Improving bone health via modulation of glycosphingolipid metabolism and autophagy

Houfu Leng

University of Oxford

Hanlin Zhang

University of Oxford

Linsen Li

Shanghai Jiao Tong University School of Medicine

Shuhao Zhang

University of Oxford <https://orcid.org/0000-0002-0301-5983>

Yanping Wang

Soochow University

Selina Chavda

University College London

Daria Galas-Filipowicz

UCL Cancer Institute

Adel Ersek

University of East Anglia

Emma Morris

The Botnar Research Centre, University of Oxford <https://orcid.org/0000-0003-1230-2586>

Erdinc Sezgin

MRC Weatherall Institute of Molecular Medicine

Yi-Hsuan Lee

University of Oxford

Yunsen Li

Soochow University

Jian-Qing Mi

State Key Laboratory of Medical Genomics, Shanghai Institute of Hematology, Rui Jin Hospital Affiliated to Shanghai Jiao Tong University (SJTU) School of Medicine, 197 Rui Jin Road II, Shanghai

Kwee Yong

University College London

Qing Zhong

Key Laboratory of Cell Differentiation and Apoptosis of Chinese Ministry of Education, Department of Pathophysiology, Shanghai Jiao Tong University School of Medicine

Claire Edwards

University of Oxford <https://orcid.org/0000-0002-1257-5659>

Anna Katharina Simon (✉ katja.simon@imm.ox.ac.uk)

University of Oxford

Nicole Horwood

University of East Anglia <https://orcid.org/0000-0002-6344-1677>

Article

Keywords: multiple myeloma, TRAF3, osteoclastogenesis

Posted Date: August 4th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-728824/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Improving bone health via modulation of glycosphingolipid metabolism and autophagy

Houfu Leng¹, Hanlin Zhang¹, Linsen Li², Shuhao Zhang^{1,3}, Yanping Wang⁴, Selina J Chavda⁵,
Daria Galas-Filipowicz⁵, Adel Ersek^{1,6}, Emma Morris⁷, Erdinc Sezgin^{8,9}, Yi-Hsuan Lee^{1,6},
Yunsen Li⁴, Jianqing Mi¹⁰, Kwee Yong⁵, Qing Zhong², Claire M Edwards^{7,11}, Anna Katharina
Simon^{1,*‡}, Nicole J. Horwood^{1,6,*‡}.

¹ Kennedy Institute of Rheumatology, University of Oxford, Roosevelt Drive, Oxford, UK,
OX3 7FY.

² Key Laboratory of Cell Differentiation and Apoptosis of Chinese Ministry of Education,
Department of Pathophysiology, Shanghai Jiao Tong University School of Medicine,
Shanghai, P.R. China.

³ Computational Biology Department, Carnegie Mellon University, Pittsburgh, Pennsylvania
15217, USA.

⁴ Institutes of Biology and Medical Sciences, Soochow University, Suzhou, P.R. China.

⁵Department of Hematology, UCL Cancer Institute, University College London, London, UK.

⁶Norwich Medical School, University of East Anglia, James Watson Road, Norwich, UK, NR4
7UQ.

⁷ Nuffield Dept. Of Surgical Sciences, Botnar Research Centre, University of Oxford, Old
Road, Oxford, UK, OX3 7LD.

⁸ Science for Life Laboratory, Department of Women's and Children's Health, Karolinska
Institute, Solna, Sweden.

⁹ MRC Weatherall Institute of Molecular Medicine, MRC Human Immunology Unit, OX3
9DS, Oxford, UK.

¹⁰ Shanghai Institute of Hematology, State Key Laboratory of Medical Genomics, National

Research Center for Translational Medicine at Shanghai, Ruijin Hospital Affiliated to Shanghai
Jiao Tong University School of Medicine, Shanghai, P.R. China.

¹¹ Nuffield Department of Orthopaedics, Rheumatology, and Musculoskeletal Sciences, Botnar
Research Centre, University of Oxford, Old Road, Oxford, UK, OX3 7LD.

* To whom correspondence should be addressed:

Prof. Nicole Horwood, n.horwood@uea.ac.uk

Prof. Katja Simon, katja.simon@imm.ox.ac.uk

‡ Senior authors.

Abstract Word Count: 149

Main Text Word Count: 3284

Methods: 998

Main Figure/Table Count: 7/0

Reference Count: 54

38 **Abstract** (149 words)

39 Patients with multiple myeloma (MM), an incurable malignancy of plasma cells, frequently
40 develop osteolytic bone lesions. In this study, glycosphingolipids were essential in promoting
41 autophagic degradation of the signaling molecule TRAF3, a key step in bone-resorbing
42 osteoclast differentiation. Specifically altering the glycosphingolipid composition with
43 eliglustat, an FDA approved glucosylceramide synthase inhibitor, arrested osteoclast
44 differentiation; this could be rescued by exogenous addition of the missing glycosphingolipids.
45 Eliglustat significantly reduced bone disease in several preclinical models of MM by inhibiting
46 osteoclastogenesis and, due to its unique mode of action, it was able to act in combination with
47 existing bone protective drugs. Furthermore, eliglustat arrested osteoclast differentiation from
48 the bone marrow of MM patients in a glycosphingolipid-dependent way. This work identifies
49 both the mechanism by which glucosylceramide synthase inhibition blocks autophagic
50 degradation of TRAF3 to reduce osteoclastogenesis as well as highlighting the translational
51 potential of eliglustat to be combined with current treatments.

Introduction

Multiple Myeloma (MM) is a haematological cancer caused by abnormal plasma cell expansion in the bone marrow (BM)¹. MM is preceded by the monoclonal gammopathy of undetermined significance (MGUS); characterized by an abnormal increase in monoclonal immunoglobulin secretion^{2,3}. Progression of disease from MGUS into active MM is accompanied by the development of osteolytic bone disease⁴, affecting 85% of MM patients⁵.

MM cells secrete factors, including receptor activator of nuclear factor kappa-B ligand (RANKL), that enhance osteoclast (OC) formation⁶. Engagement of RANKL, the OC differentiation factor, with its cognate receptor RANK leads to the activation of the NF- κ B signaling pathway and is required for OC formation. The NF- κ B pathway is regulated by tumour necrosis factor receptor-associated factors (TRAFs). In particular TRAF6 mediates RANKL-induced osteoclastogenesis⁷ whilst TRAF3 is inhibitory⁸. The bisphosphonate zoledronic acid (ZA) is commonly used to treat MM bone disease. However, therapeutic doses of ZA required to treat MM patients may result osteonecrosis of the jaw (ONJ)⁹ and can lead to suppression of bone turnover and increased fracture risk¹⁰. Novel ways to alleviate both pain and disability caused by these bone lesions without causing severe side effects represent an ongoing unmet clinical need.

Autophagy maintains eukaryotic cell homeostasis by degrading and recycling cellular waste including protein aggregates and damaged organelles¹¹. During OC differentiation Beclin-1 is upregulated¹² and Atg7 knockdown inhibits the expression of key OC proteins such as tartrate-resistant acid phosphatase (TRAP) and cathepsin K¹³. Additionally, autophagy-related proteins including ATG5, ATG7, ATG4B, and LC3 regulate a number of OC functions¹⁴. Concordantly, autophagy inhibitors such as chloroquine (CQ) can inhibit OC formation and restore bone mass⁸. Molecularly, TRAF3, as a suppressor of OC differentiation, is degraded by autophagy

76 upon RANKL induction in BM OC precursors⁸. Based on these findings, it has been proposed
77 that autophagy inhibitors may be beneficial in treating bone loss diseases in the clinic.

78 Glycosphingolipids (GSLs) are constituents of the cellular membrane that are expressed in
79 varying ratios and combinations. We previously observed that GM3, a ganglioside abnormally
80 expressed in MM cells, promotes osteoclastogenesis¹⁵ and that this can be blocked using
81 miglustat, an iminosugar and an analogue of D-glucose¹⁶. However, miglustat is involved in
82 various biological and pathological pathways and can cause deleterious side effects in the
83 gastrointestinal track including severe diarrhea and weight loss¹⁷. Eliglustat, a small synthetic
84 molecule, specifically prevents the synthesis of all GSLs by inhibition of glucosylceramide
85 synthase (GCS)¹⁸ and is used for the treatment of Gaucher disease type 1 in adults¹⁹. Gaucher
86 patients suffer from osteoporosis/osteopenia and treatment to reduce excess bioactive GSL
87 associated with the disease has been shown to alleviate bone loss²⁰. Interestingly, patients with
88 Gaucher disease have a 6–50 times increased risk of developing MM or the pre-MM MGUS
89 condition^{21,22,23}. However, it remains to be determined whether the reduction in bone symptoms
90 is due to the treatment of Gaucher disease or if eliglustat acts directly on bone cells, which
91 raised the possibility that eliglustat may be of benefit in treating MM bone complications.

92 Here we demonstrate that eliglustat reduces bone disease by directly acting on bone cells,
93 namely on the OCs, in pre-clinical MM and MM-like MGUS models, and can be used in
94 combination with ZA for superior outcomes. Mechanistically, eliglustat acts as an autophagy
95 inhibitor that prevents TRAF3 degradation in OC and modulates autophagy by altering
96 glucosylceramide (GlcCer) and lactosylceramide (LacCer) composition in OC. Lastly,
97 eliglustat effectively inhibits OCs from MM patient samples and replacement of the GSLs can
98 rescue OC formation.

Results

Eliglustat decreases MM bone disease in vivo

To investigate the effects of eliglustat in MM bone disease model, C57BL/KaLwRijHsd mice were injected with saline or 5TGM1-GFP murine MM cells on day 0. Eliglustat-containing chow was given from day 4 until sacrifice on day 23. Micro-CT reconstruction of tibiae indicated a decrease in trabecular bone in MM-bearing mice compared to saline group, whilst the eliglustat group was protected from MM-induced bone loss (Figure 1A). Treatment with eliglustat in MM-bearing mice significantly increased bone volume (BV/TV), bone surface (BS), trabecular number (Tb.N), connective density (Conn.D) and trabecular thickness (Tb.Th), accompanied by a decrease in bone surface density (BS/BV) and in trabecular separation (Tb.Sp) (Figure 1B), confirming that eliglustat inhibits bone catabolic effects. Importantly, treatment with eliglustat significantly reduced the number of cortical bone lesions in MM-bearing mice (Figure 1C-D).

Histomorphometric analysis demonstrated a significant increase in TRAP-positive OCs on the endosteal surface in MM-bearing mice compared to control mice. Treatment of MM mice with eliglustat exhibited a robust response as shown by decreased OC parameters including surface, number and perimeter (Figure 1E-F). Increased bone mass parameters were similarly observed in healthy C57BL/6J mice when fed on the eliglustat-containing diet (Figure S1A-B). Histomorphometry of tibiae from eliglustat-treated mice showed significantly lower measurements for OC parameters when compared with naïve control mice (Figure S1C-D). No difference was observed between untreated control mice and eliglustat-treated mice for OB parameters (Figure S1C and E), indicating that increased trabecular bone was a consequence of OC inhibition rather than an increase in OB.

Eliglustat treatment did not change the population of OC precursors as demonstrated by expression of surface markers (CD11b-CD3-B220-CD115+)²⁴ in MM-bearing mice (Figure S2A-C) suggesting that eliglustat blocks OC formation rather than inhibiting the generation of OC progenitors. Flow cytometry analysis of BM and spleen showed that there was no difference in GFP+ 5TGM1 cells found in BM or spleen between MM-bearing mice and mice treated with eliglustat (Figure S2D-F). Consistently, serum paraprotein IgG2b κ showed that eliglustat did not reduce systemic tumor burden (Figure S2G). Therefore, eliglustat ameliorates MM-induced bone disease by specifically inhibiting OC without exacerbating tumor burden.

Figure 1

A

B

C

D

E

F

Figure 1. Eliglustat ameliorates 5TGM1-GFP MM cell-induced bone disease. 5TGM1-GFP MM cells were injected to 8-week-old female C57BL/KaLwRij mice to generate the MM model. Eliglustat chow was administered from day 4 post tumor injection (until day 23). **(A)** Representative micro-CT reconstruction images of tibiae trabecular bones from naive control (Ctr, $n=5$), MM mice with normal chow (MM, $n=7$), or MM mice with eliglustat chow (MM+Elig, $n=7$). **(B)** Tibiae trabecular bone parameters were assessed: BV/TV, BS, Tb.N, Conn.D, Tb.Th, BS/BV and Tb.Sp. **(C/D)** Tibiae cortical bone reconstruction **(C)** and the number of cortical bone lesions **(D)**. **(E/F)** Representative TRAP/methyl green staining showing red OCs of tibial histological sections, original magnification $40\times$ in **(E)** and the quantification of OCs with Oc.S/BS (OC surface over bone surface), N.Oc/T.Ar (number of OC over total area) and N.Oc/B.Pm (number of OC over bone perimeter) in **(F)**. Error bars correspond to SEM. $*P<0.05$, $**P<0.01$, $***P<0.001$, $****P<0.0001$. Statistical analysis was performed using One-way ANOVA.

Eliglustat prevents bone loss in a diet-induced obesity model of MGUS

MGUS is a common occurrence in the elderly and carries a risk of progression to MM at approximately 1% per year. MGUS patients do not present with lytic bone lesions or hypercalcemia, but they have a greater risk of developing osteoporosis and increased fracture incidence²⁵. To expand the application of eliglustat, a well-established high fat diet (HFD)-induced murine MGUS model in C57BL/6J was used to evaluate the impact on bone loss².

Prior to 5TGM1-GFP cell injection, C57BL/6J mice were fed with 42% HFD or control diet for 7 weeks, resulting in a significant increase in body weight in HFD group (Figure 2A-B). As previously reported, C57BL/6J mice on a control diet inoculated with 5TGM1 cells showed no signs of tumor growth (Figure S3A) or evidence of bone loss². 7 weeks of HFD alone did not change the bone parameters (Figure S3B-C). Micro-CT reconstruction showed decreased tibial trabecular bone mass in HFD mice injected with MM cells (MGUS condition) compared to the mice placed on normal diet with MM cells (Figure 2C). As predicted, eliglustat reversed bone loss with a significant increase in BV/TV, BS, Tb.N and trabecular BMD, together with a decreased BS/BV ratio (Figure 2D).

Bone marrow adipose tissue (BMAT) supports OC formation²⁶. To determine whether eliglustat, a potential lipid inhibitor, impacts OC formation by suppressing HFD-induced BMAT, tibiae were stained with osmium tetroxide. Quantitative analysis of regulated BMAT (rBMAT, red area within the red rectangle) revealed a significant increase of rBMAT in HFD+MM (MGUS) compared to the MM group (control); eliglustat treatment did not alter rBMAT volume indicating that the effects of eliglustat were not due to changes in adiposity in the BM (Figure 2E). OC parameters were increased in HFD+MM (MGUS) group as compared to MM group (control) and treatment with eliglustat significantly reversed the pattern (Figure 2F-G) without affecting OB parameters or cortical bone parameters (Figure S3D-E).

Figure 2

A

B

C

D

E

F

G

Figure 2. Eliglustat reduces bone loss in a diet-induced obesity MGUS model. (A) Schematic overview and experiment design. Female C57BL6 mice were divided into MM injection with normal diet (MM, $n=4$), MM with HFD (MM+HFD, $n=10$) and MM with HFD plus eliglustat (MM+HFD+Elig, $n=10$). (B) Body weight (g) was measured over the duration of the experiment. (C) Representative micro-CT reconstruction images of tibiae trabecular bones from respective groups. (D) Micro-CT analysis parameters of tibiae: BV/TV, BS, Tb.N, Conn.D, Tb.Th, BS/BV, Tb.Sp and trabecular bone mineral density (BMD). (E) Osmium tetroxide staining to detect regulated BMAT (in red box) by micro-CT in MM, HFD+MM, and HFD+MM+Elig groups. (F) Representative TRAP/methyl green stained tibial histological sections showing red OCs. (G) Bone histomorphometry parameters including Oc.S/BS, N.Oc/T.Ar and N.Oc/B.Pm. Error bars correspond to SEM. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$. ns means non-significant. Statistical analysis was performed using One-way ANOVA.

Eliglustat combined with ZA has a superior effect compared to ZA alone

ZA leads to OC apoptosis via blockade of the mevalonate pathway²⁷, whilst our data show that eliglustat inhibits OC via an as yet unknown mechanism. The efficacy of combining these two OC inhibitors was evaluated to ascertain any potential additive effect that would also allow ZA to be used at lower concentrations to mitigate the potential to develop ONJ.

C57BL/KaLwRijHsd mice were divided into 5 groups: untreated control mice (Ctr); 5TGM1-GFP MM-bearing mice (MM); 5TGM1-GFP MM-bearing mice treated with ZA (MM+ZA); MM-bearing mice treated with eliglustat (MM+Elig); or MM-bearing mice treated with both ZA and eliglustat (MM+ZA+Elig) (Figure 3A). Treatment with the combination of eliglustat and ZA resulted in a 2-fold increase in BV/TV compared to MM-bearing mice and a 1.5-fold increase when compared with mice treated with eliglustat alone or ZA alone. Importantly, the combination strategy increased BS, BS/BV and Tb.N when compared with ZA given alone, whilst Tb.Sp was significantly decreased (Figure 3B-C). Consistently, the combination strategy significantly decreased the number of osteolytic lesions (holes) compared to single use of eliglustat or ZA (Figure 3D-E). Histomorphometric analysis revealed that the combination strategy was superior to single use of each compound in inhibiting OC surface (Figure 3F-G). Neither eliglustat nor ZA demonstrated a significant effect on OB parameters (Figure S4A). Thus, the combination of eliglustat and ZA may provide a better clinical strategy than ZA alone for MM patients.

Overall, treatment with eliglustat inhibited osteoclastogenesis in healthy mice, MM susceptible mice and the HFD-induced MGUS murine model. Additionally, eliglustat combined with ZA produced an even greater bone-sparing effect. To elucidate how eliglustat inhibits OC, further investigation of the molecular mechanisms was undertaken.

Figure 3

A

B

C

D

E

F

G

Figure 3. Eliglustat combined with ZA reduces MM bone disease with greater effect than either agent alone. (A) Cartoon illustrating the time line and experimental design in investigating how eliglustat and ZA impact on bone measurements in 5TGM1-GFP MM bearing male mice. (B) Representative images of micro-CT reconstruction of proximal tibiae from each group, including naive control (Ctr, $n=6$), MM mice (MM, $n=7$), MM mice with eliglustat chow (for 19 days) (MM+Elig, $n=7$), MM mice with single dose ZA injection (0.01 mg/kg, MM+ZA, $n=7$), or MM mice with single dose ZA injection (0.01 mg/kg) and eliglustat chow (for 19 days) (MM+ZA+Elig, $n=7$). (C) Dot plots of BV/TV, BS, BS/BV, Tb.N, Conn.D and Tb.Sp from each group. (D) Representative reconstruction images of proximal cortical bone from each group. (E) Bone lesions (holes) on the cortical bones from each mouse were counted. (F) Representative TRAP/0.2% methyl green stained tibial sections showing red OCs on the endocortical bone surface from each group. (G) Bone histomorphometry parameters including Oc.S/BS, N.Oc/T.Ar and N.Oc/B.Pm. Error bars correspond to SEM. $*P<0.05$, $**P<0.01$, $***P<0.001$, $****P<0.0001$. ns means non-significant. Statistical analysis was performed using One-way ANOVA.

OC inhibition with eliglustat depends on TRAF3

RAW264.7 cells differentiated with M-CSF and RANKL *in vitro* were used as osteoclast precursors in mechanistic experiments due to the ability to obtain high numbers of OC without other contaminating cell types in the BM. Using a dose range below or equivalent to that used clinically (50 μ M)²³, eliglustat inhibited TRAP-positive OC formation in a dose-dependent manner (Figure 4A), as quantified by OC number, nuclei number per OC and OC area (Figure 4B). In contrast, OC precursor viability was not affected (Figure 4B). Similarly, eliglustat inhibited OC formation from murine BM cells (Figure S5A).

RANKL triggers OC formation via the canonical and non-canonical NF- κ B pathways (Figure 4C), both essential for OC formation. In the canonical NF- κ B pathway, activation of the adaptor protein TRAF6 leads to proteasome-mediated I κ B α degradation²⁸ followed by nuclear translocation of p65 and p50; in the non-canonical NF- κ B pathway, the adaptor protein TRAF3 is degraded in an autophagosome/lysosome-dependent mechanism⁸, that induces nuclear translocation of p52 and RelB²⁹. To test whether these pathways were affected by eliglustat, TRAF6 and TRAF3 levels were measured. Both eliglustat and the autophagic flux inhibitor BafilomycinA1 (BafA1) led to the accumulation of TRAF3 protein in RANKL-stimulated RAW264.7 cells and in primary murine BM cell-derived OCs, whereas *traf3* mRNA was not affected (Figure 4D-E and S5B). This indicates that eliglustat blocks TRAF3 lysosomal degradation elicited by RANK activation. As expected, RANKL up-regulated TRAF6 in murine primary BM cells (Figure S5C)³⁰. However, this was not altered by eliglustat treatment (Figure 4F). Moreover, TRAF6-regulated I κ B α protein levels were not affected by eliglustat (Figure 4G). These observations indicate that eliglustat does not regulate TRAF6-mediated canonical NF- κ B pathway.

244 To confirm the role of TRAF3 in mediating eliglustat's OC-inhibitory effects *in vivo*, BM
245 chimeric mice were generated using BM donor cells from LysM-Cre+, TRAF3^{fl/fl} mice that
246 harbor a myeloid specific deletion of *traf3* (Figure S5D-E)⁸. Chimeric mice were fed eliglustat-
247 containing diet for 19 days prior to micro-CT analysis. As previously demonstrated (Figure
248 S1), eliglustat administration led to an increase of BV/TV% in mice that received wild-type
249 BM donor cells, however this was no longer apparent in chimeric mice that received BM from
250 TRAF3-deficient mice (Figure 4H and S5F), further confirming that eliglustat suppresses OC
251 formation in a TRAF3-dependent manner.

Figure 4

A

B

C

D

E

F

G

H

Figure 4. Eliglustat inhibits OC formation in a TRAF3-dependent manner. (A) RAW264.7 cells were differentiated into OCs with 50 ng/ml M-CSF and 75 ng/ml RANKL. Different doses of eliglustat (0.1, 1, 10, 25, 50 μ M) were present throughout the culture period and OCs were identified by TRAP staining on day 5. Scale bar represents 200 μ m. (B) Number of OCs per well, number of nucleus per TRAP positive cell, % OC area over total area and Alamar blue viability assay were quantified. ($n=3$) (C) Schematic graph illustrating that the binding of RANKL to its receptor activates the TRAF6-dependent canonical NF κ B pathway, which leads to proteasome-mediated I κ B α degradation and then nuclear translocation of p65 and p50 whilst in the non-canonical NF κ B pathway, binding downregulates TRAF3 via autophagy/lysosome-mediated degradation, which induces nuclear translocation of p52 and RelB. (D/E) RAW264.7 cells were treated with M-CSF, RANKL, and eliglustat for 1 day. TRAF3 protein levels were quantified by Western blot and mRNA levels were quantified by qRT-PCR. Treatment with BafA1 was for the last 2 hours ($n=6$). (F/G) Primary murine BM cells were treated with RANKL together with BafA1 or MG132 or eliglustat for 2 hours. TRAF6 (F) and I κ B α (G) levels were quantified by Western blot ($n=8$). (H) Lethally irradiated recipient mice were reconstituted with littermate (WT) or myeloid specific TRAF3 knockout (LysM-Cre+, Traf3^{fl/fl}) BM cells. After 19 days of treatment with eliglustat, bone volume of tibiae was quantified by micro-CT ($n=4-5$ per group). Data represented as mean $\pm$ SEM. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$, ns, non-significant. Statistical analysis was performed using One-way ANOVA.

Eliglustat is a novel autophagy inhibitor that prevents autolysosomal degradation

The degradation of TRAF3 in RANKL-induced OC formation is mediated by autophagy machinery⁸. Therefore, whether eliglustat acts as an autophagic inhibitor was investigated. Upon eliglustat treatment, increased accumulation of the autophagosomal and autolysosomal marker LC3-II was observed in RAW264.7 cells; similar to treatment using the autophagic flux inhibitor BafA1. BafA1 did not increase LC3-II levels in eliglustat-treated RAW264.7 cells any further suggesting that the accumulated LC3-II in eliglustat-treated cells was due to autophagic flux inhibition (Figure 5A). p62 is an autophagic receptor that attracts cargo into the autophagosomal lumen. Like LC3-II, it is degraded when the autophagosomal content is delivered to the lysosome. In this study, p62 degradation was inhibited by eliglustat as well, indicating that eliglustat acts as an autophagy inhibitor, most likely at the lysosomal step of the autophagic flux (Figure 5A).

Consistently, eliglustat treatment led to significant accumulation of GFP-LC3 puncta in transfected cell system (Figure 5B)³¹. Moreover, a tandem-tagged RFP-GFP-LC3 reporter system was used to determine whether the accumulated LC3 puncta are on autophagosomes or autolysosomes. In this system, GFP but not RFP is quenched upon autophagic delivery to the acidic lysosome³¹. Eliglustat treatment led to increased yellow puncta and reduced red puncta (Figure 5C), indicating that the accumulation of autophagosomes (or dysfunctional autolysosomes) was associated with reduced lysosomal degradation. Furthermore, transmission electronic microscopy revealed that eliglustat accumulated single-membrane autolysosomes rather than double-membrane autophagosomes (Figure 5D), similar to the effect of CQ and BafA1 treatment. Eliglustat also significantly increased the area of the lysosomal marker LAMP2, suggesting an increase of lysosomes and/or autolysosomes (Figure 5E). Taken

296 together, these data support a role for eliglustat as an autophagy inhibitor that blocks
297 autophagic flux by inhibiting autolysosomal degradation.

A

Figure 5. Eliglustat is an autophagy inhibitor. (A) RAW264.7 cells were treated with RANKL and M-CSF either with or without eliglustat for 24 hours. 10nM BafA1 was added 2 hours before protein harvest. LC3II and p62 levels were quantified by western blot. (B) BafA1, CQ and eliglustat increased LC3 puncta in U2OS cell transfected with GFP-LC3 plasmid after 2 hours treatment. (C) Eliglustat increased GFP/RFP ratio and GFP+RFP+ puncta number in RFP-GFP-LC3 transfected U2OS cell after 6 hours treatment. (D) Electron microscope images indicated eliglustat induced autolysosome (red arrow) formation in RAW264.7 cells after 2 hours treatment (25,000 $\times$). (E) Quantification of the LAMP2 puncta area per U2OS cell (arbitrary units). CQ and BafA1 served as positive control in above experiments. $n=3-7$. Error bars correspond to SEM. $*P<0.05$, $**P<0.01$, $***P<0.001$, $****P<0.0001$, ns means non-significant. Statistical analysis was performed using One-way ANOVA.

Eliglustat's effect on autophagy is mediated by the inhibition of GSL synthesis

Autophagy is critical to osteoclastogenesis and gangliosides are involved in autophagosomal biogenesis^{32, 33}. It was hypothesized that eliglustat prevents the conversion of ceramide to certain GSLs that are required for autophagy.

To evaluate the overall lipid changes on the cellular membranes, the lipid organization of eliglustat-treated RAW264.7 cells was evaluated by spectral imaging (Figure 6A). The generalized polarization (GP) value reveals how packed the membrane lipids are³⁴. Eliglustat significantly decreased the GP value (Figure 6B), indicating a higher fluidity of the plasma membrane. It is possible that overall fluidity including that of endomembranes, such as autophagosome or lysosome membranes, may be affected in a similar way. However, due to technical limitations, it was difficult to specifically measure the fluidity of endomembranes.

To profile the specific GSL composition changes, LTQ-ESI-MS glycosphingolipidomics was applied. Similar to D-PDMP, a known GCS inhibitor³⁵, eliglustat-treated RAW264.7 cells showed significant reduction of GlcCer, LacCer and overall GSLs (Figure 6C-E). To investigate whether exogenous GlcCer or LacCer may be sufficient to rescue eliglustat-induced autophagy deficiency, three commercially available lipids, LacCer (C16 and C24) and GlcCer (C16), were investigated. The LacCer and GlcCer significantly reversed, albeit not fully, the accumulated LC3II protein levels (Figure 6F) and the accumulation of TRAF3 caused by eliglustat (Figure 6G). Hence, by removing certain GSLs from OC, eliglustat inhibits the process of autophagy and maintains TRAF3 protein levels thus inhibiting OC formation.

A

Figure 6. Eliglustat blocks autophagy by inhibiting the GlcCer and LacCer in RAW264.7 cells. (A) Membrane fluidity during early OC formation process. RAW264.7 cells were treated with RANKL and M-CSF together with eliglustat (25 μ M or 50 μ M) for 12 hours and stained with NR12S dye. Representative images of the cells from DMSO group and eliglustat group at 50 μ M. Histograms of GP distribution illustrate ordered red end (1) and disordered blue end (-1). (B) The quantification for GP value was evaluated. (C) Schematic illustrating the pathway of lipid, GSL and ganglioside metabolism. Eliglustat proposed to block the GlcCer formation from Ceramide, subsequently blocking the formation of LacCer, Gangliosides and other GSLs (including globosides, isoglobosides, and [neo]lacto-series). (D) LTQ-ESI-MS glycosphingolipidomics indicating the profile of RAW264.7 cell from DMSO group and eliglustat group (50 μ M, 12 hours) with 1:1 sample mix to indicate the change of specific GSL composition (blue indicates DMSO, red indicates eliglustat) (*m/z* means *mass charge ratio*). (E) GlcCer (cluster of molecular ion peaks between *m/z* 807 and 935; including C16, C22, C24) and LacCer (cluster of molecular ion peaks between *m/z* 1011 and 1150; including C16, C22, C24) aggregate abundance in DMSO, eliglustat and D-PDMP groups. Overall GSLs including GlcCer, LacCer and Lc3. (F) RAW264.7 cells were treated with 25 μ M eliglustat and 1 μ M C16 LacCer, 1 μ M C16 GlcCer or 1 μ M C24 LacCer for 24 hours. LC3II levels were quantified by western blot. (G) RAW264.7 cells were treated with RANKL and M-CSF in addition to eliglustat and GSLs for 24 hours. TRAF3 levels were quantified by western blot. $n \geq 3$ for all the experiments shown in this figure. Error bars correspond to SEM. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. ns means non-significant. Statistical analysis was performed using One-way ANOVA.

Eliglustat inhibits OCs derived from MM patients

MM patients frequently suffer from bone lesions resulting from increased OC-mediated bone resorption. To assess the potential clinical efficacy of eliglustat, OCs from MM patient BM aspirates were treated with eliglustat. Consistent with our findings in RAW264.7 cells (Figure 4A-B), eliglustat effectively inhibited primary human OC formation in a dose dependent manner (Figure 7A-B). Moreover, eliglustat-inhibited OC formation from MM patients' BM cells was reversed by exogenous LacCer or GlcCer (Figure 7C-D). These data provide promising evidence that through modulating LacCer and GlcCer levels, eliglustat may be used to inhibit MM patient OC formation for resolving bone lesions.

Figure 7

Figure 7. Eliglustat inhibits OC formation from MM patients whilst exogenous GSLs

reverse eliglustat inhibition. (A) BM cells from MM patients were seeded at 1.5×10^5 cells/well into 48-well-plate and differentiated to OCs with 50 ng/ml human M-CSF and 75 ng/ml human RANKL in α MEM media. BM cells were treated with increasing doses of eliglustat (0.1, 1, 10, 25 μ M) and OCs were identified by TRAP staining on day 11. (B) Number of OCs per well, number of nucleus per TRAP positive cell and % OC area over total area were quantified. (C) Eliglustat was applied to the BM cells from MM patients with or without C16 LacCer, C16 GlcCer and C24 LacCer during *in vitro* OC formation process and mature OCs were recognized by TRAP staining. (D) Number of OCs per well, number of nucleus per TRAP positive cell and % OC area over total area were quantified. Scale bar represents 200 μ m. $n=9$ patients, each with 5-7 replicates. error bars correspond to SEM. ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$, ns: non-significant. Statistical analysis was performed using One-way ANOVA.

Discussion

As MM bone disease is primarily caused by increased number and over-activation of bone-resorbing OC, compounds that inhibit OC formation or function are of key clinical therapeutic importance. In this study, eliglustat reversed bone loss in C57BL/KaLwRijHsd MM model and C57BL/6J HFD MGUS model, and showed additive effect when combined with ZA. By inhibiting OC formation, eliglustat at clinically relevant doses, prevented further bone loss *in vivo* without affecting OC progenitors, OBs, BMAT or MM cells.

The combination of eliglustat and ZA could prevent osteoclastogenesis and provide extra bone protection in MM mice by using ZA (0.01 mg/kg) at a ten-fold lower concentration than previous reports³⁶; thereby allowing further increases in bone volume to be observed. This combination resulted in significantly greater preservation of bone mass than either drug alone. This finding is particularly important as a known complication of high dose ZA is osteonecrosis

of the jaw in MM patients⁹. Combination of eliglustat with ZA for optimal bone protection can be achieved with lower amounts of ZA leading to a potential novel clinical strategy with reduced side effects and thus accelerate eliglustat's translational use into the clinic. In addition, following work will be conducted to combine eliglustat with anabolic drugs to further evaluate eliglustat's role in bone disease models.

Based on the potent effect of eliglustat on OC observed *in vivo*, mechanistic studies were conducted that will underpin future studies in this area. The lipid structure of RAW264.7 cell was more fluid after the administration of eliglustat, which can be achieved by reducing cholesterol or increasing the unsaturated lipids in the plasma membrane³⁷. Lipid rafts are a functional membrane unit in charge of signal transduction that are composed of cholesterol and saturated lipids³⁷. Ha *et al.* discovered that lipid raft domains play an essential role in OC differentiation and affect RANK-TRAF6 signaling³⁸. Likewise, TRAF3 has been located in the lipid raft microdomain by several groups^{39, 40, 41}. Whilst this study does not exclude that eliglustat may interfere with other signaling pathways by affecting lipid rafts, eliglustat did not disrupt canonical NF- κ B signaling as TRAF6 was unaffected. Instead, the lipid structure altered by eliglustat may have an effect on the formation and fusion of intracellular vesicles. Some GSLs including GD3³³, GM2 and GM3 were reported to play an essential role in autophagy⁴². Hence autophagy, required for the degradation of TRAF3, was investigated.

Due to the accumulation of single-membrane autolysosomes rather than double-membrane autophagosomes in RAW264.7 cells treated with eliglustat, it is likely that eliglustat prevents autolysosome degradation thereby maintaining TRAF3 levels. Whether this is due to impaired lysosomes remains to be elucidated. For example, lysosomal disruption induced by CQ and other treatments leads to TFEB activation and lysosome biogenesis⁴³. In line with our findings,

it was reported that the GSL inhibitor D-PDMP acts as an autophagy inhibitor in neuroblastoma cells⁴⁴.

CQ and BafA1, similar to eliglustat, both maintain TRAF3 levels and suppress OCs^{8, 45}. However, CQ has considerable ophthalmic side effects and its substitute hydroxychloroquine, now used more widely in the clinic, had no inhibitory effect on human OC differentiation^{8, 46}. On the other hand, BafA1 is not an FDA approved drug and its OC inhibition effect was only observed *in vitro*⁴⁵. Therefore, eliglustat is a more suitable drug than either BafA1 or CQ to treat bone lesions in patients. Our findings pave the way to further investigate if the autophagy inhibitor eliglustat can be used in combination with other compounds to treat diseases beyond bone including refractory myeloma and melanoma^{47, 48}.

The unprecedented finding that LacCer and GlcCer are essential for functional autophagy, underscores the vital contribution of lipid membrane composition to autophagy. Furthermore, these GSLs reversed the OC inhibition effect caused by eliglustat. While it was known that some gangliosides induce autophagy⁴⁹, this study further identified LacCer and GlcCer (precursors of all gangliosides) to promote autophagy dependent OC differentiation. These GSLs partially rescued autophagy, therefore different concentrations or combinations of lipids may be required to show a complete rescue.

Excitingly, eliglustat inhibited OC formation in patients with MM, and was dependent upon GSLs in human cells. Therefore, there is now tremendous potential for the translational use of eliglustat as a therapeutic in MM bone diseases. Diseases that involve bone loss such as breast cancer and prostate cancer metastasis to bone, post-menopausal osteoporosis and arthritis may all exhibit improvement in bone indices as well. Thus, eliglustat, a clinically approved drug for Gaucher disease, could be repurposed for patients who suffer from various bone loss diseases.

Material and Methods

Experimental design

The objective of the study was to investigate if eliglustat increases bone mass and decreases MM bone disease in several pre-clinical models, and to decipher the underlying mechanism. Using a combination of *in vitro* assays and *in vivo* murine models, we revealed the role of eliglustat in the OC differentiation and evaluated the specificity and efficacy of eliglustat. Specifically (i) we used healthy murine models to investigate the cell type that eliglustat affects, (ii) we treated MM and HFD induced MGUS pre-clinical models with eliglustat to evaluate its therapeutic potential, (iii) we administrated eliglustat and ZA to MM mice to compare the treatment efficacy (iv) myeloid specific TRAF3 knockout (LysM-Cre+, Traf3^{fl/fl}) chimeric mice were used to confirm eliglustat's effect is dependent on TRAF3, (v) we verified the GlcCer and LacCer findings in OCs derived from MM patients. Sample size was determined by the authors based on power calculations. The exact *n* numbers used in each experiment are indicated in the figure legends. For *in vivo* experiments, animals were assigned randomly to the experimental and control groups. Animal allocation, data acquisition and data analysis *in vivo* or *in vitro* were performed in a blinded manner.

Patient samples

BM aspirates were donated after informed consent and covered by research ethics UCL Biobank study "Biology of Myeloma" (07/Q0502/17). Baseline characteristics of MM patients are shown in Table S1. Briefly, BM mononuclear cells were isolated by Ficoll Paque (GE Healthcare) density centrifugation from fresh BM aspirates as previously described⁵⁰.

Cell culture and reagents

5TGM1-GFP, RAW264.7 and U2OS cells were cultured as previously described^{15, 31}. For *in vitro* OC assay, murine and human BM cells or RAW264.7 cells were differentiated with M-

CSF and RANKL in α MEM media. TRAP experiments were conducted as previously described¹⁵. All reagents are listed in Table S2.

Mouse models

Animal experiments were undertaken under UK Home Office Project Licenses 30/3218 and 30/3388 in accordance with the UK Animal (Scientific Procedures) Act 1986. Eliglustat was mixed into either a standard rodent diet, or a 42% high fat diet at 0.075% w/w (150mg/kg/day for all *in vivo* experiments) by TestDiet²³. Sex and age matched C57BL/6J mice and CD45.1⁺ B6.SJL mice were purchased from Charles River, UK. C57BL/KaLwRijHsd mice were purchased from Harlan, The Netherlands. Myeloid specific TRAF3 knockout (LysM-Cre+, Traf3^{fl/fl}) BM cells were used for chimeric mouse generation⁸. All mice were fed and housed under specific pathogen-free conditions at Kennedy Institute of Rheumatology, University of Oxford. Group size was determined based on experimental experience and *n* numbers are indicated in the figure legends. Animals were randomly assigned, and data acquisition and analysis were performed in a blinded manner.

Micro-CT analysis

Tibiae were scanned with the PerkinElmer QuantumFX scanner. The region of interest was defined as 100 slices below the growth plate of proximal tibia and was scanned at energy of 90KV; 200uA; field of view 10 for 3 minutes. Scanned bone data was analyzed and reconstructed by using Analyse-12.0 software.

Chimera blood lineages analysis

BM chimera was conducted on 11Gy lethally-irradiated female CD45.1⁺ B6.SJL mice using BM cells from CD45.2 OC-specific deletion of *traf3* female mice or their littermates. Reconstitution efficiency was confirmed by evaluating CD45.2 cells in the CD45.1 mice 6-week post-irradiation⁵¹. 50 μ l of blood from tail vein bleeding was collected and treated with

Red Blood Cell Lysis Buffer for 5 minutes. After centrifugation, the supernatant was removed and cells were stained with antibodies (Table S3) for flow cytometry.

Immunofluorescence staining

Cells were fixed using 4% paraformaldehyde and incubated with 0.1% saponin and 10% FBS for 60 minutes. For LAMP2 staining, cells were incubated in primary antibody with 10% FBS for overnight at 4°C and secondary antibody for 1 hour then stained with DAPI for 5 minutes. Coverslips were added with mounting medium prior to examination under Olympus Fluoview FV3000 or Leica TCS SP8. LAMP2 area analysis was performed using MATLAB software.

Transmission electron microscopy

1×10⁸ RAW264.7 cells were fixed with 0.1M sodium cacodylate buffer solution (pH=7.4) containing 2.5% glutaraldehyde for 1 hour at 4°C. Cells were then scraped and centrifuged. Samples were submitted to the Core Facility at Shanghai Jiao Tong University for ultrastructural analyses (HITACHI 7650).

Spectral imaging

RAW264.7 cells were seeded on glass bottom dishes. After treatment, cells were incubated with 1μM NR12S dye for 5 minutes. Spectral imaging was performed by Zeiss LSM 780 microscope, WIMM, University of Oxford. Laser light at 488nm was selected for NR12S excitation and the λ-detection range was set between 490 and 691 nm. Images were analyzed with the custom GP plugin of Fiji software as described⁵².

GSLs extraction and methylation

5×10⁷ RAW264.7 cells were sonicated in chloroform/methanol 1:1 (v:v) for 3 times and another 3 times with isopropanol/hexane/water 55:25:20 (v:v:v) in glass tubes. After centrifugation, the supernatant was collected and dried with centrifugal vacuum. Anion-exchange chromatography of DEAE Sephadex A-25 (Sigma) was used to separate the total dried crude lipids, the neutral lipid

fraction was enriched by chloroform/methanol/water 30:60:10 (v:v:v). Neutral lipids were then dissolved with sodium hydroxide (Iodomethane-d₃ was added to DMSO group). After quenched with 2ml water, the permethylated products were extracted by adding 2ml of dichloromethane and washed with water. The dichloromethane phase was then collected and dried under vacuum^{53, 54}.

Statistical analyses

All data shown are represented as mean $\pm$ standard error of the mean (SEM). Two-tailed t-test was applied when comparing two experimental groups. For comparisons between two normally distributed data sets with equal variances, paired or unpaired two-tailed Student's t-test was applied. For the experiments containing ≥ 3 experimental groups, one-way analysis of variance (ANOVA) and multiple comparisons were used with Tukey's correction. Paired or unpaired one-way ANOVA was used for multiple comparisons of normally distributed datasets with one variable. To evaluate the statistical significance of the hypothesis being tested, *p* value was applied. Statistical analyses were performed using GraphPad Prism (San Diego, CA).

Additional details about tissue processing, flow cytometry, osmium tetroxide staining, histomorphometry analysis, PCR, ELISA, western blot and LTQ-ESI-MS analyses are described in the supplemental document.

Acknowledgments:

Zhenqiang Yao and Akram Ayoub from Brendan Boyce's lab in University of Rochester Medical Center provided scientific advice and prepared the TRAF3 KO mouse BM cells –it is greatly appreciated. We thank Xiaoxia Liu and members from Qing Zhong's lab helped on experiments. 5TGM1-GFP murine MM cells was provided by B.O. Oyajobi, The University of Texas Health Science Center, San Antonio. Jin Wang and Duohui Jing from Ruijin Hospital, Bin Xie from Kennedy Institute of Rheumatology, Ge Zhang from Hong Kong Baptist University all provided constructive comments.

Funding:

University of Oxford Medical and Life Sciences Translational Fund BRR00060, BRR00140 and BRR00141 from Wellcome ISSF fund and the MRC confidence in concept (KS, HL) Versus Arthritis grant 20372 (NH, AE) Genzyme grant GZ-2015-11433 (NH) NSFC grant 91754205, 91957204 and 31771523 (QZ) China Scholarship Council (HL) China Scholarship Council-Nuffield Department of Medicine Scholarship and the Oxford-Elysium Prize Fellowship (HZ) Blood Cancer UK 17012 and 15026 (CE, EVM) SciLifeLab fellowship and Wellcome Trust ISSF (ES)

Author Contributions:

Conceptualization and design: HL, HZ, QZ, CME, AKS, NJH
Performed and analyzed experiments, methodology and investigation: HL, HZ, LL, SZ, YW, SJC, G-FD, ES
Discussed analyses: HL, HZ, LL, SZ, YW, SJC, G-FD, AE, EM, ES, Y-HL, YL, JM, KY
Supervision and interpretation: QZ, CME, AKS, NJH
Writing – original draft: HL, AKS, NJH
Writing – review & editing: HL, HZ, CEM, AKS, NJH

Competing interests: The authors declare that they have no competing interests.

Data and materials availability: All data are available in the main text or the supplementary materials.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. *CA Cancer J Clin* **66**, 7-30 (2016).
2. Lwin ST, Olechnowicz SW, Fowler JA, Edwards CM. Diet-induced obesity promotes a myeloma-like condition in vivo. *Leukemia* **29**, 507-510 (2015).
3. Rajkumar SV, Kyle RA, Buadi FK. Advances in the diagnosis, classification, risk stratification, and management of monoclonal gammopathy of undetermined significance: implications for recategorizing disease entities in the presence of evolving scientific evidence. *Mayo Clin Proc* **85**, 945-948 (2010).
4. Greipp PR, *et al.* International staging system for multiple myeloma. *J Clin Oncol* **23**, 3412-3420 (2005).
5. Terpos E, *et al.* Soluble receptor activator of nuclear factor kappaB ligand-osteoprotegerin ratio predicts survival in multiple myeloma: proposal for a novel prognostic index. *Blood* **102**, 1064-1069 (2003).
6. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. *Nat Med* **19**, 1423-1437 (2013).
7. Armstrong AP, Tometsko ME, Glaccum M, Sutherland CL, Cosman D, Dougall WC. A RANK/TRAF6-dependent signal transduction pathway is essential for osteoclast cytoskeletal organization and resorptive function. *J Biol Chem* **277**, 44347-44356 (2002).
8. Xiu Y, *et al.* Chloroquine reduces osteoclastogenesis in murine osteoporosis by preventing TRAF3 degradation. *J Clin Invest* **124**, 297-310 (2014).
9. Badros A, *et al.* Natural history of osteonecrosis of the jaw in patients with multiple myeloma. *J Clin Oncol* **26**, 5904-5909 (2008).
10. Kennel KA, Drake MT. Adverse effects of bisphosphonates: implications for osteoporosis management. *Mayo Clin Proc* **84**, 632-637; quiz 638 (2009).
11. Clarke AJ, Simon AK. Autophagy in the renewal, differentiation and homeostasis of immune cells. *Nat Rev Immunol* **19**, 170-183 (2019).
12. Chung YH, *et al.* Beclin-1 is required for RANKL-induced osteoclast differentiation. *J Cell Physiol* **229**, 1963-1971 (2014).
13. Lin NY, *et al.* Inactivation of autophagy ameliorates glucocorticoid-induced and ovariectomy-induced bone loss. *Ann Rheum Dis* **75**, 1203-1210 (2016).
14. DeSelm CJ, *et al.* Autophagy proteins regulate the secretory component of osteoclastic bone resorption. *Dev Cell* **21**, 966-974 (2011).
15. Ersek A, *et al.* Glycosphingolipid synthesis inhibition limits osteoclast activation and myeloma bone disease. *J Clin Invest* **125**, 2279-2292 (2015).
16. Abian O, Alfonso P, Velazquez-Campoy A, Giraldo P, Pocovi M, Sancho J. Therapeutic strategies for Gaucher disease: miglustat (NB-DNJ) as a pharmacological chaperone for glucocerebrosidase and the different thermostability of velaglucerase alfa and imiglucerase. *Mol Pharm* **8**, 2390-2397 (2011).
17. Belmatoug N, *et al.* Gastrointestinal disturbances and their management in miglustat-treated patients. *J Inherit Metab Dis* **34**, 991-1001 (2011).

18. Ersek A, Karadimitris A, Horwood NJ. Effect of glycosphingolipids on osteoclastogenesis and osteolytic bone diseases. *Front Endocrinol (Lausanne)* **3**, 106 (2012).
19. Poole RM. Eliglustat: first global approval. *Drugs* **74**, 1829-1836 (2014).
20. Kamath RS, *et al.* Skeletal improvement in patients with Gaucher disease type 1: a phase 2 trial of oral eliglustat. *Skeletal Radiol* **43**, 1353-1360 (2014).
21. Rosenbloom BE, Becker P, Weinreb N. Multiple myeloma and Gaucher genes. *Genet Med* **11**, 134 (2009).
22. Nair S, Branagan AR, Liu J, Boddupalli CS, Mistry PK, Dhodapkar MV. Clonal Immunoglobulin against Lysolipids in the Origin of Myeloma. *N Engl J Med* **374**, 555-561 (2016).
23. Pavlova EV, *et al.* Inhibition of UDP-glucosylceramide synthase in mice prevents Gaucher disease-associated B-cell malignancy. *J Pathol* **235**, 113-124 (2015).
24. Jacquin C, Gran DE, Lee SK, Lorenzo JA, Aguila HL. Identification of multiple osteoclast precursor populations in murine bone marrow. *J Bone Miner Res* **21**, 67-77 (2006).
25. Gaudio A, Xourafa A, Rapisarda R, Zanolli L, Signorelli SS, Castellino P. Hematological Diseases and Osteoporosis. *Int J Mol Sci* **21**, (2020).
26. Muruganandan S, Ionescu AM, Sinal CJ. At the Crossroads of the Adipocyte and Osteoclast Differentiation Programs: Future Therapeutic Perspectives. *Int J Mol Sci* **21**, (2020).
27. Russell RG. Pharmacological diversity among drugs that inhibit bone resorption. *Curr Opin Pharmacol* **22**, 115-130 (2015).
28. Ang E, *et al.* Proteasome inhibitors impair RANKL-induced NF-kappaB activity in osteoclast-like cells via disruption of p62, TRAF6, CYLD, and IkappaBalpha signaling cascades. *J Cell Physiol* **220**, 450-459 (2009).
29. Oeckinghaus A, Hayden MS, Ghosh S. Crosstalk in NF-kappaB signaling pathways. *Nat Immunol* **12**, 695-708 (2011).
30. Kong X, *et al.* Triterpenoid Saponin W3 from *Anemone flaccida* Suppresses Osteoclast Differentiation through Inhibiting Activation of MAPKs and NF-kappaB Pathways. *Int J Biol Sci* **11**, 1204-1214 (2015).
31. Diao J, *et al.* ATG14 promotes membrane tethering and fusion of autophagosomes to endolysosomes. *Nature* **520**, 563-566 (2015).
32. Pierrefite-Carle V, Santucci-Darmanin S, Breuil V, Camuzard O, Carle GF. Autophagy in bone: Self-eating to stay in balance. *Ageing Res Rev* **24**, 206-217 (2015).
33. Matarrese P, *et al.* Evidence for the involvement of GD3 ganglioside in autophagosome formation and maturation. *Autophagy* **10**, 750-765 (2014).
34. Sezgin E, *et al.* Polarity-Sensitive Probes for Superresolution Stimulated Emission Depletion Microscopy. *Biophys J* **113**, 1321-1330 (2017).
35. Vykoukal J, *et al.* Caveolin-1-mediated sphingolipid oncometabolism underlies a metabolic vulnerability of prostate cancer. *Nat Commun* **11**, 4279 (2020).
36. McDonald MM, *et al.* Inhibiting the osteocyte-specific protein sclerostin increases bone mass and fracture resistance in multiple myeloma. *Blood* **129**, 3452-3464 (2017).

37. Sezgin E, Levental I, Mayor S, Eggeling C. The mystery of membrane organization: composition, regulation and roles of lipid rafts. *Nat Rev Mol Cell Biol* **18**, 361-374 (2017).
38. Ha H, *et al.* Membrane rafts play a crucial role in receptor activator of nuclear factor kappaB signaling and osteoclast function. *J Biol Chem* **278**, 18573-18580 (2003).
39. Meckes DG, Jr., Menaker NF, Raab-Traub N. Epstein-Barr virus LMP1 modulates lipid raft microdomains and the vimentin cytoskeleton for signal transduction and transformation. *J Virol* **87**, 1301-1311 (2013).
40. Higuchi M, Izumi KM, Kieff E. Epstein-Barr virus latent-infection membrane proteins are palmitoylated and raft-associated: protein 1 binds to the cytoskeleton through TNF receptor cytoplasmic factors. *Proc Natl Acad Sci U S A* **98**, 4675-4680 (2001).
41. Ardila-Osorio H, *et al.* TRAF interactions with raft-like buoyant complexes, better than TRAF rates of degradation, differentiate signaling by CD40 and EBV latent membrane protein 1. *Int J Cancer* **113**, 267-275 (2005).
42. Bedia C, Badia M, Muixi L, Levade T, Tauler R, Sierra A. GM2-GM3 gangliosides ratio is dependent on GRP94 through down-regulation of GM2-AP cofactor in brain metastasis cells. *Sci Rep* **9**, 14241 (2019).
43. Ballabio A, Bonifacino JS. Lysosomes as dynamic regulators of cell and organismal homeostasis. *Nat Rev Mol Cell Biol* **21**, 101-118 (2020).
44. Wei J, *et al.* Protective role of endogenous gangliosides for lysosomal pathology in a cellular model of synucleinopathies. *Am J Pathol* **174**, 1891-1909 (2009).
45. Zhu S, *et al.* Bafilomycin A1 Attenuates Osteoclast Acidification and Formation, Accompanied by Increased Levels of SQSTM1/p62 Protein. *J Cell Biochem* **117**, 1464-1470 (2016).
46. Lee CK, Lee EY, Chung SM, Mun SH, Yoo B, Moon HB. Effects of disease-modifying antirheumatic drugs and antiinflammatory cytokines on human osteoclastogenesis through interaction with receptor activator of nuclear factor kappaB, osteoprotegerin, and receptor activator of nuclear factor kappaB ligand. *Arthritis Rheum* **50**, 3831-3843 (2004).
47. Vogl DT, *et al.* Combined autophagy and proteasome inhibition: a phase 1 trial of hydroxychloroquine and bortezomib in patients with relapsed/refractory myeloma. *Autophagy* **10**, 1380-1390 (2014).
48. Rangwala R, *et al.* Phase I trial of hydroxychloroquine with dose-intense temozolomide in patients with advanced solid tumors and melanoma. *Autophagy* **10**, 1369-1379 (2014).
49. Garcia-Ruiz C, Morales A, Fernandez-Checa JC. Glycosphingolipids and cell death: one aim, many ways. *Apoptosis* **20**, 607-620 (2015).
50. Kassen D, *et al.* The bone marrow stromal compartment in multiple myeloma patients retains capability for osteogenic differentiation in vitro: defining the stromal defect in myeloma. *Br J Haematol* **167**, 194-206 (2014).
51. Zhang H, *et al.* Polyamines Control eIF5A Hypusination, TFEB Translation, and Autophagy to Reverse B Cell Senescence. *Mol Cell*, (2019).
52. Sezgin E, Waithe D, Bernardino de la Serna J, Eggeling C. Spectral imaging to measure heterogeneity in membrane lipid packing. *Chemphyschem* **16**, 1387-1394 (2015).
53. Wang Z, *et al.* High expression of lactotriaosylceramide, a differentiation-associated glycosphingolipid, in the bone marrow of acute myeloid leukemia patients. *Glycobiology* **22**, 930-938 (2012).

54. Zhu J, *et al.* Aberrant fucosylation of glycosphingolipids in human hepatocellular carcinoma tissues. *Liver Int* **34**, 147-160 (2014).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [BioRxivsupplemental2.pdf](#)
- [Lengetal.SupplementaryMaterialsNatComm.pdf](#)