Improving bone health via modulation of glycosphingolipid metabolism and autophagy

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Improving bone health via modulation of glycosphingolipid metabolism and autophagy

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Patients with multiple myeloma (MM), an incurable malignancy of plasma cells, frequently develop osteolytic bone lesions. In this study, glycosphingolipids were essential in promoting autophagic degradation of the signaling molecule TRAF3, a key step in bone-resorbing osteoclast differentiation. Specifically altering the glycosphingolipid composition with eliglustat, an FDA approved glucosylceramide synthase inhibitor, arrested osteoclast differentiation; this could be rescued by exogenous addition of the missing glycosphingolipids. Eliglustat significantly reduced bone disease in several preclinical models of MM by inhibiting osteoclastogenesis and, due to its unique mode of action, it was able to act in combination with existing bone protective drugs. Furthermore, eliglustat arrested osteoclast differentiation from the bone marrow of MM patients in a glycosphingolipid-dependent way. This work identifies both the mechanism by which glucosylceramide synthase inhibition blocks autophagic degradation of TRAF3 to reduce osteoclastogenesis as well as highlighting the translational 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 disease\(^4\), affecting 85% of MM patients\(^5\).

MM cells secrete factors, including receptor activator of nuclear factor kappa-B ligand (RANKL), that enhance osteoclast (OC) formation\(^6\). Engagement of RANKL, the OC differentiation factor, with its cognate receptor RANK leads to the activation of the NF-\(\kappa\)B signaling pathway and is required for OC formation. The NF-\(\kappa\)B pathway is regulated by tumour necrosis factor receptor-associated factors (TRAFs). In particular TRAF6 mediates RANKL-induced osteoclastogenesis\(^7\) whilst TRAF3 is inhibitory\(^8\). 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 risk\(^10\). 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 organelles\(^11\). During OC differentiation Beclin-1 is upregulated\(^12\) and Atg7 knockdown inhibits the expression of key OC proteins such as tartrate-resistant acid phosphatase (TRAP) and cathepsin K\(^13\). Additionally, autophagy-related proteins including ATG5, ATG7, ATG4B, and LC3 regulate a number of OC functions\(^14\). Concordantly, autophagy inhibitors such as chloroquine (CQ) can inhibit OC formation and restore bone mass\(^8\). Molecularly, TRAF3, as a suppressor of OC differentiation, is degraded by autophagy.
upon RANKL induction in BM OC precursors⁸. Based on these findings, it has been proposed that autophagy inhibitors may be beneficial in treating bone loss diseases in the clinic.

Glycosphingolipids (GSLs) are constituents of the cellular membrane that are expressed in varying ratios and combinations. We previously observed that GM₃, a ganglioside abnormally expressed in MM cells, promotes osteoclastogenesis¹⁵ and that this can be blocked using miglustat, an iminosugar and an analogue of D-glucose¹⁶. However, miglustat is involved in various biological and pathological pathways and can cause deleterious side effects in the gastrointestinal track including severe diarrhea and weight loss¹⁷. Eliglustat, a small synthetic molecule, specifically prevents the synthesis of all GSLs by inhibition of glucosylceramide synthase (GCS)¹⁸ and is used for the treatment of Gaucher disease type 1 in adults¹⁹. Gaucher patients suffer from osteoporosis/osteopenia and treatment to reduce excess bioactive GSL associated with the disease has been shown to alleviate bone loss²⁰. Interestingly, patients with Gaucher disease have a 6–50 times increased risk of developing MM or the pre-MM MGUS condition²¹,²²,²³. However, it remains to be determined whether the reduction in bone symptoms is due to the treatment of Gaucher disease or if eliglustat acts directly on bone cells, which raised the possibility that eliglustat may be of benefit in treating MM bone complications.

Here we demonstrate that eliglustat reduces bone disease by directly acting on bone cells, namely on the OCs, in pre-clinical MM and MM-like MGUS models, and can be used in combination with ZA for superior outcomes. Mechanistically, eliglustat acts as an autophagy inhibitor that prevents TRAF3 degradation in OC and modulates autophagy by altering glucosylceramide (GlcCer) and lactosylceramide (LacCer) composition in OC. Lastly, eliglustat effectively inhibits OCs from MM patient samples and replacement of the GSLs can 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+) 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 IgG2bκ 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

Ctr  MM  MM + Elig

B

Ctr  MM  MM + Elig

C

Ctr  MM  MM + Elig

D

Number of holes / Tibia

E

Ctr  MM  MM + Elig

F

Ctr  MM  MM + Elig
**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× 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 incidence\(^2\). 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 loss\(^2\).

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 loss\(^2\). 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 formation\(^2\). 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

Day -49
Day 0
Day 4
Day 30

4-week-old C57BL6 Start HFD

STG1M-GFP i.v.

Start Elig in HFD at 150mg/kg/day or maintain normal HFD or control diet

Sacrifice

MicroCT

Histology

EUSA

B

Weight change compare to MM baseline

| Time | MM | HFD + MM | HFD + MM + Elig |
|------|----|----------|----------------|
| Day 14 | ns | ** | *** |
| Day 28 | *** | **** | **** |
| Day 30 | **** | **** | **** |

C

MM
HFD + MM
HFD + MM + Elig

D

BV/TV\%*

BV/TV\%*

Tb.N (1/mm)*

Tb.Sp (mm)*

Trabecular BMD (mg/cm^3)*

Regulated BMAT

E

Regulated BMAT

F

MM
HFD + MM
HFD + MM + Elig

G

Oc.S/B.S (%)***

N.Oc/T.Ar***

N.Oc/B.Pm***

Conn.D (mm-3)ns

Tb.Th (mm) ns
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 pathway\(^\text{27}\), 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) Diagram showing the timeline and experiments. Day 0: 8-week-old C57BL/KaJ-wRJhsd STC1-1-GFP or PBS i.v. Day 1: ZA 0.01 mg/kg i.p. once. Day 4: Elig diet at 150 mg/kg/day or control diet. Day 23: Sacrifice. MicroCT Histology

(B) Images of bone samples: Ctr, MM, MM + Elig, MM + ZA, MM + ZA + Elig

(C) Graphs showing BV/TV %, BS (mm^2), Conn.D (mm^-3), and Holes / Tibia

(D) Images of bone samples: Ctr, MM, MM + Elig, MM + ZA, MM + ZA + Elig

(E) Graphs showing Oc.S/BS (%), N.Oc/T.Ar, and N.Oc/B.Pm

(F) Images of histological sections: Ctr, MM, MM + Elig, MM + ZA, MM + ZA + Elig

(G) Graphs showing Oc.S/BS (%), N.Oc/T.Ar, and N.Oc/B.Pm
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 (50µM)\(^3\), 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-κB pathways (Figure 4C), both essential for OC formation. In the canonical NF-κB pathway, activation of the adaptor protein TRAF6 leads to proteasome-mediated IkBα degradation\(^2\) followed by nuclear translocation of p65 and p50; in the non-canonical NF-κB pathway, the adaptor protein TRAF3 is degraded in an autophagosome/lysosome-dependent mechanism\(^8\), that induces nuclear translocation of p52 and RelB\(^9\). 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)\(^3\). However, this was not altered by eliglustat treatment (Figure 4F). Moreover, TRAF6-regulated IkBα protein levels were not affected by eliglustat (Figure 4G). These observations indicate that eliglustat does not regulate TRAF6-mediated canonical NF-κB pathway.
To confirm the role of TRAF3 in mediating eliglustat’s OC-inhibitory effects in vivo, BM chimeric mice were generated using BM donor cells from LysM-Cre+, TRAF3<sup>fl/fl</sup> mice that harbor a myeloid specific deletion of <i>traf3</i> (Figure S5D-E)<sup>8</sup>. Chimeric mice were fed eliglustat-containing diet for 19 days prior to micro-CT analysis. As previously demonstrated (Figure S1), eliglustat administration led to an increase of BV/TV% in mice that received wild-type BM donor cells, however this was no longer apparent in chimeric mice that received BM from TRAF3-deficient mice (Figure 4H and S5F), further confirming that eliglustat suppresses OC formation in a TRAF3-dependent manner.
Figure 4

A

|          | Ctr   | Elig 0.1 µM | Elig 1 µM | Elig 10 µM | Elig 25 µM | Elig 50 µM |
|----------|-------|-------------|-----------|------------|------------|------------|
| BV/TV%   |       |             |           |            |            |            |

B

|          | Ctr   | Elig 0.1 µM | Elig 1 µM | Elig 10 µM | Elig 25 µM | Elig 50 µM |
|----------|-------|-------------|-----------|------------|------------|------------|
| Number of OCs/well |       |             |           |            |            |            |
| Number of TRAF3+ cells |       |             |           |            |            |            |
| % OC area |       |             |           |            |            |            |
| % Alamar blue cell viability on Day 3 |       |             |           |            |            |            |

C

D

E

F

G

H

p=0.3585

WT

WT+Elig

traf3 KO

traf3 KO+Elig
**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 μM) were present throughout the culture period and OCs were identified by TRAP staining on day 5. Scale bar represents 200μm. (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 NFκB pathway, which leads to proteasome-mediated IkBα degradation and then nuclear translocation of p65 and p50 whilst in the non-canonical NFκB 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 IkBα (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 ± 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 machinery\(^4\). 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)\(^3\). 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 lysosome\(^3\). 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).
together, these data support a role for eliglustat as an autophagy inhibitor that blocks autophagic flux by inhibiting autolysosomal degradation.
Figure 5

A

RAW264.7 Day 1

M-CSF        +        +        +        +
RANKL(50ng/ml) +        +        +        +
DMSO         -        -        -        -
Elig(μM)     -        25       50       25
Baf A1(10nM) -        -        +        +
P62
\[
\begin{array}{c}
\text{RAW264.7 Day 1} \\
\text{M-CSF} & + & + & + & + \\
\text{RANKL(50ng/ml)} & + & + & + & + \\
\text{DMSO} & - & - & - & - \\
\text{Elig(μM)} & - & 25 & 50 & 25 \\
\text{Baf A1(10nM)} & - & - & + & + \\
\end{array}
\]

B

DMSO  BafA1 (10nM)  CQ (20μM)  Elig (50μM)

GFP-LC3 puncta per cell

GFP+/RFP+

DMSO  BafA1 (10nM)  CQ (20μM)  Elig (50μM)

LAMP2 puncta area per cell

GFP-/RFP+

DMSO  BafA1 (10nM)  CQ (20μM)  Elig (50μM)

Number of puncta per cell

GFP+,RFP+

DMSO  BafA1 (10nM)  CQ (20μM)  Elig (50μM)

Autolysosome number/cell

GFP-,RFP+

E

LAMP2
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×). (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\textsuperscript{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 are\textsuperscript{34}. 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 inhibitor\textsuperscript{35}, 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.
Figure 6

A. DMSO and Elig(50 µM)

B. Lipid packing of RAW264.7 cells

C. Overview of Glycosphingolipids (GSLs)

D. Overall GSLs aggregate abundance expression level

E. Aggregate abundance expression level

F. LC3II / β-Actin

G. TRAF3 / β-Actin
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 (25µM or 50µM) for 12 hours and stained with NR12S dye. Representative images of the cells from DMSO group and eliglustat group at 50µM. 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 (50µM, 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 25µM eliglustat and 1µM C16 LacCer, 1µM C16 GlcCer or 1µM 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≥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. Eliglustat inhibits OC formation from MM patients whilst exogenous GSLs reverse eliglustat inhibition. (A) BM cells from MM patients were seeded at $1.5 \times 10^5$ cells/well into 48-well-plate and differentiated to OCs with 50 ng/ml human M-CSF and 75 ng/ml human RANKL in αMEM media. BM cells were treated with increasing doses of eliglustat (0.1, 1, 10, 25 μM) 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 μm. 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 reports; 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 patients. 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 membrane. Lipid rafts are a
functional membrane unit in charge of signal transduction that are composed of cholesterol and
saturated lipids. Ha et al. discovered that lipid raft domains play an essential role in OC
differentiation and affect RANK-TRAF6 signaling. Likewise, TRAF3 has been located in the
lipid raft microdomain by several groups. Whilst this study does not exclude that
eliglustat may interfere with other signaling pathways by affecting lipid rafts, eliglustat did not
disrupt canonical NF-κB 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 GD3, GM2 and GM3 were reported to play an essential role in
autophagy. 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 biogenesis. In line with our findings,
it was reported that the GSL inhibitor D-PDMP acts as an autophagy inhibitor in neuroblastoma cells\(^4^4\).

CQ and BafA1, similar to eliglustat, both maintain TRAF3 levels and suppress OCs\(^8,4^5\). 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\(^8,4^6\). On the other hand, BafA1 is not an FDA approved drug and its OC inhibition effect was only observed \textit{in vitro}\(^4^5\). 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\(^4^7,4^8\).

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 autophagy\(^4^9\), 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 

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 described.

Cell culture and reagents
5TGM1-GFP, RAW264.7 and U2OS cells were cultured as previously described. For in vitro OC assay, murine and human BM cells or RAW264.7 cells were differentiated with M-
CSF and RANKL in αMEM media. TRAP experiments were conducted as previously described\textsuperscript{15}. 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. Elaglustat 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 TestDiet\textsuperscript{23}. Sex and age matched C57BL/6J mice and CD45.1\textsuperscript{*} B6.SJL mice were purchased from Charles River, UK. C57BL/KaLwRijHsd mice were purchased from Harlan, The Netherlands. Myeloid specific TRAF3 knockout (LysM-Cre\textsuperscript{+}, Traf3\textsuperscript{fl/fl}) BM cells were used for chimeric mouse generation\textsuperscript{8}. 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\textsuperscript{*} 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-irradiation\textsuperscript{51}. 50μl 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**

$1 \times 10^8$ 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 $\lambda$-detection range was set between 490 and 691 nm. Images were analyzed with the custom GP plugin of Fiji software as described.​

**GSLs extraction and methylation**

$5 \times 10^7$ 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 vacuum53,54.

**Statistical analyses**

All data shown are represented as mean ± 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.

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