) osteoblast surface/endocortical surface (Ob.S/Ec.S). (H) TRACP5B and (I) P1NP levels were measured in mouse sera by ELISA. (J) mRNA expression of tumour-derived factors that contribute to myeloma bone disease were measured in the bone marrow by qPCR using human-specific primers. Slides were scanned at 40X on a Pannoramic 250 (3DHistech). Data are mean±SD (n=7 mice). *p=0.05, **p=0.01, ***p=0.001 and ****p=0.0001 (one-way ANOVA with Tukey's multiple comparisons test).

Figure 6: Chemotherapy reduces osteocytic PTHrP and VEGFA is expressed during bone healing. mRNA expression of microenvironment-derived (A) Vegfa and (B) Pthlh were measured in the bone marrow by qPCR using mouse-specific primers. PTHrP and VEGFA protein expression was identified by immunohistochemistry of decalcified paraffin-embedded tibiae. (C) The proportion of PTHrP+ osteocytes was determined for cortical bone. (D-F) Representative images of sections stained for PTHrP for (D) vehicle, (E) btz+len and (F)

btz+len+SD-208 treated mice show PTHrP positive osteocytes (black arrowheads) and PTHrP negative osteocytes (white arrowheads), scale bar is 20 µm. (G-E) Representative images of sections stained for VEGFA for (G) vehicle, (H) btz+len and (I) btz+len+SD-208 treated mice show VEGFA expression in woven bone (WB), the periosteum (Ps) and in megakaryocytes (grey arrowheads), scale bars are 400 µm, 200 µm (inset) and 100 µm (dotted line inset). Slides were scanned at 40X on a Pannoramic 250 (3DHistech). Data are mean±SD (n=7 mice, for Pthlh n=4-7 due to insufficient mRNA for some mice). *p=0.05, ***p=0.001 and ****p=0.0001 (one-way ANOVA with Tukey's multiple comparisons test).

Figure 7: SD-208 enhances matrix maturation and improves femoral fracture resistance. Three-point bending was performed on the femur to determine the (A) ultimate force to failure and (B) stiffness. Compression testing was performed on (C) L3 and (D) L4 vertebrae to determine the ultimate force. (E) Raman spectroscopy was performed in 3 regions of the cortical bone (yellow dots) referred to as endocortical (Ec), middle (Mid) and periosteal (Ps) in mice treated with vehicle, btz+len or btz+len+SD-208. (F) Example Raman spectrum showing the primary phosphate ($\boldsymbol{\nu}_1PO_4$), carbonate (CO_3) and amide I peaks. (G) Example sub-peak analysis of the amide I peak. Raman peak analysis of the (H) mineral to matrix ratio ($\boldsymbol{\nu}_1PO_4$:amide I), (I) carbonate to phosphate ratio (CO_3:$\boldsymbol{\nu}_1PO_4$) and (J) matrix maturity ratio from amide I subpeak analysis (1668 cm^{-1}:1692 cm^{-1}). Data are mean±SD (-Raman n=3 mice, mechanical loading n=4-7 mice). *p=0.05, **p=0.01, ***p=0.001 and ****p=0.0001 (one-way ANOVA with Tukey's multiple comparisons test).

Figure 8: SD-208 promotes formation of alkaline phosphatase positive osteoblasts from normal and myeloma patient BMSCs. Adherent $CD138^-$ BMSCs from normal donor (N-BMSCs) and myeloma patient (MM-BMSCs) were cultured in osteogenic media containing

vehicles or recombinant TGFβ (rTGFβ) and/or SD-208. (A-B) After 3, 7 and 10 days cells were alkaline phosphatase (ALP) assays were performed on (A) N-BMSCs and (B) MM-BMSCs. After 7 and 14 days qPCR of osteoblastic genes RUNX2, SP7, ALPL, PTH1R and BGLAP was performed on (C-G) N-BMSCs and (H-L) MM-BMSCs. ND = not detected. Data are mean±SD (n=3 independent patients) *p=0.05, **p=0.01, ***p=0.001 and ****p=0.0001 (two-way ANOVA with Tukey's multiple comparisons test).