

Translational Potential of a Novel Osteoclast Inhibitor, Eliglustat, in Myeloma Bone Disease



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Abstract

Multiple myeloma is a fatal haematological malignancy, in which the majority of patients either present with or develop a destructive and debilitating osteolytic bone disease. The bone disease is characterised by an increase in osteoclastogenesis and by osteoblast suppression. Current treatments for MM bone disease include bisphosphonates and denosumab, both of which act to inhibit osteoclast formation and thus limit bone resorption. Recently the glycosphingolipid inhibitor miglustat was found to be effective in the treatment of myeloma bone disease. Eliglustat is small molecule drug that inhibits glucosylceramide synthase and is approved by the Food and Drug Administration for the treatment of Gaucher's Disease. Patients with Gaucher's disease have an increased risk of MM development, raising the possibility that this drug may be of benefit in MM. This thesis aimed to investigate the efficacy of eliglustat in myeloma bone disease in vivo, and to determine its mechanism of action.

Preliminary data revealed that treatment with eliglustat in a murine MM model reduced osteolytic bone lesion development, associated with a reduction in osteoclast number but no change in osteoblastic bone formation. Eliglustat was also found to be effective in reducing myeloma bone disease in both diet-induced obesity and ageing models of myeloma. The combination of eliglustat and the bisphosphonate, zoledronic acid, was found to have a greater effect to reduce myeloma bone disease than either agent alone, suggesting the potential for combination therapies that allow for dose reductions. Mechanistic studies demonstrated that Eliglustat is likely to inhibit OC

formation via preventing degradation of TRAF3 in OC precursors. This could be achieved either by disrupting GSL integrity in the cell membrane or working as an autophagy inhibitor to exert its effect in OC by maintaining TRAF3 protein level.

Taken together, this project identifies a novel mechanism by which GSL inhibition impacts osteoclastogenesis and highlights the translational potential of eliglustat for the treatment of myeloma bone disease.

Declaration

This thesis is submitted to the University of Oxford in support of my application for the degree of Doctor of Philosophy. The work described herein was performed when I was a graduate student at the Kennedy Institute of Rheumatology, under the supervision of Professor Katja Simon, Associate Professor Claire Edwards, and Associate Professor Nicole Horwood. All the work presented in this thesis is the result of my own work unless otherwise stated. No part of my thesis has been accepted or is currently being submitted for any degree, diploma or certificate or other qualification in the University of Oxford or elsewhere.

Author's Publications

Leng H, Hanlin Zhang, Shuhao Zhang, Claire Edwards, Nicole Horwood, Anna Katharina Simon. March 2020. A novel glycosphingolipid inhibitor reveals a role for lipids in autophagy regulation. Gordon Research Conferences (GRC) 2020 meeting of Autophagy in Stress, Development and Disease. Los Angeles, CA, USA [invited oral presentation]

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Numerous brilliant minds have been thriving in Oxford, and just in this week in 2019, several of them are awarded the Nobel Prize. I feel very lucky to have an opportunity to come here, meet them and follow their footsteps.

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List of Abbreviations

ALP	Alkaline phosphatase
AMP-DNM	N-(5-adamantane-1-yl-methoxy-pentyl)-deoxynojirimycin
ANOVA	One-way analysis of variance
AV/TV	Adipose volume over total volume
BafA1	Bafilomycin A1
Bcl 2	B-cell leukaemia/lymphoma 2
BM	Bone marrow
BMAT	Bone marrow adipose tissue
BMD	Bone mineral density
BMP	Bone morphogenetic proteins
BP	Bisphosphonate
BS	Bone surface
BTZ	Bortezomib
BV/TV	Bone volume over total volume
c-fms	colony-stimulating factor-1 receptor
Conn.D	Connective density
Ct.Ar	Cortical area
Ct.Ar/Tt.Ar	The ratio of cortical area over total area
Ct.Th	Cortical bone thickness
CTSK	Cathepsin K
CYP	Cytochrome P450
CYP2D6	Cytochrome P4502D6

DC-STAMP	Dendritic cell-specific transmembrane protein
DKK1	Dickkopf-related protein 1
DLBCL	Diffuse large B-cell lymphoma
DXA	Dual-energy x-ray absorptiometry
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FACS	Flow cytometry
FOV	Field of view
GBA	Glucocerebrosidase
GC	Germinal center
GCS	Glucosylceramide synthase
GD	Gaucher's Disease
GlcCer	Glucosylceramide
GP	Generalized polarization
GSL	Glycosphingolipid
GTPase	Guanosine-5'-triphosphatase
HCl	Hydrochloric acid
HFD	High fat diet
IGF1	Insulin-like growth factor 1
IKK	I κ B kinase
IκB	The inhibitor of κ B
LacCer	Lactosylceramide
LC3	Microtubule-associated light chain 3

LGL-1	Lyso-glucosylceramide
LTβR	Lymphotoxin β receptor
M-CSF	Macrophage-colony stimulating factor
MCP-1	Monocyte chemotactic protein-1
MGUS	Monoclonal gammopathy of undetermined significance
micro-CT	Micro-computed tomography
MIP-1α	Macrophage inflammatory protein 1 α
MM	Multiple myeloma
MM cell	Abnormal plasma cell
mRNA	messenger RNA
MSC	Mesenchymal stem cell
N.Ob/B.Pm	Number of OBs per bone perimeter
N.Ob/T.Ar	Number of OBs over total bone area
N.Oc/B.Pm	Number of OCs per bone perimeter
N.Oc/T.Ar	Number of OCs over total bone area
NB-DNJ	N-butyl-deoxynojirimycin
NF-κB	Nuclear factor κ B
NIK	NF- κ B-inducing kinase
NSG	NOD-scid IL2rnull
OB	Bone forming osteoblast
Ob.S/BS	Percentage of OB surface over total bone surface
OC	Osteoclast
OC-STAMP	Osteoclast-stimulatory transmembrane protein
Oc.S/BS	Percentage of OC surface over total bone surface

OCN	Osteocalcin
OPG	Osteoprotegerin
PC	Plasma cell
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PFA	Paraformaldehyde
PI3P	Phosphatidylinositol 3-phosphate
qPCR	quantitative polymerase chain reaction
RANK	Receptor activator of nuclear factor κ B
RANKL	Receptor activator of nuclear factor κ B ligand
RBC	Red blood cell
SEM	Standard error of the mean
SMM	Smouldering multiple myeloma
SRBC	Sheep red blood cell
STED	Super-resolution stimulated emission depletion
FCS	Fluorescence correlation spectroscopy
TAK1	Transforming growth factor β -activated kinase 1
Tb.N	Trabeculae number
Tb.Sp	Trabeculae separation
Tb.Th	Trabeculae thickness
TNFR	Tumour necrosis factor receptor
TRAF	Tumor necrosis factor receptor-associated factor
TRAP	Tartrate-resistant acidic phosphatase
UDP	Uridine diphosphate

UDP	Uridine diphosphate
ULK	UNC-51-like kinase
WAT	White adipose tissue
WHO	World Health Organisation
WIPI	WD-repeat protein interacting with phosphoinositide
Wnt signaling	Wingless Signaling
ZA	Zoledronic acid

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Chapter 1. General Introduction:

Bone Homeostasis, GSL Inhibitors and Bone Diseases

1.1 Bone homeostasis

1.1.1 Bone remodeling

Bone is the mineralized tissue that holds up the body by providing attachment sites for muscles, tendons and ligaments to allow for locomotion and functions as a shelter for many organs such as brain, bone marrow (BM) and heart. Through the life time, the bone is under dynamic equilibrium during which damaged bone is removed from the bone surface and new bone is subsequently formed, a process defined as bone remodeling¹. Bone remodeling can be divided into different phases including: resorption phase, reversal phase and formation phase^{2,3}. The phases of bone remodeling are primarily coordinated by two main cell types; namely bone resorbing osteoclasts (OCs) and bone forming osteoblasts (OBs).

1.1.2 Osteoclasts

OC, located on endosteal surfaces to remove mineralized bone matrix, are terminally differentiated multinucleated giant cells derived from mononuclear hematopoietic myeloid lineage⁴. Preliminary initiated in BM niche, flattened and non-polarized OC migrate to the bone surface⁵. The attachment of pre-OC to the bone surface is mainly mediated by $\alpha v \beta 3$ -integrin and CD44⁶⁻⁹. Only after contact with extracellular mineralized matrix, the actin cytoskeleton is reorganized and pre-OC become polarized OC as characterized by specific structures including sealing zone, ruffled border, functional secretory domain, and basolateral membrane¹⁰⁻¹².

Each of these structures in OCs have their own functions. The sealing zone, located at the border of the OC next to the bone matrix, is an area devoid of organelles defined by an actin ring that creates an isolated acidic microenvironment between the OC and the bone surface^{8,12}. The ruffled border, a membrane domain next to sealing zone, is formed for trafficking of protons and enzymes into the Howship lacuna component¹³. OC secrete protons via an electrogenic H⁺ATPase (proton pump) to make hydrochloric acid (HCl) to dissolve the inorganic bone matrix and to lower the pH to allow the OC specific enzymes to cleave the organic components of the bone matrix¹⁴. By dissolving the inorganic components such as hydroxyapatite, enzymes including tartrate-resistant acid phosphatase (TRAP), cathepsin K, and matrix metalloproteinase-9 are able to then degrade the proteins, primarily composed of type I collagen. Specifically, researchers can make use of TRAP to visualize OCs as TRAP stained cells become red¹⁵. After degradation, degradants are released to functional secretory domain at the basolateral membrane by endocytosis across the ruffled border^{12,16} (Figure 1.1¹⁷).

In addition to the unique structural features of the OC, an important step for proper OC function is the cell-cell fusion of OCs¹⁸. Dendritic cell-specific transmembrane protein (DC-STAMP)¹⁹⁻²¹ and osteoclast-stimulatory transmembrane protein (OC-STAMP)^{22,23} are two essential proteins for OC cell-cell fusion. DC-STAMP is expressed in monocyte/macrophage lineage^{18,20,24}. It is reported that DC-STAMP-deficient mice have no multinucleated OC formation both *in vivo* and *in vitro*, while the expression level of OC differentiation markers is normal²¹. This finding clearly indicates that DC-STAMP is essential for OC cell–cell fusion. DC-STAMP also plays a role in maintaining bone resorbing efficiency, as evidenced by the increased bone

mass in DC-STAMP-deficient mice²¹ and decreased bone mass in DC-STAMP-overexpressing mice²⁵. Different from DC-STAMP, OC-STAMP specifically expressed on multinucleated RANKL-induced OCs²³. Mice with OC-STAMP deficiency encounter OC fusion defect²⁶. Interestingly, OC-STAMP and DC-STAMP may regulate OC cell fusion independently, as evidenced by the expression of OC-STAMP is normal in DC-STAMP-deficient OCs, and *vice versa*²². In addition to OC-STAMP and DC-STAMP, factors including CD44, CD47 and CD9 also affect OC fusion^{27,28}.

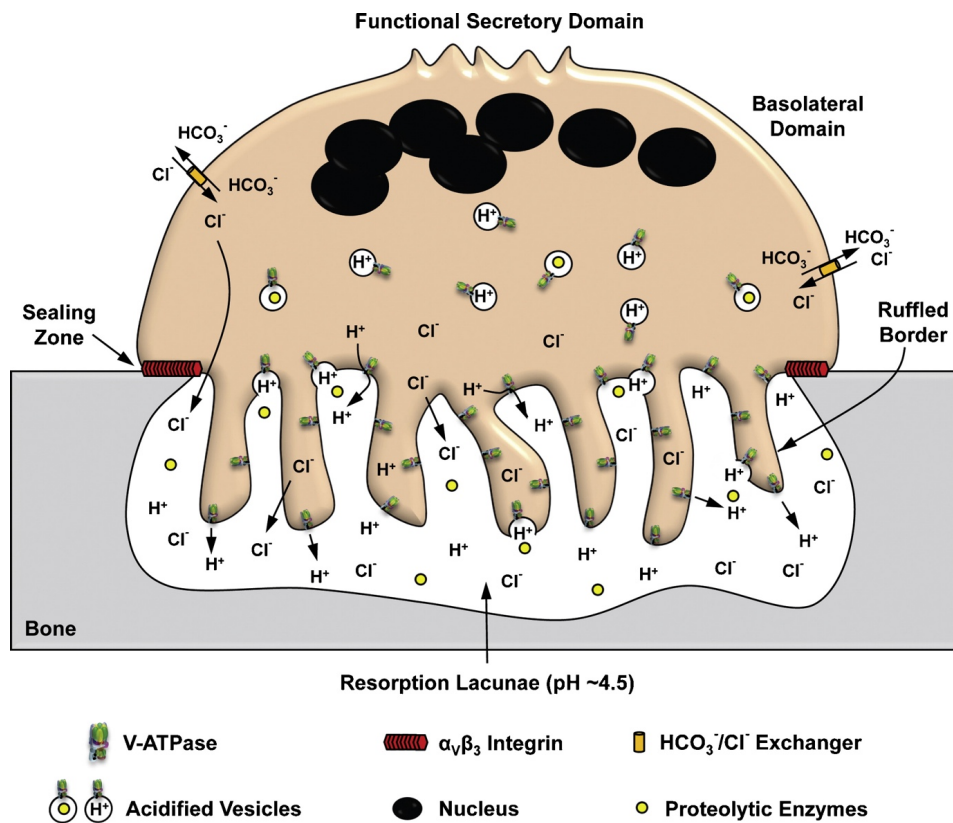


Figure 1.1 A functional OC cell. Cartoon illustrates the structures of OC, including sealing zone, ruffled border, functional secretory domain, and basolateral membrane.

This figure was adapted from a review by Qin *et al.*, 2012.

1.1.3 Osteoblasts

The OB, a cuboidal cell type which is located along the bone surface, is responsible for bone formation and works as the counterpart of OC²⁹. Originally differentiated from mesenchymal stem cells (MSC), OBs are formed with the help of bone morphogenetic proteins (BMPs) and molecules from Wingless (Wnt) signaling³⁰. In addition, Runx2, an important gene for OB differentiation^{31,32}, is capable of upregulating other OB-related genes such as alkaline phosphatase (ALP) and osteocalcin (OCN)³³. Different genes and proteins expression levels define the cell type during OB differentiation. For example, the expression of Runx2 in OB progenitors indicates a proliferation phase will subsequently occur, which is characterized by the presence of ALP activity and these cells are then considered as pre-OBs²⁹. The pre-OBs further progress to mature OBs characterized by an increase of bone matrix proteins and collagen proteins such as OCN, collagen type I, proteoglycan and osteoid^{31,34-36}. These proteins secreted by mature OBs deposit the organic matrix and subsequently follows the mineralization of the organic matrix¹³. Eventually, mature OBs can go with three fates including apoptosis, become osteocytes, or turn into bone lining cells^{13,37}.

1.1.4 RANKL/RANK/OPG signaling

OC formation, or osteoclastogenesis, is the process which mononuclear hematopoietic myeloid cells (monocytes and macrophages) are stimulated by cytokines to form OCs. Macrophage-colony stimulating factor (M-CSF) and membrane-bound receptor activator of nuclear factor κ B ligand (RANKL) are the two major players responsible for OC formation³⁸.

M-CSF is secreted from OB and osteoprogenitor mesenchymal cell³⁹. M-CSF binds to colony-stimulating factor-1 receptor (c-fms) present on the OC precursor, which stimulates cells to differentiate onto the macrophage lineage, facilitates cell proliferation and inhibits cell apoptosis^{39,40}.

RANKL is an essential cytokine for OC formation that is known as the OC differentiation factor. RANKL is expressed by OB, osteocyte and stromal cells⁴¹. Normally, OC formation is induced when RANKL binds to receptor activator of nuclear factor κ B (RANK), which expressed in osteoclast precursors⁴². In addition, the binding of RANKL with RANK induces other OC formation factors such as NFATc1, the master regulatory transcription factor of OC⁴³. For example, NFATc1 can be influenced by RANKL/RANK interaction and induces DC-STAMP, which is essential for osteoclast fusion^{44,45}. Moreover, NFATc1 also regulates TRAP and cathepsin K, both of which are OC specific genes crucial for OC activity⁴⁶.

OC formation can be negatively regulated by osteoprotegerin (OPG), a soluble decoy receptor of RANKL⁴⁷ which mainly produced by OBs and stromal cells⁴⁸. The binding of OPG with RANKL prevents RANKL interaction with RANK and subsequently inhibits OC formation⁴⁸. Therefore, RANKL/RANK/OPG axis regulates OC differentiation and imbalance between RANKL and OPG can lead to diseases such as osteopetrosis or osteoporosis⁴⁹ (Figure 1.2⁴¹).

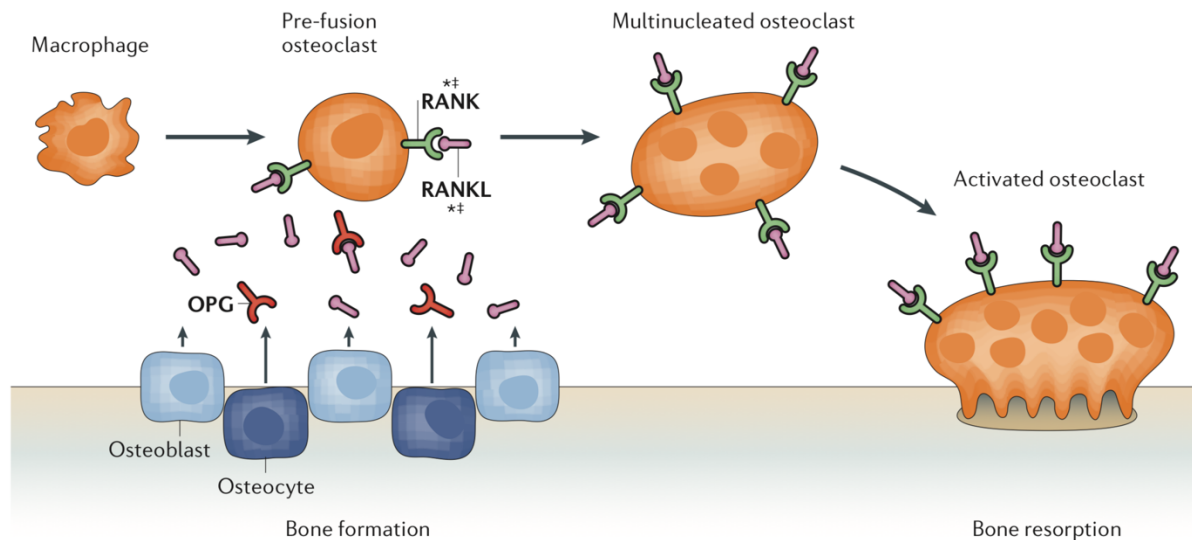


Figure 1.2 RANK/RANKL/OPG system. RANK/RANKL/OPG signalling in OC

formation and function. This figure was adapted from a review by Richards *et al.*, 2012.

1.2 NF- κ B signaling pathway

As a group of inducible transcription factors, nuclear factor κ B (NF- κ B) is involved extensively in cell growth and survival, inflammation and immune responses, and cancers⁵⁰⁻⁵². The activation of NF- κ B is mediated by two signaling pathways; the canonical pathway and non-canonical pathways⁵³.

NF- κ B signaling is important for bone biology as it regulates OC formation and function. Activated by RANKL binds to RANK receptor, NF- κ B signaling regulates OC formation⁵⁴. It is reported that NF- κ B KO mice failed to form OCs resulting in increased bone mass, which indicates NF- κ B signaling is essential for the signaling transduction of OC formation⁵⁵. Moreover, NF- κ B KO cells stimulated with cytokines including

RANKL failed to form OC, which emphasizes the essential role of NF- κ B in regulating cytokine-induced OC formation⁵⁶ (Figure 1.3⁵⁷).

1.2.1 Canonical NF- κ B signaling

Triggered by signals from various immune receptors to exert its catalytic activation and signaling function, the kinase transforming growth factor β -activated kinase 1 (TAK1) activates a trimeric I κ B kinase (IKK) complex (including catalytic subunits IKK α and IKK β , and regulatory subunit IKK γ) via phosphorylation of IKK β ^{58,59}. Phosphorylated IKK β then degrades I κ Bs (I κ B α , p105), the inhibitor of κ B (I κ B) family, in a ubiquitin-dependent manner in the proteasome which eventually leads to the formation of RelA-p50, C-Rel-p50, and p50-p65. The canonical NF- κ B signaling pathway is rapid and transient which is essential for the proper regulation of NF- κ B functions^{52,53,60} (Figure 1.3⁵⁷).

1.2.2 Non-canonical NF- κ B signaling

The activation of the non-canonical NF- κ B pathway only depend on a limited members from tumour necrosis factor receptor (TNFR) superfamily (CD40, BAFF receptor, lymphotoxin β receptor [LT β R], RANK, etc.^{61,62}), that are capable of activating NF- κ B-inducing kinase (NIK)⁶². Although non-canonical NF- κ B pathway is independent from TAK1 and IKK complex, it still requires IKK α ^{53,63,64}. Activated NIK directly phosphorylates IKK α , which subsequently phosphorylates and degrades P100. The processing of p100 regulates RelB and leads to the generation of p52. Finally, nuclear translocation of p52 and RELB happens⁶³. Non-canonical NF- κ B signaling is slower than canonical as NIK is degraded via the ubiquitin/proteasome pathway under normal

conditions, which prevents the processing of p100^{62,65}. The non-canonical NF- κ B pathway plays an active role in B cell development, OC formation and lymphoid organ maturation⁶⁶ (Figure 1.3⁵⁷).

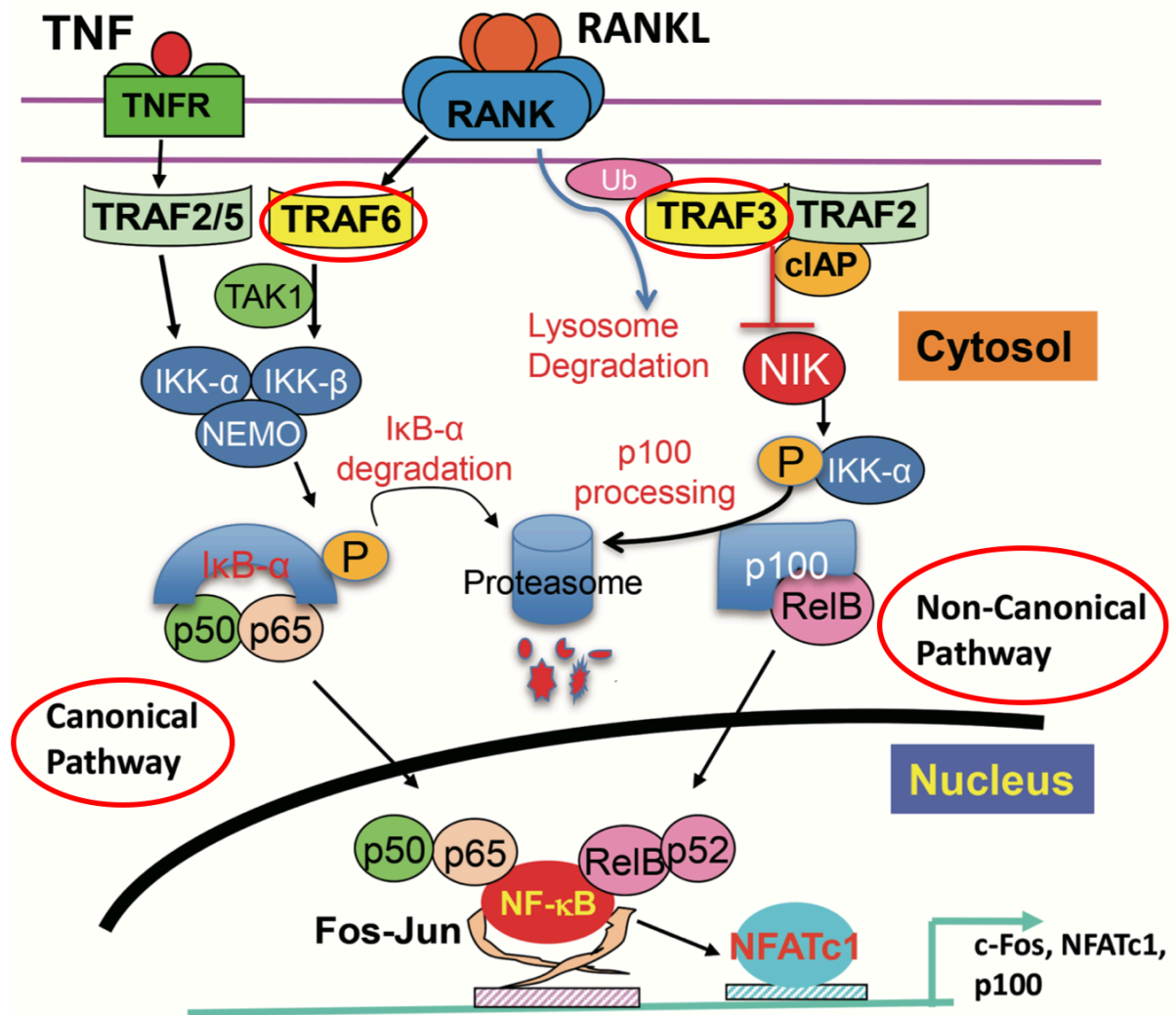


Figure 1.3 NF- κ B signalling regulating OC formation. Canonical NF- κ B signalling and non-canonical NF- κ B signalling regulate RANKL-mediated OC formation. This figure was adapted from a review by Boyce *et al.*, 2015.

1.2.3 TRAF adaptor proteins related with osteoclast formation

Tumor necrosis factor receptor (TNFR)-associated factors (TRAFs) consists of six members (TRAF1–6) and an atypical member (TRAF7)^{67,68}. All TRAF proteins have a homologous C-terminal domain (TRAF domain), and have mutations in the numbers of zinc fingers. It is summarized that the TRAF proteins are oligomerized by TRAF domains, and then the TRAF protein is capable of interacting with upstream receptors and downstream effector proteins⁶⁷. In addition, on the N-terminal of TRAF2–6, the RING domain mediates TRAF protein ubiquitination via E3 ubiquitinase ligases⁶⁹. As the NF- κ B pathway is regulated by TRAFs, moreover, TRAF6⁷⁰ and TRAF3⁷¹ are confirmed to regulate RANKL-induced OC formation and function.

1.2.3.1 TRAF6

TRAF6 is a representative E3 ligase which conjugates lysine (K) 63-linked polyubiquitin chains⁴³. As an essential downstream mediator of RANK signaling, TRAF6 can subsequently activates NF- κ B signaling⁷²⁻⁷⁴. Moreover, TRAF6 is required for the expression of NFATc1^{75,76}, cytoskeletal rearrangement⁷⁰, and essential for OC formation and OC function⁷⁷. Interestingly, different from closely related CD40 receptor, RANK receptor has more TRAF6 binding sites, which brings stronger TRAF6-mediated OC formation signaling⁷⁸. Moreover, it is reported that target TRAF6 can inhibit RANK signaling and hence deteriorate OC function⁷⁹⁻⁸¹. One group reported that TRAF6 is dispensable for RANKL-independent OC formation initiated by TNF α ⁸², while the other group reported TRAF6 facilitate OC formation additively with RANKL⁸³. Therefore, it remains unclear whether TRAF6 is essential for TNF α -mediated OC formation.

1.2.3.2 TRAF3

NIK binds to the TRAF-C domain of TRAF3 for the progression of non-canonical NF- κ B pathway⁸⁴. It is reported that mice over-expressing NIK lack the TRAF3 binding domain and develop osteoporosis due to the over activation of OCs⁸⁵. In the non-canonical NF- κ B pathway, RANKL processes p100 into p52 that induces OC formation. By preventing p100 processing into p52, TRAF3 effectively inhibits OC formation⁸⁶ as well as decreasing OC formation markers such as NFATc1 and c-Fos⁷¹. Consequently, as a suppressor for both canonical and non-canonical NF- κ B signaling^{87,88}, TRAF3 knockdown promotes TNF-induced OC formation⁸⁶. Moreover, TRAF3 has anti-inflammatory and anti-B cell lymphomas effects⁸⁹.

1.3 Autophagy and osteoclasts

1.3.1 Autophagy

Macroautophagy (here after referred as “autophagy”) is a conserved pathway which maintains eukaryotic cell homeostasis by degrading and recycling cellular waste including protein aggregates and damaged organelles⁹⁰⁻⁹².

Autophagy process can be artificially divided into several stages including induction (or nucleation), phagophore elongation, autophagosome formation and autolysosome maturation^{91,93}. In detail, upstream signaling pathways such as mTORC1 de-activation initiate autophagy, which leads to UNC-51-like kinase (ULK) activation and the formation of ULK1 complex (including ULK1, ATG13, FIP200 and Atg101)⁹⁴⁻⁹⁸. Triggered by ULK1 complex, the Class III PI3K complex I (including AMBR, Belclin-1, ATG14L, Vps34 and Vps15) is then translocated from the cytoskeleton to a pre-

autophagosomal structure⁹³. Downstream of Vps34, phosphatidylinositol 3-phosphate (PI3P) binds to WD-repeat protein interacting with phosphoinositides (WIPs) to mediate the formation of the phagophore⁹⁹ together with ATG9 (a lipid carrier for membrane extension)¹⁰⁰⁻¹⁰². The elongation and closure of the phagophore requires ATG12 conjugation system (ATG12–ATG5/ATG16)¹⁰³ and the LC3 conjugation system, that conjugates phosphatidylethanolamine [PE] to microtubule-associated light chain 3 [LC3]¹⁰⁴. As an integral membrane protein present in autophagosomes, LC3-PE (LC3-II) is used as a marker for autophagy. The closed autophagosome fuses with lysosome to form autolysosome, in which the cargos are degraded by hydrolases and metabolites are produced for cells⁹³ (Figure 1.4⁹³).

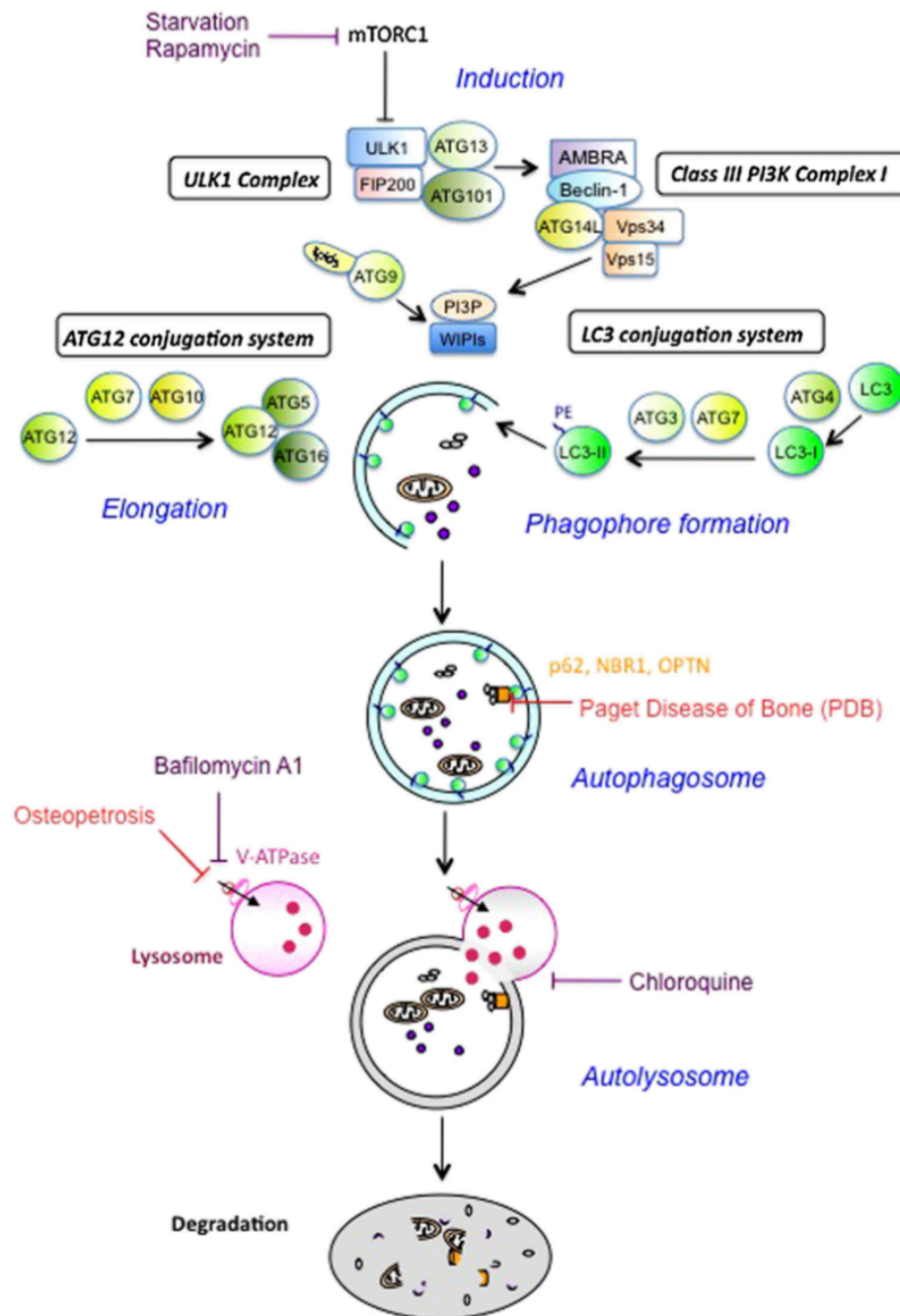


Figure 1.4 The process of autophagy. Autophagy process can be divided into several stages including induction (or nucleation), phagophore elongation, autophagosome formation and autolysosome maturation. Different molecules are involved in the process and autophagy is related with bone diseases including osteoporosis. This figure was adapted from a review by Pierrefite-Carle *et al.*, 2015.

1.3.2 Autophagic flux measurement

The degradation activity in the autophagic system is measured as autophagic flux¹⁰⁵. Autophagic flux can be determined by several LC3II-based assays such as Western blot. Cells are treated with or without the autophagosome-lysosome fusion inhibitor bafilomycin A1 (BafA1) to quantify the amount of LC3II, a marker of autophagosomes, accumulation during a certain period. Other autophagy adaptors such as p62 are also used as autophagic flux markers. A high protein level of p62 usually indicates low or blocked autophagic flux, although increased autophagic flux may also up-regulate p62 expression¹⁰⁶.

1.3.3 Autophagy is required for osteoclast formation and function

Autophagy positively regulates OC formation. Atg7 knockdown during OC formation inhibits the expression of TRAP and cathepsin K (CTSK)^{93,107}. It has been reported that monocyte chemoattractant protein-1 (MCP-1), an inducer of TRAP- and CTSK- double positive pre-OCs^{108,109}, up regulates Beclin-1 (ATG6) and autophagy during OC formation^{110,111}. Recently, researchers showed that Beclin-1 upregulates NFATc1 to promote OC formation in primary BM cells¹¹². Moreover, autophagy is found to positively regulate OC formation in both hypoxia¹¹³ and microgravity¹¹⁴ conditions. TRAF3, a suppressor of OC differentiation, is degraded by autophagy upon RANKL induction in BM OC precursors⁷¹. In sum, the above findings suggest that autophagy is extensively involved in OC formation process.

Autophagy is known to regulate a number of OC functions. It is reported that ruffled border formation, secretory function, and bone resorption ability of OC all require

autophagy-related proteins including ATG5, ATG7, ATG4B, and LC3¹¹⁵. In detail, ATG5 is essential for LC3II targeting to the ruffled border and actin ring¹¹⁶, two vital organelles required for lysosomal secretion in bone resorption process. In animal models, specific deletion of *Atg5* in the macrophage lineage not only increases bone mass but also ameliorates bone loss in ovariectomized mouse. This indicates that modulating autophagy could be a potential therapeutic strategy for osteoporosis. Another group showed that LC3B deletion has no impact on TRAP-positive multinucleated cell formation. However, the bone resorption ability, actin ring formation and CTSK release are all suppressed. They concluded that OC function is correlated with the switch of LC3I to LC3II¹¹⁷.

BafA1 is an autophagy inhibitor by inhibiting V-ATPase activity. BafA1 causes accumulation of autophagosomes and autolysosomes by preventing lysosome acidification, thereby inhibiting autophagosome-lysosome fusion and autolysosome degradation¹¹⁸. It is reported that BafA1 inhibits OC formation *in vitro*^{119,120}. Other autophagy inhibitors such as chloroquine were also found to inhibit OC formation and restore bone mass⁷¹.

Taken together, autophagy is required for OC formation and OC functions. Based on these findings, various autophagy inhibitors can be translational applied to treat bone loss diseases.

1.4 Glycosphingolipid inhibitors

1.4.1 Glycosphingolipids

Sphingolipids are composed of a long chain sphingoid amine¹²¹ and ceramide, and refer to a lipid containing a sphingoid base that comes from acylation of fatty acid. Glycosphingolipids (GSLs) are so named due to the addition of sugars to the ceramide core structure, which are the glycosylated derivatives of sphingolipids¹²². The number of sugars can vary from 1 to more than 20, and GSLs are now found in all vertebrate tissues.

GSLs are initiated at the cytoplasmic face of the endoplasmic reticulum (ER) by serine and palmitoyl-CoA condensation which generates ceramide. Then, glucosylceramide synthase (GCS) converts ceramide to glucosylceramide (GlcCer), which is recognized as an important and rate limiting biochemical step in GSL biosynthesis process^{123,124}. After that, directed by galactosyltransferase I, β -(1,4), the transfer of galactose from UDP-galactose helps GlcCer convert to lactosylceramide (LacCer)¹²⁵. LacCer is the main component for the synthesis more complex GSL¹²⁶. After biosynthesis, GSLs are transported by exocytosis to the plasma membrane. Metabolic recycling of the building blocks is the so-called metabolic salvage pathway for GSL synthesis, in which GSL is released from catabolic degradation^{127,128}. The degradation of GSLs takes place in endosomes and lysosomes. After endocytosis to the acidic compartment, the terminal carbohydrate residues of GSLs are cleaved off by lysosomal glucosidases. Ceramide can then be recycled via salvage pathways or be further degraded^{129,130}. Whilst in the non-lysosomal degradation process, with the help of ceramidases, ceramide can be degraded to sphingosine and a fatty acid¹²⁹ (Figure 1.5).

GSL inhibitors, by their name, are designed to inhibit the synthesis of GSLs. Most GSL inhibitors such as miglustat or eliglustat comes to effect by inhibiting GCS.

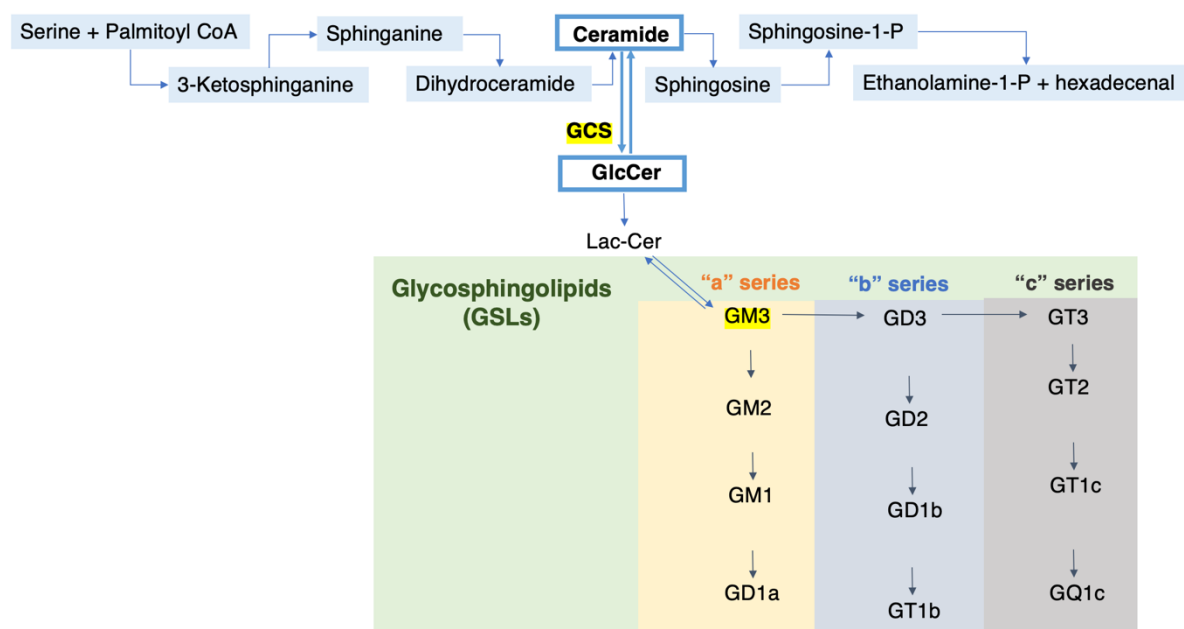


Figure 1.5 GSLs biogenesis.

1.4.2 Eliglustat

Eliglustat, a synthetic small molecule with molecular weight at 404.543g/mol, was marketed by Genzyme as CERDELGA® and globally approved for the treatment of long-term Gaucher disease type 1 in adults given at the dosage of 84 mg capsules once or twice daily in 2014¹³¹ (Figure 1.6).

Gaucher disease type 1 belongs to the class of lysosomal diseases known as glycosphingolipidoses, which due to insufficient production of the enzyme glucosylceramidase that results the accumulation of GSLs, most GSLs are derived from glucocerebroside. The first step in GSL biosynthesis is the formation of

glucocerebroside, the primary storage molecule in Gaucher disease, via glucocerebroside synthase (uridine diphosphate [UDP] – glucosylceramide glucosyl transferase). As a drug belongs to substrate reduction therapy, eliglustat is an oral glucosylceramide analogue (Figure 1.2¹³²) that inhibits glucocerebroside synthase which can further inhibit GSLs.

Clinically, with an IC₅₀ of 10 ng/mL for inhibition of GCS, eliglustat is mainly metabolized by cytochrome P450 (CYP) 2D6 (maximum plasma concentration at 2 hours and terminal elimination half-life was 5-6 hours) and is capable of reducing the complications caused by accumulation of the lipid glucosylceramide, including hepatosplenomegaly, anemia, thrombocytopenia and bone lesions¹³¹. Compared to miglustat, eliglustat is more specific, shares higher potency and less side effects as it is an analogue of the ceramide part. However, eliglustat has several adverse effects (<10%) including arthralgia, headache, nausea and fatigue¹³¹.

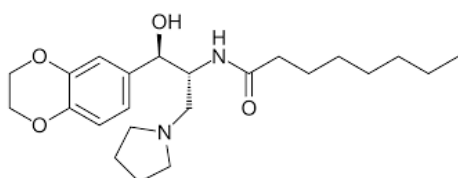


Figure 1.6 Eliglustat. Chemical structure of eliglustat.

1.4.3 Miglustat

Miglustat (C₁₀H₂₁NO₄, 219.28 g/mol), or *N*-butyl-deoxynojirimycin (NB-DNJ), was developed by Oxford GlycoSciences and approved by FDA in 2003, marketed under the trade name Zavesca®. Miglustat is an iminosugar as well as a synthetic analogue of D-glucose¹³³. Miglustat, a GCS inhibitor subsequently inhibits GSL, was found to treat type I Gaucher disease in adults for whom enzyme replacement therapy is unsuitable¹³⁴. Interestingly, patients with Gaucher's disease have a 6–50 times

increased risk of developing myeloma or the pre-myeloma MGUS condition¹³⁵. Moreover, miglustat can dramatically inhibited RANKL-induced OC formation in murine MM model¹⁵. However, miglustat has severe side effects in gastrointestinal track and hence is limited for clinical applications¹³¹.

1.5 Bone diseases and treatment strategies

1.5.1 Myeloma, myeloma induced bone disease and vicious cycle

Multiple myeloma (MM), the second most common haematological cancer, is caused by abnormal plasma cells (MM cells) primarily reside in BM niche¹³⁶. Originated from monoclonal gammopathy of undetermined significance (MGUS) and subsequently progress to smouldering multiple myeloma (SMM), MM is finally reached as characterized by the infiltration of clonal plasma cells into the BM niche and the secretion of monoclonal immunoglobulin M protein. MM induces signs of end-organ damage including hypercalcemia, renal failure, anemia, and bone complications (CRAB features)¹³⁷.

The MM bone complications are particularly important for three reasons as they are a major cause of pain for the patients. Firstly, bone lesions are one of the disease-defining features, as progression of disease from a premalignant state into active MM is characterized by the development of osteolytic bone disease¹³⁸. Secondly, more than 85% of MM patients suffer from skeletal complications including osteopenia, pathological fracture, spinal cord compression and vertebral collapse¹³⁹. Thirdly, the other complication including hypercalcemia is caused due to excessive calcium released to blood stream from bone by OC.

From a physiological perspective, BM is a soft viscous tissue that is located in the bone (tibia, femur) cavities¹⁴⁰. BM consists of extracellular matrix compartment including blood vessels as well as different population of cells (stromal cells like mesenchymal stem cells, immune cells, adipocytes, endothelial cells, fibroblasts, OBs, OCs and chondrocytes) that are involved in haematopoiesis and support haematopoietic cell function¹⁴¹. Moreover, haematopoiesis in BM niche is protected by the bone, which is undergoing a dynamic equilibrium between bone formation OB and bone resorption OC.

From MM pathological perspective, BM is altered to a unique supportive microenvironment for tumour growth and to develop the associated bone disease due to interactions of MM cells with BM resident cells. In detail, MM cells are capable of secreting factors like membrane bounded receptor activator of nuclear factor kappa-B ligand (RANKL), insulin-like growth factor 1 (IGF1) and pro-inflammatory cytokine Macrophage Inflammatory Proteins-1 α (MIP-1 α) that enhance OC proliferation¹⁴². In return, the resorbed bone and OC directly secrete tumour growth factors including IL-6 that stimulates MM cell proliferation. The interactions between MM cells and OCs forms the so-called “vicious cycle”¹⁴³, which further contributes to MM progression and bone disease. In addition to stimulating OC, MM cells can also directly suppress OB by various factors including Dickkopf-related protein 1 (DKK1), which further exacerbates the bone lesions¹⁴⁴. (Figure 1.7¹⁴⁵)

Moreover, BM adipocytes, or bone marrow adipose tissue (BMAT), contribute to the development of MM bone disease¹⁴⁶. Mechanistically, adipokines, growth factors¹⁴⁷ and inflammatory mediators such as IL-6 and TNF α are all released from BMAT, which eventually creates a permissive microenvironment for tumor development¹⁴⁸, drives bone marrow inflammation¹⁴⁹ and may accelerate bone resorption and osteoporosis¹⁵⁰. It is reported BMAT is significantly increased with obesity, which can cause MM engraftment in MM unsusceptible C57BL6 strain and bring significantly low bone quality¹⁵¹.

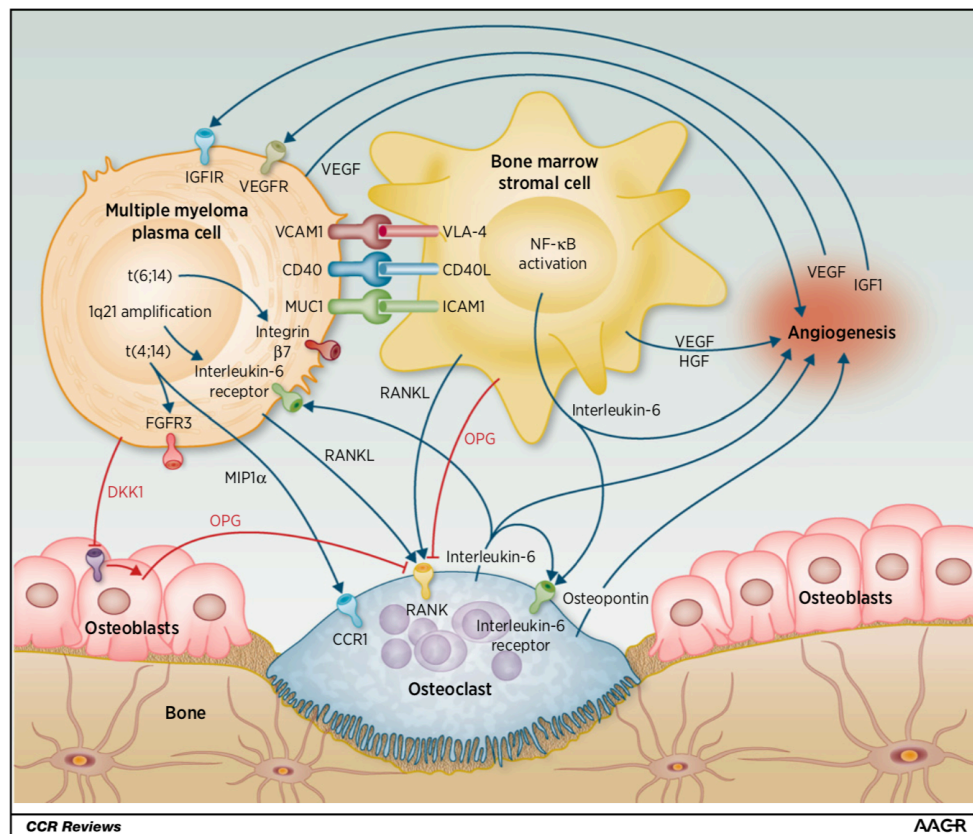


Figure 1.7 MM interaction with OC and OB in BM niche. OCs in MM disease are greatly stimulated by MIP1 α and RANKL which released by MM cells. As a vicious cycle, increased OCs activity causes IL-6 production, a pro-MM factor for MM further proliferation. Adhesion of plasma cells to stromal cells upregulates many cytokines with angiogenic activity. This figure was adapted from a review by Raje *et al.*, 2019.

1.5.2 Current anti-resorptive agents for MM bone disease

Traditionally, the current clinical strategy used to treat MM bone disease can be categorized into either antiresorptive agents or anabolic reagents. The development of bone anabolic agents to treat myeloma bone disease, with many targeting the Wnt signalling pathway' is an area of great research interest, however is beyond the scope of this thesis and therefore will not be discussed in detail. In this section, current clinical antiresorptive strategy will be introduced.

Bisphosphonates (BPs) are currently used as first-line treatments to prevent destructive bone disease in MM. BPs are stable chemical analogues of inorganic pyrophosphate (PPi), in which the P-O-P bond structure is replaced by the chemically stable P-C-P structure. Inorganic pyrophosphate is one of the body's endogenous regulators of biological mineralization¹⁵². BPs, unlike PPi, can also inhibit bone resorption, and have become the basis of the clinical strategy underlying antiresorptive treatment¹⁵³ in diseases in which bone resorption is excessive, such as in Paget's disease, osteoporosis and MM.

BPs as a group can be further divided into non-nitrogen-containing BPs and nitrogen-containing BPs. Clodronate, a member of the non-nitrogen BP subgroup, is believed to cause apoptosis in OCs by becoming incorporated into non-hydrolyzable ATP analogues¹⁵⁴. Pamidronate and zoledronic acid (ZA) are examples of the nitrogen-containing BPs subgroup which bind to hydroxyapatite, and lead to impairment of OC function by inhibiting the enzyme farnesyl diphosphate synthase in the mevalonate pathway of cholesterol biosynthesis¹⁵⁵⁻¹⁵⁷ (Figure 1.8¹⁵⁸).

According to one of the MM research trial, ZA is much more superior in decreasing bone lesions than the non-nitrogen-containing BPs¹⁵⁹. Moreover, different from non-nitrogen-containing BPs, ZA is also capable of reducing MM tumour burden in several clinical trials which make it as the first choice for MM bone lesions at bedside¹⁶⁰. In addition, ZA is more acceptable than pamidronate in reducing infusion time and has relatively less side effects^{157,161,162}. In short, all these factors contribute and justify ZA as the top choice for antiresorptive treatment in MM.

Admittedly, there are several shortages of ZA in clinical use thus novel antiresorptive reagents need to be developed in order to overcome the undesirable side effects. The first and most important side effect is jaw osteonecrosis mainly happens in MM patients¹⁶³, moreover, ZA can even bring femur fracture and suppression of bone turnover in some cases¹⁶⁴. These hazardous effect on bone happens in 4% of patients¹⁵⁹. Secondly, ZA leads to renal toxicity in patients with renal impairment. As renal failure is one of the prominent complications in MM patients, thus limit the actual application of ZA in MM patients¹⁶⁵. Furthermore, some MM patients report they

suffered from gastrointestinal complications, atrial fibrillation and flu-like symptoms since receive the treatment of ZA¹⁵⁹. All these factors limit the consistent ZA administration to MM bone disease patients. Therefore, currently indication for ZA treatment is up to 2 years, and continuation of ZA treatment should follow an interval or break, which jeopardize the treatment expectations.

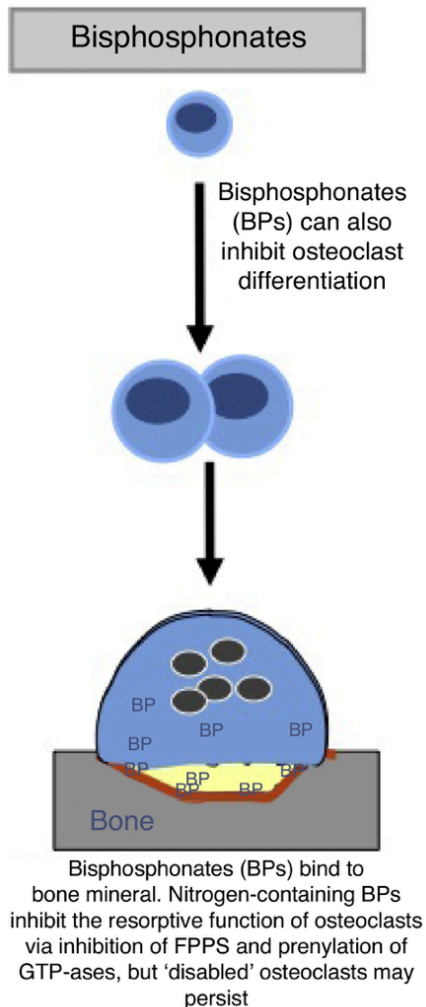


Figure 1.8 Bisphosphonates (BPs)

inhibit OCs. Current findings reveal BPs can 1) inhibit OC activity and promote OC apoptosis, 2) released locally during bone resorption, 3) concentrated under OCs. This figure was adapted from a review by Russell *et al.*, 2015.

1.5.3 Proteasome inhibition as a therapeutic treatment for MM

Bortezomib (BTZ), patented as VELCADEO®, is a kind of proteasome inhibitor that binds reversibly and specifically to the 26S proteasome, a multicatalytic enzyme which involves in ubiquitinated protein degradation¹⁶⁶. Such binding disrupts the proper regulation of important proteins including the p53¹⁶⁷, the oncogenes c-Myc, c-Jun and

c-Fos¹⁶⁸, B-cell leukaemia/lymphoma 2 (Bcl 2)¹⁶⁹ and the inhibitory protein kappa B (I κ B)¹⁷⁰, which ultimately leading to cell cycle arrest and cell death^{171,172}. Interestingly, in BM niche, BM stromal cells seems less sensitive to BTZ as compared to MM cells with the IC50 of BTZ 170-fold higher in BM stromal cells than in MM cells¹⁷². BTZ is given intravenously at 1.3mg/m², then, metabolized by the CYP3A4, CYP2C19 and CYP1A2, with a minor amount of metabolism occurring via CYP2D6¹⁷³ (which is similar as eliglustat).

BTZ not only inhibits MM cell, but also showing anabolic effects on bone according to laboratory findings and clinical observations. First, *in vitro* study show BTZ inhibits OC formation and function^{174,175}. Moreover, BTZ is reported to help restore OB population by decrease dickkopf-1 expression¹⁷⁶. Second, *in vivo* study reveals BTZ upregulates OBs with downregulates of OCs in nonmyelomatous implanted bones¹⁷⁵. Thirdly, clinical trials demonstrate BTZ elevation in bone-specific alkaline phosphatase and osteocalcin, which are the indicators of bone formation¹⁷⁷⁻¹⁷⁹ (Figure 1.9¹⁸⁰).

However, BTZ still encounters some shortages¹⁸¹⁻¹⁸⁷. First, drug resistance was observed in patients, no matter inherent resistance or acquired resistance. This severe limitation makes up to half of patients with MM may not respond to BTZ monotherapy, and the time confirm the patient not respond to BTZ normally at a late stage which may cause detrimental consequence. Second, there are undesired BTZ related toxicities and off-target actions observed at bedside.

Mechanistically, BTZ resistance can be caused by several reasons. Some researchers reported the downstream effectors Bcl-2¹⁸⁸ or NF- κ B¹⁸⁹ are overactivated or overexpressed in some patients which compromise the therapeutic effects. In another research, it is reported the BTZ-binding proteasome β 5 submit encounters mutations or overexpression which decrease the binding affinity and jeopardize the goal of treatment^{190,191}. Moreover, anti-apoptotic proteins (Bcl-2, AKT, MKP-1) show an increase after the BTZ treatment which may eventually set up a pro-MM environment^{192,193}. In view of above listed severe consequences, novel anti-MM agents or drugs assist BTZ in anti-MM arena are needed in order to decrease the sides effects and increase the therapeutic outcomes.

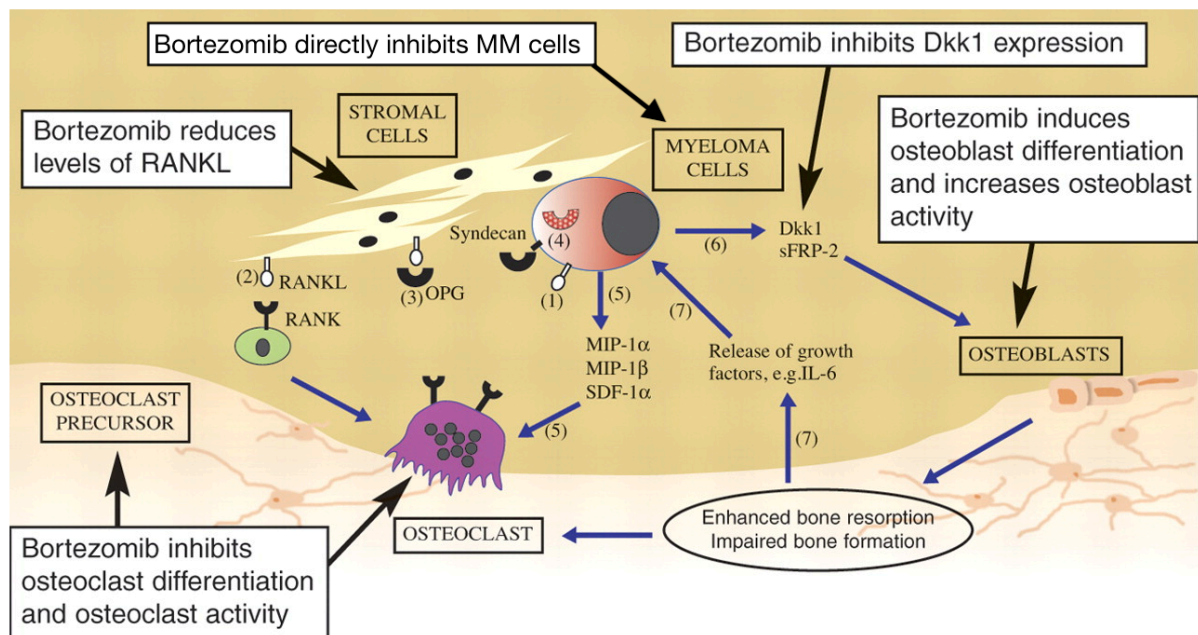


Figure 1.9 BTZ inhibit MM and OCs. According to current findings, BTZ can 1) directly inhibits MM cells, 2) inhibits Dkk1 expression, 3) induces OB differentiation and increase OB activity, 4) reduce RANKL, 5) inhibits OC differentiation and activity. This figure was adapted from a review by Terpos *et al.*, 2007.

1.6 Aims and thesis summary

The treatment strategies for MM bone disease have been extensively investigated in the past few decades. Identifying a novel reagent with translational potential brings benefits both to MM patients and the disease-caused socioeconomic burden. Previous work revealed that miglustat, a GCS inhibitor, ameliorated MM bone disease by inhibiting OC formation¹⁵. However, the precise mechanism is still unknown and miglustat brings severe side effects. Therefore, eliglustat, an FDA recent approved small molecule drug that inhibits GCS for the treatment of Gaucher's Disease¹³¹, is investigated in this thesis to determine whether it can ameliorate MM bone disease and what is the underlying mechanism.

During the course of this thesis, I will therefore, seek to test the following hypotheses:

Hypothesis / Aim 1: choose a suitable preclinical model with bone lesions in order to check whether GSL inhibition with eliglustat ameliorates bone loss status (Chapter 3).

Hypothesis / Aim 2: Eliglustat can ameliorate MM bone disease with negligible side effects (Chapter 4).

Hypothesis / Aim 3: Eliglustat modulates TRAF3 to inhibit OCs via interfere with lipid composition of the cell membrane and work as an autophagy inhibitor (Chapter 5).

Hypothesis / Aim 4: Eliglustat increases the therapeutic effect of ZA and can have translational effect in inhibiting MM development (Chapter 6).

C57BL/KaLwRij strain is well-established MM model after inoculated with murine MM cells, while closely related C57BL/6 strain cannot get MM cells engrafted¹⁵¹. As C57BL/6 mice are used for most genetic models, hence various optimization methods get C57BL/6 engrafted with MM were titrated (Chapter 3), which will open up the utility of this strain to study MM in genetically modified backgrounds in future. Next, eliglustat was tested *in vitro* and *ex vivo* to evaluate its impact on OCs and MM cells. Moreover, eliglustat was administrated *in vivo* to reduce osteolytic bone lesion development (Chapter 4). The subsequent mechanistic studies used primary cells and cell lines revealed that eliglustat is likely to inhibit OC formation via preventing TRAF3 degradation in OC precursors. In addition, eliglustat may come to effect by inhibiting autophagy to stop OC formation and by altering cell membrane lipid orders (Chapter 5). To extend the translational application, eliglustat was found to increase the therapeutic effect of bisphosphonate ZA in reducing MM bone disease. Moreover, eliglustat is likely to inhibit BM MM cells when combined with BTZ and inhibits germinal center on its own (Chapter 6).

Therefore, this project identified a novel small molecule anti-resorptive agent, justified the feasibility of translational use eliglustat in MM bone disease and characterized novel mechanisms of how eliglustat affects OC formation, which brings benefits above and beyond the current treatment strategies.

Chapter 2. Materials and Methods

2.1 Cell line culture

RAW264.7 cell is a murine macrophage cell line obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). RAW 264.7 cells were cultured in DMEM (Sigma) supplemented with 10% FBS (Gibco), 1% Penicillin-Streptomycin (P/S, Sigma, P0781, 1:100) and 1% L-Glutamine (L/G, Sigma, G7513, 1:100) in 5% CO₂ at 37°C.

The 5TGM1-GFP murine cell line was obtained from Prof. Claire Edwards, Botnar Research Centre, NDORMS, University of Oxford. 5TGM1-GFP cells were cultured in RPMI-1640 (Sigma, R8758-500ML) supplemented with 10% FBS (Gibco), 1% Penicillin-Streptomycin (P/S, Sigma, P0781, 1:100), 1% L-Glutamine (L/G, Sigma, G7513, 1:100), 1% Minimum Essential Medium Non-Essential Amino Acids (Gibco, 11140050) and 1% Sodium Pyruvate (Gibco, 11360-070) in 5% CO₂ at 37°C.

Jurkat cells (TIB-152™, ATCC) were cultured in RPMI-1640 Medium (Sigma, R8758-500ML) supplemented with 10% FBS (Sigma), Penicillin-Streptomycin (P/S, Sigma, P0781, 1:100), and L-Glutamine (Sigma, G7513, 1:100) in 5% CO₂ at 37°C.

HEK293T cells (CRL-3216, ATCC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS (Sigma), Penicillin-Streptomycin (P/S, Sigma, P0781, 1:100), and L-Glutamine (Sigma, G7513, 1:100) in 5% CO₂ at 37°C.

2.2 Osteoclast formation assay

RAW264.7 cell can differentiate into OC upon 75 ng/ml RANKL and 50 ng/ml M-CSF stimulation in Minimum Essential Medium Alpha (Gibco, 12571063) supplemented with 10% FBS (Gibco), 1% Penicillin-Streptomycin (P/S, Sigma, P0781, 1:100) and 1% L-Glutamine (L/G, Sigma, G7513, 1:100) in 5% CO₂ at 37°C.

Fresh BM cells were collected from 4-week-old C57BL6 mice. Cells were plated in a 96-well-plate at a density of 2×10^4 cells (after treatment with red blood cell lysis buffer) per well in Minimum Essential Medium Alpha (Gibco, 12571063) supplemented with 10% FBS (Gibco), 1% Penicillin-Streptomycin (P/S, Sigma, P0781, 1:100) and 1% L-Glutamine (L/G, Sigma, G7513, 1:100) in 5% CO₂ at 37 °C. BM cells were first treated with 100 ng/ml M-CSF for 2 days then treated with 20 ng/ml M-CSF and 50 ng/ml RANKL with or without eliglustat for the remaining 6 days with the media changed every 3 days.

To detect the autophagic flux *in vitro*, 10 nM Bafilomycin A1 (Cayman Chemical) was added to cells for 2 hours for autophagic flux analysis.

2.3 Osteoclast TRAP staining

After 6 days, OCs were fixed and TRAP stained. In detail, the culture media was removed from the 96-well-plate and the cells washed with ice cold PBS twice. Cells were then fixed with 80 μ l of 4% paraformaldehyde (PFA) for 1 minute. The PFA was then removed and 100 μ l of the premixed 1:1 ethanol-acetone solution was added to the cells for 5 seconds in order to permeabilize the cells. The solution was then poured

off and the plate was air-dried briefly in the hood. Once dry, 100 μ l TRAP solution was added to each well and the plate was incubated in the dark at 37°C incubator for 15 minutes or until adequately developed. Staining was stopped by removing the TRAP solution and washing the plate under running tap water. Once dry, plates were stored wrapped with foil paper at room temperature.

TRAP-positive cells were counted, and OCs were identified as multinucleated (≥ 3 nuclei) TRAP-positive cells. The size of the OCs was measured using Osteomeasure (Osteometrics) software. Data analysis was performed using Prism software (GraphPad Software).

Table 2.1 Tartrate-resistant acid phosphatase (TRAP) buffer				
Chemical Name	Chemical Formula	Molecular Weight	Concentration (mM)	g/1000ml
Sodium acetate	$C_2H_3NaO_2$	82.0343	50mM	4.1
Potassium sodium tartrate	$KNaC_4H_4O_6 \cdot 4H_2O$	282.22	40mM	11.3
Remarks				
1, Adjust pH to 5.0 with Glacial Acetic Acid.				
2, Adjust the final volume to 1000ml.				
3, Store at 4°C up to 6 months.				

Table 2.2 TRAP Solution				
Chemical Name	Chemical Formula	Molecular Weight	Volume (ml)	mg/50ml
Naphthol AS-MX phosphate	C ₁₉ H ₁₈ NO ₅ P	371.32	NA	5
Dimethylformamide	C ₃ H ₇ NO	73.09	0.5	NA
Mix above two compounds well and only then add following compounds				
Fast red violet LB salt	C ₁₄ H ₁₁ Cl ₂ N ₃ O•1/2ZnCl ₂	376.31	NA	25
See Table 2.1	TRAP buffer	NA	50ml	NA
Remarks				
1, Only use 50ml Corning polypropylene tube for TRAP solution preparation.				
2, Adjust the final volume to 50ml.				
3, Mix well/vortex and syringe filter with 0.45um filter.				
4, Store at 4°C up to 7 days, avoid light.				

2.4 Osteoblast formation assay

Newborn C57BL6 mice (born within 48 hours) were sacrificed with 200µl pentobarbital *i.p.* and then decapitated after cervical dislocation. The skulls were stored in DMEM GlutaMAX media (Gibco by life technologies, 32430-027) on ice. Sterile scissors and forceps were used to remove the soft tissues by first cutting from the base of the skull and around the edge then removing the scalp. The extraneous tissues were further trimmed and the processed calvarial bones were placed in PBS before being transferred into 10ml Calvaria digest solution (Table 2.3) to extract osteoblasts. The

initial digest solution was shaken for 5 minutes at 37°C, and discarded. Subsequent digestion steps were collected and pooled. The digestion process was repeated twice with shaking for 10 min each time with new 10ml Calvaria digest solution. Next, the 20ml of digest solution was centrifuged at 350g for 5 minutes to pellet the cells. The pellet was resuspended with 5ml Red Blood Cell Lysing Buffer (Sigma-Aldrich, R7757) for 5 minutes. After washing off the lysis buffer with DMEM GlutaMAX media (Gibco by life technologies, 32430-027), the cells were counted using a hemocytometer. The cell concentration was adjusted to $5 \times 10^4/\text{ml}$ and $200 \mu\text{l}$ of the media was added to each well of a 48-well-plate. The cells were cultured in DMEM GlutaMAX media (gibco by life technologies, 32430-027) supplemented with 10% FBS (Gibco), 1% Penicillin-Streptomycin (P/S, Sigma, P0781, 1:100) and 1% L-Glutamine (L/G, Sigma, G7513, 1:100) in 5% CO₂ at 37 °C. On the second day, the media was replaced with 1ml Osteogenic Media: DMEM with 10% FBS, 1% P/S, 1% L/G, 10mM β -glycerol phosphate (Sigma-Aldrich, G9422), 0.05nM Ascorbic acid (Sigma-Aldrich, A8960) and 100 nM Dexamethasone (Sigma-Aldrich, D2915) to generate OB. Media was changed every 3 days.

Table 2.3 Calvaria digest solution	
Chemical Name	mg/100ml
Collagenase Type II	100
Dispase	200
PBS	NA
Remarks	
1, Dissolve in PBS.	
2, Prepare immediately before use.	

3, filter the solution through a 0.2 μm Acrodisc 32 Supor before use.

2.5 Glycosphingolipid inhibitors

Eliglustat, a small molecule GSL inhibitor, was provided by Genzyme, a Sanofi Company or purchased from Sigma. For *in vitro* experiments, eliglustat was originally dissolved in DMSO at 10 mM upon arrival and stored at -20°C until diluted immediately before use in culture medium (0.1–50 μM). For *in vivo* experiments, eliglustat was mixed into either a standard rodent diet, or a 42% high fat contained diet at 0.075% w/w, during manufacture by TestDiet. These special diets were kindly provided by Genzyme Corporation (USA).

2.6 *In Vivo* Studies

2.6.1 Study approval

Animal care and experimental procedures were approved by the Home Office and the University of Oxford Local Ethical Review Committee under PPL 30/3218 and PPL 30/2306. Experiments were conducted in accordance with the UK Home Office Animals Act of 1986.

2.6.2 Strains

In each experiment, sex and age matched wild type C57BL/6 mice were purchased from Charles River UK Ltd. Margate, UK. Age and sex matched C57BL/KaLwRijHsd mice were purchased from Harlan for each experiment. They were fed and housed under specific-pathogen-free conditions in the Biological Services Unit, Kennedy Institute of Rheumatology, University of Oxford.

2.6.3 Drug administration

Animals were administered with normal chow, 42% high fat diet (HFD), eliglustat chow (0.075% w/w) or 42% HFD with 0.075% w/w eliglustat started from respective day as described in each of the experiment until sacrifice. All of the above mentioned diets were manufactured by TestDiet and provided by Genzyme Corporation (USA). Zoledronic acid (ZA) was obtained from Sigma (1724827-150MG). Bortezomib (BTZ) was purchased from Cambridge Bioscience (10008822-5mg-CAY). The administration of eliglustat to animals and injections were approved and performed under UK project licenses PPL 30/2306 and PPL 30/3218 as mentioned above.

2.6.4 Tissue and serum processing

Mouse blood sampling was collected by tail vein bleeding using capillary blood collection tubes (16.440, Microvette® CB 300 Z). These 10ul samples were stored on ice for 2 hours and centrifuge at full speed (16,000 g) for 5 minutes to extract serum (supernatant). On the culling day, serum was collected by intracardiac puncture. The serum was stored at -80°C until further analysis.

Mouse spleens were crushed with a syringe plunger through 70 μ m cell strainers (542070, greiner bio-one) with PBS, 0.1% BSA and 2mM EDTA for single-cell suspensions. After centrifugation for 3 minutes at 400g, 4°C, red blood cells (RBCs) were lysed with RBC Lysis Buffer (Sigma, Red Blood Cell Lysing Buffer Hybri-Max™, R7757-100ML) for 5 minutes at room temperature. RBC-lysed splenocytes were washed once more with PBS, 0.1% BSA, 2mM EDTA and then used for experiments.

Mouse bone marrow cells were collected from femur and in some experiments from tibia. Bones were crushed with a pestle and mortar in PBS-0.1% BSA-2 mM EDTA and filtered through 70 μ m cell strainers (542070, greiner bio-one), then stored on ice ready to be used for other experiments without RBC lysis.

Tibiae, were first scanned for micro-CT analysis. Then fixed in 4% PFA at 4°C overnight. On the second day, the tibiae were washed in PBS 4 times for 10 minutes each time. After that, the bones were decalcified and set in paraffin for histological analysis.

Table 2.4 Ammonium chloride lyse (RBC lysing solution, 1X concentration)			
Chemical Name	Chemical Compound	Molecular Weight	g/2000ml
Ammonium Chloride	NH ₄ Cl	53.491	16.04
Sodium bicarbonate	NaHCO ₃	84.007	1.68
Ethylenediaminetetraacetic acid	C ₁₀ H ₁₆ N ₂ O ₈	372.2	0.74
Remarks			
1, Dissolve in 2000ml Millipore water.			
2, Store at 4°C for six months.			

2.6.5 5TGM1-GFP cell inoculation

C57BL/6 mice were sublethally irradiated at 5.5 Gy twice with 4 hours in between before injection of 5TGM1-GFP murine MM cells in 200 μ l HBSS via the tail vein. Mice *i.v.* injected with 200 μ l HBSS served as naive controls. 0.67% Baytril was added to the drinking water for 14 days to avoid infections.

For myeloma cell inoculation, the C57BL/KaLwRij mice were *i.v.* inoculated with a designated number of 5TGM1-GFP MM cells (number as described in each experiment) in 200 μ l HBSS via the tail vein. For the mice in control group, they were *i.v.* injected with 200 μ l HBSS without cells¹⁵¹.

To prepare the 5TGM1-GFP cells for injection, cells from culture flasks in 37°C incubator with 5% CO₂ were centrifuged at 350g for 5 minutes to pellet the cells. The pellet was resuspended with ice cold HBSS and centrifuged again at 350g for 5 minutes as the first wash. This process was repeated for another time in order to completely wash away the remaining FBS in the culture media. Finally, the cell number was counted and the concentration was adjusted for injection^{15,151}.

2.7 Antibodies

Table 2.5 Antibodies used for Western blot and flow cytometry				
Name / Target	Company	Clone	Cat #	Dilution (1:)
Western Blot				
GAPDH	Millipore	6C5	MAB374	5000
LC3	Sigma	Poly	L8918	1000
TRAF3	Cell Signaling	Poly	4729	1000
β Actin	Cell Signaling	8H10D10	3700	5000
P62 (SQSTM1)	MBL	NA	PM045	1000
Flow Cytometry				
CD16/CD32 Monoclonal Antibody (FcR Block)	Thermo Fisher	93	14-0161-85	200
APC Annexin V	BioLegend	NA	640920	50

Zombie Aqua™ Fixable Viability Kit	BioLegend	NA	423102	400
Brilliant Violet 605™ anti-mouse/human CD11b Antibody	BioLegend	M1/70	101237	200
PE/Cy7 anti-mouse Ly-6C Antibody	BioLegend	HK1.4	128018	200
Brilliant Violet 785™ anti-mouse Ly-6G Antibody	BioLegend	1A8	127645	200
APC anti-mouse CD115 (CSF-1R) Antibody	BioLegend	AFS98	135510	200
PE anti-mouse CD117 (c-Kit) Antibody	BioLegend	2B8	105808	200
Alexa Fluor® 700 anti-mouse/human CD45R/B220 Antibody	BioLegend	RA3-6B2	103232	200
Brilliant Violet 421™ anti-mouse CD3 Antibody	BioLegend	17A2	100227	200
Brilliant Violet 421™ anti-mouse Ly-6A/E (Sca-1)	BioLegend	D7	108127	400

APC/Cyanine7 anti-mouse CD117 (c-kit) Antibody	BioLegend	2B8	105826	200
PE/Cy7 anti-mouse CD150 (SLAM) Antibody	BioLegend	TC15-12FL12.2	115913	100
APC anti-mouse CD48 Antibody	BioLegend	HM48-1	103411	200
Alexa Fluor 700 CD34 Monoclonal Antibody	eBioscience	RAM34	56-0341-82	200
PE anti-mouse CD16/32 Antibody	BioLegend	93	101308	200
Brilliant Violet 421™ anti-mouse CD138 (Syndecan-1) Antibody	BioLegend	281-2	142508	200
FITC anti-mouse/human CD45R/B220 Antibody	BioLegend	RA3-6B2	103206	200
Alexa Fluor® 700 anti-mouse IgD Antibody	BioLegend	11-26c.2a	405730	200
PE-Cy™7 Hamster Anti-Mouse CD95 (FAS)	BD Pharmingen	Jo2	557653	200
Alexa Fluor® 647 anti-mouse/human GL7	BioLegend	GL7	144606	200

Antigen (T and B cell Activation Marker) Antibody				
PE/Cy5 anti-mouse CD4 Antibody	BioLegend	GK1.5	100410	800
PE/Cy5 anti-mouse CD5 Antibody	BioLegend	53-7.3	100610	800
PE/Cy5 anti-mouse CD8a Antibody	BioLegend	53-6.7	100710	800
PE/Cy5 anti- mouse/human CD11b Antibody	BioLegend	M1/70	101210	800
PE/Cy5 anti- mouse/human CD45R/B220 Antibody	BioLegend	RA3-6B2	103210	200
PE/Cy5 anti-mouse TER-119/Erythroid Cells Antibody	BioLegend	TER-119	116210	300
PE/Cy5 anti-mouse Ly- 6G/Ly-6C (Gr-1) Antibody	BioLegend	RB6-8C5	108410	400
PE/Cy5 anti- mouse/human CD11b Antibody	BioLegend	M1/70	101210	800

PE/Cy5 anti- mouse/human CD45R/B220 Antibody	BioLegend	RA3-6B2	103210	200
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2.8 Flow cytometry (FACS)

2.8.1 Surface marker staining

Cells prepared as mentioned in section 2.6.4 were first stained with fixable Live/Dead staining (BioLegend) and FcR block in PBS for 20 minutes. Then, the samples were topped up with PBS, 0.1% BSA, 2mM EDTA as a wash. After centrifugation for 3 minutes at 400g, 4 °C, the samples were stained with surface marker antibodies in PBS at 4°C for another 20 minutes. The cells were washed once with PBS, 0.1% BSA, 2mM EDTA. Stained cells were analysed using four-laser LSR Fortessa X-20 (BD Bioscience). For some experiments, cells were fixed with 4% paraformaldehyde (PFA) in PBS at room temperature for 5 minutes, then washed once and stored in PBS at 4 °C for up to 3 days. Acquired data was analysed using FlowJo 10.2. For Annexin V staining, cells were washed with PBS to remove medium. Then, cells were stained for Live and dead in Annexin V (1:50, Biolegend) binding buffer at 4 °C for 20 minutes. The cells were washed again with Annexin V binding buffer and analyzed by flow cytometry in Annexin V binding buffer without fixation.

2.8.2 Intracellular staining

The plasma cells (PCs) were intracellularly stained before analysis by flow cytometry. Specifically, and different from other intracellular staining, Fc blocker at 1:50 for 125

$\mu\text{l/well/sample}$ was required for PCs. The spleens were digested in freshly prepared digestion buffer including: collagenase IV (0.5mg/ml), DNase at 1:250 in RPMI buffer; 1.5ml digestion buffer was used for half a spleen dissected into pieces. After 30 minutes at 37°C° on a shaker, the small pieces were mashed through a 100 μm strainer (Greiner bio-one). The digestion was stopped by adding 2mM EDTA (1:250 from 0.5M stock) to the buffer and the samples were incubated for 5 minutes at 37 C° on a shaker. Then, the samples were topped up by 5% FBS in PBS as a wash and the cell numbers were counted accordingly.

5×10^6 cells were loaded into each well of a 96-well-plate and the plates were centrifuged (350 g, 3 minutes, 4 C°) to collect the pellets. After that, the pellets were re-suspended in FC block buffer and after another wash the pellets were re-suspended in antibody mixture. Each staining step took 20 minutes and the cells were kept on ice and placed in a dark place. Finally, the cells were washed another two times with 5% FBS in PBS ready for flow cytometry analysis.

2.9 Western blot

Cells from primary tissue cultured *in vitro* and cell lines were washed once with PBS and lysed using NP-40 lysis buffer together with proteinase inhibitors (Sigma) on ice.

After spinning down the debris, the quantification of protein concentration in the supernatant was measured by BCA Assay (23227, Thermo Fisher). The supernatant was then moved to new eppendoffs. Reducing Laemmli Sample Buffer (NP0008, life technologies) was then added to the supernatant and heated at 100°C for 10 minutes.

15-30 μg of protein was loaded per well for SDS-PAGE analysis. For non-LC3 western blot, NuPAGE Novex 4-12% Bis-Tris gradient gel (Thermo Fisher) with MES running buffer (Thermo Fisher) was used. Specifically, 15% Tris-HCl gel and SDS running buffer was used for LC3 western blot in order to separate LC3-I and LC3-II.

After that, proteins on the gel were transferred to the PVDF membranes (IPFL00010, Millipore). Next, the membranes were blocked with 5% skimmed milk-TBST (TBS+0.1% Tween 20) for one hour. After pouring off the blocking buffer, membranes were incubated with primary antibodies in 1% milk at 4°C for 12 hours. After washing, the membranes were incubated with secondary antibodies in 1% milk with 0.01% SDS for one hour at room temperature. After another wash, the membranes were imaged using the Odyssey CLx Imaging System. Acquired data were analysed using Image Studio Lite.

Table 2.6 NP-40 lysis buffer (2X)

Chemical Name	Chemical Compound	Molecular Weight	Concentration (Mm)	g/1000ml
Tris Base	$\text{C}_4\text{H}_{11}\text{NO}_3$	121.1	20	2.4
Sodium chloride	NaCl	58.4	300	17.5
Ethylenediaminetetraacetic acid (EDTA)	$\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_8$	372.2	2	0.746
ethylene glycol-bis (β-aminoethyl ether) - N,N,N',N'-tetraacetic acid	$\text{C}_{14}\text{H}_{24}\text{N}_2\text{O}_{10}$	380.4	2	0.77

(EGTA)				
Remarks				
1, Adjust pH to 7.5.				
2, Adjust the volume to 1000ml.				

Table 2.7 15% Tris-HCl polyacrylamide gels		
For 4 gels	5% (Stacking)	15% (Separating)
H ₂ O (ml)	9.6	9.2
30% Acrylamide/Bis-acrylamide (ml)	2.4	20
Buffer (ml)	1.75	10
10% SDS (μ l)	140	400
10% Ammonium persulfate (μ l)	140	400
TEMED (μ l)	14	16
Total (ml)	14	40
Remarks		
1, Stacking buffer: 1M Tris (pH=6.8).		
2, Separating buffer: 1.5M Tris (pH=8.8).		

2.10 Quantitative PCR

Polymerase chain reaction (PCR) technology was used to detect the change in gene expression in cells. Primary cells or cell lines were washed three times with ice cold PBS. RNA was extracted using the RNeasy Plus Mini Kit (74134, Qiagen). The

concentration of RNA was measured using NanoDrop1000 (Thermo Fisher). High Capacity RNA-to-cDNA Kit (Thermo Fisher, 4387406) was used to reverse transcribed the RNA to cDNA. Taqman probes (Thermo Fisher) and TaqMan Gene Expression Master Mix (Thermo Fisher, 4369016) were used for the qPCR reactions according to the manufacturer's instructions. The ViiA 7 Real-Time PCR System (Thermo Fisher) was used to conduct the quantitative PCR. The quantification of target mRNAs expression was based on GAPDH. $\Delta\Delta C_t$ method was used in all of the experiments.

Table 2.8 TaqMan® probes for quantitative PCR	
Gene	Assay ID
TRAF3	Mm00495752_m1
GAPDH	Mm99999915_g1
β Actin	Mm00607939_s1

2.11 Micro-CT analysis

After the tibiae were harvested, they were scanned with a Perkin Elmer Quantum FX scanner, Rigaku micro-computed tomography (CT) technology, Caliper Life Science. In detail, the region beneath the growth plate of proximal tibia was scanned at energy of 90KV; 200uA; field of view (FOV) 10 for 3 min. Scan data was analysed by using Analyse 12.0 software. In detail, the analysis can be divided into two parts: the generation of standard curve and analyse bone scans.

Firstly, a standard curve was generated by scanning the photons of known density, provided by the manufacturer. The readout of scanned photons and the known density can be correlated with a linear function. Then the slope value of this linear function is

SigmaCT and the offset of this linear function is BetaCT. According to the manufacturer's instructions, the SigmaCT and BetaCT is unique in each experiment as the energy generated by micro-CT is slightly different from time to time and each time such calculation secured the consistency across different experiments.

For the bone analysis, the previously scanned 3D bones were loaded and straightened. Then, the region of interest was defined as 100 slices below the growth plate. To ensure the region to be analysed was composed purely of bone, the threshold calculated from above the standard curve was applied to define and clean up bone images. Next, the bones were measured and analysed together by the threshold, SigmaCT and BetaCT from the above calculation. Lastly, the bone 3D reconstruction images were exported.

2.12 Osmium tetroxide stain

Osmium tetroxide (Honeywell Fulka, 251755-2ml) staining was used to visualize the adipose tissue in the right tibiae. After the muscles and tendons were removed, the tibiae were fixed in 4% PFA overnight. Then, the bones were scanned at 90KV; 200 μ A; FOV10 for 3 minutes. The acquired images would be used later to overlay with the Osmium stained tibiae.

Then the bones were decalcified by EDTA decalcification solution at 4 °C. The EDTA decalcification solution was changed every three days for two weeks. After two weeks, the EDTA decalcification solution was washed away with tap water and the tibiae were stained by osmium tetroxide staining solution.

For osmium tetroxide staining, the tibiae were transferred into osmium tetroxide which was composed of $K_2Cr_2O_7$ and OsO_4 (Sigma, 75632) for 48 hours at room temperature in the fume hood. Then, the tibiae were washed five times with water. All the waste solution was considered dangerous and needed to be handled carefully. 4% osmium tetroxide solution was fully neutralized by four times the volume of corn oil as corn oil has a high concentration of unsaturated bonds. In detail, four times the volume of corn oil was poured into one volume of the osmium tetroxide solution in fume hood. After the oil completely turned black, the completion of neutralization was checked by suspending a filter paper just soaked in clean corn oil over the solution. If the filter paper turned black, then, the osmium tetroxide was not fully neutralized and more corn oil was added until the filter paper did not become black any more. Finally, the corn oil dissolved with osmium tetroxide was collected in a container and dispose through the chemical safety office.

Finally, the tibiae stained with Osmium tetroxide were embedded in 1% agarose gel and scanned by micro-CT using 90KV; 200 μ A; FOV10 for 3 minutes.

Table 2.9 EDTA decalcification solution			
Chemical Name	Chemical Compound	Molecular Weight	g/1000ml
Ethylenediaminetetraacetic acid (EDTA)	$C_{10}H_{16}N_2O_8$	372.2	41.4
Sodium hydroxide	NaOH	39.997	4.4

Remarks
1, Adjust pH to 7.0–7.4.
2, Adjust the volume to 1000ml and gentle heating to dissolve EDTA.

Table 2.10 Osmium tetroxide staining solution

Chemical Name	Chemical Compound	Molecular Weight	Concentration	Volume (ml)
Potassium dichromate	K ₂ Cr ₂ O ₇	294.185	5% (250 mg to 5 ml water)	4
Osmium tetroxide	OsO ₄	254.23	4%	2
Milli-Q Water	H ₂ O	18		2
Remarks				
1, prepare in the fume hood on the day of use.				
2, Above compounds mixed in glass scintillation vials.				
3, Above 8 ml solution can be used for 6 tibiae.				

2.13 Histomorphometry analysis of tibiae

Following micro-CT scanning, the left tibiae were immersed into 10% EDTA decalcification solution for two weeks and the solution was changed every 3 days. Two weeks later, the left tibiae were paraffin embedded and were sectioned with a Leica RM2235 microtome at 5 μ m per section to generate histology slides (VWR 631-1553).

After the histology slides were generated, they were incubated at 65°C for 12 hours in order to dewax. After 12 hours, the slides were rehydrated in graded ethanol in the following sequence: 100% ethanol, 90% ethanol, 80% ethanol, 60% ethanol and finally water. The slides were immersed in each graded ethanol for 3 minutes before moving to the lower alcohol percentage.

The slides were stained for TRAP activity using freshly prepared TRAP solution as described previously for 30 minutes at 37 °C incubator. After 30 minutes, the slides were taken out and gently washed with running tap water for 3 minutes. To better visualize the OC stained with TRAP, the slides were then counterstained with 0.2% methyl green solution (Bioenno Lifesciences, 003027) for 30 seconds as the background. Then, the slides were washed again with running tap water for 3 minutes and left to air dry overnight. Finally, one drop of DePex mounting medium (VWR, 361254D) was added to each glass slides and covered with coverslips (VWR, 631-0137) for histomorphometry analysis. Slides were analysed and pictured using an Olympus BX51 microscope. Osteomeasure (Osteometrics) software was used to quantify OC and OB parameters by using standard methods^{194,195}. Moreover, the *in vitro* OC area quantification in this project was measured by Osteomeasure (Osteometrics) software.

2.14 ELISA analysis

Enzyme-linked immunosorbent assay (ELISA) technology was used to detect the expression level of specific proteins from murine serum samples in experiments.

The IgG2bk ELISA was conducted as per the manufacturer's instructions (Bethyl Laboratories, E90-109). Serum from C57BL6 strain was diluted at 1:50,000 while the serum from C57BL/KaLwRijHsd strain was diluted at 1:100,000 in order to obtain the IgG2bk expression level within the best detectable range.

For the mouse IgG isotype detection, the mouse Ig isotyping uncoated ELISA kit was used according to manufacturer's protocol (Thermo Fisher, 88-50630-88). The measurement of serum urea, serum creatinine and serum calcium were analysed by MRC Harwell Institute.

2.15 Alkaline phosphatase measurement

ALP is regarded as an early marker of OB differentiation. As a byproduct of OB activity, the high level of ALP indicates the increased active bone formation. On day 7, the OBs monolayer in a 48-well-plate was washed with ice cold PBS twice. After carefully aspirating the PBS, the OBs were fixed with acetone-ethanol at a ratio of 1:1 for 5 minutes. Then, the fixation media was taken off and OBs were stained with SIGMA FAST™ BCIP®/NBT (Sigma-Aldrich, B-5655) (1 tablet dissolved in 10ml dH₂O) for 30 minutes at 37°C. After the incubation, the media was removed and cells expressing ALP turned a dark purple colour. On day 7, ALP can be quantified using the LabAssay™ ALP Kit (Wako Pure Chemical Industries, 291-58601) according to manufacturer's instructions.

2.16 Alizarin red stain

Alizarin red solution stains mineral deposits from OBs a bright red colour that is easily visualized by the naked eye. On day 21, before alizarin red stain, the culture medium was carefully aspirated from the 48-well-plate and the adherent cells were washed with ice cold PBS twice. The cells were fixed using 70% ethanol from the -20°C freezer for 1 hour at 4°C. The ethanol was then carefully removed from the wells and the cells were gently rinsed with water three times without disturbing the monolayer. After air dry briefly, 1% aqueous Alizarin Red Solution (Sigma, A5533-25G) with pH adjusted to 4.2 by the addition of 10% ammonium hydroxide. After 20 minutes' incubation at room temperature, the excess dye was removed and the monolayer was washed for five times with water. Stained OBs were then imaged.

2.17 Maximum load measurement

The muscles were removed from femurs on the day of sacrifice and the femurs were stored at -80°C until the day of maximum load analysis. On the day of analysis, the femurs were taken out of the freezer and rehydrated in PBS for 4 hours. The maximum extension load measurements for the femurs were conducted using a testing machine obtained from Instron Calibration Laboratory (5942K9817) according to manufacturer's instructions.

2.18 Plasma membrane generalized polarization measurement

The plasma membranes are highly ordered membrane compartment consists of cholesterol, sphingomyelin and GSLs (cite). To evaluate the cholesterol and saturated lipid content in the outer leaflet of the plasma membrane, NR12S, the

molecular probe was used in this project. Based on the modification of Nile Red solvatochromic fluorescent dye with an amphiphilic anchor group, NR12S dye can reversible redox switching of its fluorescence at outer leaflet using sodium dithionite. By using fluorescence microscopy, NR12S stained plasma membrane show the emission colour between blue (liquid ordered phase) and red (liquid disordered phase) spectrum.

RAW264.7 cells were plated at a density of 5×10^4 cells per small glass petri dish. After each treatment, the cells were washed once with Opti-MEM media and NR12S in Opti-MEM media was added at $0.05 \mu\text{M}$ and incubated for 5 to 10 minutes in 37°C incubator.

Then, the unique NR12S emission spectrum of plasma membrane precisely differentiates the liquid ordered phases and the disordered phases under the fluorescence microscopy, which makes it as a feasible tool to evaluate the generalized polarization of the cell membranes.

2.19 Statistical analyses

The data analysis was conducted using Prism (GraphPad) software. All the data shown in the thesis are represented as mean $\pm$ standard error of the mean (SEM).

Two-tailed t-test were 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 two normally distributed data sets have unequal variances, Welch's unequal variances t-test was used. In Chapter

4 and Chapter 6, t-test was used in comparisons between treated and non-treated mice with the null hypothesis “eliglustat makes no difference between MM mice and MM+Elig mice”, and between MM+ZA mice and MM+Elig+ZA mice with the null hypothesis “eliglustat does change the bone parameter/function in MM+ZA mice”. Since above two null hypotheses are independent with each other, and each of them leads to an independent conclusion. Therefore, the statistical analyses were conducted individually without combining analyses together. In above mentioned cases, two independent t-tests were used without adjust P value correction. The data from all experimental groups were pooled together in one graph for clear visualization without altering statistics.

For the experiments that contain more than three experimental groups, One-way analysis of variance (ANOVA) and multiple comparisons was 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. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, ns, not significant.

Chapter 3. Establishing Animal Models

3.1 Introduction

Bone loss is common under pathological conditions. For example, in cancer related bone diseases including MM, bone loss is determined by low bone mineral density (BMD) (T-score ≤ -2.5) measured from dual-energy x-ray absorptiometry (DXA)¹⁹⁶.

As bone loss appears in many conditions, the aim of this chapter was to choose a suitable preclinical model with bone lesions in order to check whether GSL inhibition with eliglustat ameliorates bone loss status.

Eliglustat is preliminary designed for the treatment of Gaucher's Disease (GD), and it is reported that GD is linked with MM^{197,198} a haematological cancer accompanied with bone loss¹⁹⁹. Therefore, MM could be a disease which is potentially suitable to check the function of eliglustat both from a pathogenesis perspective and bone loss perspective.

To select a suitable MM preclinical model with bone lesions for the study of eliglustat, both the common C57BL/6 strain and closely related C57BL/KaLwRij strain were evaluated individually.

C57BL/KaLwRij is an immunocompetent strain closely related to the C57BL/6 strain. The C57BL/KaLwRij mouse has a distinct microenvironment that can spontaneously develop benign idiopathic paraproteinemia, which is similar to MGUS in humans²⁰⁰. Interestingly, the mouse can progress to murine myeloma at a similar rate to humans, around 1% annually. Moreover, the MM bearing C57BL/KaLwRij mouse has the typical bone loss phenotype and bone lesions. The 5TGM1 murine myeloma cell was

derived from a C57BL/KaLwRij mouse that spontaneously developed MM²⁰¹. In this study, the work related with C57BL/KaLwRij strain is reproduced to exam whether this mature preclinical model is a reliable MM model for future studies.

As C57BL/6 mice are widely used for most genetic studies, hence researchers have tried several different MM models in C57BL/6 strain, which will allow the study of MM in genetically modified backgrounds in the future. Since the normal C57BL/6 strain does not establish MM engraftment²⁰², researchers have employed the immunocompetent Vk*MYC transgenic mouse for the preclinical study of MM; a modified strain based on C57BL/6. Mechanistically, the activation of MYC in specific murine plasma cells (Vk12653 and Vk12598) leads to immunophenotypic markers of MM²⁰²⁻²⁰⁴.

Using C57BL/6 mice that have not been genetically modified, Fowler *et al.* have injected BM stromal cells derived from a MM mouse together with 5TGM1-GFP MM cells into the C57BL/6 strain and observed MM-like conditions²⁰⁵. In other experiments it has been shown that feeding C57BL/6 mice with 42% HFD leads to the MGUS or pre-MM condition in B57BL6 mice after 5TGM1-GFP cell inoculation¹⁵¹. Furthermore, aged C57BL/6 mice become ill 2 to 3 months after injection with 5T33 MM cells. Under these conditions there was no detectable serum IgG2b level change²⁰⁶ however, there were some differences in IgG2b CDR3 mRNA and IgH CDR3 mRNA²⁰⁶. Glick *et al.* conclude that C57BL/6 strain resistance to MM was a constitutional capacity of certain aged mice and is not a random event²⁰⁶ (inoculated 3 times and still no MM engraftment).

Chapter 3. Establishing Animal Models

3.2 Results

3.2.1 MM susceptibility and bone lesions in C57BL/KaLwRij mice

The C57BL/KaLwRij strain is a MM susceptible strain, developing typical MM complications including bone lesions²⁰². To confirm that 5TGM1-GFP cells can engraft in C57BL/KaLwRij mice, mice were divided into two groups, a control group without 5TGM1-GFP cell injection, and a MM group with 5TGM1-GFP cell injection. Serum was collected on day -2 as the baseline and mice from the MM group received 5TGM1-GFP cell inoculation on day 0. On day 14, serum from all the mice was collected again and the mice were culled on day 21 (Figure 3.1).



Figure 3.1 Experiment time line for inoculat 5TGM1-GFP cells to C57BL/KaLwRij

mice. Mice were divided into 2 groups a control group and MM group. 8-week-old C57BL/KaLwRij mice were injected with 5TGM1-GFP MM cells and sacrificed depending on weight loss or lameness. The serum was collected several times during the experiment to monitor the tumour progression.

The *in vivo* tumour burden of BM and spleen were examined by flow cytometry under the GFP channel to detect the presence of 5TGM1-GFP cells. According to the GFP positive cell percentage shown in BM samples and spleen samples, there was a significant in tumour burden in the MM group. In BM samples, around 30-40% of the total cells in the MM group were tumour cells. In the spleen, the average number of tumour cells was around 5% of total cells (Figure 3.2A). This model has 30-40% BM MM cells which makes tumour burden quite obvious. From above perspectives, the

C57BL/KaLwRij model seems to be a good candidate model for the study of elglustat's role in MM and its associated bone lesions. Furthermore, the IgG2b κ levels show a significant increase in the MM group confirming the finding from the flow cytometry data (Figure 3.2B).

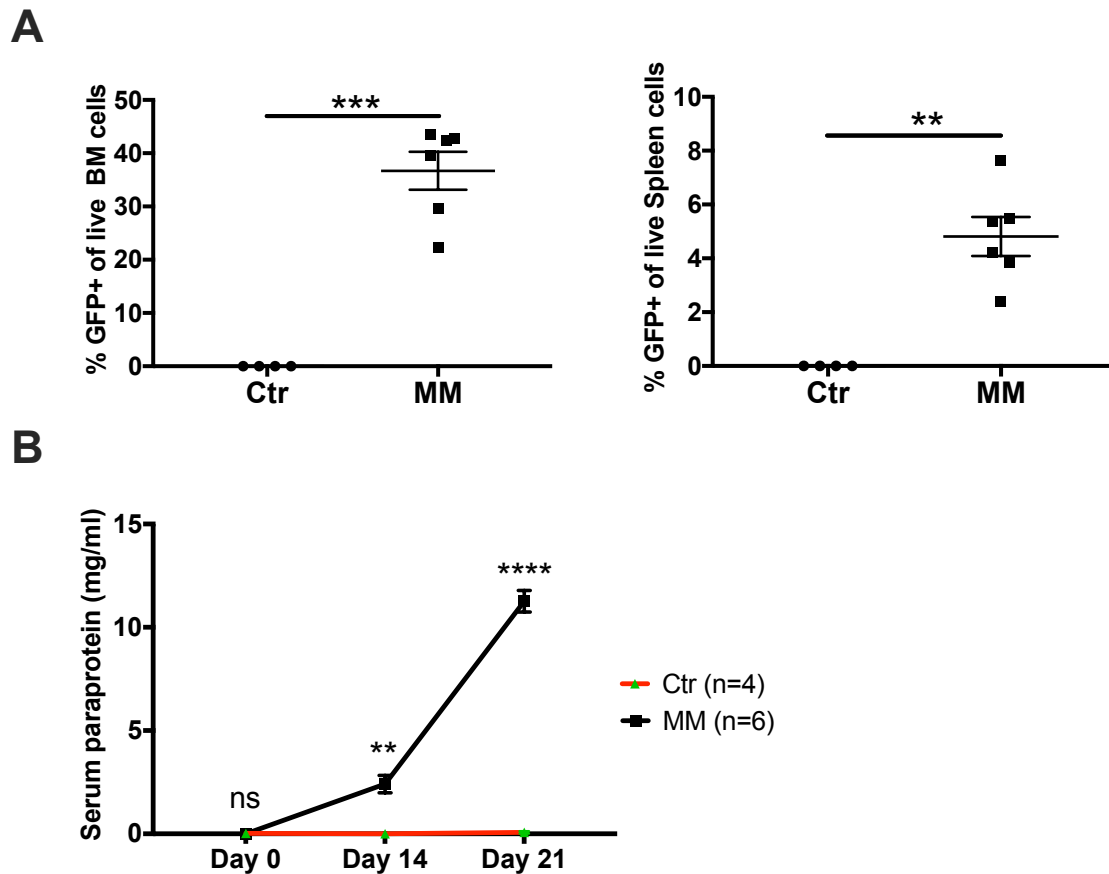


Figure 3.2 MM burden measurements following 5TGM1-GFP cell inoculation in

C57BL/KaLwRij mice. Mice were divided into 2 groups, a control group and MM group.

8-week-old C57BL/KaLwRij mice were injected with 5TGM1-GFP MM cells and sacrificed depending on weight loss or lameness. **(A)** The BM cells and spleen cells from each

mouse were collected for flow cytometry analysis on day 21. **(B)** The serum IgG2b κ

paraprotein was measured by ELISA. Error bars correspond to SEM. **P<0.01, ***P<0.001,

****P<0.0001, ns means non-significant. Statistical analysis was performed using Student *t*

test.

In addition, the weight of C57BL/KaLwRij after inoculation with 5TGM1-GFP MM cells was monitored and showed that the mice did not differ significantly in weight from the non-myeloma bearing control mice (Figure 3.3).

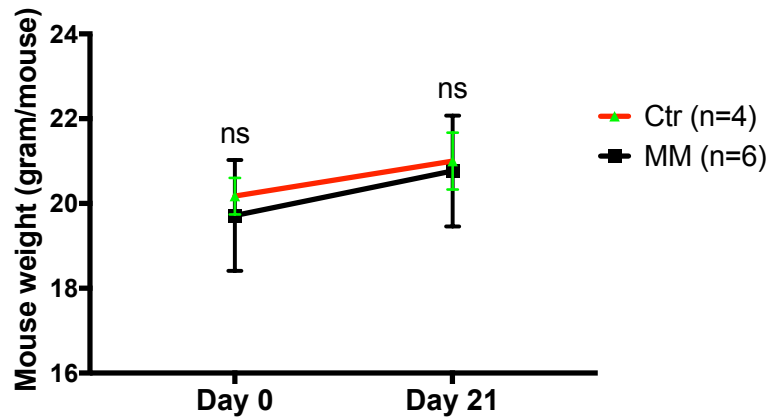


Figure 3.3 Body weights of C57BL/KaLwRij mice before and after 5TGM1-GFP cell inoculation. 8-week-old C57BL/KaLwRij mice were divided into 2 groups a control group and MM group.

Next, bone lesion development was evaluated following 5TGM1-GFP MM cell inoculation. The tibiae were collected and analysed by micro-CT. According to the 3D reconstruction images, the MM bone had more cortical bone holes compared with the bone from control group (Figure 3.4A). This difference demonstrates that 5TGM1-GFP cells were capable of generating bone lesions in cortical bone. To quantify the bone lesions in the cortical bone, each hole on the cortical bone of tibia was counted manually. According to the statistical analysis, the MM group had a significant increase of the holes as compared with control group, which reveals that the 5TGM1-GFP MM cells cause cortical bone lesions in the C57BL/KaLwRij strain (Figure 3.4B).

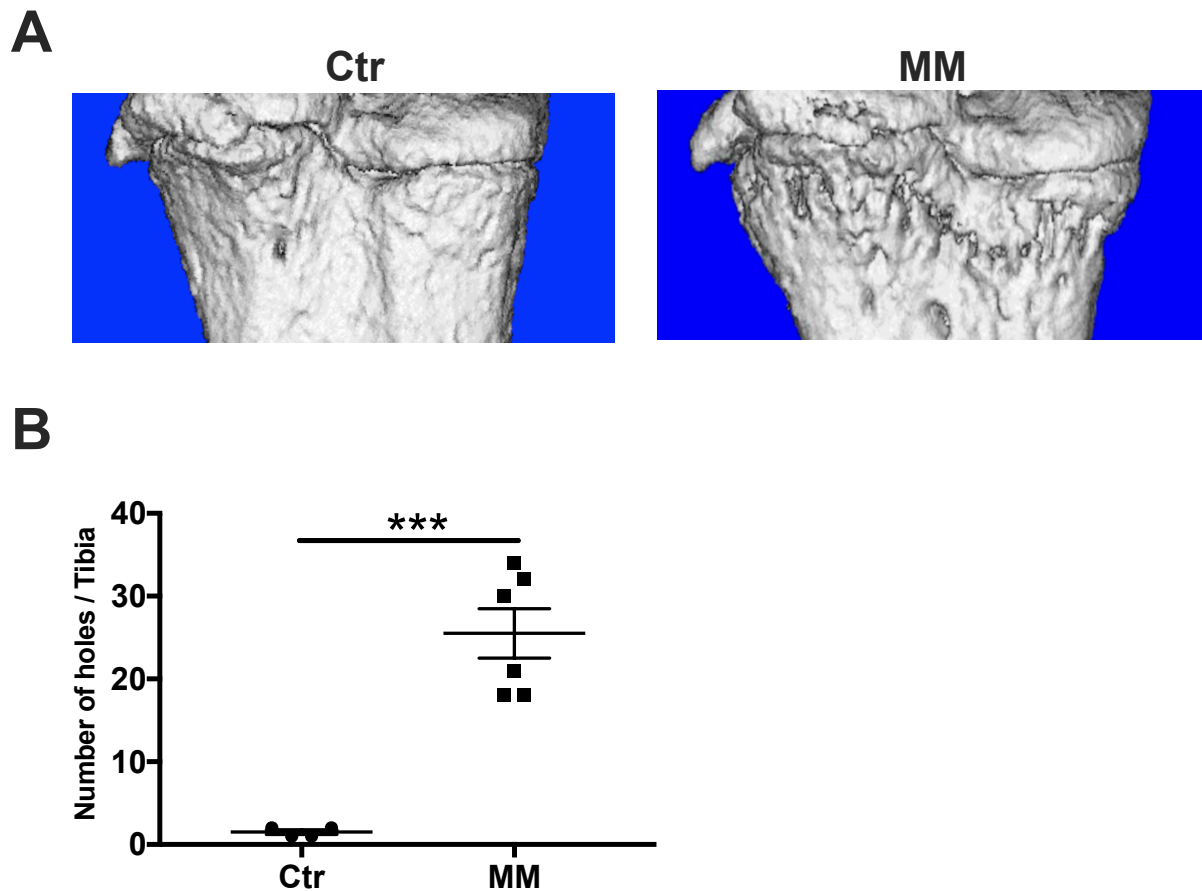


Figure 3.4 Number of cortical bone lesions Holes on tibia were counted from each group, naive control group (n=4) and 5TGM1-GFP MM group (n=6). **(A)** 3D reconstruction of the cortical bone from respective groups. **(B)** The holes on cortical bones were counted and compared. Error bars correspond to SEM. ***P<0.001. Statistical analysis was performed using Student *t* test.

To further confirm the finding, different micro-CT bone were used to quantitatively evaluate the bone quality. Bone volume over total volume (BV/TV) represents bone volume fraction, which provides information on “how much” of the bone contains mineralized tissue distributed in the interior compartment. Therefore, decrease or increase of BV/TV reveals the loss or gain of mineralized bone. Bone surface (BS) means the surface of overall bone, the higher of BS indicates more bone in the region of interest. Bone surface over bone volume (BS/BV) indicates the relative ratio of bone surface over bone volume, the higher the ratio means the lower the bone volume. Trabeculae separation (Tb.Sp) is an indicator of the ability to participate in a bone’s resistance to bending and compression. The higher Tb.Sp, the more likely the bone is able to be bended and compressed. However, Trabeculae number (Tb.N) is the inverse of the mean spacing of Tb.Sp, indicating the number of trabecular bone. So the higher Tb.N, the more unlikely the bone is to fracture. Trabeculae thickness (Tb.Th) means trabecular thickness, the thicker the trabecular, the better it can resist the bending. Connective density (Conn.D) represents how trabeculae contributes to bone strength by the interconnectivity. The higher the Conn.D, the more robust the bone. In sum, all above mentioned parameters indicate the MM group has the bone with less integrity as compared with control group (Figure 3.5).

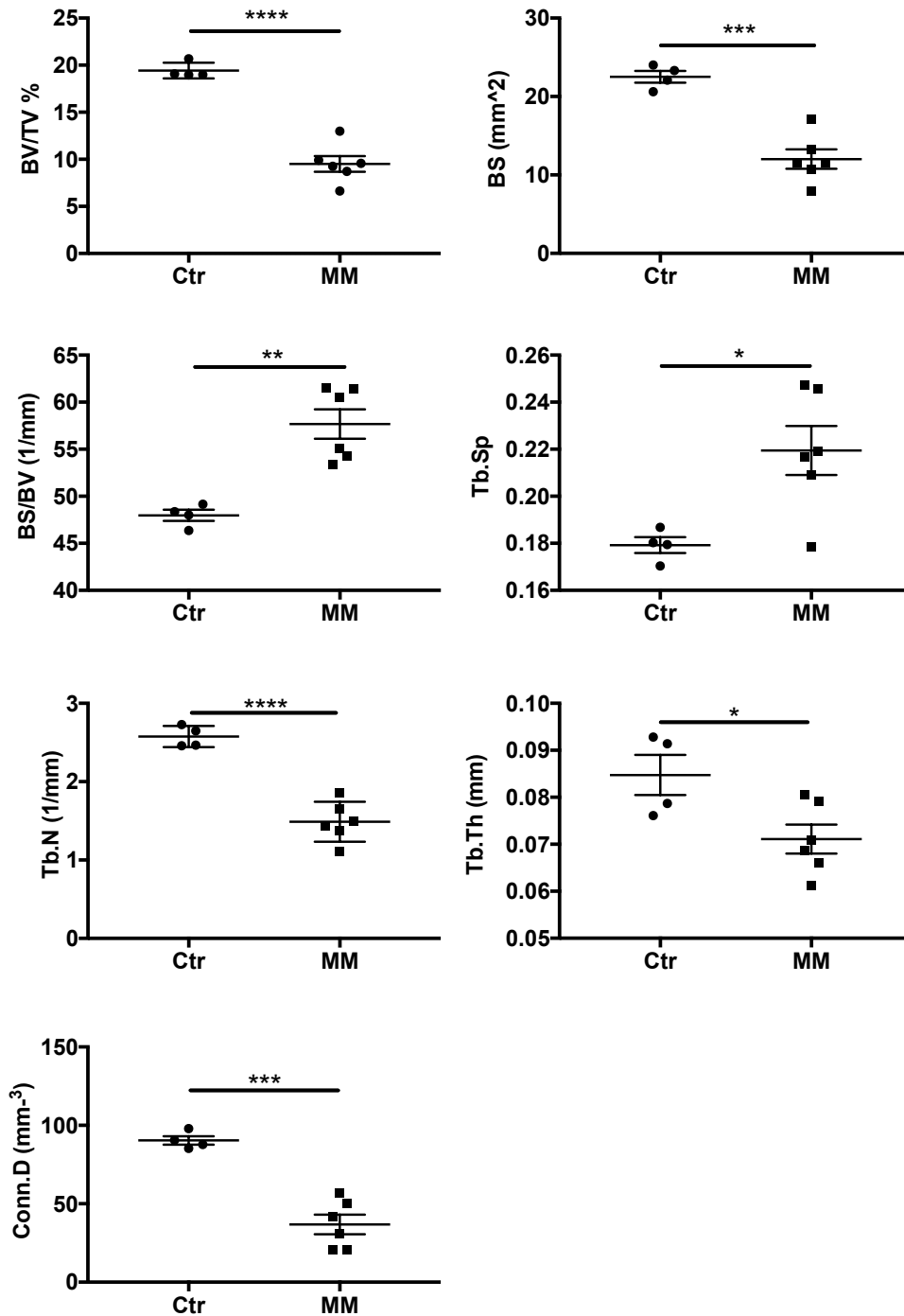


Figure 3.5 Bone quality parameters from 5TGM1-GFP MM cell induced bone disease in 8-week-old C57BL/KaLwRij mice. Micro-CT analysis parameters of tibiae including BV/TV, BS, BS/BV, Tb.Sp, Tb.N, Tb.Th and Conn.D indicate the bone quality is significantly deteriorated in 5TGM1-GFP MM group. Error bars correspond to SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Statistical analysis was performed using Student t test.

In conclusion, C57BL/KaLwRij model has a medium level of MM engraftment and has a typical phenotype of MM bone lesions. Therefore, C57BL/KaLwRij model is ideal for the study of eliglustat's function *in vivo*.

3.2.2 Bone conditions in HFD C57BL/6 mice compared to normal mice

Before assessing whether eliglustat can reduce MM burden in C57BL/6 mice on HFD, the baseline effect of HFD on bone quality was investigated. It has been reported that HFD may cause bone loss and osteoporosis^{207,208}. 13-week-old female C57BL/6 mice were divided into 2 groups a normal diet group and mice administrated with 42% HFD for 7 weeks. All the mice were sacrificed after 7 weeks and tibiae were collected for micro-CT bone analysis (Figure 3.6).



Figure 3.6 HFD experiment design. Time line illustrating the experiment design in investigating whether HFD leads to bone loss in 13-week-old female C57BL/6 mouse.

To assess the effect of 42% HFD, body weight was measured once a week (Figure 3.7). Importantly, a significant increase in body weight was observed 5-weeks after the HFD was initiated, with a further increase at the 7-week time point.

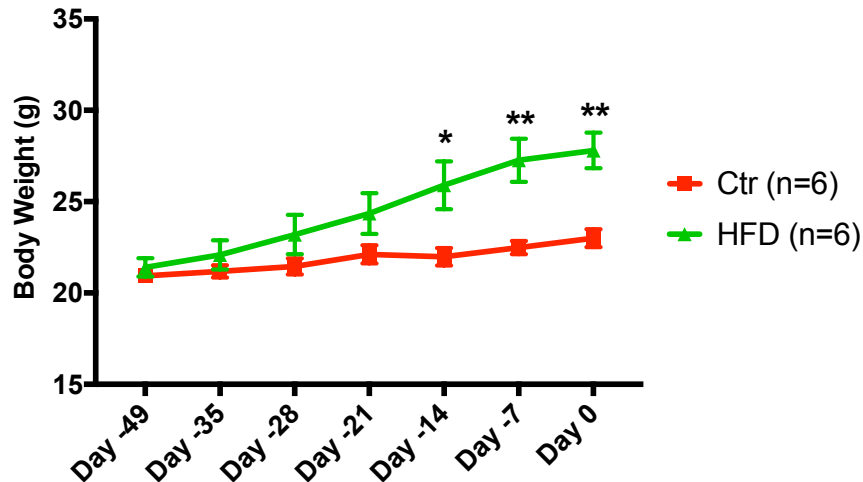


Figure 3.7 Weight change of HFD C57BL/6 mice experiment. 13-week-old female C57BL/6 mice were divided into normal control diet group (Ctr, n=6) or HFD group (HFD, n=6). Weight change of each mouse was recorded during the experiment. In HFD group, a statistical weight gain is observed. Error bars correspond to SEM. *P<0.05, **P<0.01. Statistical analysis was performed using Student t test.

According to the micro-CT analysis parameters including BV/TV, BS/BV, Tb.Sp, Tb.N and Tb.Th there was no statistical difference observed between HFD group and normal diet control group (Figure 3.8A). Moreover, BMD of the inner trabecular bone, outer cortex bone and overall bone were analysed individually. According to data, all above parameters from the HFD group remained at a relatively similar level as the control group, indicating that HFD does not change the bone quality (Figure 3.8B).

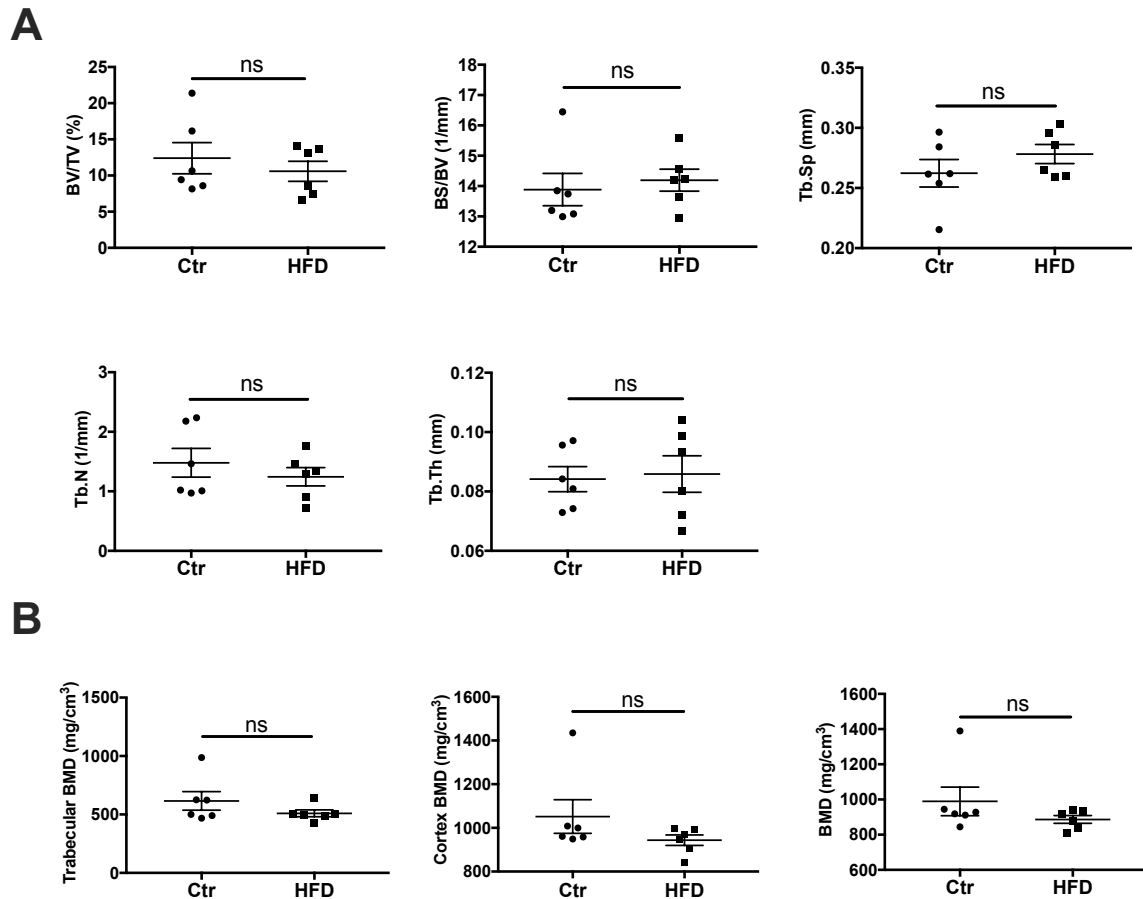


Figure 3.8 7-weeks' HFD has no significant impact on bone quality in female C57BL/6

mice. (A) Micro-CT analysis parameters of tibiae including BV/TV, BS/BV, Tb.Sp, Tb.N and

Tb.Th indicates the bone quality has no significant difference between normal control diet

group (n=6) and HFD group (n=6). **(B)** The BMD from different bone compartments including

trabecular BMD, cortical BMD and overall BMD were analyzed. Error bars correspond to SEM.

ns means non-significant. Statistical analysis was performed using Student *t* test.

To determine whether HFD alters bone marrow adiposity, the BMAT of each tibia was evaluated following labelling with osmium tetroxide. The ratio of adipose volume (AV) over total volume (TV) was calculated by micro-CT generating the proportion of adipose volume. 3D reconstruction images show that the tibia from the HFD group has more AV (red area) as compared with the normal diet group (Figure 3.9A),

demonstrating a significant increase in adipose volume in response to the HFD (Figure 3.9B).

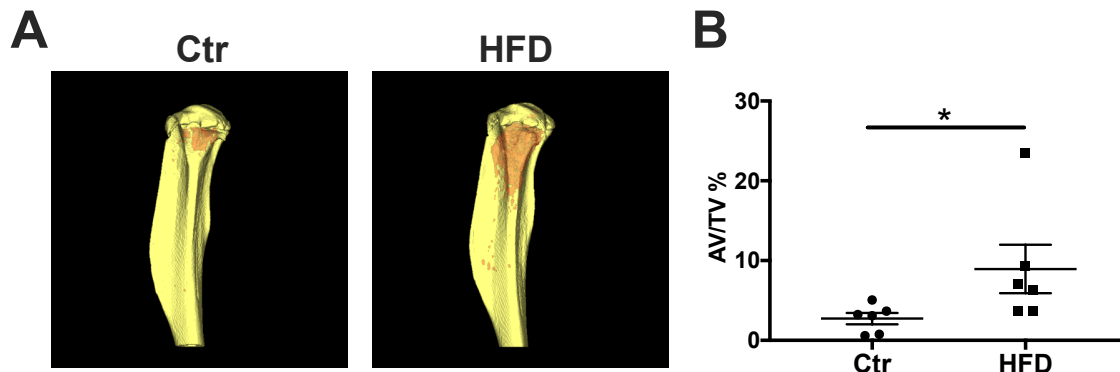


Figure 3.9 HFD increases BMAT in C57BL/6 mice. (A) Micro-CT reconstruction images show BMAT (red) distribution in the tibiae of control diet group (Ctr, n=6) and HFD group (HFD, n=6). (B) The quantitative analysis of AV/TV%. Error bars correspond to SEM. *P<0.05.

Statistical analysis was performed using Student *t* test.

In conclusion, the HFD caused an elevation in body weight and BMAT, while there was no difference observed between control group and HFD group in bone quality parameters.

3.2.3 MM susceptibility in C57BL/6 mice under HFD conditions

Lwin *et al.* shown that a HFD can create a permissive microenvironment for MM development in C57BL/6 mice, resulting in an MGUS-like condition where accumulation of MM cells in BM, increase in serum paraprotein as well as bone loss¹⁵¹. As GD has an underlying connection with MM^{197,198} and MM has the typical phenotype of bone loss¹⁹⁹, this MM preclinical model could be a platform to study eliglustat both from a bone lesion perspective and from a MM tumour burden perspective. Therefore, the following experiment was designed to investigate whether this model may be

useful to study the function of eliglustat *in vivo* undertaking initial experiments to replicate this model to ensure adequate development of myeloma and the associated bone disease for subsequent evaluation of eliglustat.

On day -42, C57BL/6 mice, a strain that does not normally develop MM disease when inoculated with 5TGM1-GFP murine MM cells, were fed either on normal chow (Ctr, n=8) or 42% HFD (HFD, n=12) for 6 weeks. After 6 weeks, all the mice were inoculated with 5TGM1-GFP murine MM cells. The mice were fed on their respective diets for another 6 weeks, prior to sacrifice at day 42 for evaluation (Figure 3.10).



Figure 3.10 Schematic overview of the experiment design. Experiment time line for 13-14-week-old C57BL/6 mice inoculated with 5TGM1-GFP cells $\pm$ HFD. 13-14 weeks old C57BL/6 mice were fed either with normal chow or HFD for 6 weeks. 2 days before MM cell injection, serum sample was collected from each mouse. All the mice were then inoculated with 5TGM1-GFP cells at 2×10^6 cells/mouse and sacrificed after another 6 weeks depending on health condition.

In this experiment, body weight confirmed that the HFD was effective and tumour burden was evaluated to ensure effective engraftment of myeloma cells. Following confirmation of tumour burden, bone disease was assessed by micro-CT to determine whether the model was appropriate to evaluate the effect of eliglustat on myeloma bone disease. A significant increase in body weight was demonstrated in response to the HFD (Figure 3.11).

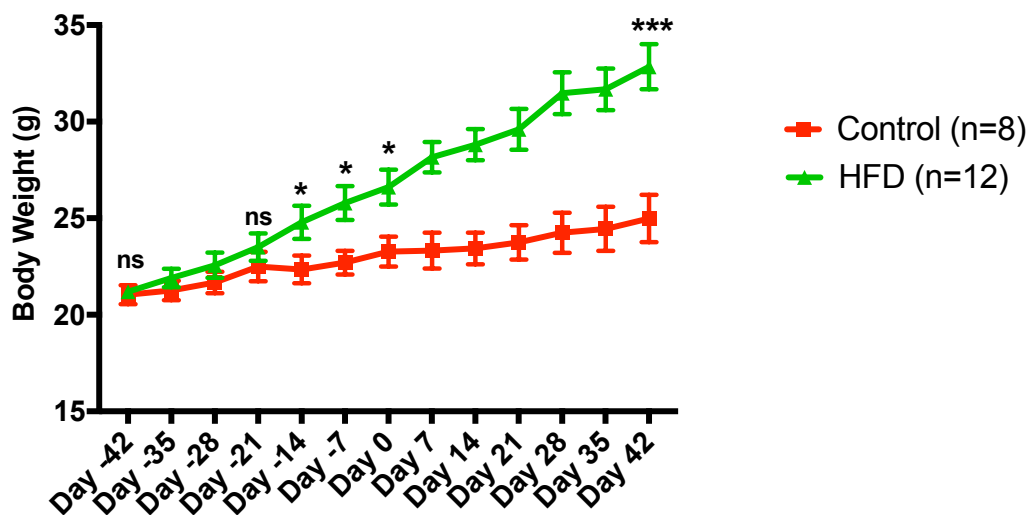


Figure 3.11 Weight change of the C57BL/6 mice inoculated with 5TGM1-GFP cells

+/- HFD. 13-14 weeks old C57BL/6 mice were fed either with normal chow or HFD for 6 weeks. Then, all the mice were inoculated with 5TGM1-GFP cells at 2×10^6 cells/mouse and sacrificed after another 6 weeks depending on health condition. The body weight change was recorded over the period of experiment. Error bars correspond to SEM.

* $P < 0.05$, ** $P < 0.01$, ns means non-significant. Statistical analysis was performed using Student *t* test.

Figure 3.12 MM tumour burden in C57BL/6 mice inoculated with 5TGM1-GFP cells +/- HFD. 13-14 weeks old C57BL/6 mice were fed either with normal chow or HFD for 6 weeks. Then, all mice were inoculated with 5TGM1-GFP cells (2×10^6 cells/mouse) and sacrificed after another 6 weeks depending on health condition. **(A)** BM and spleen from each mouse were collected for flow cytometry analysis. **(B)** Serum IgG2bk paraprotein was measured by ELISA. Error bars correspond to SEM. * $P < 0.05$, ns indicates non-significant. Statistical analysis was performed using Student *t* test.

Taken together, the HFD combined with MM cell inoculation in C57BL/6 strain model had low MM cell engraftment, which may potentially lead to less bone lesions, this is in line with previous studies that observed a mild bone loss phenotype instead of a severe bone loss phenotype¹⁵¹. As such, it is not the best model to study the treatment of MM bone disease. However, this HFD/MM model still has merit for use with eliglustat or other GSL inhibitors as these agents are involved in lipid metabolism. In view of the effect of HFD on lipid metabolism, it would be interesting to know whether HFD will interact with eliglustat *in vivo*. In addition, it would be interesting to evaluate whether eliglustat performs differently in bone lesions with different severity (for example, whether eliglustat performs better in mild bone loss condition as compared to severe bone loss condition).

3.2.4 MM susceptibility in C57BL/6 mice with the BM cells inoculated from C57BL/KaLwRij strain after sublethal irradiation

As previously described HFD/MM C57BL/6 murine model exhibited low MM cell engraftment and may not be perfect to study myeloma bone disease, a new MM model with irradiation is introduced in this section. Here, C57BL/6 strain, a strain that normally does not develop MM disease¹⁵¹ even after inoculated with MM cells was used to receive sublethal dosage of irradiation before MM cell inoculation. To test out if irradiation alone would allow MM engraftment, or whether BM cells from the C57BL/KaLwRij mice assist MM engraftment, the following experiment was designed.

5TGM1-GFP murine MM cells were mixed with BM cells from the MM permissive strain C57BL/KaLwRij before inoculation into C57BL/6 mice. Here, the 5TGM1-GFP murine MM cells are for MM initiation, while the BM cells from C57BL/KaLwRij mice may provide a supportive microenvironment for the MM cells (as shown in section 3.2.3, C57BL/6 BM cells are not supportive to MM cells). As it is reported that inoculating C57BL/6 mice with BM stromal cells from C57BL/KaLwRij mice will create a permissive microenvironment for MM development²⁰⁵, thus here freshly isolated whole BM from C57BL/KaLwRij mice which included different sub-populations of stromal cells (mesenchymal stem cells and immune cells) were used. However, the BM stromal cells only lead to a relatively medium tumour burden which is less severe and acceptable²⁰⁵. In view of the fact that complete BM cells differs from BM stromal cells, and MM cells may not engraft, both the irradiation and non-irradiated groups were investigated. Moreover, if both groups engraft, the irradiated group can serve as a

control to determine whether inoculating MM cells with C57BL/KaLwRij BM cells has less engraftment ability than inoculating MM cells alone.

C57BL/6 mice were divided into 4 groups; non-irradiated+MM, non-irradiated+MM+BM, irradiated+MM and irradiated+MM+BM. In this experiment, 1 million 5TGM1-GFP MM cells and 1 million C57BL/KaLwRij BM cells were used in order to be consistent with previous experiments and provide sufficient cells for microenvironment set up. On day -2, the serum was collected for IgG2 κ paraprotein baseline. On day 0, designated mice were irradiated with sublethal dosage (5.5Gy). The serum was collected on day 14 in order to monitor the MM progression. The mice were culled depending on the weight loss and presence of lameness. (Figure 3.13)

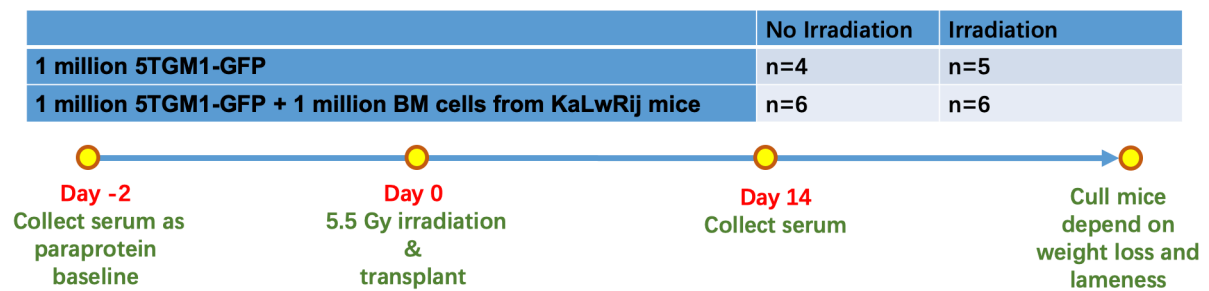


Figure 3.13 Experiment design and time line of C57BL/6 mouse with irradiation and cell inoculation. 7-month-old C57BL/6 mice were divided into 4 groups, non-irradiated+MM, non-irradiated+MM+BM, irradiated+MM and irradiated+MM+BM. 2 days before MM cell injection, serum samples were collected from each mouse. Designated groups of mice were irradiated with 5.5Gy and inoculated with 5TGM1-GFP murine cells and C57BL/KaLwRij BM cells. The mice were sacrificed depending on weight loss and lameness.

To compare the survival percentage of each group, the time point of mouse lameness and sacrifice was recorded. Interestingly, neither of the non-irradiated groups showed

any sign of MM engraftment and no mice were lost in these 2 groups. This is probably due to non-irradiated C57BL/6 mouse having an intact immune system and any foreign cells even from the same species cannot invade the system. Moreover, as this experiment inoculated whole BM cells instead of the BM stromal cells, so each mouse may receive a small population of BM stromal cell which is different from 0.5 million as mentioned in the literature²⁰⁵. Irradiated+MM and irradiated+MM+BM share similar end point percentage around similar time point which is around day 26-27 post 5TGM1-GFP cell injection. This finding illustrates that the supportive BM cells from MM permissive C57BL/KaLwRij strain does not alter MM engraftment when C57BL/6 mice are irradiated at a sublethal dosage (Figure 3.14).

End point of BL6 mice after MM cell injection

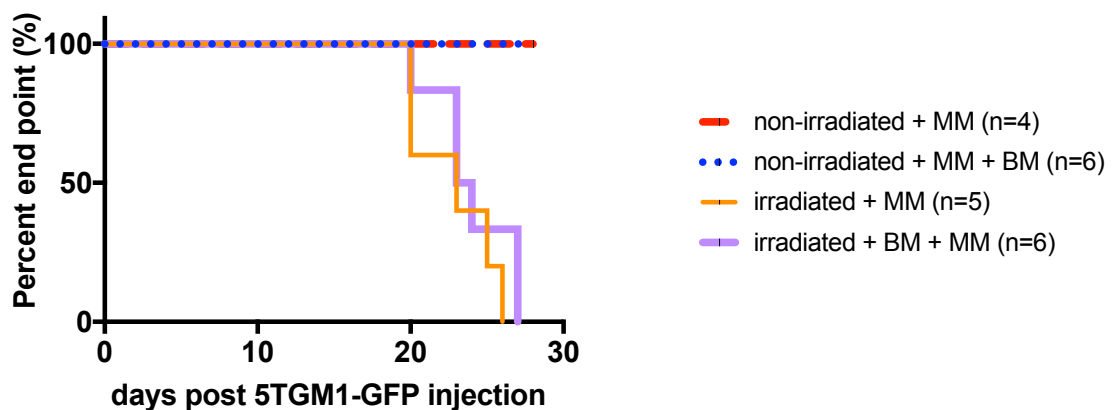


Figure 3.14 End point percentage of C57BL/6 mouse with irradiation and cell

inoculation. C57BL/6 mice are divided into 4 groups: non-irradiated+MM, non-irradiated+MM+BM, irradiated+MM and irradiated+MM+BM. After designated groups of mice are irradiated with 5.5Gy and inoculated with 5TGM1-GFP murine cells and C57BL/KaLwRij BM cells, the date of mice sacrificed depending on weight loss and lameness are recorded and the percent end point (%) are calculated accordingly.

After the mice were sacrificed, the BM cells and spleen cells were collected accordingly and the cells were fixed immediately. After the last mouse was culled in this experiment, all the cells were treated exactly the same as the cells collected from other mice previously. Then, all the cells were analysed by flow cytometry and the GFP channel is applied in order to track the 5TGM1-GFP cells. According to the 5TGM1-GFP MM cell percentage, both the non-irradiated+MM group and non-irradiated+MM+BM group reveal there is almost no 5TGM1-GFP MM that survived after inoculation. And, the BM and spleen MM cell percentage in irradiated+MM group and irradiated+MM+BM group are relatively within similar range which validates the previous findings (Figure 3.15A). Consistent with the MM cell percentage, both the irradiated+MM group and the irradiated+MM+BM group had elevated serum paraprotein levels (Figure 3.15B). The irradiated+MM group showed a significant increase in serum paraprotein as compared with the non-irradiated+MM group. Moreover, the irradiated+MM+BM group also showed a significant increase of serum paraprotein as compared with non-irradiated+MM+BM group (Figure 3.15B).

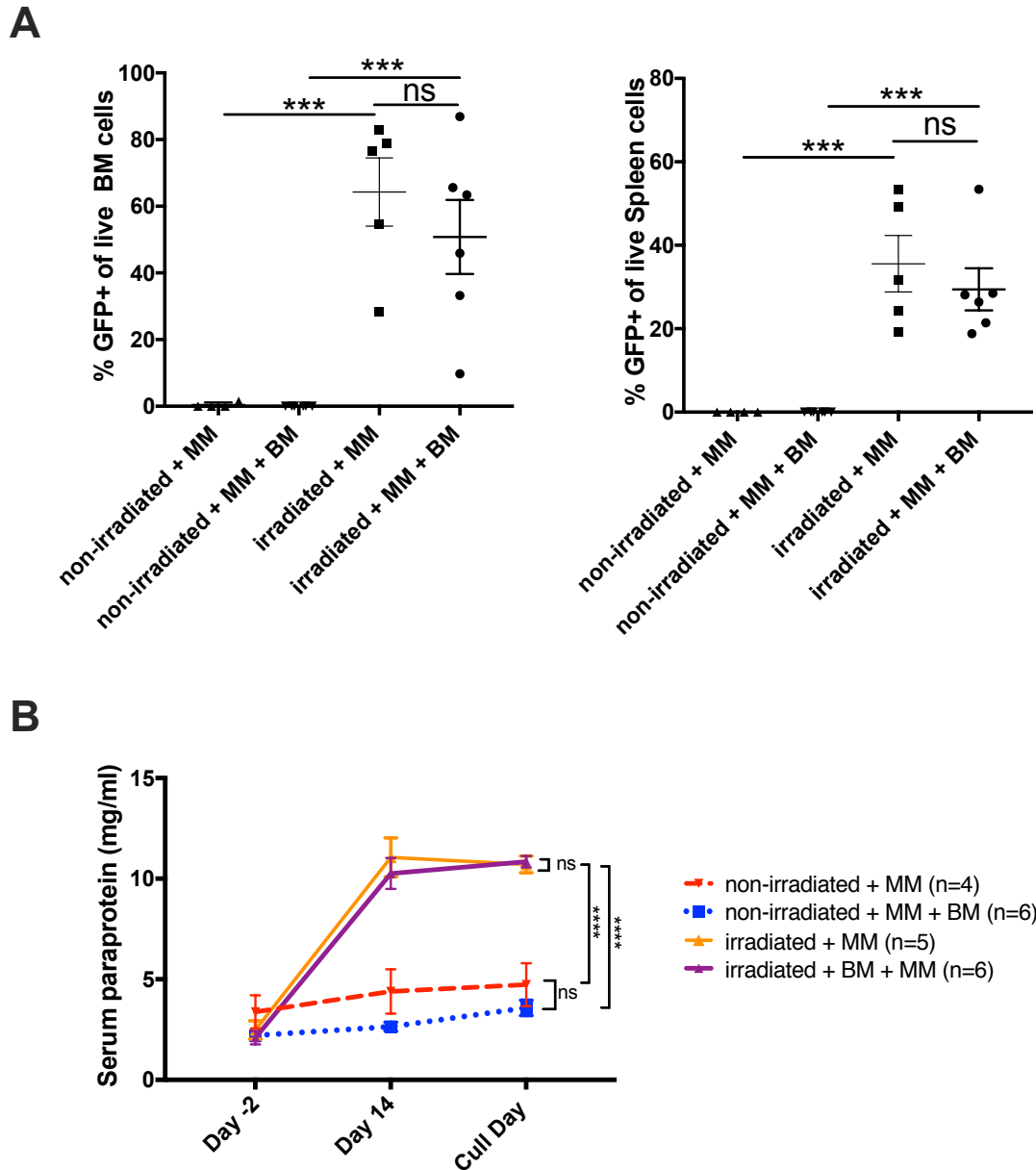


Figure 3.15 MM burden in C57BL/6 mouse with irradiation and cell inoculation.

C57BL/6 mice are divided into 4 groups: non-irradiated+MM, non-irradiated+MM+BM, irradiated+MM and irradiated+MM+BM. After designated groups of mice are irradiated with 5.5Gy and inoculated with 5TGM1-GFP murine cells and C57BL/KaLwRij BM cells, mice are then sacrificed depending on weight loss. **(A)** The BM cells and spleen cells from each mouse were collected for flow cytometry analysis. **(B)** The serum IgG2b κ paraprotein was measured by ELISA. Error bars correspond to SEM. *** $P < 0.001$, ns means non-significant. Statistical analysis was performed using Ordinary one-way ANOVA.

In sum, BM cells from was C57BL/KaLwRij mice were not required for C57BL/6 mice MM engraftment. Indeed, irradiation alone was sufficient for C57BL/6 mice to develop MM. Both the irradiated+MM group and irradiated+MM+BM group had high tumour burden. In this experiment, the mice were sacrificed based on lameness instead of sacrificed at the same time point, which could be the cause of high MM burden. To potentially decrease the MM burden and possibly alleviate tremendous suffering to the animals, the following experiment in 3.2.5 was designed.

3.2.5 MM susceptibility in C57BL/6 mice with sublethal irradiation

To investigate whether the MM burden from the irradiated groups will be at a relatively low level when all the mice are sacrificed at the same time point, the following experiment was conducted.

The control group received sublethal irradiation (5.5Gy) without injection of MM cells, while the experimental group received the same dosage of irradiation followed by inoculation of 5TGM1-GFP MM cells. To set up the model, the serum of 8-week-old male C57BL/6 mice were collected on day-2 as the baseline of IgG2bk paraprotein. On day 0, both of the groups receive irradiation and the mice from MM group receive the murine MM cells as well. All mice were subsequently culled depending on the health condition of the mice in MM group (Figure 3.16).

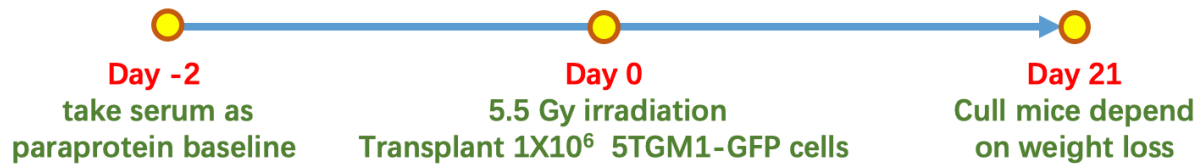


Figure 3.16 Experiment time line for 8-week-old male C57BL/6 mice inoculated with 5TGM1-GFP MM cells after sublethal irradiation. Schematic overview of the experiment design. 8-week-old male C57BL/6 mice were divided into 2 groups, control group and MM group. 2 days before MM cell injection, serum sample was collected from each mouse. All the mice were then irradiated with 5.5Gy and MM group receives 5TGM1-GFP MM cell inoculation. All the mice were sacrificed after 21 days depending on health condition.

To evaluate the *in vivo* tumour burden in BM and spleen, 5TGM1-GFP cells were quantitated by flow cytometry. There was a significant increase in the MM burden with approximately 90% MM cells detected in the bone marrow and 20% in the spleen (Figure 3.17A). The tumour burden was further confirmed by serum IgG2bk paraprotein ELISA according to manufacturer's protocol. In accordance with the findings from flow cytometry, IgG2bk increased significantly to 10 mg/ml (Figure 3.17B).

These findings reveal that the sublethal irradiation generates a rapid and severe MM model with as much as 90% of the BM being MM cancer cells. In view of the animal welfare, this model may be a less optimal model to study the function of eliglustat.

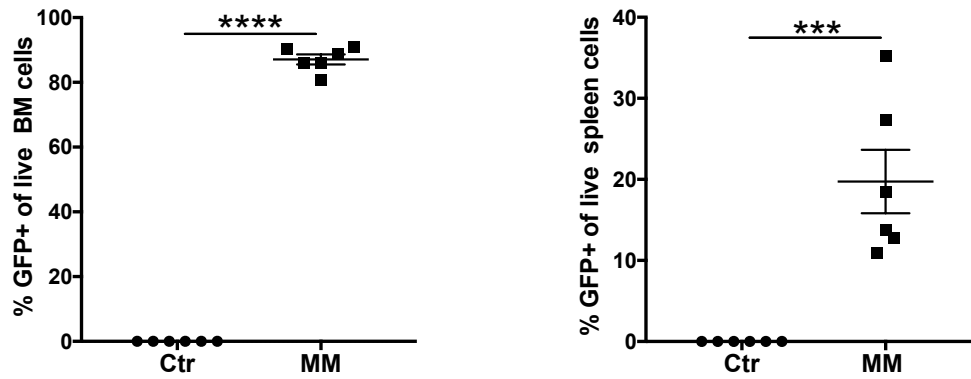
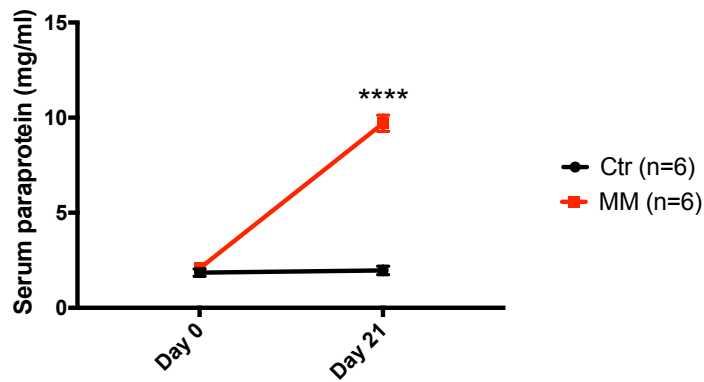
A**B**

Figure 3.17 MM tumour burden in 8-week-old male C57BL/6 mice inoculated with 5TGM1-GFP MM cells after sublethal irradiation. 8-week-old male C57BL/6 mice were divided into 2 groups, control group and MM group. All the mice were irradiated with 5.5Gy and MM group receives 5TGM1-GFP MM cell inoculation. **(A)** The BM cells and spleen cells from each mouse were collected for flow cytometry analysis on day 21. **(B)** The serum IgG2b κ paraprotein was measured by ELISA. Error bars correspond to SEM.

P<0.001, *P<0.0001. Statistical analysis was performed using Student *t* test.

Chapter 3. Establishing Animal Models

3.3 Discussion

3.3.1 Does HFD contributes to bone loss?

Previous literature has shown that HFD leads to bone loss and osteoporosis in several murine models^{207,208}, while this is not the case as mentioned in section 3.2.1. This could be due to several reasons. First, the mice age of the experiment conducted in section 3.2.1 is 13-week-old C57BL/6 mice, which is different from 12-month-old female C57BL/6 mice as mentioned in the literature²⁰⁷. This can lead to the possibility that different age of the mice will respond differently to the HFD. Second, the tibiae of the mice were measured in this project while the other literature are focus on mandible bone²⁰⁹, vertebrae and femur²¹⁰. As different bones may react quite distinctly from other bones under HFD condition so others finding may not be applicable universally. Thirdly, female mice were used for the HFD experiment, which was different from the male as mentioned in the literature²⁰⁹. Such difference could led to different bone sensitivity to the HFD²⁰⁹. Fourthly, the during of the HFD administration is quite different from other experiments. As this project is using a dosage which can cause MGUS¹⁵¹, while other paper administrate the HFD for 6 months²⁰⁷. Moreover, the HFD used here was 42% HFD, while others use 10% corn oil²⁰⁷ or 60% HFD²⁰⁸. The difference in the HFD components could also be an important factor caused different bone mass. In short, different researchers used different conditions or systems to evaluate the bone quality under HFD which makes direct compare with others' finding not applicable.

3.3.2 Can irradiation dosage be optimized in order to set up the ideal model?

As using sublethal irradiation methods to generate MM disease in C57BL/6 mice has never been reported before, hence this is a novel model which could be potentially

used for future study (especially in studies involving high MM cell population in the BM niche). However, this model is not ideal for this project as high level (80%) of BM MM engraftment may bring extra suffering to the mice, which is not expected and described in the initial research plan. The question here to be address is whether the irradiation model can be optimized in order to fit a medium MM engraftment percentage. Theoretically, this may be reached by decreasing the irradiation dosage. However, there is no existing protocol ready to follow. Moreover, animal work is strictly conducted under the Home Office Licence and only lethal and sublethal dosage is approved. Therefore, reduction of irradiation dosage is not reachable in this project. Moreover, irradiation may bring other unexpected or unobserved symptoms which may alter the actual results.

Chapter 4. Eliglustat Improves MM Bone quality by Inhibiting Osteoclastic Bone Resorption

4.1 Introduction

MM, is a malignancy of plasma cells, which is always accompanied by osteolytic bone disease. The bone complication is characterized by increased activity of OCs and OCs can promote MM cell proliferation by secreting IL-6 via the vicious cycle¹⁴³. In return, MM cells secrete RANKL, which is the prime mechanism of OC formation and activation¹⁴⁵.

It was reported that some GSLs inhibitors including D-PDMP²¹¹ and Miglustat¹⁵ can inhibit OCs without a clear mechanism. Interestingly, NB-DGJ, another kind of GCS inhibitor cannot inhibit OC formation in some conditions the reasons for this remain unclear.¹⁵. Moreover, D-PDMP is a nonspecific GCS inhibitor¹⁵, and miglustat has severe gastrointestinal complications (85%) and causes weight loss (65%)²¹². These nonspecific inhibition and side effects indicate that the above two compounds may interfere with other factors to suppress OC instead of regulating GCS or GSLs to inhibit OC. Compared with previous GSL inhibitors, eliglustat is more specific in inhibiting GCS, hence may clearly reveal whether GSL and GCS are involved in OC formation or not.

Ersek *et al.* reported that miglustat can be beneficial for MM bone quality¹⁵, therefore, it is hypothesized that eliglustat can ameliorate MM bone disease with negligible side effects. Based on above hypothesis, this chapter aims to investigate whether eliglustat improves MM bone quality.

Chapter 4. Eliglustat Improves MM Bone quality by Inhibiting Osteoclastic Bone Resorption

4.2 Results

4.2.1 Eliglustat inhibits osteoclast formation *in vitro* without affecting viability

To determine whether eliglustat inhibits OC formation *in vitro*, eliglustat was added to an OC formation assay from C57BL/6 mouse BM cells. 6 days after eliglustat was added to the media, the cells were fixed and identified by tartrate-resistant acidic phosphatase (TRAP) staining for OCs.

A dose-dependent inhibition in OC formation was observed in the presence of eliglustat (Figure 4.1). Quantitative analysis of cell viability and OC formation were subsequently conducted. First, cells with respective treatments were evaluated by Alamar Blue assay to check OC progenitors' viability at day 3 time point according to manufacturer's instruction. According to the data, cell viability was not compromised with increased eliglustat dose up to 50 μ M (Figure 4.2A). Based on TRAP staining on day 6, the number of nuclei per TRAP+ cell (Figure 4.2B), the percentage OC area over the total area (Figure 4.2C) and the number of OC per well (Figure 4.2D) showed that eliglustat reduced OC formation in a dose-dependent manner.

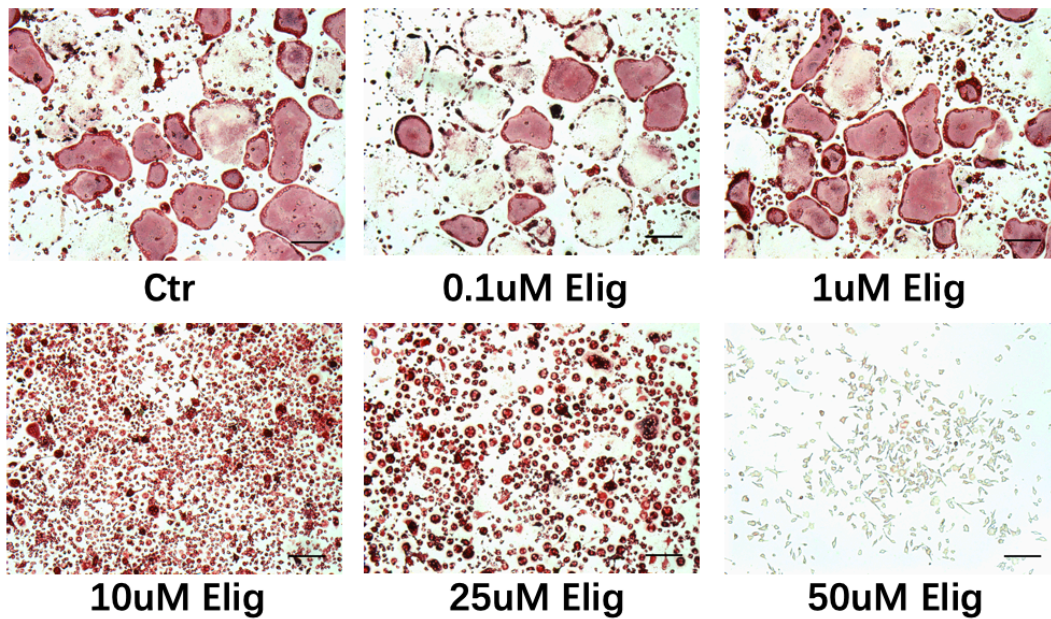


Figure 4.1 Eliglustat inhibits *in vitro* TRAP+ OC formation in a dose-dependent manner. OC differentiation assay on day 6. 8-week-old C57BL/6 mouse BM cells were treated with 20 ng/ml M-CSF and 50 ng/ml RANKL to form OCs in 96-well plates (200ul, 2×10^4 BM cells/well) since day -2. On day 0, different doses of eliglustat (0.1, 1, 10, 25, 50 μ M) were added and OCs can be identified by TRAP staining on day 6 (original magnification, X10). Scale bars represent 200 μ m. (n=3)

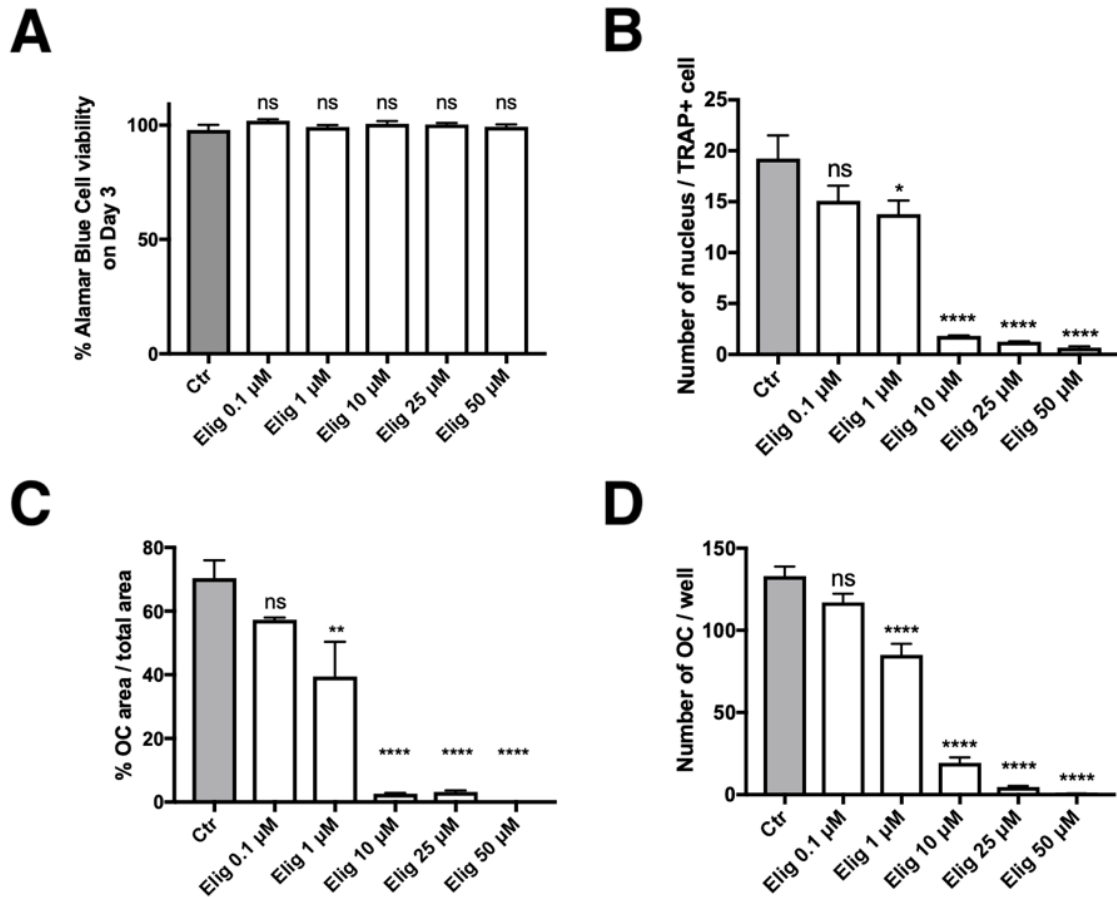


Figure 4.2 Without affecting cell viability, eliglustat inhibits *in vitro* TRAP+ OC

formation. (A) 72 hours after different doses of eliglustat (0.1, 1, 10, 25, 50 μ M) was added to respective groups, mouse BM cells were evaluated by Alamar Blue assay to check the OC progenitors' viability (n=3). On day 6, quantitative analysis of TRAP stained OCs indicates the number of nucleus per TRAP+ cell (B), percentage OC area over total area (C) and number of OC per well (D). Error bars correspond to SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, ns means non-significant by One-way ANOVA.

4.2.2 Eliglustat inhibits osteoblast formation *in vitro*

OBs, the bone forming cells, are critical players in maintaining bone homeostasis. OBs act as the counterpart of OCs thus it is important to characterize if eliglustat has any direct effect on OBs. OBs were generated from calvaria of 2-day-old C57BL/6 mice and a dose response of eliglustat was conducted in osteogenic media.

On day 7, alkaline phosphatase (ALP) staining (Figure 4.3A) and ALP quantification assay (Figure 4.3B) revealed that eliglustat also exerted a dosage-dependent inhibition of OB.

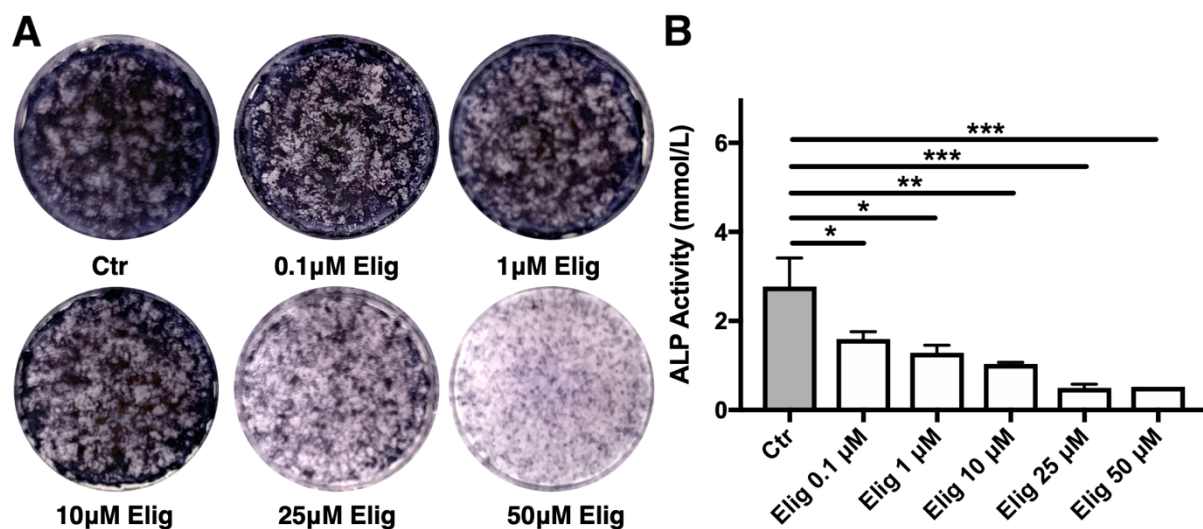


Figure 4.3 ALP staining and quantification of eliglustat dosage response with OB.

The calvaria from C57BL/6 new born pups (within 48 hours) were digested and cells were cultured in culture media (CM) in 48-well plate until confluent (500ul, 1×10^4 cells/well). Then, different doses of eliglustat (0.1, 1, 10, 25, 50 μ M) in osteogenic media were added. ALP staining (A) and quantification assay (B) were performed on day 7. (n=3) *P<0.05, **P<0.01, ***P<0.001. Statistical analysis by One-way ANOVA.

Above finding was further confirmed by a decrease of bone nodule formation stained by Alizarin Red on day 21 (Figure 4.4).

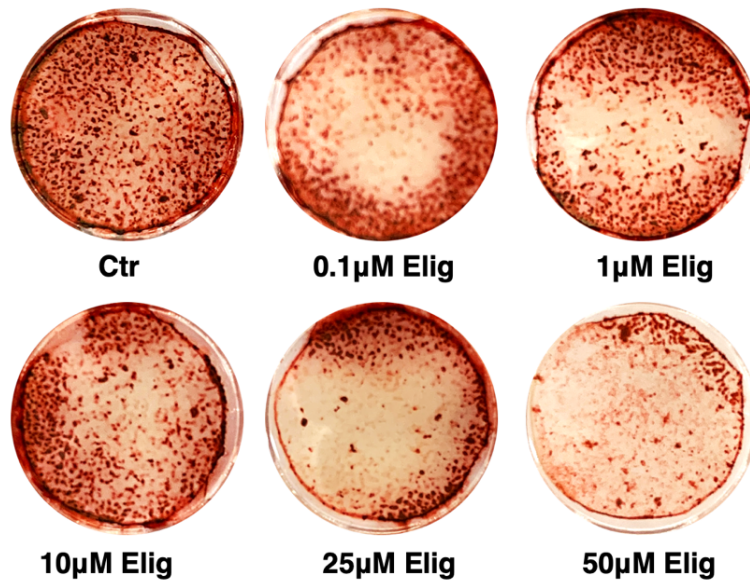


Figure 4.4 Alizarin Red staining of eliglustat dosage response with OB. The calvaria from C57BL/6 new born pups (within 48 hours) were digested and cells were cultured in culture media (CM) in 48-well plate until confluent (500ul, 1×10^4 cells/well). Then, different doses of eliglustat (0.1, 1, 10, 25, 50 μ M) in osteogenic media were added. Bone nodules can be identified by Alizarin Red Staining on day 21 (n=3).

4.2.3 Eliglustat inhibits MM cells *in vitro*

After investigating eliglustat's effect on *in vitro* OC formation, the next hypothesis to be proposed is whether eliglustat inhibits MM tumour burden. This hypothesis is based on 2 existing findings. Firstly, it is reported some GSL inhibitors including eliglustat can decrease the progression of MM development from Gaucher's Disease^{213,214,197}, which may be due to GSL inhibitors ability to decrease MM cells either directly or indirectly. Secondly, OCs were inhibited by eliglustat (Figure 4.2.1) which may disrupt the "MM vicious cycle" thus decreasing MM cell growth and survival.

To investigate if eliglustat has direct effects on MM cells *in vitro*, 5TGM1-GFP cells were used to conduct a dose response experiment of eliglustat at 72-hour time point. In this experiment, eliglustat concentrations previously used for *in vitro* culture of OC and OB were applied in order to have a cross comparison. There was a dosage dependent decrease of live 5TGM1-GFP MM cells (Figure 4.5A), with bortezomib (BTZ), a proteasome inhibitor used as positive control.

MM cells were stained with live/dead zombie aqua dye and Annexin V for flow cytometry analysis (Figure 4.5B). The proportion of live cells was analysed and a significant inhibition of cell viability was observed at the dosage of 10 μ M or above (Figure 4.5C). Therefore, when eliglustat was used at the dosage of 10 μ M or above, it effectively inhibits 5TGM1-GFP murine MM cell viability while the same concentration had no effect on murine OC viability at 72-hour time point.

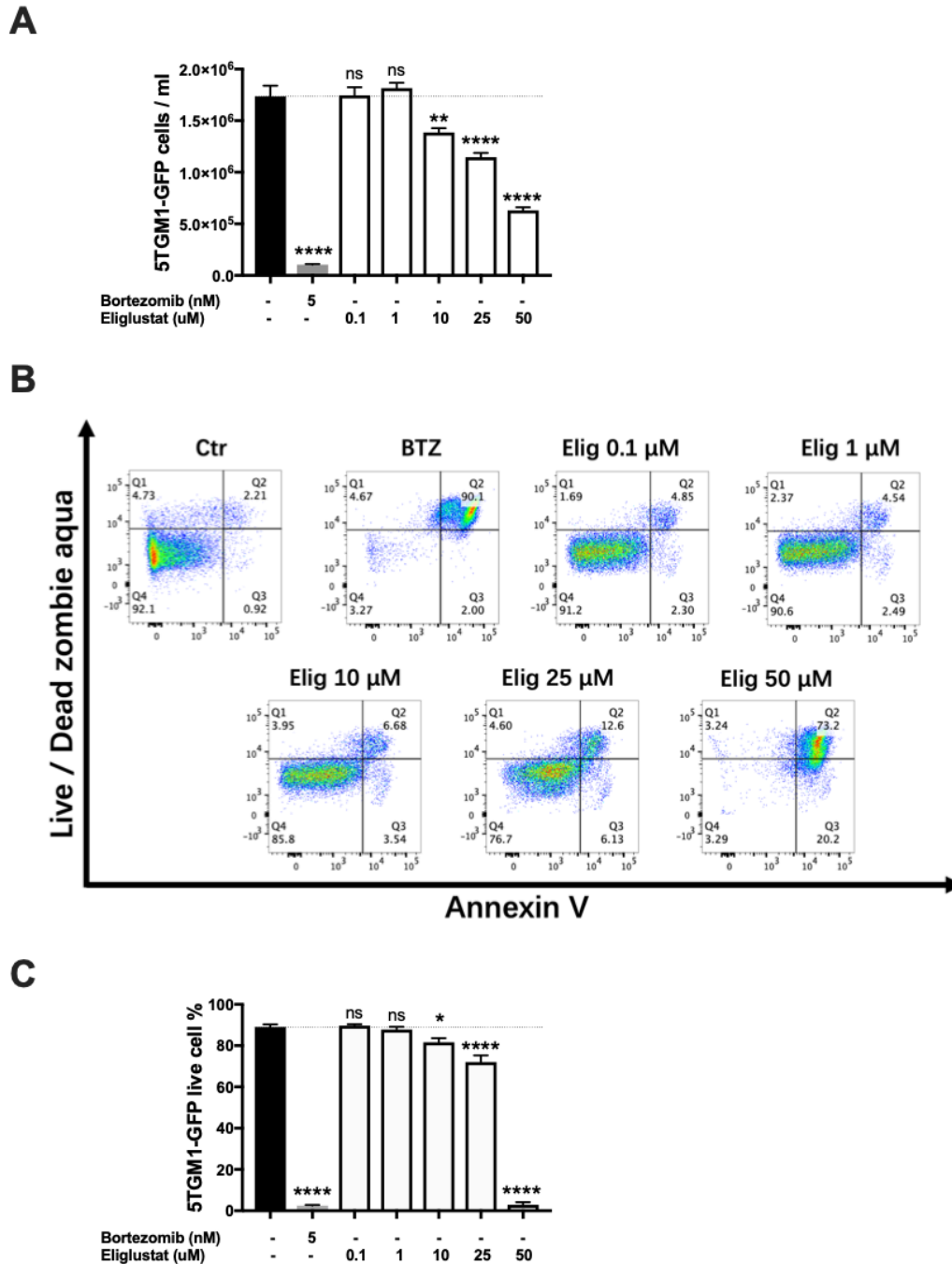


Figure 4.5 Eliglustat inhibits 5TGM1-GFP murine MM cells *in vitro*. (A) 5TGM1-GFP cell number following 72hrs of eliglustat treatment (B), flow cytometry gating strategy using live / dead zombie aqua and Annexin V (C) quantification of live cell percentage using different dosage of eliglustat (0.1 μ M, 1 μ M, 10 μ M, 25 μ M, 50 μ M). Error bars correspond to SEM. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. ns means non-significant. Statistical analysis was performed using one-way ANOVA.

As human MM disease can be categorized into different subtypes including IgG MM (52%), IgA MM (21%), light chain MM (16%), IgM MM (0.5%) and IgD MM (2%)²¹⁵, specific immunotherapy maybe conducted based on these categories. To investigate whether eliglustat inhibits specific subtypes of immunoglobulin in 5TGM1 murine MM disease, each individual immunoglobulin subtype was examined. Given the finding of eliglustat inhibits 5TGM1 MM viability, IgG isotype kit was used to see whether immunoglobulin was affected at 72-hour time point. According to the IgG isotype ELISA data, IgG2a, lambda light chain, IgG3 and IgM show a dosage-dependent decrease pattern which means the cells secrete these IgG isotypes may be inhibited by eliglustat (Figure 4.6A). Interestingly, 5TGM1-GFP cells did not express IgA and IgG1. Moreover, it was surprising to see IgG2b and kappa light chain, indicators used to evaluate murine MM tumour burden, were not affected even at the highest dosage of eliglustat (50 μ M) and in the bortezomib group. This phenomenon can be interpreted as these two isotypes may not change so rapidly as their degradation was slower compared to other isotypes (Figure 4.6B).

A

4.2.4 Eliglustat increases bone quality in healthy mice

To evaluate if eliglustat has any effect on bone quality *in vivo*, healthy C57BL/6 mice were administrated with eliglustat for 19 days at the dosage of 150mg/kg/day. After the mice were sacrificed, both of the tibiae were then collected. Bone quality was evaluated by micro-CT, OC parameters were analyzed by bone histomorphometry. According to the 3D reconstruction images, the trabecular bone of tibia had a significant increase as evident by different micro-CT parameters including increased BV/TV, BS, Tb.N as well as Conn.D, and decreased BS/BV as well as Tb.Sp (Figure 4.7A and 4.7B).

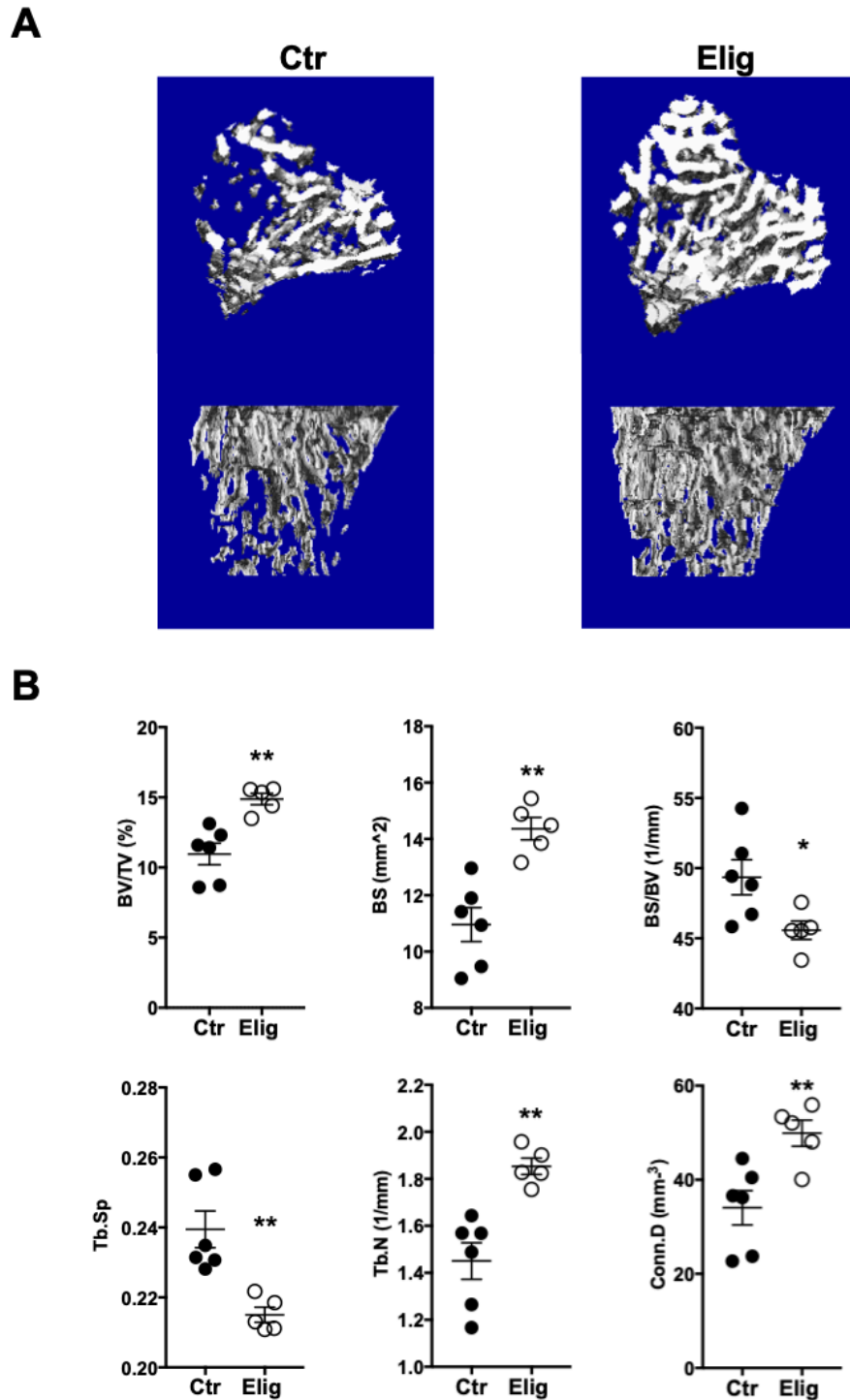


Figure 4.7 Eliglustat increases bone conditions in 8-week-old normal C57BL/6 mice. (A)

Representative micro-CT reconstruction images of tibiae from mice treated with normal chow (Ctrl) (n=6) or Elig chow (150 mg/kg/day for 19 days) (Elig) (n=5). **(B)** Micro-CT analysis parameters of tibiae: BV/TV, BS, BS/BV, Tb.Sp, Tb.N and Conn.D. Error bars correspond to SEM. *P<0.05, **P<0.01. Statistical analysis was performed using Student *t* test.

In order to have a close examination of how OC changes after the administration of eliglustat *in vivo*, the tibia histology sections were then analysed by bone histomorphometry as described in 2.11. There were less TRAP positive OC beneath the growth plate in the eliglustat treated group from overall appearance. This was further confirmed after 40X zoom in the region in the boxed area as showed below the original slide. Moreover, the zoomed in OB from each group was also shown as pointed out by the black arrow head (Figure 4.8A). OC surface over bone surface, number of OC over total area and number of OC over bone perimeters are three critical parameters indicating OC actual distribution and absolute number in a defined region, thus can best describe the change of OC *in vivo*. According to the quantitative analysis, a significant decrease of all these three parameters were observed, which indicates OCs were clearly inhibited by eliglustat (Figure 4.8B).

Similarly, to the afore mentioned parameters for OC, OB parameters include OB surface over bone surface, number of OB over total area and number of OB over bone perimeters. These parameters can also define whether OBs are influenced by eliglustat. None of the three parameters changed after the administration of eliglustat indicating that eliglustat neither supported OBs nor inhibited OBs (Figure 4.8C).

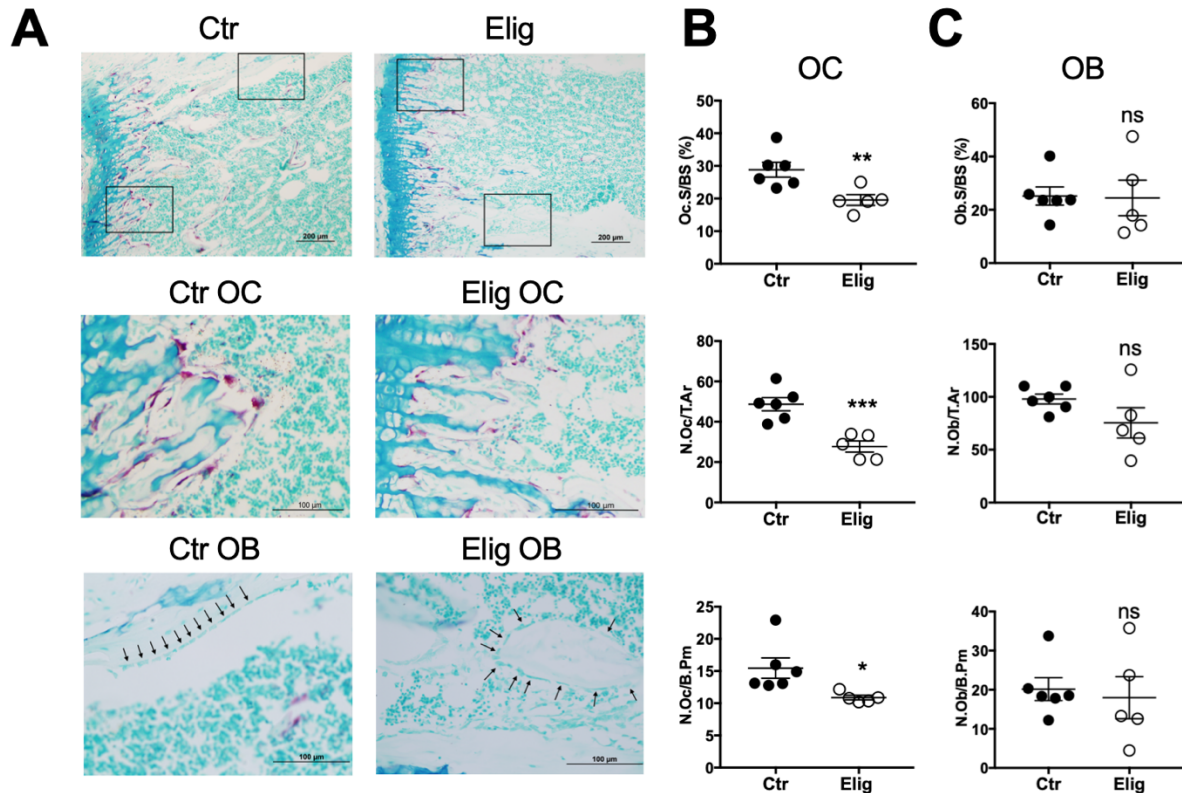


Figure 4.8 Eliglustat inhibits *in vivo* OC while not affect *in vivo* OB. (A)

Representative TRAP/0.2% methyl green stained tibial sections showing red OCs and OBs on the endocortical bone surface from control group and eliglustat group (original magnification 20X); boxed areas in lower images (original magnification 40X). **(B and C)** Bone histomorphometry analysis for OC and OB including percentage of OC surface over total bone surface (Oc.S/BS), number of OCs over total bone area (N.Oc/T.Ar), number of OCs per bone perimeter (N.Oc/B.Pm), percentage of OB surface over total bone surface (Ob.S/BS), number of OBs over total bone area (N.Ob/T.Ar) and number of OBs per bone perimeter (N.Ob/B.Pm). Error bars correspond to SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Statistical analysis was performed using Student *t* test.

Eliglustat, as a GSL inhibitor, may affect the normal lipid metabolism and may cause other phenotypes in adipocytes. To investigate if eliglustat affects adipocytes, the body weight and BMAT were examined as these two parameters represent most adipose tissue distributed in the body. Moreover, BMAT represents tissue specific adipocytes where OCs are located. Body weight change is a reflection of white adipose tissue (WAT) change, WAT is widely distributed in the body and contributes greatly to weight gain or loss. There was no change observed in body weight after 19 days administration of eliglustat, suggesting that WAT was not affected (Figure 4.9A). Moreover, the BMAT of tibia was stained by osmium tetroxide and the volume percentage of BMAT over BV was calculated by micro-CT as mentioned in 2.12. According to the data, there was no difference between control group (Ctr) and eliglustat group (Elig) in percentage of BMAT volume over BV which illustrates BMAT was not affected by eliglustat *in vivo* (Figure 4.9B).

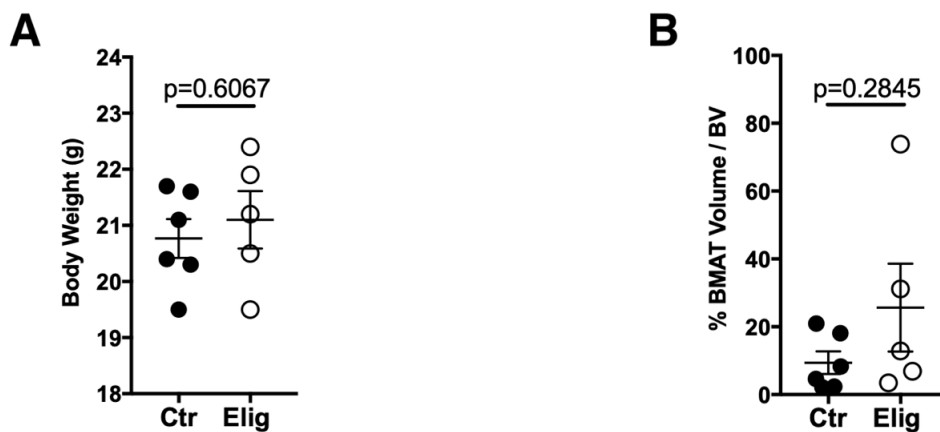


Figure 4.9 Eliglustat does not affect body weight and BMAT in 8-week-old normal C57BL/6 mice. (A) Body Weight measured on day 19 reveals there is no significant difference between Control and eliglustat. (B) Bone histomorphometry analysis for the %BMAT volume/BV. Error bars correspond to SEM. Statistical analysis was performed using Student *t* test.

4.2.5 Eliglustat reduces osteolytic bone lesions in MM murine model

Bone lesions are one of the typical complications in MM disease. To examine if eliglustat can be used as a therapeutic reagent in the MM bone disease, 8-week-old C57BL/KaLwRij mice were inoculated with 5TGM1-GFP murine MM cells and then treated with eliglustat to evaluate the bone quality and tumor burden. The mice were divided into 3 groups: non-MM control group (Ctr), MM bearing group (MM), MM with eliglustat at 150 mg/kg/day (MM+Elig). eliglustat treatment was started 4 days after the injection of MM cells until the end of the experiment 23 days after tumor inoculation (Figure 4.10).

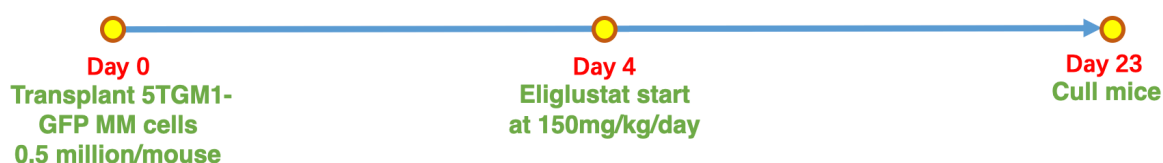


Figure 4.10 Schematic overview of the administration of eliglustat to MM bearing 8-week-old female C57BL/KaLwRij mice. (A) Mice were *i.v.* injected with 5TGM1-GFP MM cells or PBS on day 0. 4 days after injection, eliglustat group is applied to the mice at 150 mg/kg/day until the end of the experiment. Mice were culled on day 23.

Both of the tibiae from each mouse were collected for micro-CT analysis and histomorphometry analysis to evaluate the bone parameters. The serum, spleen and femurs were collected in order to measure the MM tumor burden. According to the micro-CT reconstruction images of the trabecular bone in tibia from each group, a loss of trabecular bone in MM group was observed which was partly reversed by eliglustat (Figure 4.11A).

Further quantitative analysis of different parameters from micro-CT clearly describes how bone quality was improved in MM+Elig group as compared with the MM group. Parameters including BV/TV, BS, Tb.N, Conn.D and Tb.Th all had a significant increase in the MM+Elig group compared with the MM group which indicates the bone volume, bone surface, trabecular bone number, trabecular bone connective density and trabecular thickness all have improved after the administration of eliglustat. Parameters including BS/BV and Tb.Sp both demonstrated a significant decrease after eliglustat treatment indicating the trabecular separation and ratio of bone surface over bone volume was decreased, suggesting trabecular bone quality was improved compared to the MM alone group (Figure 4.11B).

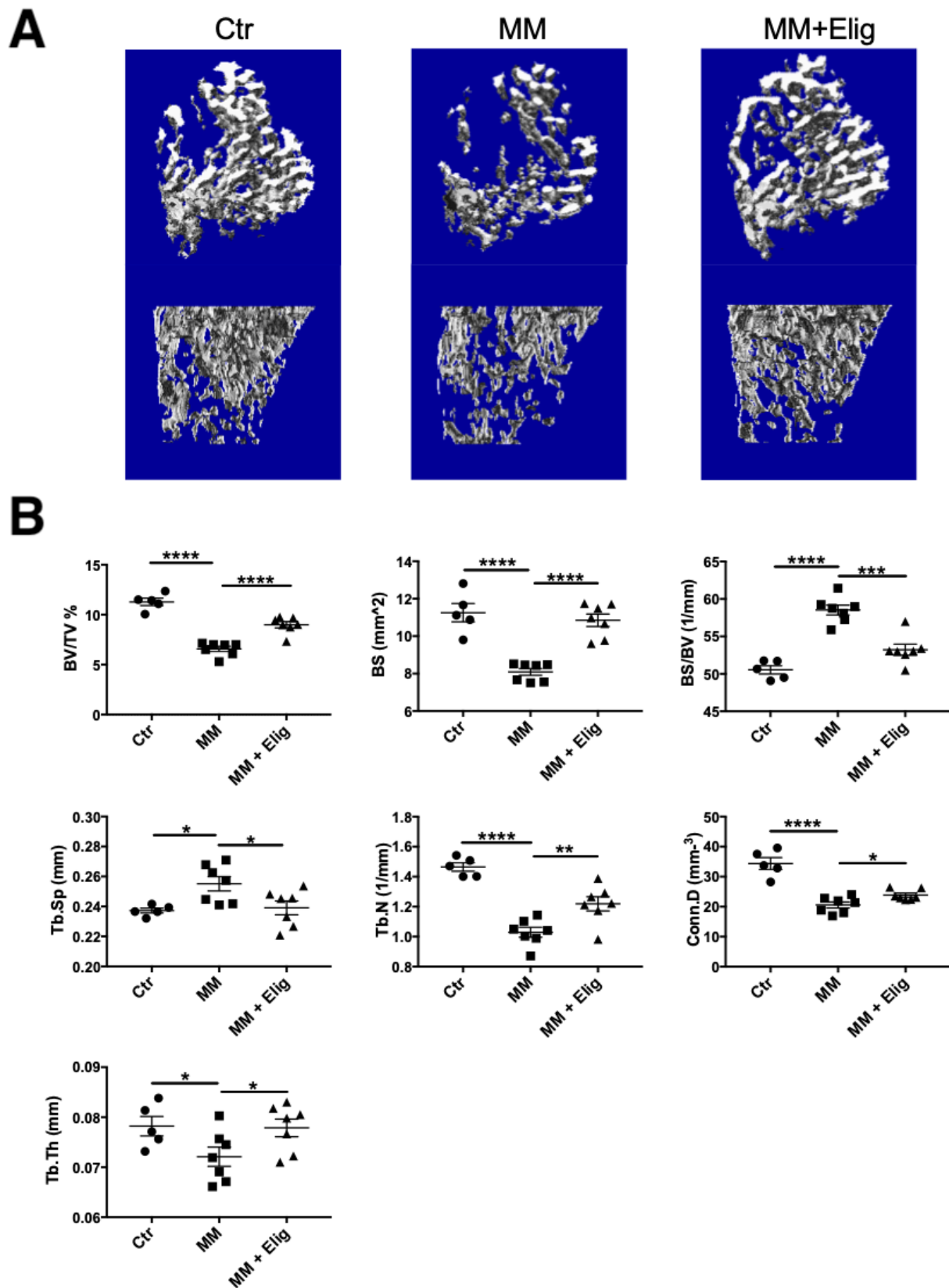


Figure 4.11 Eliglustat ameliorates 5TGM1-GFP MM cell induced bone diseases in 8-week-old female C57BL/KaLwRij mice. (A) Representative micro-CT reconstruction images of tibiae trabecular bones from naive control (Ctr) (n=5), myeloma bearing mice with normal chow (MM) (n=7), or myeloma bearing mice with eliglustat chow start from day 4 after tumor injection (150 mg/kg/d for 19 days) (MM + Elig) (n=7). (B) Micro-CT analysis parameters of tibiae including BV/TV, BS, BS/BV, Tb.Sp, Tb.N, Conn.D and Tb.Th indicates the bone quality is significantly ameliorated after eliglustat treatment. Error bars correspond to SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Statistical analysis was performed using Student *t* test.

Normal bone consists of inner trabecular bone and outer cortical bone, both of which are commonly affected in MM disease. To evaluate if eliglustat affects cortical bone in the MM, micro-CT analysis was applied to cortical bone as well. According to the micro-CT reconstruction images, a number of bone lesions were observed in the MM group when compared to normal Ctr group, whilst there was reduction of the holes in the MM+Elig group (Figure 4.12A). In contrast to MM group, there was a significant decrease in osteolytic lesions in tibia in MM+Elig group, which indicates that eliglustat may be beneficial for cortical bone quality (Figure 4.12B).

Micro-CT analysis of the cortical bone included three parameters: cortical area (Ct.Ar), the ratio of cortical area over total area (Ct.Ar/Tt.Ar) and cortical bone thickness (Ct.Th). These three parameters can together describe the overall integrity of the cortical bone. The analysis shows a significant decrease of all these three parameters in the MM group and MM+Elig group when compare with Ctr group. There was no significant improvement of all the parameters in MM+Elig group as compared to MM

group (Figure 4.12C). This could be that the sheer mass of cortical bone swamps out the smaller changes as there is so much of it. Similarly, previous calvaria and joints samples where clear differences could be visualised also did not come up on the micro-CT software, which is one of the limitations of Analyze 12.0.

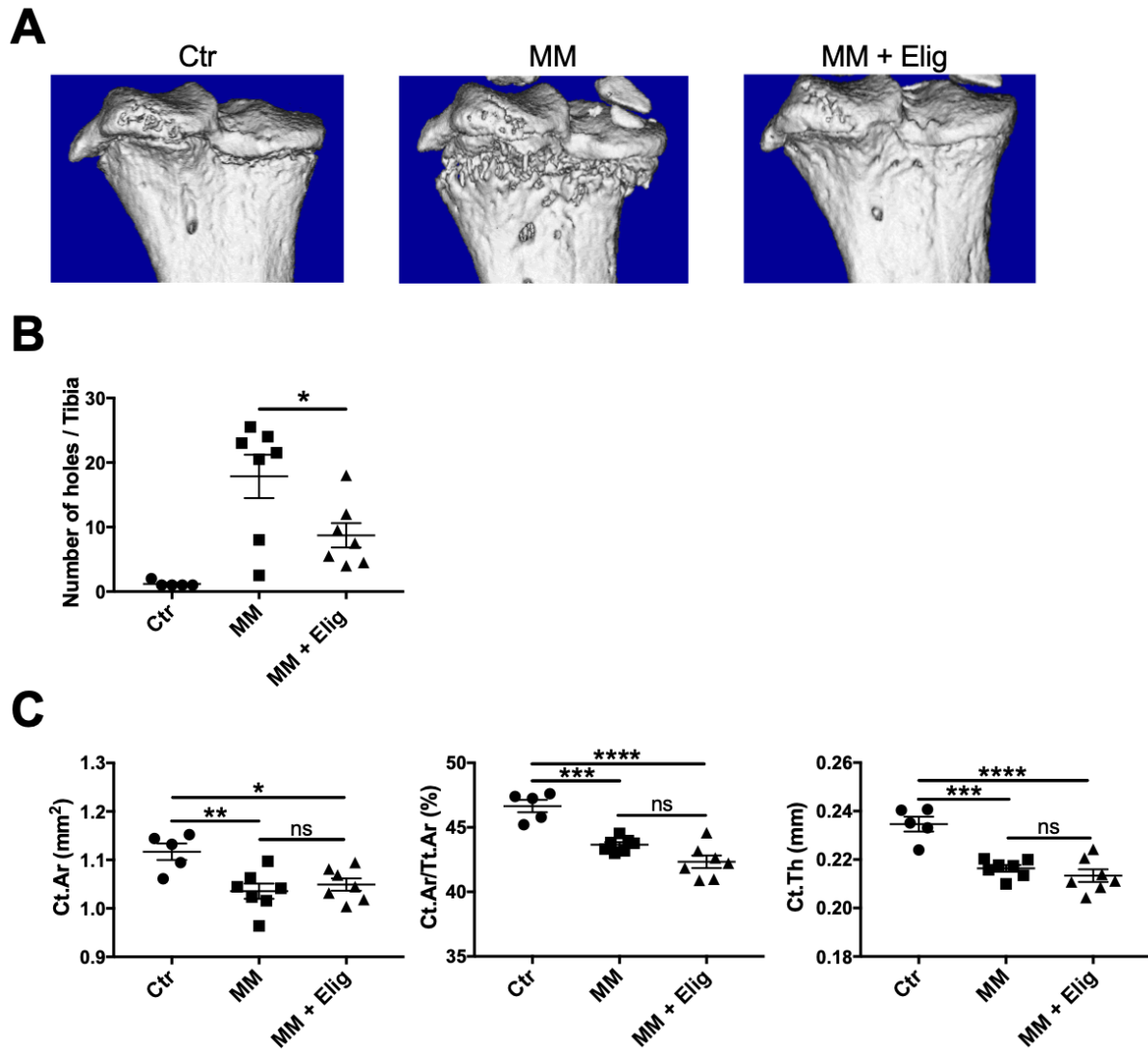


Figure 4.12 Eliglustat ameliorates 5TGM1-GFP MM cell induced cortical bone holes without affecting micro-CT cortical bone parameters in 8-week-old female

C57BL/KaLwRij mice. (A) Representative micro-CT reconstruction images of tibiae trabecular bones from naive control (Ctr) (n=5), myeloma bearing mice with normal chow (MM) (n=7), or myeloma bearing mice with eliglustat chow start from day 4 after tumor injection (150 mg/kg/d for 19 days) (MM + Elig) (n=7). (B) The number of bone lesions on cortical bones were counted. (C) micro-CT parameters including Ct.Ar, Ct.Ar/Tt.Ar and Ct.Th are measured. 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 Student *t* test.

Then, the tibiae were decalcified using the method as described in 2.13 and paraffin embedded to evaluate if there was any histomorphometry change in OC population. Compared to Ctr group, there was an increase of the TRAP positive area in MM group. In contrast to MM group, there was less TRAP positive area in MM+Elig group indicating eliglustat did decrease the OC population in murine MM model (Figure 4.13A).

This pattern was further confirmed by the bone histomorphometry quantitative analysis, with Oc.S/BS and N.Oc/B.Pm significantly increasing in MM as compared to Ctr group. Following treatment with eliglustat, all osteoclast parameters demonstrated a significant decrease, which can be observed from the comparison between MM+Elig and MM (Figure 4.13B). Put together, eliglustat significantly inhibits OC area, number and population on MM tibia.

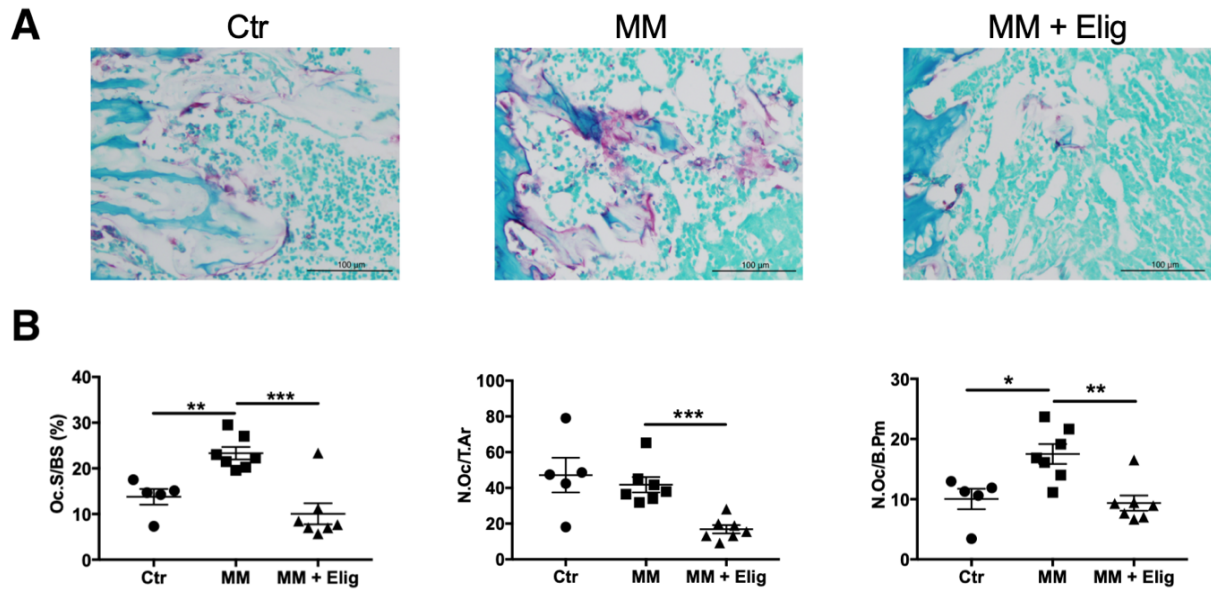


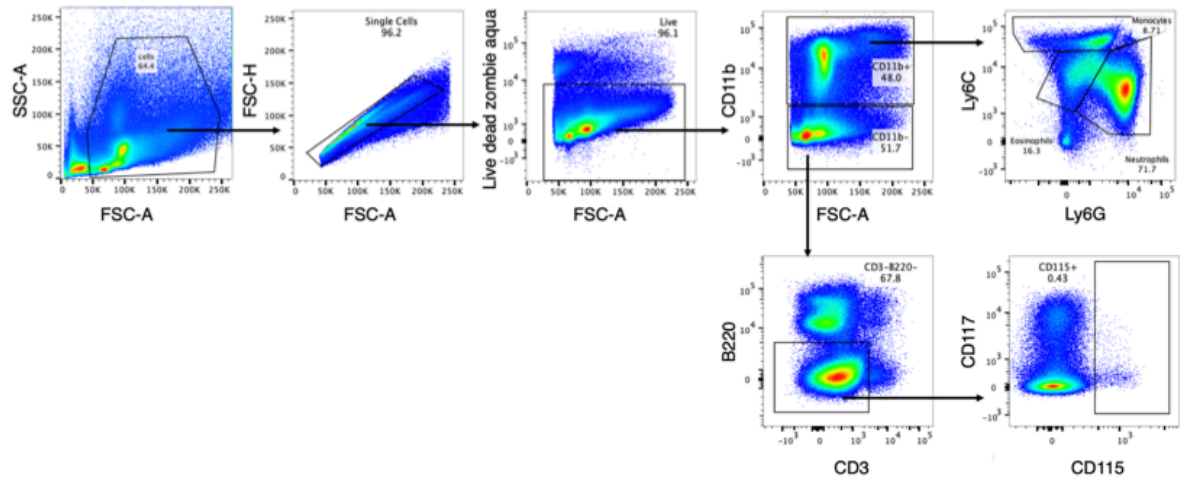
Figure 4.13 Eliglustat decreases OC area and number in 5TGM1-GFP MM cell induced murine model. (A) Representative TRAP/0.2% methyl green stained tibial histological sections showing red OCs (original magnification 40X) from naive control (Ctr) (n=5), myeloma bearing mice with normal chow (MM) (n=7), or myeloma bearing mice with eliglustat chow start from day 4 after tumor injection (150 mg/kg/d for 19 days) (MM + Elig) (n=7). (B) Bone histomorphometry parameters including percentage of OC surface over total bone surface (Oc.S/BS), number of OCs over total bone area (N.Oc/T.Ar) and number of OCs per bone perimeter (N.Oc/B.Pm). *P<0.05, **P<0.01, ***P<0.001. Statistical analysis was performed using Student *t* test.

As eliglustat improves bone quality by decreasing OC area and number, thus it needs to be determined whether this decrease was due to the inhibition of OC progenitors, or, due to interference with the fusion of macrophage precursors, or, both. To examine if eliglustat affects OC progenitors, flow cytometry technique and a widely recognised OC progenitor panel was applied²¹⁶. In short, the OC progenitors were regarded as CD115+ from CD3-B220- population based on CD11b- live BM cells. Moreover, an overall myeloid panel was also applied in order to broadly check if other cell populations were altered by eliglustat in MM²¹⁷. From BM CD11b+ population, it was accepted that Ly6C^{low}Ly6G- cells were eosinophils, Ly6G-Ly6C^{high} cells were monocytes and Ly6G+Ly6C^{low} cells were neutrophils. The combined gating strategy of OC progenitor panel and myeloid panel is shown as follows (Figure 4.14A).

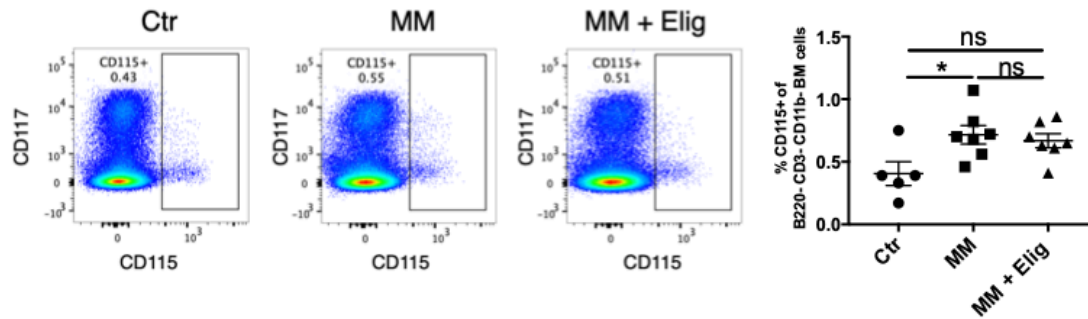
Based on afore mentioned gating strategy, the FACS plot of OC progenitors from each group was shown and quantitative analysis conducted (Figure 4.14B). According to the data, no significant difference was observed between MM group and MM+Elig group. These data indicates MM can generate more OC progenitors whilst eliglustat does not affect OC progenitors, which suggests that eliglustat may interfere with the formation of the OC rather than inhibiting the generation of OC progenitors.

To examine whether eliglustat has a broad effect on the myeloid population, three typical myeloid populations including monocytes, eosinophils and neutrophils were gated by flow cytometry (Figure 4.14A). The statistical analysis of each individual cell type across different treatment groups revealed that eliglustat had no effect on the CD11b+, eosinophil, monocyte and neutrophil population (Figure 4.14C).

A



B



C

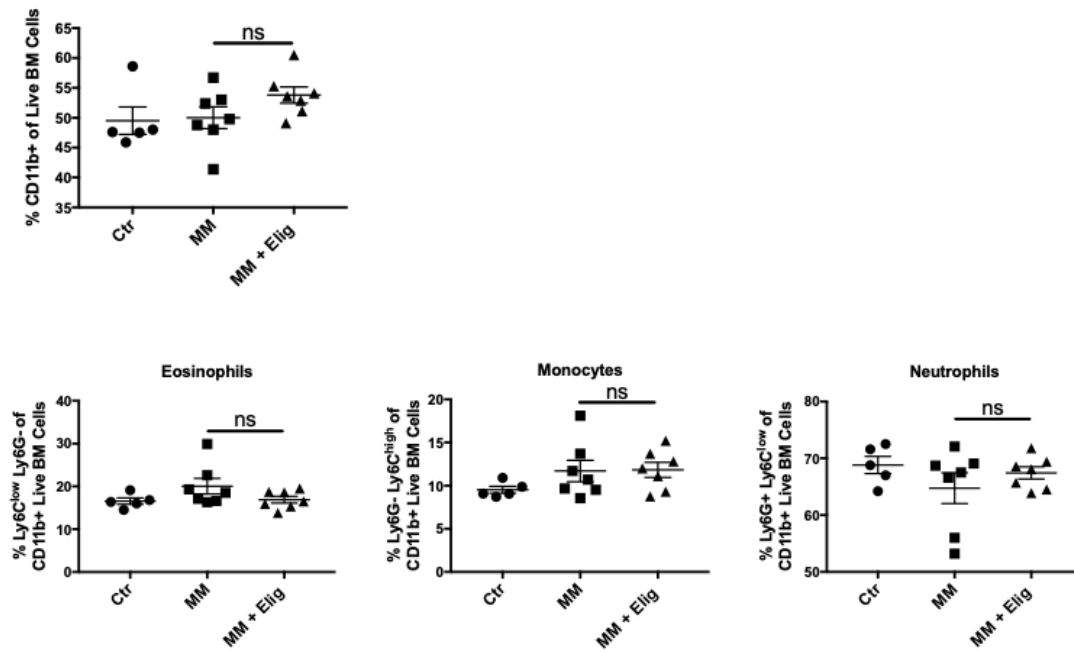


Figure 4.14 Eliglustat does not affect OC progenitor and myeloid cell population in 5TGM1-GFP MM cell induced murine model. BM of the mice from naive control (Ctr) (n=5), myeloma bearing mice with normal chow (MM) (n=7), or myeloma bearing mice with eliglustat chow start from day 4 after tumor injection (150 mg/kg/d for 19 days) (MM + Elig) (n=7) were collected for flow cytometry analysis. **(A)** Flow cytometry gating strategy of the OC progenitor (CD115+CD3-B220-CD11b-) and myeloid cells (including monocytes, eosinophils and neutrophils, which are identified based on Ly6C and Ly6G from CD11b+ gate). **(B)** Flow cytometry plots and quantitative analysis of OC progenitors. **(C)** Flow cytometry plots and quantitative analysis of CD11b+ population, eosinophils, monocytes and neutrophils between MM group and MM+Elig group. *P<0.05, ns means non-significant. Statistical analysis was performed using Student *t* test.

To investigate if there is any actual difference in OC progenitors caused by eliglustat, HSC, the precursor of the OC progenitor was analysed by flow cytometry. A widely recognized HSC panel is selected and applied in the HSC analysis²¹⁸. In short, the GMP, MEP and CMP populations were defined based on Lin-cKit+Sca1- gate (Figure 4.15A). Then, each individual population under the Lin- gate was analysed. According to the data, eliglustat had no effect on L-S+K+, L-S-K+ population, nor on GMP, MEP and CMP population in the MM condition (Figure 4.15B). These results may further suggest the OC progenitor and its precursor was not affected by eliglustat and eliglustat was likely to affect the transformation of OC progenitor to mature OC.

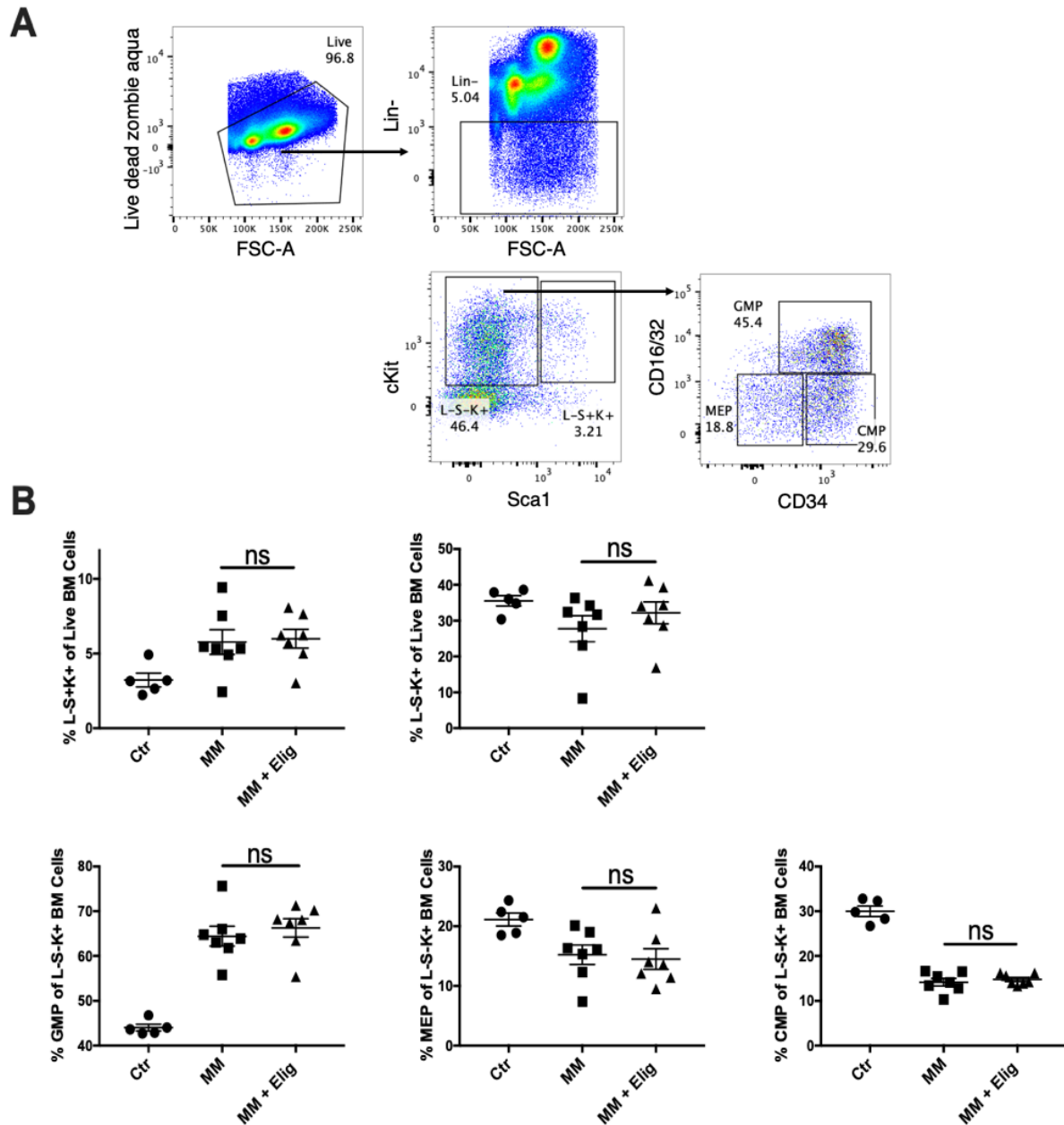


Figure 4.15 Eliglustat does not affect HSC population in 5TGM1-GFP MM cell induced murine model. The BM of the mice from naive control (Ctr) (n=5), myeloma bearing mice with normal chow (MM) (n=7), or myeloma bearing mice with eliglustat chow start from day 4 after tumor injection (150 mg/kg/d for 19 days) (MM + Elig) (n=7) were collected for flow cytometry analysis. **(A)** Flow cytometry gating strategy of the LSK and HSC. For the HSC, it can be further divided into GMP, MEP and CMP subpopulations. **(B)** Quantitative analysis show eliglustat can't alter the LSK, GMP, MEP and CMP population in MM. ns means non-significant. Statistical analysis was performed using Student *t* test.

As this bone lesion murine model was caused by MM, thus it is important to examine whether eliglustat can ameliorate the MM tumour burden. In the murine model, the MM cells mainly reside in BM and spleen. First the BM cells from femur and spleen cells were analysed by flow cytometry to detect the GFP positive 5TGM1-GFP cells. According to the FACS plots and quantitative analysis of BM cells (Figure 4.16A) and spleen cells (Figure 4.16B), eliglustat did not inhibit the MM tumour burden *in vivo*.

In addition to directly measuring the cells residing in the BM and spleen, the systemic MM tumour burden can be evaluated by measuring the serum IgG2bk paraprotein¹⁵¹, which is specifically released by MM cells. The IgG2bk paraprotein level measured from ELISA showed that MM greatly increase the serum IgG2bk level and that eliglustat was incapable of decreasing the IgG2bk level in MM (Figure 4.16C). In short, eliglustat had no anti-MM effect *in vivo*.

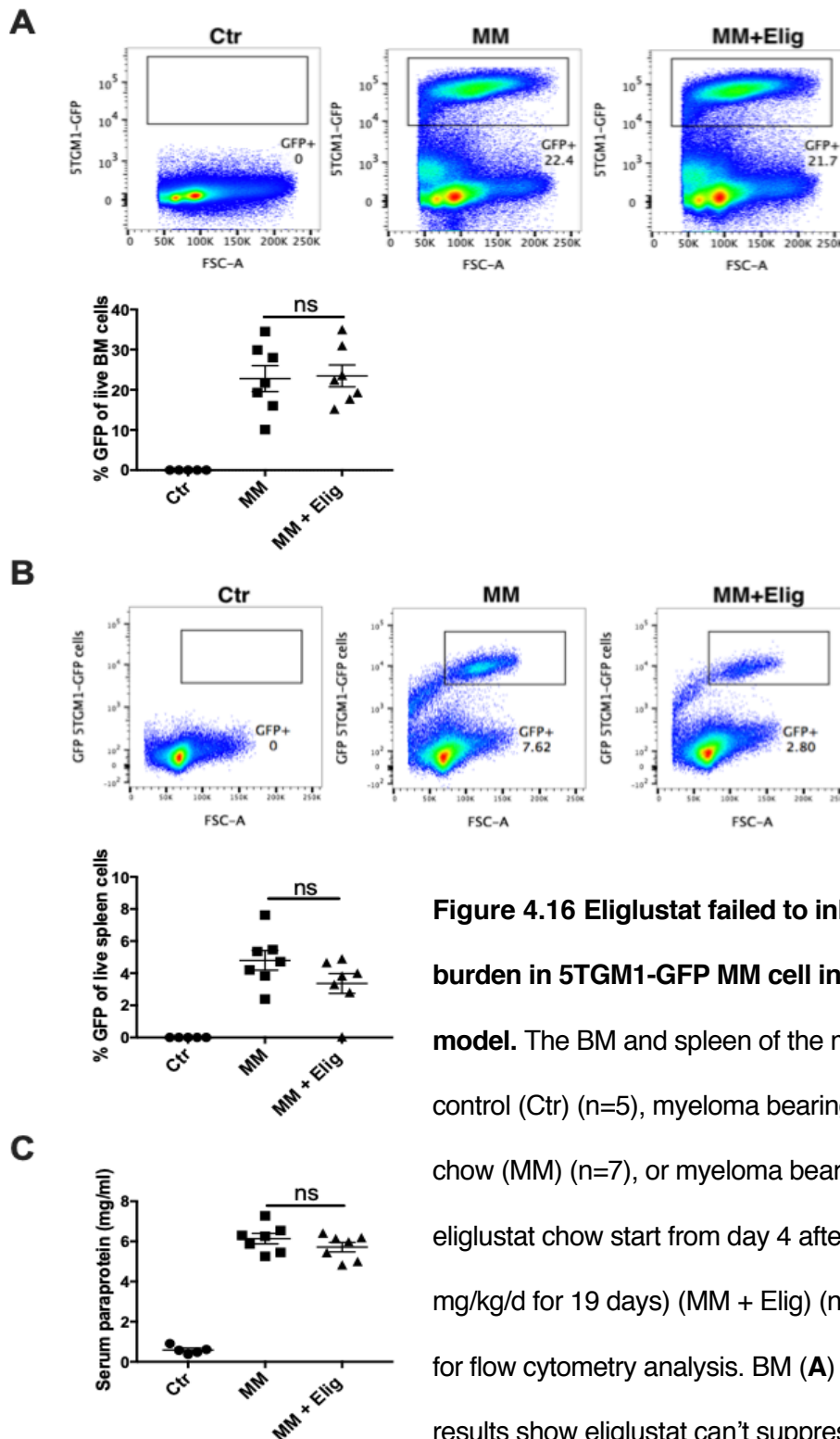


Figure 4.16 Eliglustat failed to inhibit MM tumor burden in 5TGM1-GFP MM cell induced murine model.

The BM and spleen of the mice from naive control (Ctr) (n=5), myeloma bearing mice with normal chow (MM) (n=7), or myeloma bearing mice with eliglustat chow start from day 4 after tumor injection (150 mg/kg/d for 19 days) (MM + Elig) (n=7) were collected for flow cytometry analysis. BM (**A**) and spleen (**B**) results show eliglustat can't suppress tumor cells. (**C**) Serum from each mouse was collected and IgG2bk was measured by ELISAs means non-significant. Statistical analysis was performed using Student *t* test.

4.2.6 Prophylactic administration of eliglustat ameliorates murine MM bone disease

As illustrated in previous section, eliglustat was capable of ameliorating bone lesions in MM. To determine whether administration of eliglustat 7 days before MM cell injection can maintain (instead of ameliorate) the bone quality in the treatment group as compared with control group, the following experiment was designed.

Eliglustat administration was started 7 days before the injection of 5TGM1-GFP murine MM cells and was maintained until the mice were culled 23 days after the MM cell injection. After the mice were sacrificed, tibiae were collected for micro-CT bone analysis, and femurs as well as spleen were harvested for flow cytometry analysis (Figure 4.17).

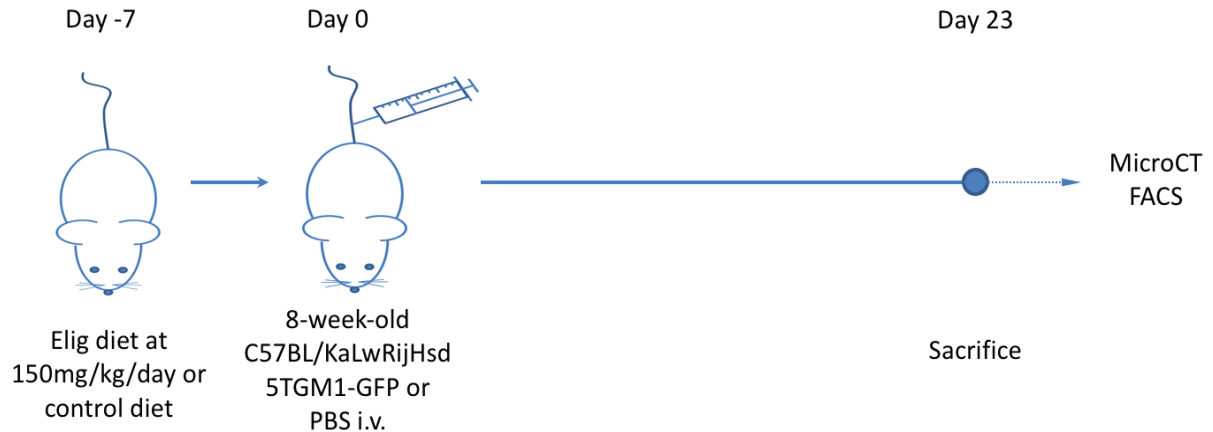


Figure 4.17 Prophylactic administration of eliglustat (Elig) 7 days before 5TGM1-GFP MM cell injection. Schematic cartoon timeline of the experiment design.

Quantitative analysis of trabecular bone by micro-CT precisely describes the bone quality in respective experimental groups. Similarly as shown in previous experiments, parameters like BV/TV, BS, Tb.N and Conn.D were all significantly increased in the

MM+Elig group compared with the MM group. Parameters like BS/BV and Tb.Sp both showed a significant decrease after eliglustat treatment indicating that trabecular bone was improved compared to the MM group (Figure 4.18). As the value of a critical parameter like BV/TV in eliglustat treatment group did not fully reach the value in Ctr group, this indicates that prophylactic treatment may not be superior to the therapeutic treatment regime.

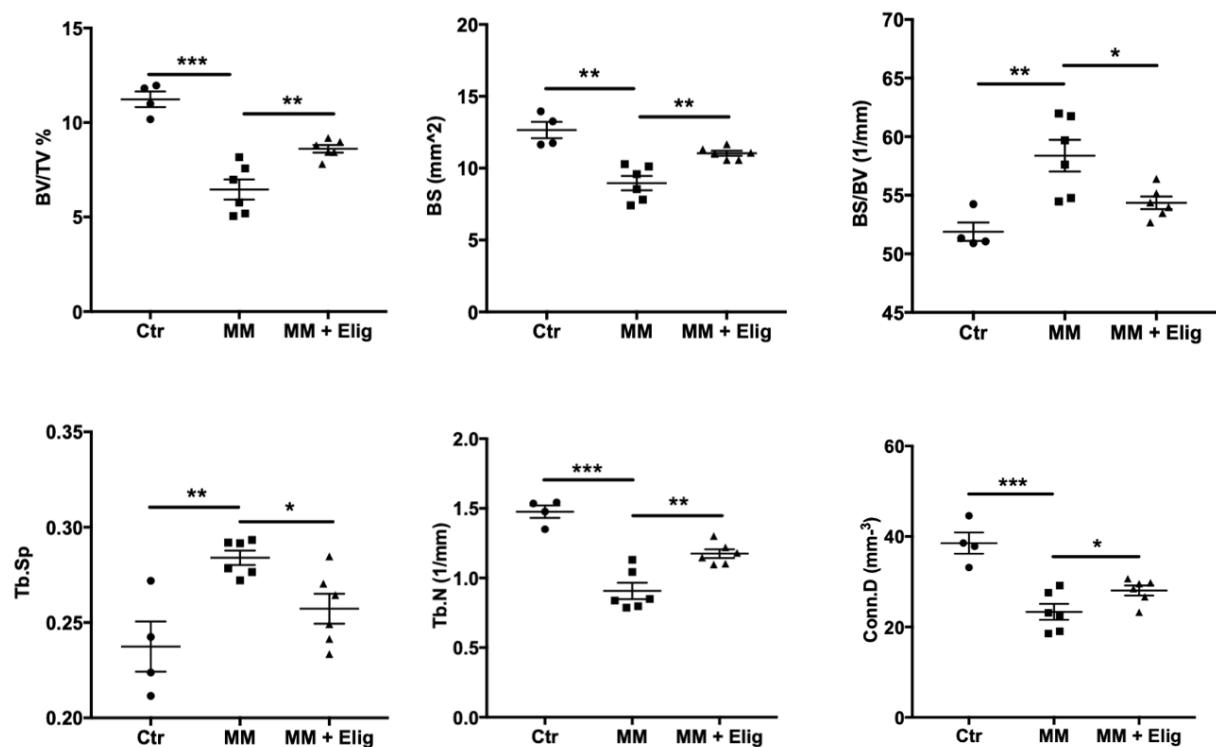


Figure 4.18 Prophylactic administration of eliglustat 7 days before 5TGM1-GFP MM cell injection ameliorates bone diseases in 8-week-old C57BL/KaLwRij mice. Micro-CT analysis parameters of tibiae including BV/TV, BS/BV, BS, Tb.Sp, Tb.N and Conn.D from naive control (Ctr) (n=4), MM bearing mice with normal chow (MM) (n=6), or MM bearing mice with Elig chow (150 mg/kg/d for total 30 days) (MM + Elig) (n=6). Error bars correspond to SEM. *P<0.05, **P<0.01, ***P<0.001. Statistical analysis was performed using Student t test.

To evaluate if eliglustat affects MM tumour burden in prophylactic treatment, the BM cells from femur and spleen cells were collected for flow cytometry analysis. The MM cells are 5TGM1-GFP cell so the GFP positive population was gated and analysed. According to the data, eliglustat showed no effect on MM tumour burden in the spleen (Figure 4.19).

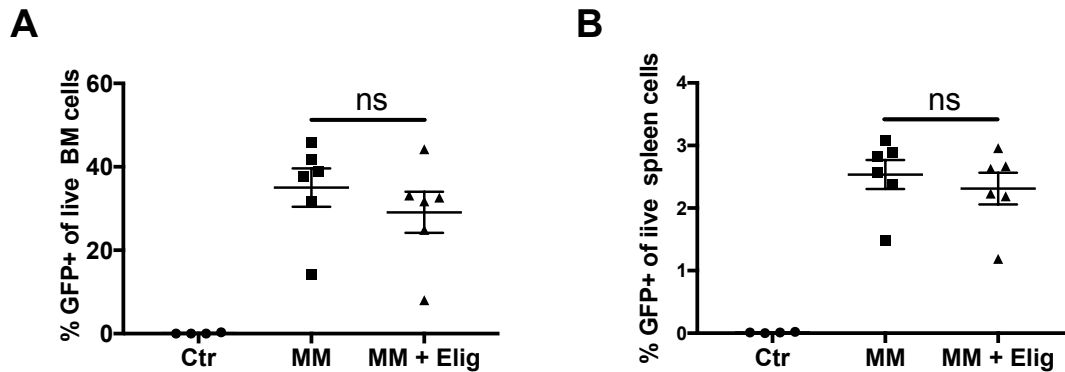


Figure 4.19 Prophylactic administration of Elig 7 days before 5TGM1-GFP MM cell injection in 8-week-old C57BL/KaLwRij mice cannot exacerbating tumor burden.

Prophylactic treatment with eliglustat does not exacerbate the tumor burden of BM (**A**) and spleen (**B**) from flow cytometry analysis. Error bars correspond to SEM. ns means non-significant. Statistical analysis was performed using Student t test.

4.2.7 Eliglustat improves bone quality in HFD mice without causing detrimental effect

Miglustat, another GSL inhibitor from a previous generation, caused detrimental effect to healthy mice under HFD condition (data not shown). To expand the potential applications and justify the reason why eliglustat is superior to miglustat, whether eliglustat affects the viability of mice under HFD condition needs to be evaluated.

Mice were divided into 3 groups including C57BL/6 mice feed on normal diet injected with 5TGM1-GFP cells, C57BL/6 mice fed with 42% HFD for 7 weeks before 5TGM1-GFP cell injection and C57BL/6 mice fed with 42% HFD for 7 weeks then injected with 5TGM1-GFP cells with eliglustat starts 4 days later. All the mice were sacrificed 30 days after MM cell inoculation. Serum, tibiae and femurs were collected for micro-CT, histology and ELISA analysis (Figure 4.20).

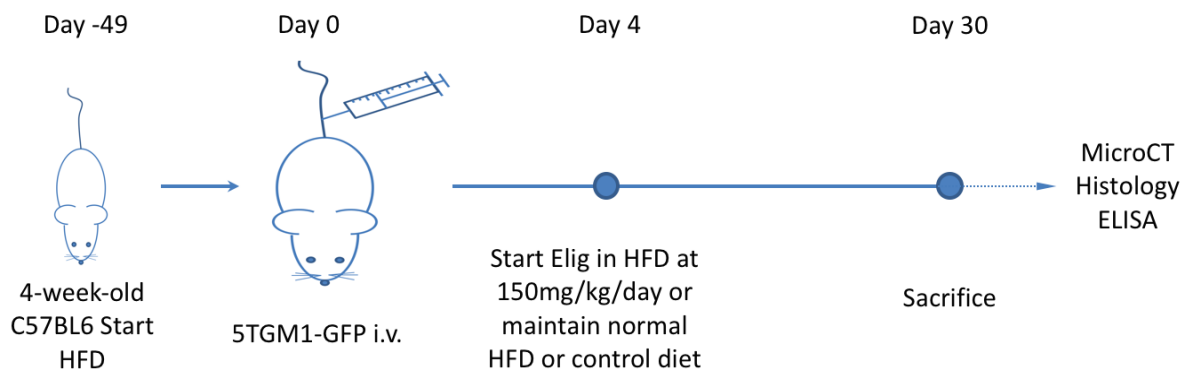


Figure 4.20 Evaluate eliglustat bone quality in HFD 5TGM1-GFP MM bearing C57BL/6 mice.

Schematic overview and experiment design. C57BL/6 mice were divided into MM injection with normal diet (MM)(n=4), MM injection with HFD (MM + HFD) (n=10) and MM injection with HFD plus Elig treatment (MM + HFD + Elig) (n=10).

To evaluate if the HFD works properly and to monitor if eliglustat causes detrimental effects, the body weight of each mouse was collected. From the data, HFD+MM group and HFD+MM+Elig both increased the body weight significantly when compared to MM group. There was a decrease of the body weight in HFD+MM+Elig group compared to the HFD+MM group however the average of body weight was still higher than Ctr group (Figure 4.21). Put together, HFD did successfully increased the body weight and eliglustat did not adversely affect the viability of the mice.

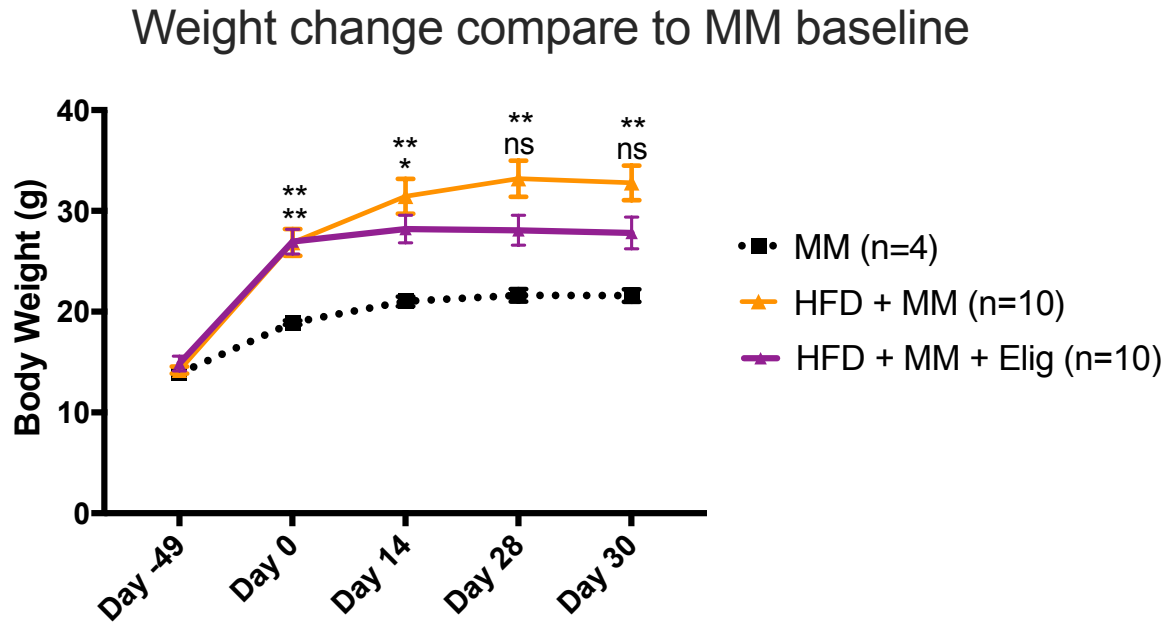


Figure 4.21 Weight change of HFD 5TGM1-GFP MM bearing C57BL/6 mice experiment.

C57BL/6 mice were divided into MM injection with normal diet (MM)(n=4), MM injection with HFD (MM + HFD) (n=10) and MM injection with HFD plus Elig treatment (MM + HFD + Elig) (n=10). Weight change of each group was observed during the experiment. In MM + HFD + Elig group, no detrimental weight loss was observed during the experiment. Error bars correspond to SEM, HFD+MM group data and HFD+MM+Elig group data are compared with MM group. *P<0.05, **P<0.01, ns means non-significant. Statistical analysis was performed using Student t test.

Next, the micro-CT reconstruction images showed that the HFD+MM group encountered a decrease in trabecular bone quality as compared to both MM group and HFD+MM+Elig group (Figure 4.22A).

Quantitative analysis of trabecular bone parameters BV/TV, BS, Tb.N and Trabecular BMD all had a significant increase in HFD+MM+Elig group when compared with HFD+MM group which indicated the bone volume, bone surface, trabecular bone number and trabecular bone mineral density all had improved after the administration of eliglustat under HFD condition. Moreover, Tb.Th was likely to increase in the eliglustat treatment group as compared with HFD+MM, which was another indicator for the better bone. Parameters like BS/BV had a significant decrease after eliglustat treatment reveals the ratio of bone surface over bone volume was decreased, which were indicators of trabecular bone quality was ameliorated (Figure 4.22B). In short, eliglustat safely ameliorates the MM bone lesions under HFD condition.

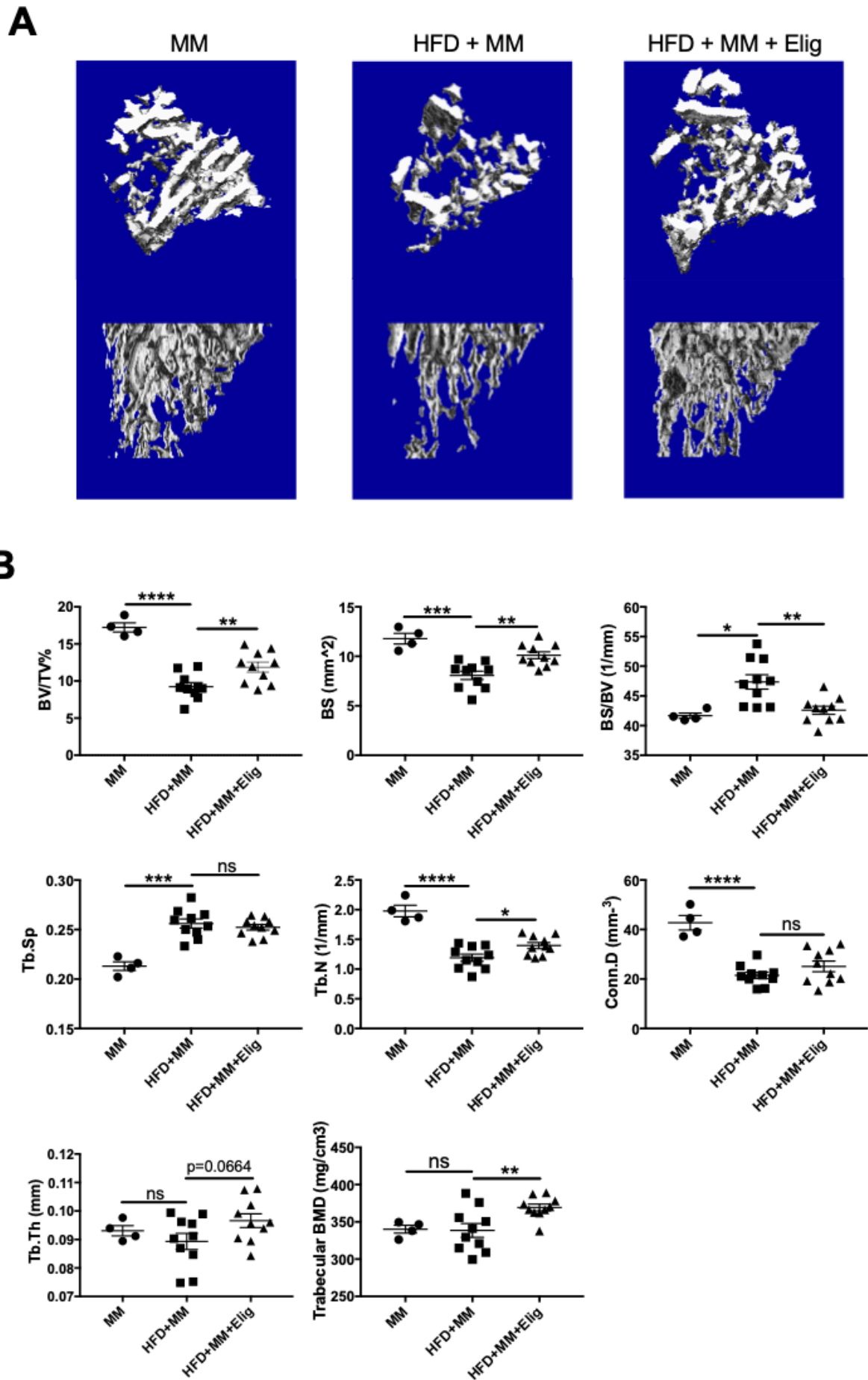
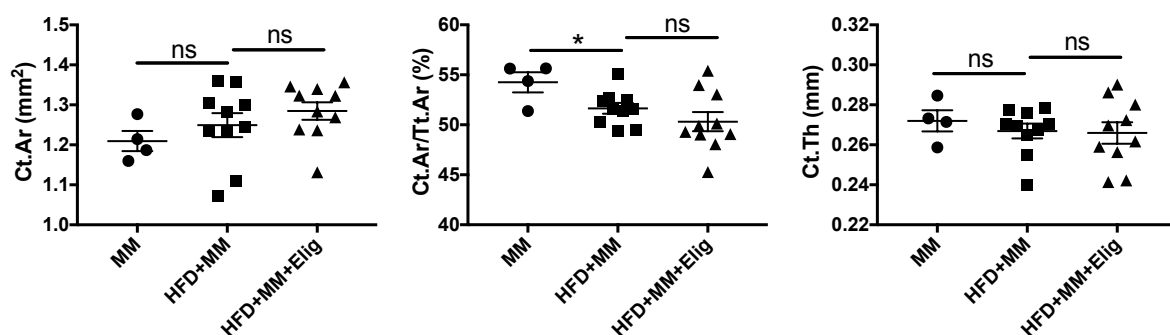


Figure 4.22 Eliglustat ameliorates bone conditions in HFD 5TGM1-GFP MM bearing

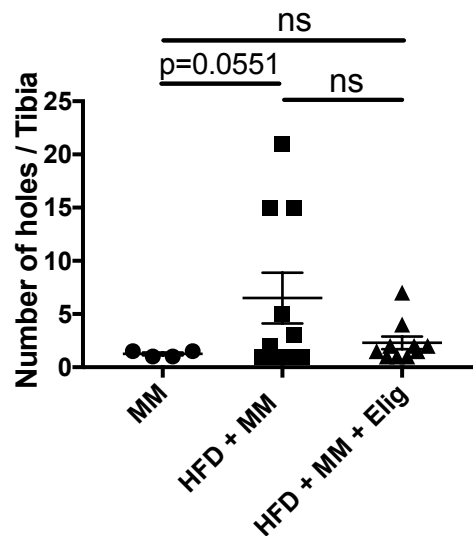
C57BL/6 mice without causing detrimental effects. C57BL/6 mice were divided into MM injection with normal diet (MM)(n=4), MM injection with HFD (MM + HFD) (n=10) and MM injection with HFD plus Elig treatment (MM + HFD + Elig) (n=10). **(A)** Representative micro-CT reconstruction images of tibiae trabecular bones. **(B)** micro-CT trabecular parameters including BV/TV, BS, BS/BV, Tb.Sp, Tb.N, Conn.D, Tb.Th and Trabecular BMD are measured. 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 Student *t* test.

Similar as previous experiments, micro-CT analysis of the cortical bone parameters including Ct.Ar, Ct.Ar/Tt.Ar and Ct.Th were applied in this experiment. Only Ct.Ar/Tt.Ar showed a significant change between MM control group (MM group) and HFD group (HFD+MM group), while the rest of the comparisons from these 3 parameters indicated there was no difference neither between MM group and HFD+MM group nor between HFD+MM group and HFD+MM+Elig group (Figure 4.23). The possible reason could be due to the limitation of the Analyze 12.0 software.

**Figure 4.23 Eliglustat has no impact on cortical bone in HFD 5TGM1-GFP MM bearing**

C57BL/6 mice. C57BL/6 mice were divided into MM injection with normal diet (MM)(n=4), MM injection with HFD (MM + HFD) (n=10) and MM injection with HFD plus Elig treatment (MM + HFD + Elig) (n=10). Cortical bone micro-CT parameters including Ct.Ar, Ct.Ar/Tt.Ar and Ct.Th are measured. Error bars correspond to SEM. *P<0.05. ns means non-significant.

Statistical analysis was performed using Student *t* test.



As the mice are fed on HFD, and MM is closely related with BM microenvironment including BM adipocytes, so it is necessary to see if BMAT is altered by eliglustat under HFD condition or not. Use the micro-CT technology, the BMAT on tibiae can be clearly visualized by Osmium Staining as described in 2.12. According to the micro-CT reconstruction of BMAT, both HFD+MM group and HFD+MM+Elig group showed

an increase of regulated BMAT (red area within rectangle) as compared with MM group (Figure 4.25A). The quantitative analysis of the regulated BMAT over bone volume reveals there was a significant increase of the regulated BMAT in HFD+MM group as compared with MM group. Moreover, there was no significant difference between HFD+MM group and HFD+MM+Elig group which indicates that eliglustat does not regulate BMAT metabolism (Figure 4.25B).

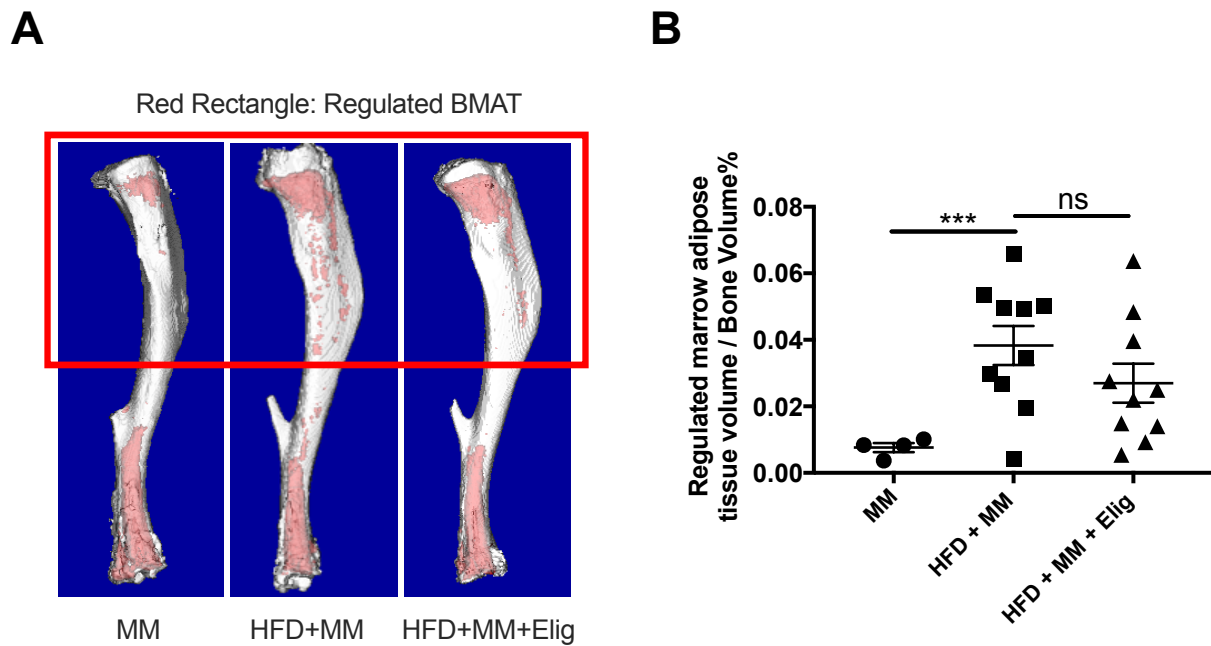


Figure 4.25 Eliglustat does not affect BMAT in HFD 5TGM1-GFP MM bearing C57BL/6

mice. C57BL/6 mice were divided into MM injection with normal diet (MM)(n=4), MM injection with HFD (MM + HFD) (n=10) and MM injection with HFD plus Elig treatment (MM + HFD + Elig) (n=10). **(A)** Representative images from each group show osmium stained regulated BMAT (red area on bone within red rectangle). **(B)** Quantification analysis of regulated BMAT from respective groups. Error bars correspond to SEM. ***P<0.001. ns means non-significant. Statistical analysis was performed using Student *t* test.

After micro-CT analysis, the tibiae were decalcified as described in 2.13 and paraffin embedded to evaluate the histomorphometry change in OC population. From the histology slides, large adipocytes were observed in HFD+MM group and HFD+MM+Elig group indicating the HFD did influence the BM niche. In addition, compared to HFD+MM group, the TRAP stain reveals there was a decrease of the TRAP positive area in HFD+MM+Elig group, indicating eliglustat may decrease the OCs (Figure 4.26A).

This observation was further confirmed by the histomorphometry quantitative analysis using the parameters including Oc.S/BS, N.Oc/T.Ar and N.Oc/B.Pm. In detail, all of the parameters had significant increase in HFD+MM group as compared to MM group (serves as non-MM control group as they did not get MM engrafted¹⁵¹). After the treatment of eliglustat, all of the three parameters show significant decrease, which could be observed from the comparison between HFD+MM group and HFD+MM+Elig group (Figure 4.26B). Put together, eliglustat significantly inhibits OC area, number and population in tibia from HFD MM murine model.

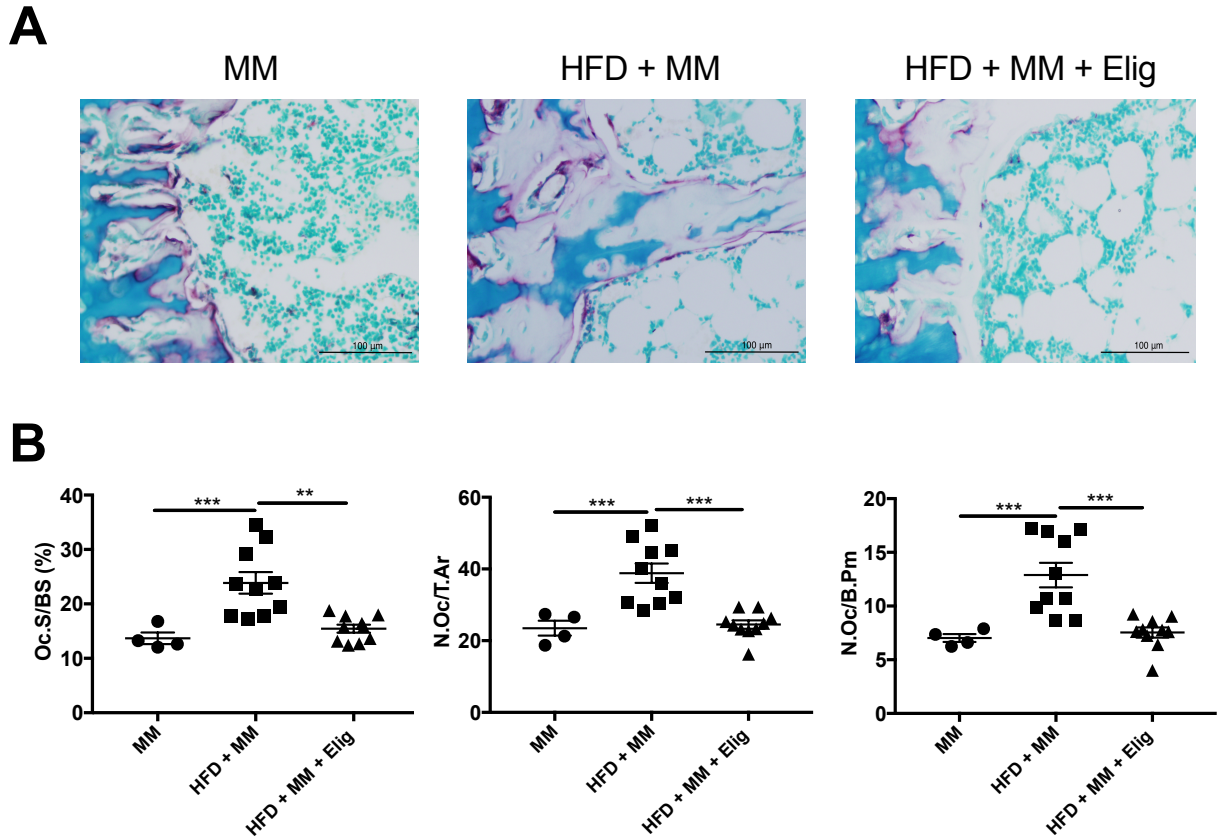


Figure 4.26 Eliglustat significantly inhibits OCs in HFD 5TGM1-GFP MM bearing

C57BL/6 mice. (A) Representative TRAP/0.2% methyl green stained tibial histological sections showing red OCs (original magnification 40X) from normal diet (MM) (n=4), MM injection with HFD (MM + HFD) (n=10) and MM injection with HFD plus Elig treatment (MM + HFD + Elig) (n=10). (B) Bone histomorphometry parameters including percentage of OC surface over total bone surface (Oc.S/BS), number of OCs over total bone area (N.Oc/T.Ar) and number of OCs per bone perimeter (N.Oc/B.Pm). Error bars correspond to SEM.

P<0.01, *P<0.001. Statistical analysis was performed using Student *t* test.

Compared to MM bearing C57BL/KaLwRij mice, the bone quality in MM bearing C57L6 mice caused by HFD was relatively good as there were still some observable OBs on the slides. To quantitatively measure OB area, number and population, parameters like Ob.S/BS, N.Ob/T.Ar and N.Ob/B.Pm were employed in this experiment. First, all parameters do significantly decrease in HFD+MM group as compared with MM group indicating the bone lesions were successfully set up in the disease group. Moreover, all these 3 parameters showed no significant difference between HFD+MM group and HFD+MM+Elig group which means eliglustat did not significant inhibits OB *in vivo*. In addition, the significance between MM group and HFD+MM+Elig group was less than the significance between MM group and HFD+MM group, which illustrated there was likely more OBs in the treatment group as compared with the disease group (Figure 4.27).

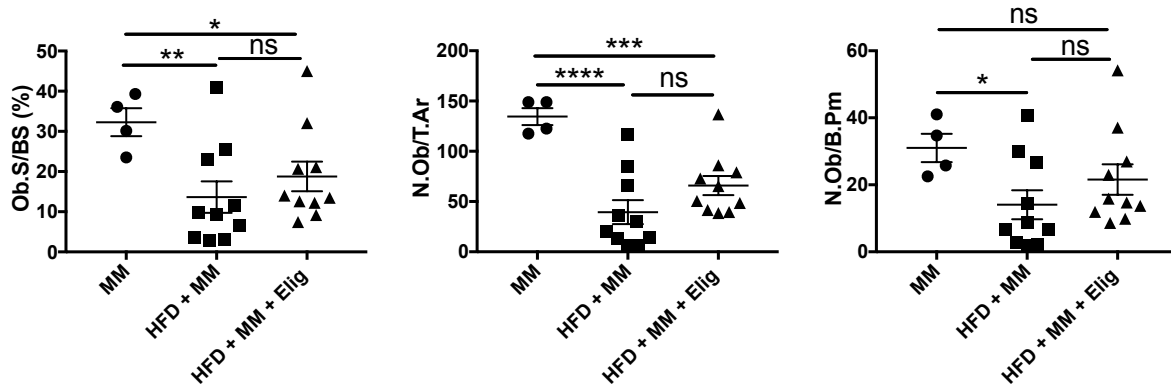


Figure 4.27 Eliglustat does not affect *in vivo* OB in HFD 5TGM1-GFP MM bearing C57BL/6

mice. Bone histomorphometry analysis for OB including percentage of OB surface over total bone surface (Ob.S/BS), number of OBs over total bone area (N.Ob/T.Ar) and number of OBs per bone perimeter (N.Ob/B.Pm) from normal diet (MM)(n=4), MM injection with HFD (MM + HFD) (n=10) and MM injection with HFD plus Elig treatment (MM + HFD + Elig) (n=10). 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 Student *t* test.

In this experiment, one femur from each mouse was collected for mechanical bending test to evaluate if eliglustat can increase the stiffness of the femur. The detailed procedures are described in 2.17. According to the maximum extension load, there was no difference between HFD+MM group and MM group, also, there was no difference between HFD+MM group and HFD+MM+Elig group (Figure 4.28). These data indicate HFD induced MM in C57BL/6 mice did not decrease the maximum extension load of the femur, moreover, eliglustat did not help in increasing the maximum extension load of femur.

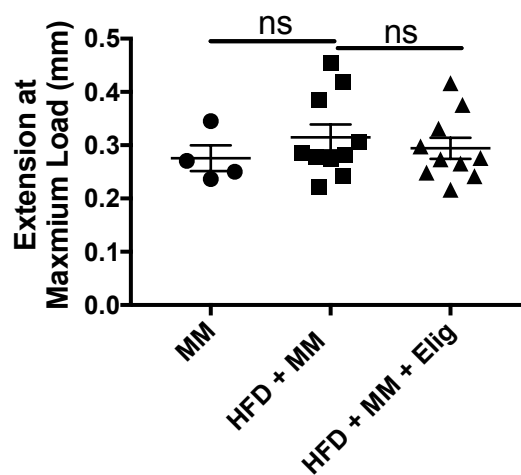


Figure 4.28 Femur maximum extension load does not decrease in HFD 5TGM1-GFP MM bearing C57BL/6. Femur maximum extension load was measured in different experimental groups, including MM injection with HFD (MM + HFD) (n=10) and MM injection with HFD plus Elig treatment (MM + HFD + Elig) (n=10). Error bars correspond to SEM. ns means non-significant. Statistical analysis was performed using Student *t* test.

To check the 5TGM1-GFP MM cells *in vivo*, spleen and BM cells were collected for flow cytometry analysis using the method as mentioned in 2.6.4 and 2.8.1. According to the spleen and BM data, there was no significant difference of tumour burden between MM group and HFD+MM group. This indicates HFD was more likely to cause a mild MM condition in C57BL/6 with limited engraftment of MM cells (Figure 4.29A and Figure 4.29B).

The serum of each mouse was collected every 2 weeks from day 0 until the day of sacrifice. To precisely verify the tumour burden from serum, IgG2bk paraprotein specifically secreted by 5TGM1-GFP MM cell was measured by ELISA. According to the data, IgG2bk paraprotein from both HFD+MM group and HFD+MM+Elig group increased significantly as compared with MM group (Figure 4.29C and Figure 4.29D).

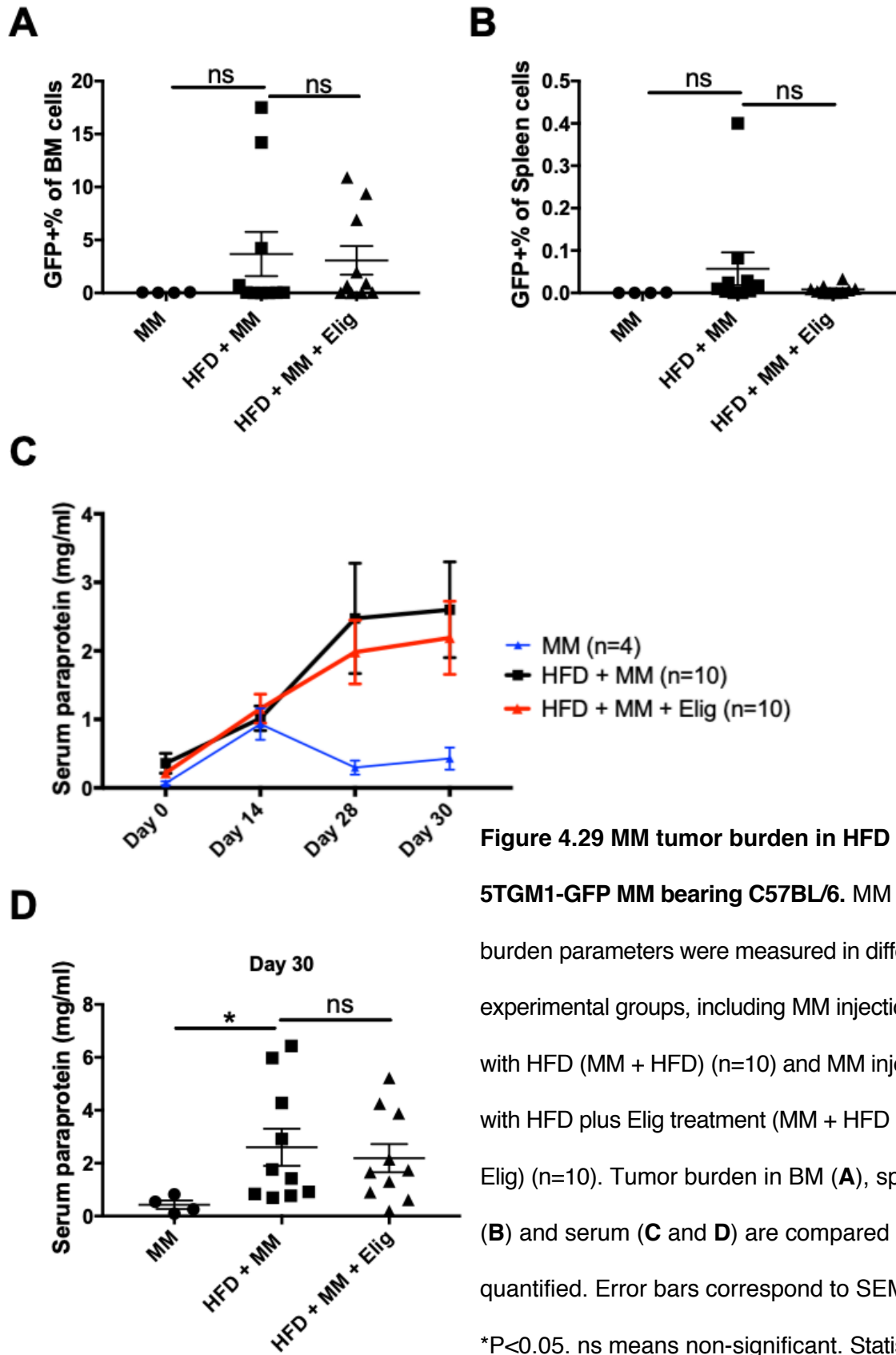


Figure 4.29 MM tumor burden in HFD

5TGM1-GFP MM bearing C57BL/6. MM tumor burden parameters were measured in different experimental groups, including MM injection with HFD (MM + HFD) (n=10) and MM injection with HFD plus Elig treatment (MM + HFD + Elig) (n=10). Tumor burden in BM (**A**), spleen (**B**) and serum (**C** and **D**) are compared and quantified. Error bars correspond to SEM.

* $P < 0.05$. ns means non-significant. Statistical analysis was performed using Student *t* test.

As a reference of the flow cytometry analysis, the cells from BM and spleen were counted and the wet spleen weight was also measured. According to the data, the BM cells had a significant decrease in HFD+MM group as compared with MM group and there was no difference between HFD+MM+Elig group and MM group (Figure 4.30A). For the spleen, both the spleen cell number and wet spleen weight had an increase in HFD+MM group and HFD+MM+Elig group, which indicates the MM and HFD condition could increase the cell number and overall weight (Figure 4.30B and Figure 4.30C).

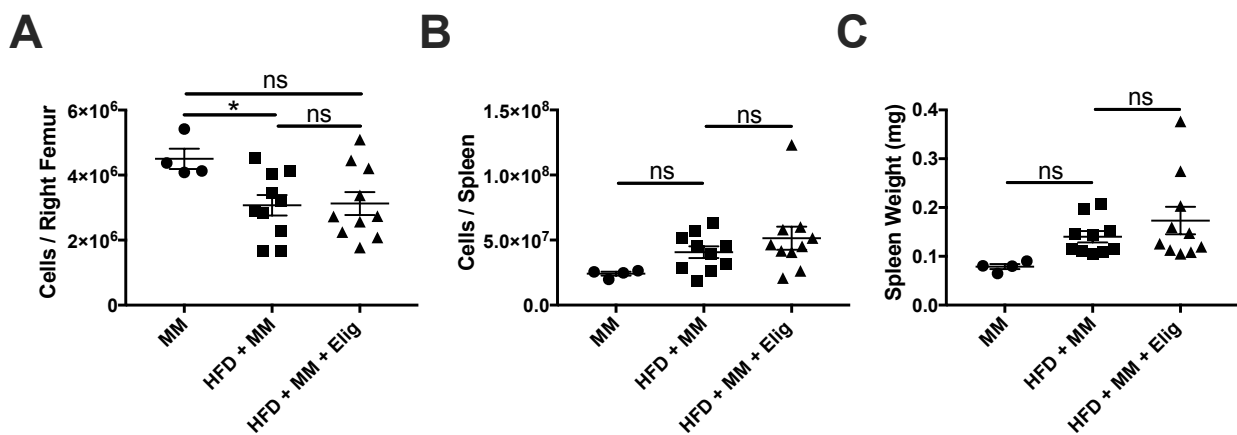


Figure 4.30 BM and spleen cell count as well as spleen weight in HFD 5TGM1-GFP MM bearing C57BL/6. BM cell number (A), spleen cell number (B) and wet spleen weight (C) are recorded and compared in different experimental groups, including MM injection with HFD (MM + HFD) (n=10) and MM injection with HFD plus Elig treatment (MM + HFD + Elig) (n=10). Error bars correspond to SEM. *P<0.05. ns means non-significant. Statistical analysis was performed using Student *t* test.

Chapter 4. Eliglustat Improves MM Bone quality by Inhibiting Osteoclastic Bone Resorption

4.3 Discussion

4.3.1 Does Eliglustat influence other cell populations in addition to osteoclast?

As shown in this chapter, eliglustat is capable of inhibiting OCs under various conditions. For example, eliglustat inhibits mouse BM derived OC *in vitro* and inhibits OC in healthy mice *in vivo*. Moreover, eliglustat also suppresses OCs in MM condition and MM with HFD condition. All of these data demonstrated that eliglustat performs as a consistent OC suppressor both *in vitro* and *in vivo*, which indicates that eliglustat may be an interesting candidate in the treatment of MM bone lesions or osteoporosis disease.

As from the bone homeostasis perspective, OBs, the only cell type that forms bone and acts as a counterpart to OCs, were examined. Similar to OCs, the potential effect of eliglustat on OBs was first evaluated *in vitro*. According to the result of experiment, eliglustat inhibits OB formation in a dose-dependent manner. As eliglustat mainly inhibits GCS and GSL, which are widely distributed in almost all kind of cells^{219,220}, thus it is not surprising that eliglustat inhibits OBs. However, there was no inhibition of OBs when eliglustat was administrated *in vivo* as evaluated by histomorphometry including OB surface ratio and OB number. It should be considered that the *in vitro* system is far less complex than the *in vivo* system with multiple cell types present so whilst eliglustat may have a direct effect on isolated OB *in vitro*, these cells may not be the primary targets *in vivo*. Additionally, different cell types may express different levels of GCS and it could be the expression of GCS in OCs is higher than OBs. In short, the *in vivo* data represents the complexity of a real biological system.

In vitro a dose-dependent inhibition of murine MM cell viability by eliglustat was observed, however eliglustat does not inhibit *in vivo* MM burden. Such phenomenon can be interpreted by two possibilities: 1) compared to OCs, MM cells express less GCS or GSL which makes eliglustat unable to inhibit the cells; 2) the treatment time and/or dosage is not suitable for MM tumour burden. The first explanation is unlikely as according to the literature report, human MM cells do express a large amount of GSL, especially GM3¹⁵. Admittedly, murine MM cells greatly differ from human MM cells and potential anti-MM work of eliglustat is suggested to be conducted in a xenograft model using human MM cells²⁰².

4.3.2 How does eliglustat improves MM bone lesions without affecting MM tumor burden?

As MM vicious cycle is defined as the interdependent action between MM and OC, which causes MM cells to stimulate OC proliferation and *vice versa*. The data in this chapter clearly demonstrated that OC number and OC area are significantly inhibited by eliglustat *in vivo*, thus it is expected the MM tumour burden can be relieved as the MM vicious cycle is interrupted. While according to the data, eliglustat was not able to inhibit MM tumour burden significantly in murine model. Such findings can be attributed to following reasons: 1) vicious cycle is one factor for MM cell proliferation (only contributes IL-6 production for MM cell proliferation); 2) the 5TGM1-GFP MM cell type is too aggressive to see all indirect effects of eliglustat within such a short time frame.

Regarding to the first possibility, according to the opinions from other researchers, the vicious cycle is one of the four pro-MM factors. Other players such as endothelial cells, impaired T cells and various MM growth factors can all contribute to MM survival and proliferation¹⁶⁰. Therefore, it seems the inhibition of OCs does not greatly affect MM cells. For the second possibility, researchers reported zoledronic acid (a kind of bisphosphonates that inhibits OC) can inhibit MM tumour burden in 5T2MM murine model. This 5T2MM model runs for 12 weeks, which is much longer than the 5TGM1 model²²¹. As the 5T2MM model is relatively longer, it allows enough time for the drug to exerts its anti-MM effect either directly by inhibiting MM growth factors or indirectly via the vicious cycle. In conclusion, the above two possibilities hinder the further observation of how eliglustat affects MM *in vivo*.

4.3.3 Difference between miglustat and eliglustat directs future translational application

Although many people prefer to simply summarize both miglustat and eliglustat as GSL inhibitors or GCS inhibitors, this is not really the case as they differ from each other in chemical structures, chemical categories, mode of reactions as well as adverse effects²¹². For example, miglustat is an iminosugar while eliglustat is a small synthesised molecule, and, the side effects of these two compounds are different which indicate they may distinctly affect other cells or physiological functions²¹².

The most prominent difference observed in this project was miglustat, which seemed to be detrimental to the health of the mice when administrated with HFD, while eliglustat was relatively compatible with HFD and less adverse effect was observed.

These findings justify the potential translational use of eliglustat, in the future, such as combination therapeutic strategy with current first-line drugs, which will be discussed in Chapter 6.

Chapter 5. Eliglustat Regulates TRAF3 Expression and Alters Lipid Membrane Organization

5.1 Introduction

Initially stimulated by RANKL, NF- κ B pathway is activated via TRAF adaptor proteins for OC formation process⁵⁷. Amongst six different subtypes of TRAF proteins located on lipid rafts, it is currently recognized that TRAF6 is essential for OC formation as mice generated with TRAF6 deficiency have marked osteopetrosis⁷⁷. Some researchers report that TRAF6 is re-localized into lipid rafts upon the addition of RANKL and stimulates nuclear accumulation of NFATc1, a pro-factor for OC formation signal²²². However, regarding the role of other TRAF proteins and of the lipid rafts in OC formation process, the complete mechanism remains to be further investigated.

Recently, it was reported that TRAF3 inhibits OC without TRAF6 being present *in vitro*. Moreover, TRAF3 is not regulated by TRAF6 upon RANKL stimulation²²³. It is known that after stimulation with RANKL during OC formation, TRAF3 protein decreased⁷¹ while TRAF6 protein increased²²². In addition to finding that TRAF3 knockout mice accrue less bone mass, the same group found that the lysosomal inhibitor, chloroquine, prevents TRAF3 degradation in a murine model and thereby inhibits OC formation⁷¹.

However, while TRAF3 protein is already present before OC formation and is independent of TRAF6, the hypothesis here is that eliglustat modulates TRAF3 to inhibit OCs. As a GSL inhibitor, eliglustat may interfere with lipid composition of the cell membrane and therefore could alter the lipid raft to consequently maintain TRAF3 protein levels upon RANKL stimulation. Alternatively, eliglustat could be an autophagy inhibitor preventing TRAF3 degradation. To investigate whether the above hypotheses are the mechanisms for eliglustat inhibition of osteoclastogenesis, the following experiments were conducted.

Chapter 5. Eliglustat Regulates TRAF3 Expression and Alters Lipid Membrane Organization

5.2 Results

5.2.1 Eliglustat prevents the degradation of TRAF3 in OCs

TRAF3 was recently found to be an important molecule in decreasing OC formation at the initial differentiation stage independently of TRAF6 signalling^{71,223}. Therefore, the protein level of TRAF3 expression was evaluated by Western blot. The BM cells collected from C57BL/6 mice were differentiated into OC according to the method described in 2.2. Proteins from different time points were collected as mentioned in 2.9. Day 1 is regarded as one day after the treatment with RANKL in addition to M-CSF, while on day 0 cells were only treated with M-CSF. The samples from day 0, day 1, day 3 and day 5 were collected. Then, all the samples with same amount (10-30 μ g) were loaded onto one NuPAGE Novex 4-12% Bis-Tris gradient gel to evaluate the level of TRAF3 protein at different time points. The result shows that TRAF3 protein levels decline during OC formation after RANKL was added. β -Actin is the house keeping gene that serves as the loading control (Figure 5.1). This result is consistent with previous findings reported by another group⁷¹.

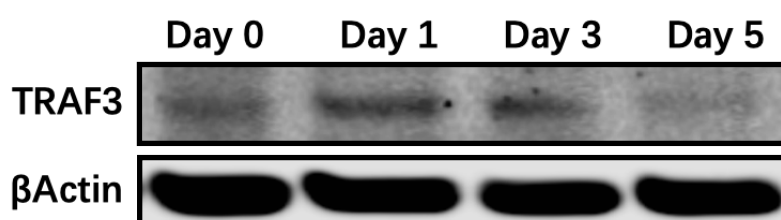


Figure 5.1 TRAF3 protein level decreases during OC formation process in primary BM cells from 4-week-old C57BL6 mice. The BM cells were first treated with 100 ng/ml M-CSF for 2 days, then treated with 20 ng/ml M-CSF and 50 ng/ml RANKL for the remaining 5 days. Culture media was changed every 3 days. Proteins were harvested at different time points and TRAF3 protein expression level was evaluated by Western blot. This experiment was repeated three times.

Next, to determine whether eliglustat affects OC formation via modulating TRAF3 expression, TRAF3 protein levels from OCs treated with eliglustat were evaluated. Same as before, fresh BM cells were harvested and differentiated into OC after stimulation with M-CSF and RANKL. On day 1, eliglustat was added at different concentrations (25 μ M and 50 μ M) together with RANKL to the culture medium. To observe the direct effect of eliglustat's impact on TRAF3, a relatively short time point like day 1 rather than day 5 was chosen.

It has been reported that chloroquine and NH_4Cl (lysosomal inhibitors) maintain TRAF3 levels and suppresses OC formation by inhibiting autophagy⁷¹. In addition, Bafilomycin A1 (BafA1) and 3-Methyladenine (autophagy inhibitors) prevent RANKL-induced TRAF3 degradation⁷¹. In contrast, MG-132 (proteasome inhibitor) did not prevent RANKL-induced TRAF3 degradation⁸⁶. In short, the degradation of TRAF3 in RANKL-treated OC progenitors is inhibited by the autophagy pathway instead of the proteasome pathway.

Here, BafA1 was used as the positive control to see if TRAF3 accumulates. The difference between chloroquine and BafA1 is that chloroquine blocks the fusion of the lysosome with the autophagosome, while BafA1 is a V type ATPase specific inhibitor (in lysosome, V-ATPases help maintain the low pH at 4.5²²⁴) and blocks the fusion of lysosome with autophagosome. In short, BafA1 has exactly the same effect as chloroquine. As TRAF3 is reported to be maintained due to the inhibition of autophagy⁷¹, there is no functional difference between the use of BafA1 and

chloroquine in this experiment. Taking into account that long-term BafA1 treatment affects the viability of cells^{225,226} and that a relatively medium to high dosage of BafA1 is needed to efficiently block the autophagic flux²²⁷, BafA1 was given at 10 nM for 2 hours before the proteins were extracted.

According to the blot, BafA1 maintained TRAF3 protein levels after 2 hours of treatment. In addition, a dose-dependent accumulation of TRAF3 after treatment with eliglustat was observed (Figure 5.2A). The bands were quantified by densitometry (Figure 5.2B). According to the quantification, eliglustat treatment significantly increased the ratio of TRAF3 over β -Actin, showing that eliglustat halted TRAF3 degradation in RANKL-treated OC progenitors (Figure 5.2B).

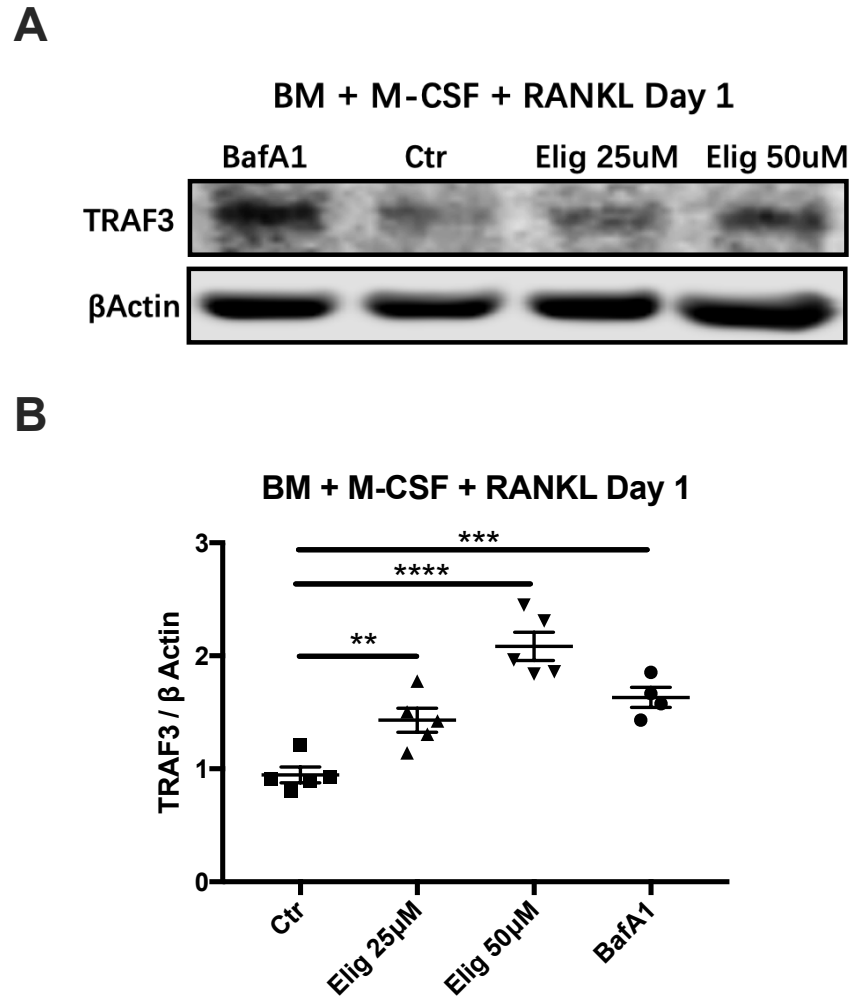


Figure 5.2 TRAF3 protein is not degraded in the presence of eliglustat. Primary BM cells from 4-week-old C57BL/6 mice were first treated with 100 ng/ml M-CSF for 2 days, then treated with 20 ng/ml M-CSF and 50 ng/ml RANKL for 1 day. BafA1 was added 2 hours before protein harvest. **(A)** Representative Western blot image of the TRAF3 protein expression level. **(B)** Quantification of the TRAF3 over β Actin ratio. Error bars correspond to SEM. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Statistical analysis was performed using One-way ANOVA. This experiment was repeated five times.

According to above findings, it was confirmed that eliglustat induces the accumulation of TRAF3 protein at the beginning of OC formation on day 1 in primary BM cells. To examine whether the TRAF3 pattern observed on day 1 is consistent during OC formation process (from pre-OC to mature OC), cells were treated for 5 days with RANKL and eliglustat. On day 5, the proteins were extracted from the cells using the same procedures as on day 1. According to the data, eliglustat can maintain the TRAF3 level on day 5 (Figure 5.3). These findings indicate that the impact of eliglustat on TRAF3 protein expression is consistent in OCs derived from primary BM cells at the start and the end of the *in vitro* differentiation process.

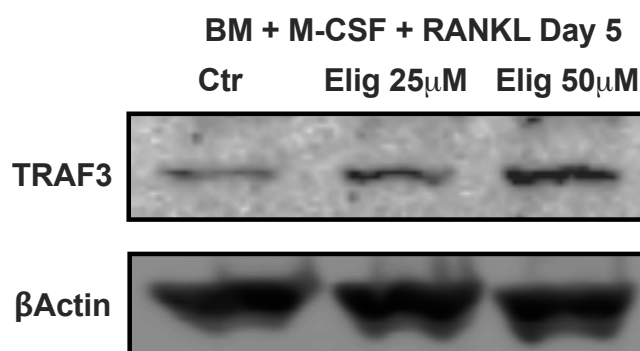


Figure 5.3 Representative Western blot showing TRAF3 protein level is accumulated by eliglustat during OC formation process in primary BM cells from 4-week-old C57BL6 mice on day 5. The BM cells were first treated with 100 ng/ml M-CSF for 2 days, then treated with 20 ng/ml M-CSF and 50 ng/ml RANKL for 5 day. Culture media was changed every 3 days.

To investigate whether the accumulation of TRAF3 protein is due to increased production or blocked degradation, the messenger RNA (mRNA) level of *Traf3* was measured by quantitative polymerase chain reaction (qPCR). On day 1, the mRNA of the cells which received the same treatment as above was extracted as described in 2.10. mRNA level of *Traf3* in the groups treated with eliglustat did not increase. Indeed, eliglustat-treated groups even showed a significant decrease of *Traf3* mRNA (Figure 5.4). This result could be due to high level of TRAF3 protein expression eliciting a negative feedback to mRNA generation. Importantly, these data suggest that the accumulation of TRAF3 protein by eliglustat may not be due to the increase of protein synthesis from mRNA but rather to an inhibition of degradation. However, there remains a possibility that *Traf3* may be post transcriptionally upregulated²²⁸.

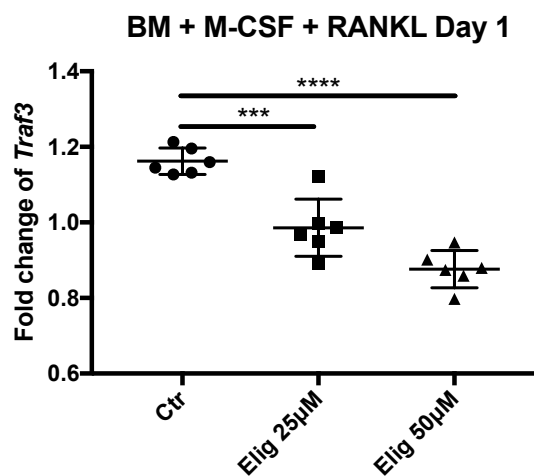


Figure 5.4 Quantification of the fold change of *Traf3* mRNA level indicates *Traf3* in BM derived OC is not increased after treated with eliglustat on day 1. The BM cells were first treated with 100 ng/ml M-CSF for 2 days, then treated with 20 ng/ml M-CSF, 50 ng/ml RANKL and eliglustat for 1 day. *Traf3* mRNA level was measured by qPCR. Error bars correspond to SEM. *** $P < 0.001$, **** $P < 0.0001$. Statistical analysis was performed using One-way ANOVA. This experiment was repeated six times.

For further biochemical analysis, whether the RAW264.7 murine macrophage cell line could be used rather than primary BM cells was tested. RAW264.7 cells readily differentiate into OCs upon stimulation with RANKL and M-CSF¹⁵. The culture and differentiation of RAW264.7 cells were described in 2.1 and 2.2. One day after RANKL and eliglustat were added to RAW264.7 cells, proteins were extracted. As before, BafA1 served as positive control for TRAF3 and was added to the cells at 10 nM 2 hours before harvesting the proteins.

According to the Western blot, similar to primary cells, RAW264.7 cells treated with eliglustat showed a dose-dependent accumulation of TRAF3 on day 1 (Figure 5.5A). As expected, the quantification of TRAF3 over β -Actin ratio was also significantly increased in the eliglustat treated group (Figure 5.5B). Taken together, the findings on TRAF3 in RAW264.7 cells treated with eliglustat confirm the previous finding in BM primary cells. We conclude that RAW264.7 cells are a valid *in vitro* model to study the role of eliglustat without the need to sacrifice mice.

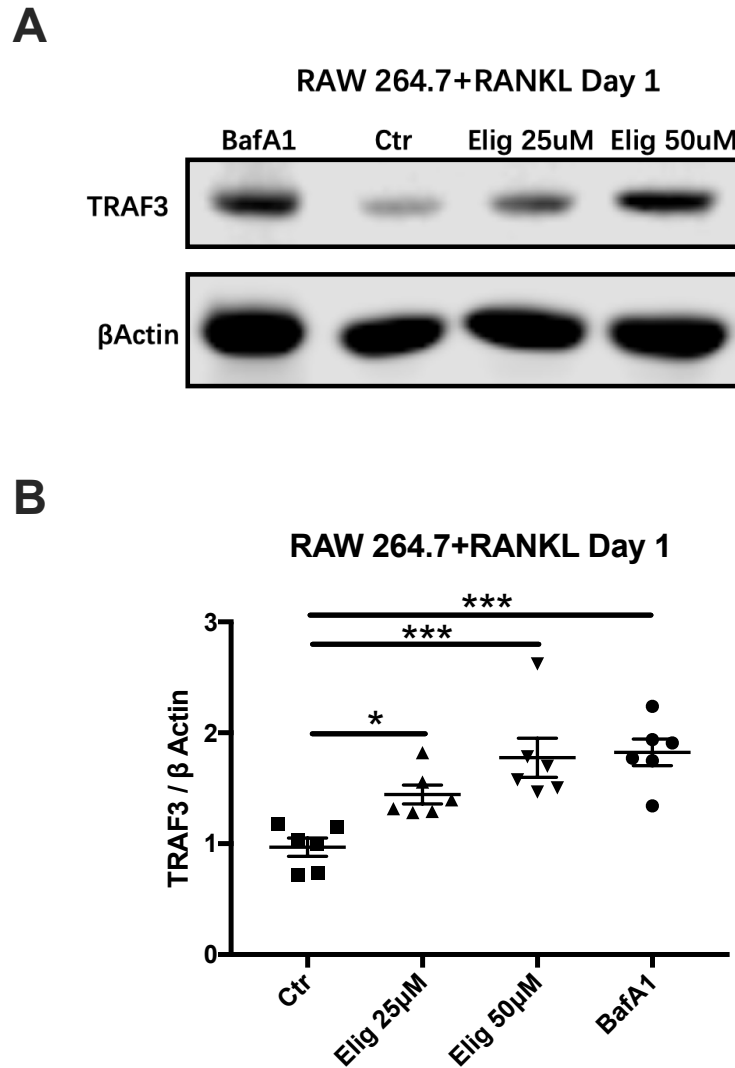


Figure 5.5 TRAF3 protein level is accumulated by eliglustat during OC formation process in RAW264.7 cells on day 1. RAW264.7 cells were treated with 75 ng/ml RANKL and 50 ng/ml M-CSF for 1 day. BafA1 was added 2 hours before harvesting the protein. **(A)** Representative Western blot image of TRAF3 protein expression level. **(B)** Quantification of TRAF3 over β Actin ratio. Error bars correspond to SEM. *P<0.05, ***P<0.001. Statistical analysis was performed using One-way ANOVA. This experiment was repeated six times.

Next, *Traf3* mRNA expression level in RAW264.7 cells after treatment with eliglustat was evaluated, no significant change of *Traf3* mRNA levels were observed after the RAW264.7 cells were treated with RANKL and eliglustat for 1 day (Figure 5.6). Consistent with previous findings in primary BM cells, TRAF3 accumulation in RAW264.7 cells after treatment with eliglustat was not due to the increase of *Traf3* mRNA.

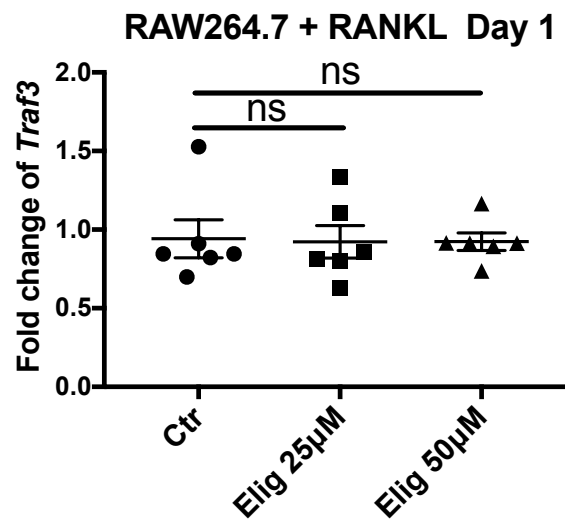


Figure 5.6 Quantification of the fold change of *Traf3* mRNA level indicates *Traf3* in RAW264.7 cells treated with RANKL and eliglustat are not increased on day 1. The RAW264.7 cells were treated with RANKL and eliglustat for 1 day. *Traf3* mRNA level was measured by qPCR. Error bars correspond to SEM. ns means non-significant. Statistical analysis was performed using One-way ANOVA. This experiment was repeated six times.

5.2.2 Eliglustat and autophagy

As described in section 5.2.1, the accumulation of TRAF3 by eliglustat is not likely to be due to the increase of *Traf3* mRNA level. Therefore, whether eliglustat has an effect by inhibiting the degradation of TRAF3 was checked. It was reported that the proteasome inhibitor MG132 marginally reduced TRAF3 degradation upon LT β R stimulation in HeLa cells²²⁹. However, this was not the case in OCs as Xiu *et al.* reported that the proteasome inhibitor MG132 had no effect on TRAF3 levels in OCs derived from primary mouse BM cells⁷¹. However, this could be due to the degradation of TRAF3 in different cell types having distinct mechanisms.

On the other hand, Xiu *et al.* reported that TRAF3 in OCs accumulates in the presence of chloroquine⁷¹. Moreover, Wei *et al.* reported that PDMP, a compound that inhibits GSL similarly to eliglustat, is an autophagy inhibitor²³⁰. To investigate whether eliglustat is an autophagy inhibitor, the autophagic flux of the cells was measured via LC3 and p62 protein expression level. As described in the autophagy introduction in Section 1.3, two forms of LC3 proteins can be detected in cells; the non-lipidated LC3I protein and lipidated LC3II protein. LC3-I migration is slower than LC3II in SDS-PAGE²²⁷. Autophagy activation involves the transition from non-lipidated LC3I protein to lipidated LC3II protein. However, a compound that increases LC3II does not necessary report on whether it is an autophagy inhibitor or autophagy inducer.

In that case, autophagic flux inhibitor and alternative markers such as p62 protein expression level are needed.

According to the Western blot results, as compared with control group treated only with RANKL (Ctr), RAW264.7 cells treated with 10 nM BafA1 for 2 hours showed an increase of LC3II. Moreover, p62 also accumulated as compared with Ctr group (Figure 5.7A). Such findings confirmed that BafA1 as the positive control worked efficiently as an autophagy inhibitor to block LC3II and p62 degradation.

In groups treated with eliglustat (25 μ M and 50 μ M), a significant increase of LC3II protein was observed (Figure 5.7A). This indicates that either the autophagic flux was enhanced in the RAW264.7 cells after treatment with eliglustat, or blocked at the degradation level. LC3II levels of eliglustat-treated (50 μ M) were quantified over several repeats (Figure 5.7B).

Next, p62 protein expression level was assessed. Similar to BafA1 treatment, eliglustat also showed increased p62 expression, suggesting that eliglustat may be an autophagy inhibitor (quantified in Figure 5.7C).

To further confirm whether eliglustat is an autophagy inhibitor, RAW264.7 cells that were treated with different doses of eliglustat for 22 hours, were cultured in 10 nM BafA1 for a further 2 hours to fully block autophagic flux. The addition of BafA1 failed to elicit a further increase the LC3II level of the RAW264.7 cells treated with 50 μ M eliglustat (Figure 5.7B), indicating that the autophagic flux was maximally blocked by eliglustat at the level of the lysosome.

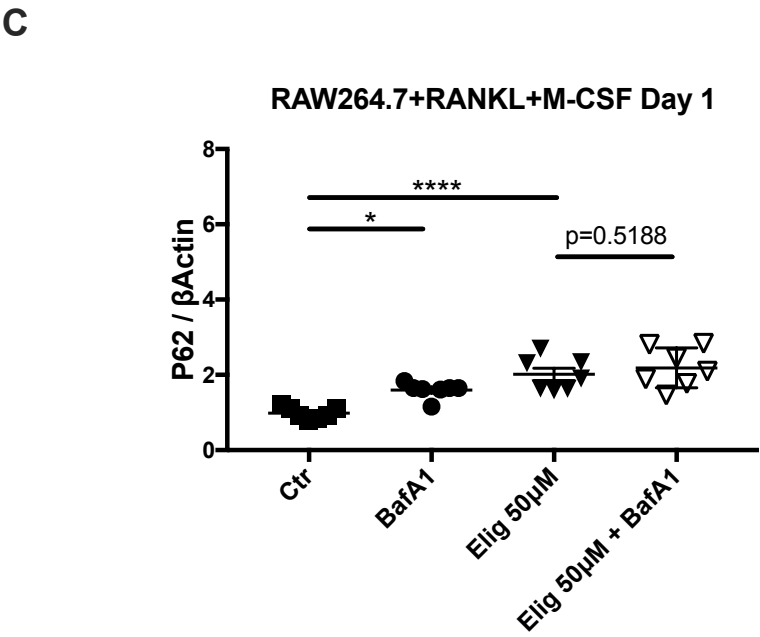
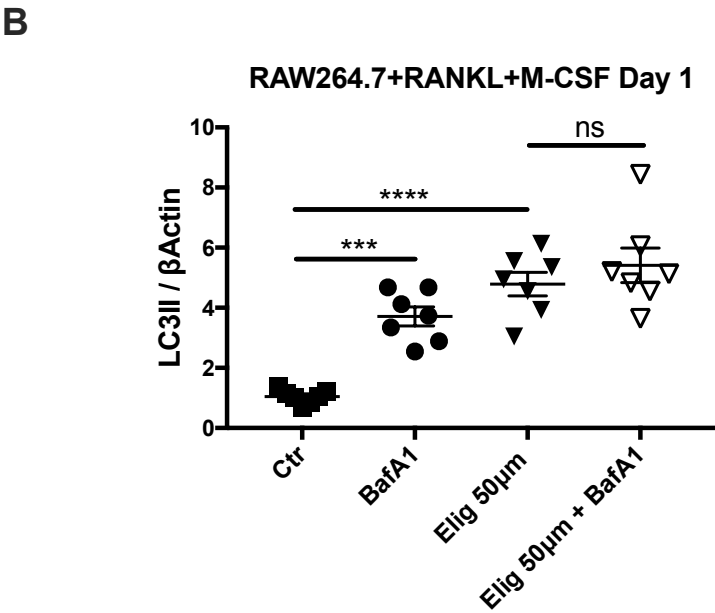
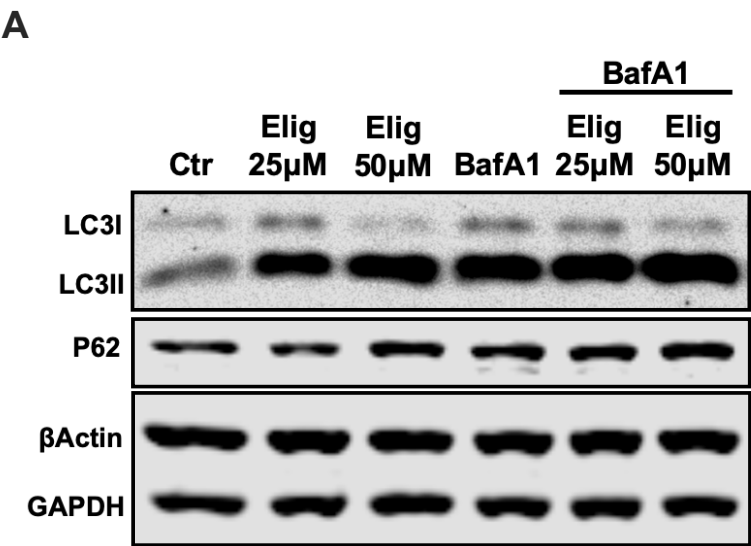


Figure 5.7 LC3II protein level and p62 protein level accumulated by eliglustat during OC formation process in RAW264.7 cells on day 1. Further LC3II protein accumulation was not observed after addition of BafA1. The RAW264.7 cells were treated with 100 ng/ml RANKL either with or without eliglustat for 1 day. 10 nM BafA1 was added 2 hours before protein harvest. **(A)** Representative Western blot of LC3 protein and p62 protein expression level. β -Actin and GAPDH served as loading control. **(B)** Quantification of the LC3II over β -Actin ratio. **(C)** Quantification of the p62 over β -Actin ratio. Error bars correspond to SEM. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$, ns means non-significant. Statistical analysis was performed using One-way ANOVA. This experiment was repeated seven times.

To validate whether the above findings regarding LC3 proteins were repeatable in mouse primary BM cells, fresh C57BL/6 murine BM cells were harvested and differentiated into OC after stimulation with M-CSF and RANKL. On day 1, eliglustat at different dosage (25 μ M and 50 μ M) was added. BafA1 was added 2 hours before the protein was harvested.

According to the Western blots, BafA1 and eliglustat both caused increase of LC3II (Figure 5.8A). Consistent with the findings in RAW264.7 cells, LC3II from the Elig 50 μ M group encountered a further increase as compared with the Elig 25 μ M group, indicating that 25 μ M is not a saturating concentration.

Interestingly, however, Elig 50 μ M treated with BafA1 group shows a further increase of the autophagic flux compared to Elig 50 μ M group (Figure 5.8B). This finding has

two possible explanations. First, different from the RAW264.7 cells, eliglustat at 50 μ M is still not at a saturating concentration to fully block the autophagic flux in primary BM cells. Therefore, a higher dosage of eliglustat may be needed. Second, eliglustat could be an autophagy inducer in primary BM cells, which is opposite to its effects on RAW264.7 cells.

Importantly, the expression levels of p62 protein on the same membranes were quantified. According to the quantification result, there was an increased accumulation of p62 protein after treatment with eliglustat as compared with baseline control group (Ctr) (Figure 5.8C), which indicates eliglustat is very likely to be an autophagy inhibitor.

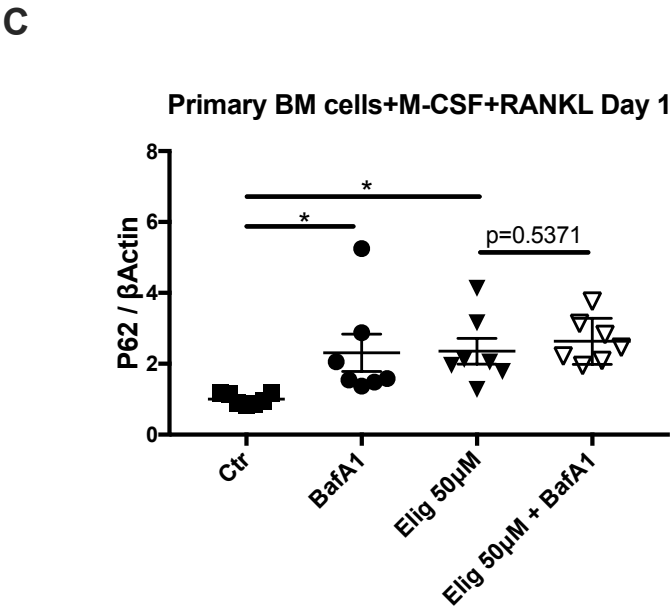
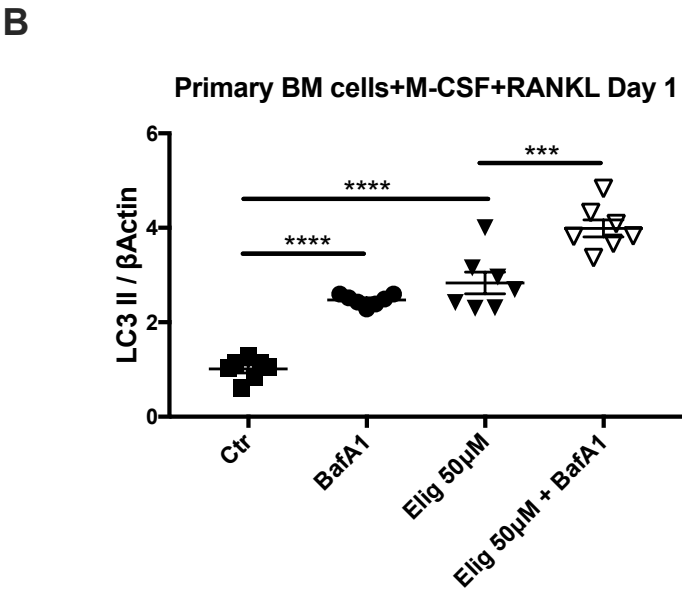
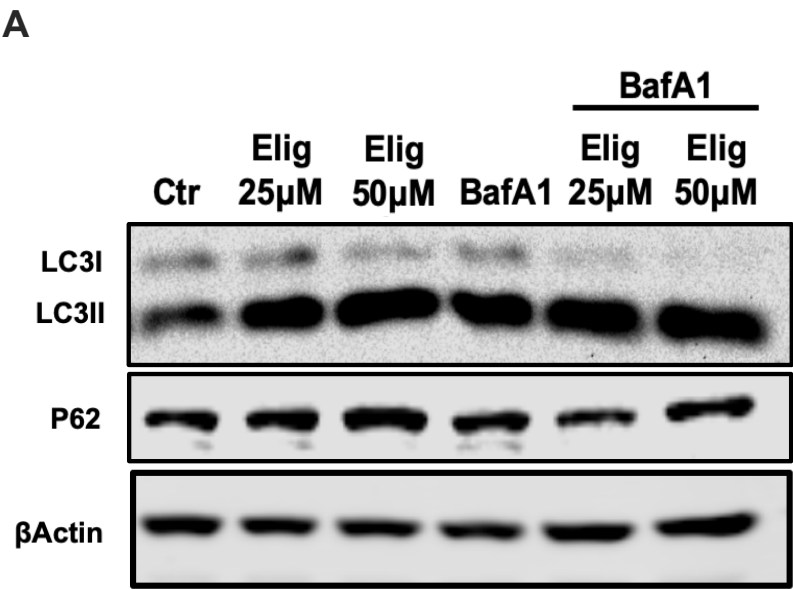


Figure 5.8 LC3II protein level was accumulated by eliglustat during OC formation process in primary BM cells on day 1. Further LC3II protein accumulation was observed after addition of BafA1. 10 nM BafA1 was added 2 hours before protein harvest. **(A)** Representative Western blot image of the LC3 protein and p62 protein expression level. β -Actin serves as loading control. **(B)** Quantification of the LC3II over β Actin ratio. **(C)** Quantification of p62 over β -Actin ratio. Error bars correspond to SEM. *** $P < 0.001$, **** $P < 0.0001$. Statistical analysis was performed using One-way ANOVA. This experiment was repeated seven times.

5.2.3 Eliglustat and OC membrane lipid

As the data from section 5.2.2 suggested that eliglustat may not fully block autophagic flux in OCs derived from primary BM cells at the concentration of 50 μ M, it remains possible that alternative mechanisms exist for the increased TRAF3 protein expression upon eliglustat treatment. Signal transduction in both canonical and non-canonical NF- κ B pathways involves the binding of RANKL to RANK receptor on the cell surface followed by downstream signalling. Eliglustat may change the membrane lipid organization (particularly GSL and subsequent lipid ordering) to alter the lipid environment where RANK is anchored²²⁰. This may lead to inhibition of RANK/RANKL signalling transduction, resulting in the failure to degrade TRAF3²³¹ and subsequently blocks the OC formation process.

To assess the lipid environment of cell membranes, the overall lipid organisation of RAW264.7 cells treated with eliglustat for 12 hours was evaluated by spectral imaging

after staining the membrane with NR12S²³² (spectral imaging reveals how packed the lipid membrane is, particularly approximate cholesterol and saturated lipid content) (Figure 5.9A). As shown in Figure 5.9B, each data point indicates the average value of generalized polarization (GP) of the cells from one image (each image has 5-10 cells). The higher the value of GP, the higher the level of lipid ordering. Similarly, the lower the value of GP indicates the higher the fluidity of the cell membrane. According to the results, there was no significant difference between RAW264.7 without treatment, RAW264.7 treated only with RANKL and RAW264.7 treated with RANKL+DMSO. However, eliglustat treatment significantly reduced the GP value (Figure 5.9B). Therefore, eliglustat fluidises the membrane, which can be achieved either by reducing cholesterol or increasing the unsaturated lipids in the plasma membrane. It is likely that the signalling transduction for OC formation may be affected as a consequence.

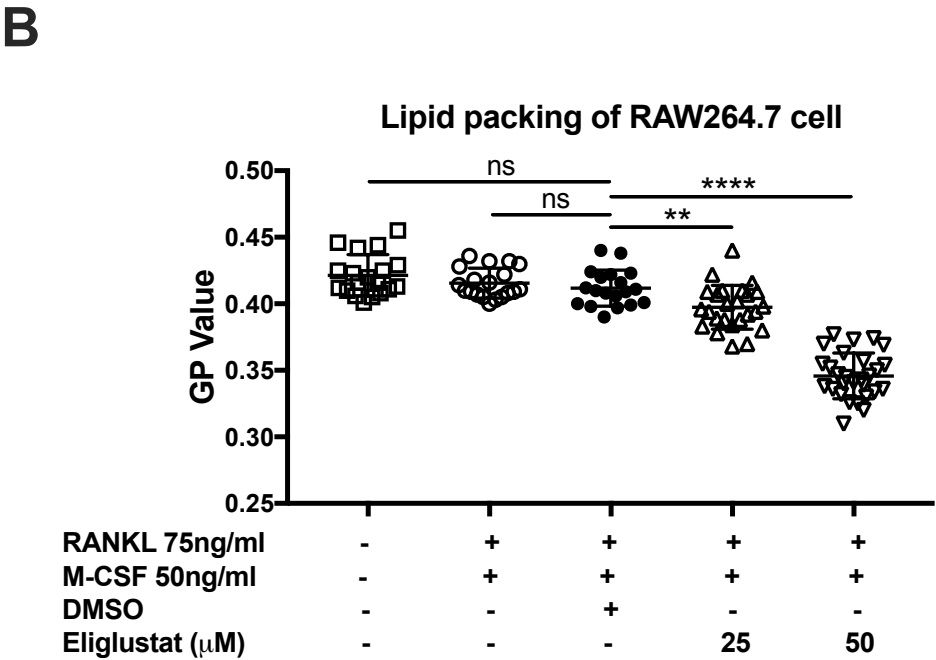
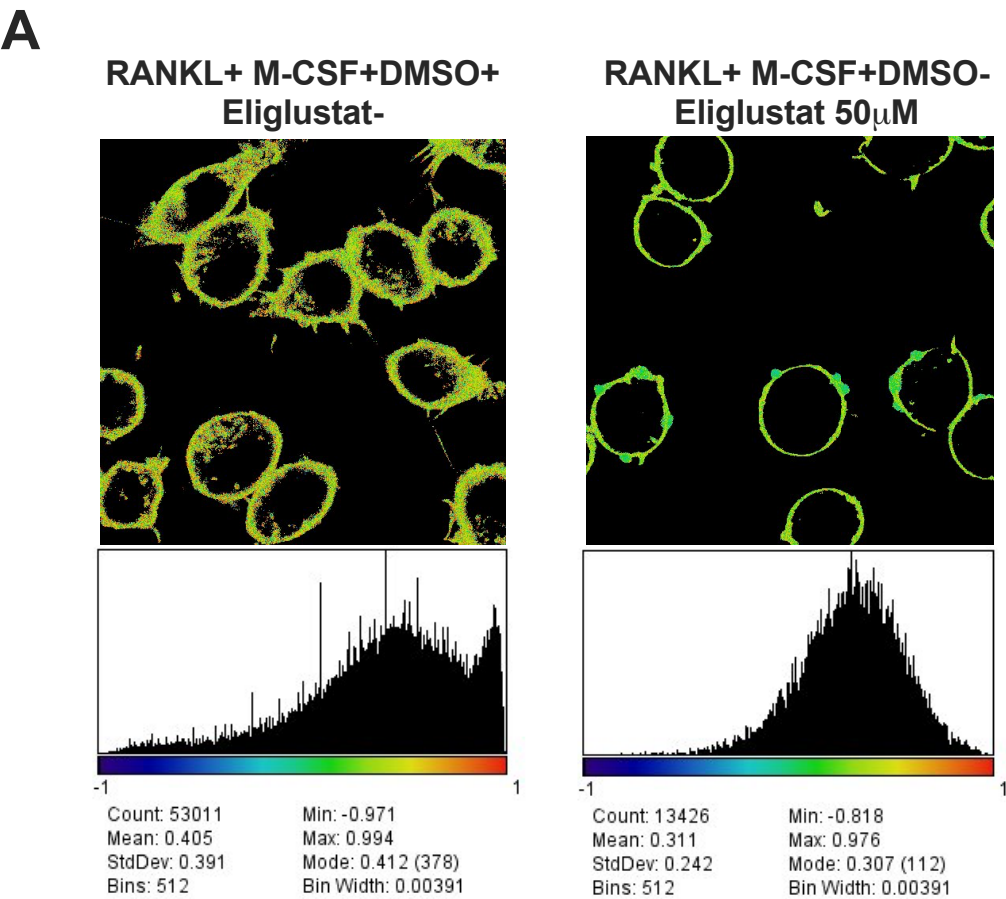


Figure 5.9 Eliglustat is fluidising the membrane during initial OC formation process in RAW264.7 cells after 12 hours. The RAW264.7 cells were treated with 75 ng/ml RANKL and 50 ng/ml M-CSF together with eliglustat or DMSO for 12 hours. Eliglustat was added to respective groups at the dose of 25 μ M and 50 μ M. **(A)** Representative images of the cells from Control group and eliglustat at 50 μ M group. Histograms of generalized polarization (GP) distribution illustrate ordered red end (1) and disordered blue end (-1). **(B)** The quantification for GP value was evaluated. Error bars correspond to SEM. **P<0.01, ****P<0.0001, ns means non-significant. Statistical analysis was performed using One-way ANOVA. This experiment was repeated two times.

Chapter 5. Eliglustat Regulates TRAF3 Expression and Alters Lipid Membrane Organization

5.3 Discussion

5.3.1 Is eliglustat an autophagy inhibitor?

As shown in Figure 5.8, eliglustat did not fully inhibit autophagy in OCs derived from primary mouse BM cells. However, it is reported that a similar member from the GSL inhibitor family called PDMP is an autophagy inhibitor in neuroblastoma cells^{230,233}. And, it can be confirmed that, eliglustat almost fully inhibits autophagic flux in the murine RAW264.7 macrophage cell line stimulated with RANKL and M-CSF.

On the other hand, Shen *et al.* concluded that another GCS inhibitor (Genz-123346) is an autophagy inducer in HEK293 cells²³⁴. However, their results are not plausible and self-contradictory. First, they concluded that there was a negative correlation between the increasing dosage of Genz-123346 and a decrease of p62 protein level without showing the original Western blot image. Therefore, the p62 data itself is not convincing. Indeed, the data presented in Figure 5.7 and Figure 5.8 did not confirm their findings regarding p62 protein neither in RAW264.7 cell line nor in primary BM cells. Secondly, their Western blot results show that Genz-123346 caused an apparent accumulation of LC3II that was not further increased when treated together with BafA1. This is indeed consistent with Figure 5.7 and suggests that both eliglustat and Genz-123346 are indeed an autophagic flux inhibitor as opposed to an inducer.

GD3 is a member of the GSL family and therefore its formation is inhibited by GSL inhibitors such as eliglustat. Recently, Matarrese *et al.* showed that GD3 ganglioside contributes to the biogenesis, formation and maturation of autophagic vacuoles²³⁵. Therefore, GD3 might be key for autolysosome formation²³⁵. As eliglustat inhibits GSL,

this may be the mechanism by which eliglustat inhibits autophagy as autophagosome neither can be formed nor can be matured without GD3.

Regarding the findings shown in Figure 5.7 and Figure 5.8, they seem to contradict themselves at first as the pattern of LC3II protein between eliglustat and eliglustat with BafA1 was different in different cell types. However, this could be due to distinct sensitivity of various cell types to eliglustat. For example, the inhibition of autophagic flux of RAW264.7 cells have already reached the plateau when eliglustat is administered at 50 μ M. Therefore, BafA1 cannot further increase the LC3II level. In contrast, 50 μ M eliglustat may not fully block the autophagic flux in primary BM cells and therefore a further increase upon BafA1 treatment can still be observed. To solve this concern, higher doses of eliglustat that does not interfere with cell viability should be applied to primary cells. Importantly, p62 protein expression was increased in both RAW264.7 cell and primary cells, which indicates eliglustat is likely to be an autophagy inhibitor.

5.3.2 Does membrane lipid alteration affects signalling transduction?

As described in section 5.2.3, the lipid structure of RAW264.7 cell was more fluid after the administration of eliglustat. This change can be achieved by reducing cholesterol or increasing the unsaturated lipids in the plasma membrane²³⁶. The lipid raft, is a functional membrane unit in charge of signal transduction and is composed of cholesterol and saturated lipids²³⁶. Therefore, eliglustat may directly affect the lipid rafts and interfere with OC formation signalling. Ha *et al.* discovered that lipid raft domains play an essential role in OC differentiation and function via RANKL-RANK-

TRAF6 signalling²²². In parallel, it was reported that TRAF3 signalling for OC formation is independent of TRAF6 signalling²²³. In addition, TRAF3 was confirmed to be located in the lipid raft microdomain by several groups²³⁷⁻²³⁹. Both TRAF6 and TRAF3 regulate NF- κ B activation to further regulate OC differentiation²²². Taken together, signalling through TRAF3, as a novel molecule regulating OC formation, may be regulated by eliglustat via altering lipid rafts to affect the formation of OCs. To further dissect the changes in the plasma membrane, it remains to be characterised whether eliglustat affects cholesterol or unsaturated lipids, or both, to interfere with OC formation. Alternatively, eliglustat may act further downstream, interfering with the membrane composition of the lysosome or autophagosome, possibly by inhibiting their fusion. All above mentioned possibilities can be tested in future work.

Unfortunately, the evaluation of GP in primary cells failed due to technical reasons. To image and measure the lipid packing properly, the cells need to be cultured on a glass surface instead of a plastic surface. As primary BM cells are extremely hard to attach on glass, different coating methods were titrated to see how to keep the cells adhered. First, Kim *et al.* reported that the glass coated with polylysine would be suitable for culture adherent cells like neurons²⁴⁰. Polylysine can be further categorised into two isomers including D-isomer and L-isomer. However, it was reported that polylysine L-isomer inhibits OCs fusion and formation²⁴¹. Therefore, the polylysine D-isomer is preferred as it escapes the breakdown by the proteases released by cells. Both changing, duration and temperature of polylysine D-isomer coating efficacy for culture OCs were tried. Different concentrations of polylysine D-isomer (0.05 mg/ml, 0.1 mg/ml and 0.2 mg/ml), coating incubation duration (0 minutes, 5 minutes, 1 hour, 2

hours and 6 hours) and temperatures (room temperature and 37 °C) were combined separately. Unfortunately, none of the above combination worked for the attachment of murine BM derived OCs. In addition, fibronectin, a constituent of extracellular matrix, was reported to be suitable for the culture fibroblasts²⁴², neurons²⁴³ and endothelial cells²⁴⁴ after coating glass dishes with it. However, fibronectin coated coverslips inhibited the expression of TRAP in murine OCs²⁴⁵, hence it was not a suitable system to study the lipid packing of OCs as these OCs may have some initial defects.

To sum up, the widely used *in vitro* model of osteoclastogenesis, the RAW264.7 cell line gave us some promising clues to the mechanisms by which eliglustat exerts its effects in that the membrane lipid packing was altered upon eliglustat treatment. This may further lead to a series of signal transduction changes or inhibit the completion of the autophagic flux.

Chapter 6. Eliglustat Combination Strategies and Translational Applications

6.1 Introduction

6.1.1 Combination hypothesis for MM bone disease and aged osteoporosis

Teriparatide and BPs are the two most common agents currently used in clinic for MM bone disease. It has been reported that the combined use of teriparatide and BPs was not superior than single use of either compound²⁴⁶. eliglustat suppresses MM cells (Figure 4.5) while same dosage of eliglustat cannot suppress OC viability *in vitro* (Figure 4.2), this led to the hypothesis that eliglustat may potentially have anti-MM effect if being assisted by BTZ *in vivo*. Therefore, the aim of this Chapter is to ascertain whether eliglustat could increase therapeutic effect when in combination with leading treatments used to control MM bone loss and tumour burden.

In addition to MM, aged related bone loss such as that caused by osteoporosis affects a huge population. According to the World Health Organisation (WHO) the official definition of osteoporosis is described as ‘progressive systemic skeletal disease characterised by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture’²⁴⁷. Compared to the MM population in the UK, the osteoporosis population is huge and around 536,000 fracture cases occur every year^{248,249}. Therefore, ageing is a semi-health state which the overall body condition is changed, thus the reason of bone loss due to aging is somehow a grey area between pure physiological and pure pathological conditions. Currently, the common treatment for osteoporosis is BPs, which the limitations of have been introduced in section 1.5.2. Therefore, a new drug is needed and it is worth to checking if eliglustat can treat age related osteoporosis in order to extend the range of its therapeutic applications and provide a novel strategy

to treat the osteoporosis. In this chapter, the C57BL6 aged preclinical model is used to evaluate if eliglustat can ameliorate the bone quality.

6.1.2 Germinal center

Under normal physiological condition, the development of B cells involves three different stages including pre-B cells, pro-B cells and immature B cells²⁵⁰. After mature native B cells leave the BM niche and encounter T cell-independent antigenic exposure in blood they differentiate into short-lived plasma cells (PCs), that are capable of generating IgM²⁵¹. These IgM-secreting PCs do not have somatically mutated immunoglobulin genes and undergo apoptosis in situ.

For mature native B cells, they migrate into secondary lymphoid tissues including spleen and lymph nodes and establish germinal centers (GC)²⁵¹. GC composed two typical zones called the dark zone and light zone. In the dark zone, mature native B cells are activated to differentiate into centroblasts and lead to somatic hypermutation²⁵². These centroblasts then migrate to the light zone and become centrocytes, which avoid the apoptotic pathway whilst centroblasts with unfavorable mutations go through apoptosis²⁵² (Figure 6.1²⁵³).

Selected centrocytes from GC then differentiate into memory B cells or PCs and leave the GC. Normally, the post-GC PCs go to the BM, in which they receive survival signals including IL-6 and become long lived. Recent studies have demonstrated MM is a malignancy of post-GC PC, which indicates these abnormal PCs are derived from GC. Some researchers have reported that eliglustat decreases the progression of

Gaucher's disease to MM, thus it is important to check whether eliglustat is doing so by inhibiting GC function and causing less PC generation, thereby decreasing the absolute value of PCs thus decreasing malignancy transformation.

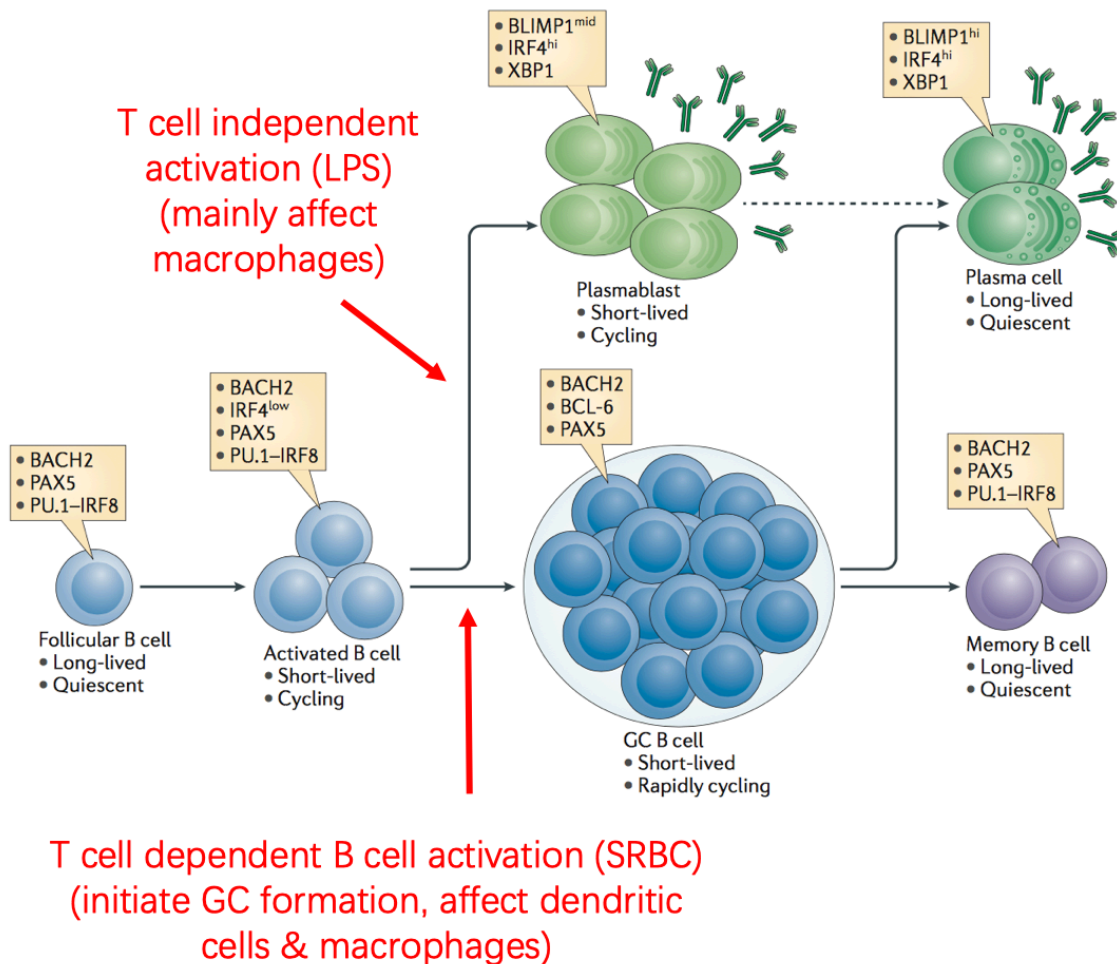


Figure 6.1. Formation of plasma cells. Plasma cells can be formed via T cell independent activation initiated by immunisation of LPS (lipopolysaccharide). Alternatively, plasma cells can also be generated from germinal center via T cell dependent B cell activation boosted by SRBC (sheep red blood cell). This figure was adapted from a review by Nutt *et al.*, 2015.

Chapter 6. Eliglustat Combination Strategies and Translational Applications

6.2 Results

6.2.1 Eliglustat combined with ZA has a superior effect compared to ZA alone in improving bone conditions

Since ZA was a common therapeutic reagent currently used in MM bone disease and osteoporosis, it is important to check whether eliglustat has a superior treatment effect when combined with ZA than ZA alone in murine MM bone disease model. To address this hypothesis, C57BL/KaLwRij mice were divided into 5 groups; control mice without MM and drug treatment (Ctr), MM bearing mice (MM), MM bearing mice treated with ZA alone (MM+ZA), MM bearing mice treated with eliglustat alone (MM+Elig) and MM bearing mice treated with both ZA and eliglustat (MM+ZA+Elig). MM bearing groups were injected with 0.5 million 5TGM1-GFP cells via *i.v.* on day 0, while the control group was injected with 200 μ l of PBS. On day 1, MM bearing mice treated with ZA alone, and MM bearing mice treated with both ZA and eliglustat received ZA *i.p.* injection at the dosage of 0.01mg/kg, while 200 μ l of PBS *i.p.* was administrated to the rest of the mice. On day 4, MM bearing mice treated with eliglustat alone and MM bearing mice treated with both ZA and eliglustat were administrated with eliglustat diet at the dosage of 150 mg/kg/day while the rest of the groups were maintained on a normal diet. All the mice in this experiment were sacrificed on day 23 and micro-CT analysis, histology analysis and FACS analysis were conducted (Figure 6.2).

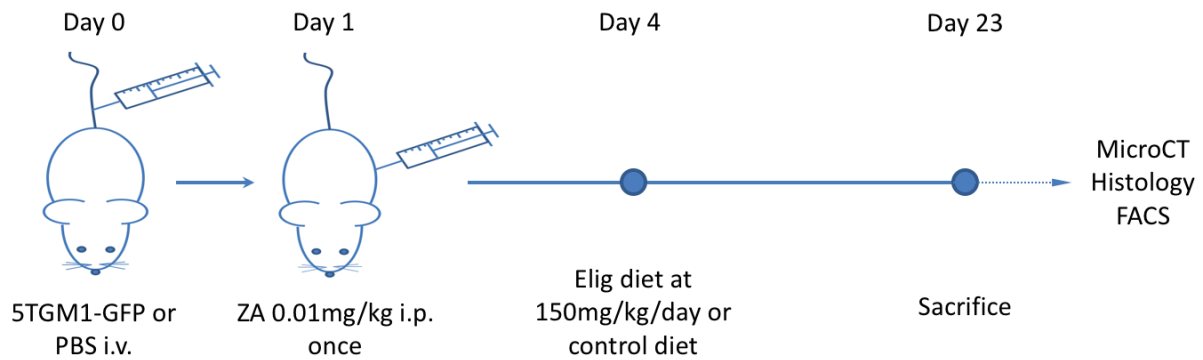


Figure 6.2 Schematic. The time line and experimental design in investigating how eliglustat and ZA impact on bone conditions in 5TGM1-GFP MM bearing mice.

The tibiae were collected and scanned by micro-CT as described in 2.11. Tibia trabecular bone reconstruction by micro-CT revealed a significant decrease of bone quality in the MM group. This decrease was reversed in both the MM+Elig group and MM+ZA group, moreover, the MM+ZA+Elig group showed further improvement in trabecular bone quality compare to the two groups with a single drug (Figure 6.3A). Quantitative analysis of the bone was conducted including BV/TV, BS, BS/BV, Tb.Sp, Tb.N and Conn.D. In short, a significant improvement of bone quality was observed both in MM+ZA group and in MM+Elig group. The treatment effect of MM+ZA and MM+Elig is relatively similar according to the quantitative measurement of various parameters. Importantly, the MM+ZA+Elig group showed a further improvement in trabecular bone quality compare either with MM+ZA group or MM+Elig group (Figure 6.3B).

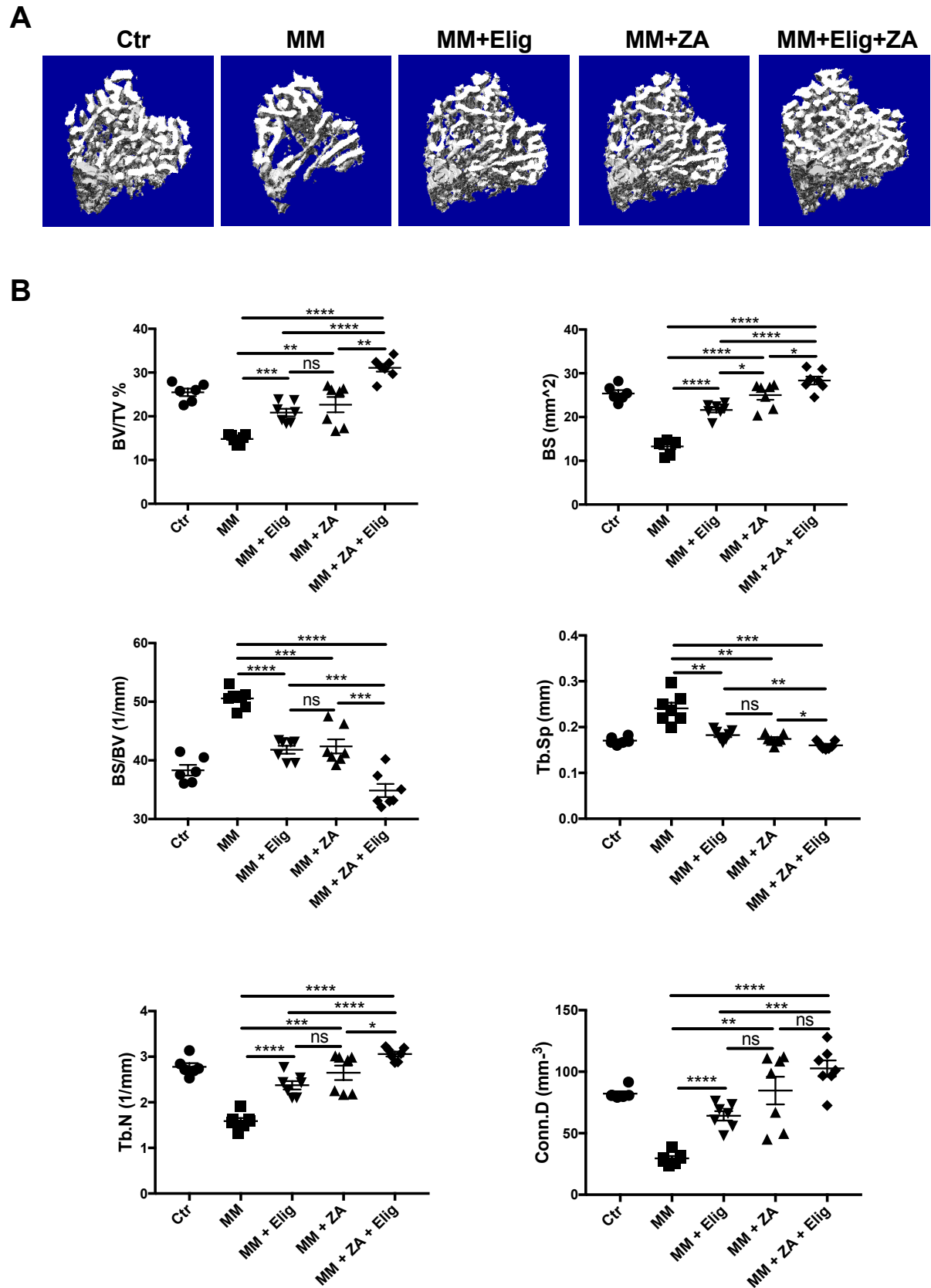


Figure 6.3 Eliglustat combined with ZA increases therapeutic effect compare to ZA alone in ameliorating bone diseases in the male 5TGM1-GFP murine myeloma

model. (A) Representative images of micro-CT reconstructions of proximal tibiae from each group, including naive control (Ctr) (n=6), myeloma bearing mice (MM) (n=7), myeloma bearing mice with eliglustat chow (150 mg/kg/d for 19 days) (MM + Elig) (n=7), myeloma bearing mice with single dose ZA injection (0.01 mg/kg) (MM + ZA) (n=7), or, myeloma bearing mice with single dose ZA injection (0.01 mg/kg) and eliglustat chow (150 mg/kg/d for 19 days) (MM + ZA + Elig) (n=7). (B) Dot plots of BV/TV, BS, BS/BV, Tb.Sp, Tb.N and Conn.D. for each group. Error bars correspond to SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Statistical analysis was performed using Student t test

To assess whether eliglustat ameliorates cortical bone lesions, micro-CT reconstruction images of the tibiae were generated as shown (Figure 6.4A). Then, the quantitative analysis of the holes on each tibia were counted based on micro-CT images. Based on the statistical analysis, both MM+Elig group and MM+ZA group have a significant decrease in the number of holes. Moreover, the MM+ZA+Elig group has a further decrease of the holes compared to either of the single treatment groups as well as the MM group (Figure 6.4B).

Thus, the single administration of eliglustat has a similar treatment effect as the single administration of ZA, moreover, the combination therapy of eliglustat with ZA showed better outcomes than ZA alone. Both of the findings indicate eliglustat is comparable with ZA making it a good candidate to be added to the current clinical treatment, which may decrease the unwanted ZA side effects.

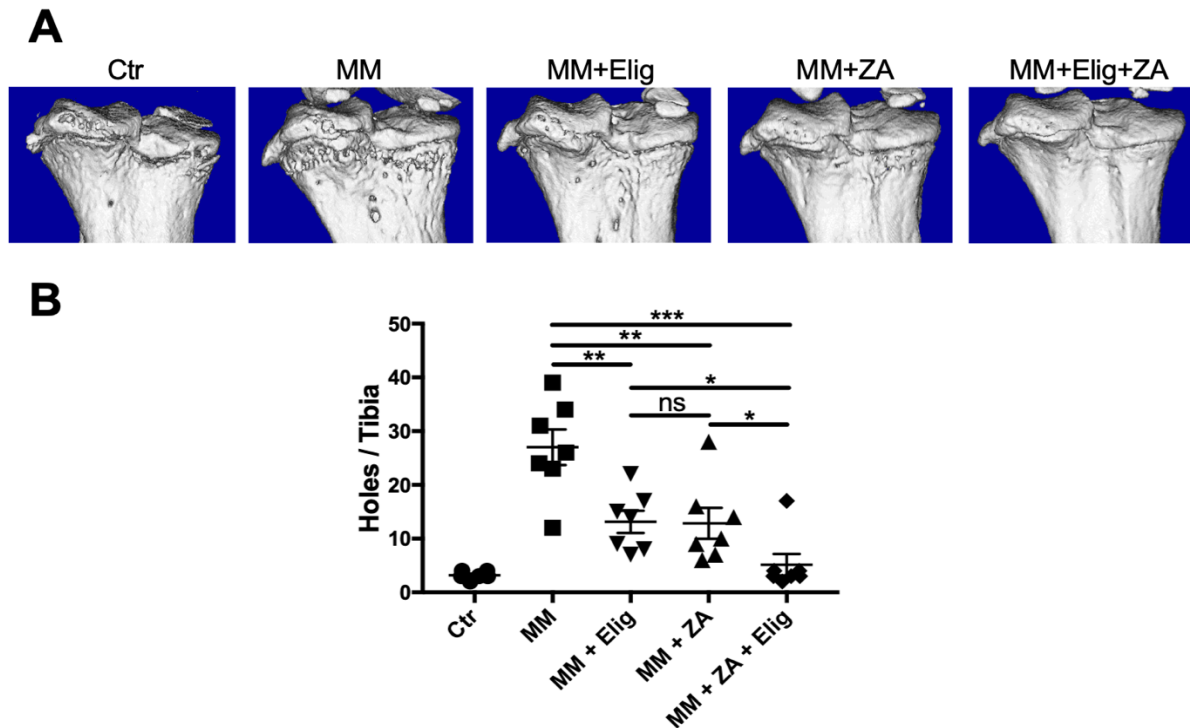


Figure 6.4 Eliglustat combined with ZA increases treatment effect compare to ZA alone in ameliorating cortical bone lesions in the male 5TGM1-GFP murine myeloma model. Holes on tibia were counted from each group, including naive control (Ctr) (n=6), myeloma bearing mice (MM) (n=7), myeloma bearing mice with eliglustat chow (150 mg/kg/d for 19 days) (MM + Elig) (n=7), myeloma bearing mice with single dose ZA injection (0.01 mg/kg) (MM + ZA) (n=7), or, myeloma bearing mice with single dose ZA injection (0.01 mg/kg) and eliglustat chow (150 mg/kg/d for 19 days) (MM + ZA + Elig) (n=7). Error bars correspond to SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Statistical analysis was performed using Student t test.

ZA improves bone condition by inhibiting the resorptive function of OC¹⁵⁸ (inhibiting farnesyl pyrophosphate synthase and prenylation of Guanosine-5'-triphosphate [GTP]-ases), however, eliglustat inhibits OCs mainly through the OC formation process. To precisely evaluate the OC inhibition level in tibiae, histology and histomorphometry analysis were conducted as described in 2.13. The images of TRAP stained OCs from respective groups are shown in Figure 6.5A.

Quantitative analysis was conducted based on the slides. In treatment groups, both the MM+Elig group and MM+ZA group have a significant decrease of OC surface and OC number compared to non-treatment groups. The inhibition of OCs is quite prominent as both OC surface and OC number are even lower than Ctr group. Moreover, the MM+ZA+Elig group has a further significant decrease of Oc.S/BS compared to single use of each individual treatment (Figure 6.5B).

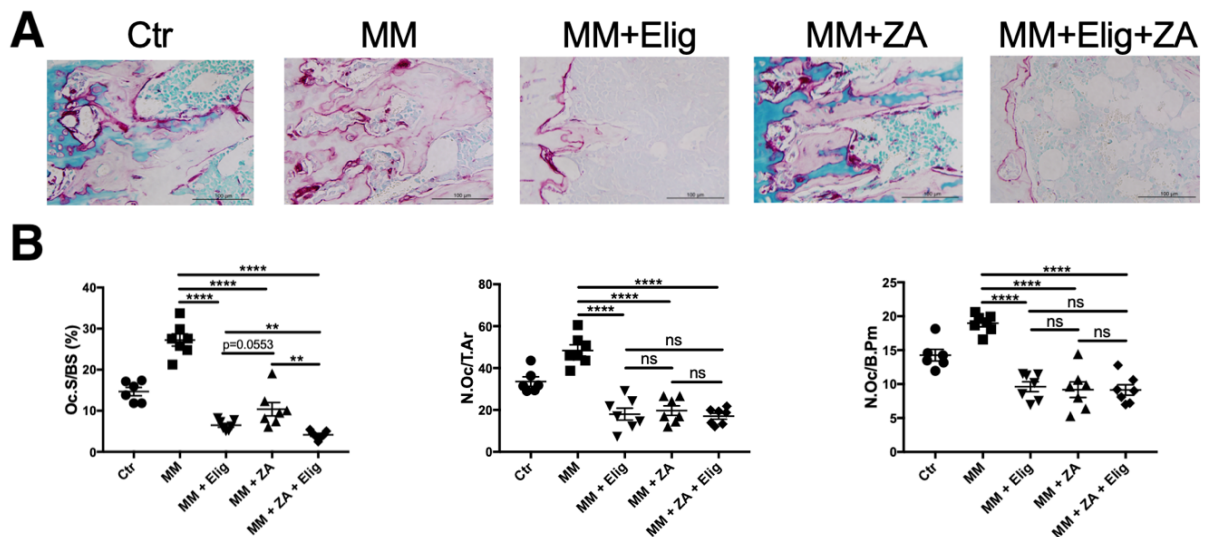


Figure 6.5 Eliglustat combined with ZA has a better inhibition of OCs compared to ZA alone in ameliorating bone diseases in the 5TGM1-GFP murine myeloma model. (A)

Representative TRAP/0.2% methyl green stained tibial sections showing red OCs and OBs on the endocortical bone surface from each group (original magnification 40X), including naive control (Ctr) (n=6), myeloma bearing mice (MM) (n=7), myeloma bearing mice with Elig chow (150 mg/kg/d for 19 days) (MM + Elig) (n=7), myeloma bearing mice with single dose ZA injection (0.01 mg/kg) (MM + ZA) (n=7), or, myeloma bearing mice with single dose ZA injection (0.01 mg/kg) and eliglustat chow (150 mg/kg/d for 19 days) (MM + ZA + Elig) (n=7).

(B) Bone histomorphometry parameters including Oc.S/BS, N.Oc/T.Ar and N.Oc/B.Pm. Error bars correspond to SEM. **P<0.01, ****P<0.0001. Statistical analysis was performed using Student t test.

To evaluate whether eliglustat has any effect on OBs, all the tibia histology slides from the respective groups underwent OB histomorphometry analysis. From the parameters of Ob.S/BS, N.Ob/T.Ar and N.Ob/B.Pm, it seems that neither ZA nor eliglustat altered OB surface or OB number (Figure 6.6).

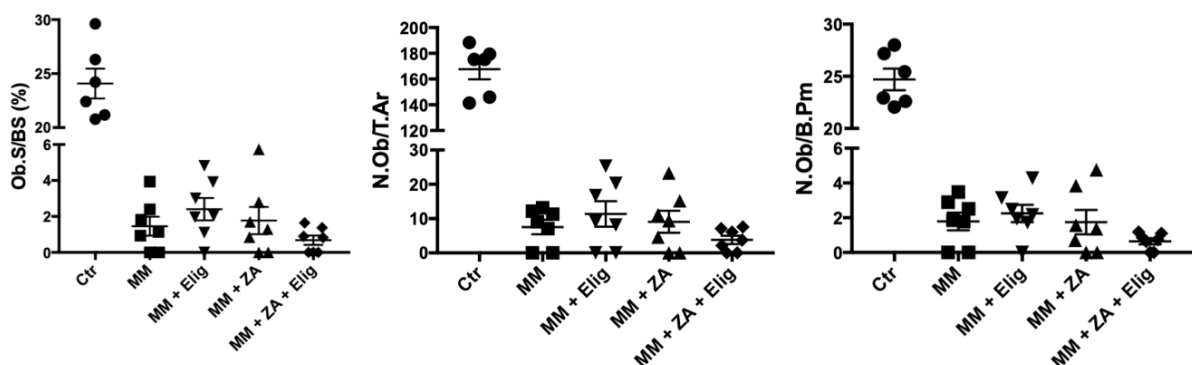


Figure 6.6 From histology and tibia histomorphometry analysis, neither eliglustat nor ZA affects OB in 5TGM1-GFP murine myeloma model.

Bone histomorphometry parameters including Ob.S/BS, N.Ob/T.Ar and N.Ob/B.Pm reveals OB parameters on the endocortical bone surface from each group (original magnification 40X), including naive control (Ctr) (n=6), myeloma bearing mice (MM) (n=7), myeloma bearing mice with eliglustat chow (150 mg/kg/d for 19 days) (MM + Elig) (n=7), myeloma bearing mice with single dose ZA injection (0.01 mg/kg) (MM + ZA) (n=7), or, myeloma bearing mice with single dose ZA injection (0.01 mg/kg) and eliglustat chow (150 mg/kg/d for 19 days) (MM + ZA + Elig) (n=7). Error bars correspond to SEM. Statistical analysis was performed using Student t test.

6.2.2 Eliglustat combined with Bortezomib may increase the treatment effect compared to Bortezomib alone in decreasing tumor burden

As eliglustat was confirmed to inhibit both murine and human MM cells *in vitro*, while it did not relieve tumour burden *in vivo* when used on its own (Figure 4.16). To investigate whether the anti-MM effect *in vitro* could be observed *in vivo* in the presence of another anti-MM reagent, eliglustat was combined with BTZ, a classical proteasome inhibitor currently used for relieving MM tumour burden.

In order to examine if the combination of eliglustat with BTZ would increase the treatment effect, first, a sub-optimal dosage of the BTZ needed to be defined. C57BL/KaLwRij mice were divided into four groups including BTZ at 0.5 mg/kg, BTZ at 0.05 mg/kg, BTZ at 0.01 mg/kg and MM control group. It is reported BTZ at 0.5 mg/kg can significantly inhibit the tumour burden²⁵⁴, thus the BTZ at 0.05 mg/kg group and BTZ at 0.01 mg/kg group is designed based on the BTZ at 0.5 mg/kg. One week

after the mice were transplanted with 0.5 million murine MM cell, BTZ treatment was started at three different dosages as mentioned above. BTZ was given three times per week by *i.v.* and the MM only group received 200 μ l PBS. On the day 23, all the mice were sacrificed and BM cells were collected to evaluate the tumour burden (Figure 6.7).

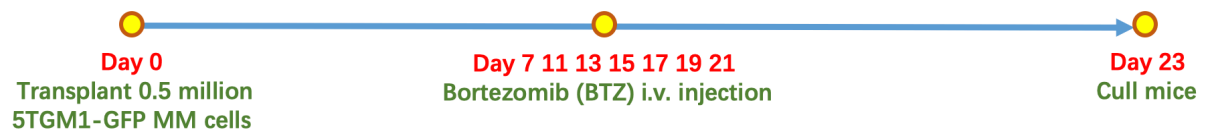


Figure 6.7 Schematic. The time line and experimental design in investigating the less optimal dosage of BTZ to inhibit the tumour burden in the 5TGM1-GFP MM murine model.

BM tumour burden reflects the typical phenotype of MM and best describes the disease state. In this titration experiment, BM tumour burden was evaluated by flow cytometry. According to the quantitative analysis of the tumour cell percentage, BTZ at 0.5 mg/kg significantly inhibits tumour burden as compared with MM group. Tumour burden was not relieved for the other two groups treated with BTZ at 0.05 mg/kg and 0.01 mg/kg compare with MM group (Figure 6.8). This experiment shows that BTZ is effective although BTZ at 10% of its original dosage had no effect on tumour burden.

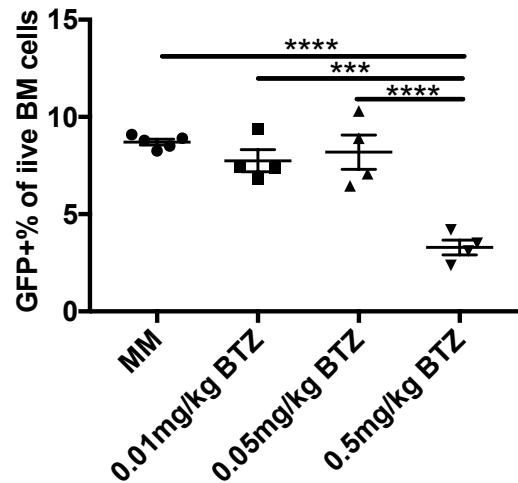


Figure 6.8 Dosage titrate of BTZ in 5TGM1-GFP MM murine model. Four groups were set up in this experiment; MM bearing control (MM) (n=5), MM bearing mice with BTZ at 0.01 mg/kg (0.01 mg/kg BTZ) (n=4), MM bearing mice with BTZ at 0.05 mg/kg (0.05 mg/kg BTZ) (n=4), MM bearing mice with BTZ at 0.5 mg/kg (0.5 mg/kg BTZ) (n=4). Error bars correspond to SEM. ***P<0.001, ****P<0.0001. Statistical analysis was performed using One-way ANOVA.

As BTZ at 0.05 mg/kg did not ameliorate tumour burden a dose between the 0.05mg/kg and the 0.5 mg/kg dose was set to examine if it can increase the treatment effect together with eliglustat. All of the mice were transplanted with 5TGM1-GFP murine MM cells and randomly assigned to four groups including MM control (MM), single treatment with BTZ at 0.3 mg/kg *i.v.* (BTZ), single treatment with eliglustat at 150 mg/kg/day (Elig) and combination therapy of BTZ at 0.3 mg/kg with eliglustat at 150 mg/kg/day (Elig+BTZ). eliglustat treatment started 4 days after the injection of MM cells until the end of the experiment. As per the previous experiment, BTZ was started 7 days after MM cell transplantation and administered at a frequency of 3 times per

week. BM, spleen and serum were harvested 20 days after the mice were transplanted with MM cells (Figure 6.9).

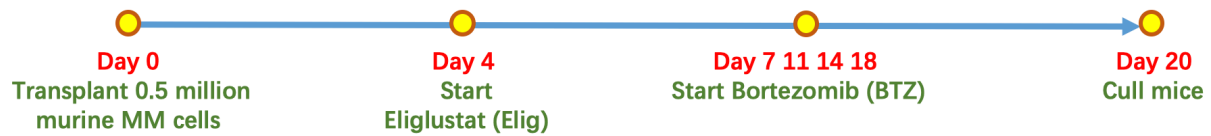


Figure 6.9 Schematic. The time line and experimental design used to investigate whether eliglustat combined with BTZ is more effective than BTZ alone in inhibiting the tumour burden in the 5TGM1-GFP MM murine model.

The tumour burden in BM and spleen was analysed by flow cytometry. From the BM result, a significant inhibition of MM tumour burden was observed in Elig+BTZ group compared with BTZ group (Figure 6.10A). However, this inhibition pattern was not observed in spleen and serum samples (Figure 6.10B, 6.10C), indicating that eliglustat may have a specific effect in suppressing BM MM burden instead of exerting a systematic anti-MM effect. In BM and serum, in contrast to the BTZ group ($p < 0.05$), Elig+BTZ had a prominent significance ($p < 0.01$) in decreasing MM burden as compared with the MM group (Figure 6.10A, 6.10C). This comparison also supports the idea that eliglustat may assist the anti-MM effect of BTZ. Moreover, concordant with previous data, eliglustat had no direct effect in controlling MM tumour burden in the BM, spleen and serum (Figure 6.10A, 6.10B, 6.10C).

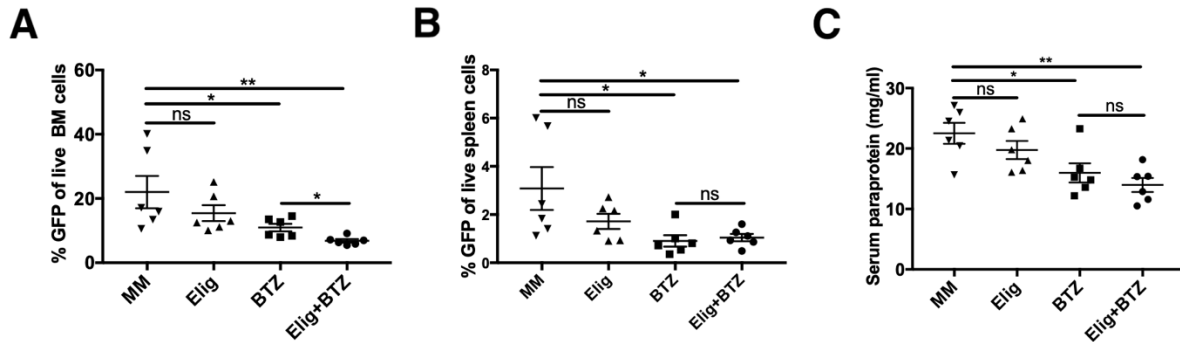


Figure 6.10 Eliglustat combined with BTZ may better ameliorate MM tumour burden

as compared with BTZ alone in 5TGM1-GFP MM murine model. Four groups were used in this experiment; MM bearing control (MM) (n=6), MM bearing mice with eliglustat at 150 mg/kg/day (Elig) (n=6), MM bearing mice with BTZ at 0.3 mg/kg (BTZ) (n=6), MM bearing mice with eliglustat at 150 mg/kg/day and BTZ at 0.3 mg/kg (Elig+BTZ) (n=6). Error bars correspond to SEM. *P<0.05, **P<0.01. Statistical analysis was performed using Mann-whitney test.

6.2.3 Eliglustat in aged osteoporosis preclinical model

As aged osteoporosis is a disease with severe bone loss, to extend the application of eliglustat to the bone field, the anti-OC function of eliglustat was evaluated in aged osteoporosis preclinical model²⁵⁵. Moreover, it is reported that aging may contribute to the MM engraftment in preclinical models²⁰⁶. Therefore, the engraftment ability of 5TGM1-GFP in aged mice was examined and the therapeutic effect of eliglustat was evaluated. Due to the limited number of aged mice, all of above hypotheses are addressed in one experiment. First, C57BL6 female mice with different ages including 12-week old, 28-week old and 80-week old were used. This is to provide the information as to whether aged mice (80-week old) have a lower bone quality as

compared with young mice. Secondly, a group of 80-week old aged mice were administered with eliglustat at 150mg/kg/day for 28 days to evaluate whether eliglustat can ameliorate the bone quality. Thirdly, some aged mice were inoculated with MM cells to see whether MM can further exacerbate the bone in the aged mice. Lastly, eliglustat was administrated to the aged mice with MM. During the experiment, no adverse effects were observed in each experimental group.

After the mice were sacrificed, the tibiae were collected for micro-CT bone parameter analysis. According to the BV/TV data, a significant decrease of bone quality was observed in 28-week old group and 80-week old group as compared with 12-week old mice. This indicates there was an age-related reduction of bone quality in the C57BL6 preclinical model. For the 80-week old mice, there was no significant difference between treatment with eliglustat and the non-treatment group. This finding reveals 28 days of eliglustat treatment is not helpful in increasing the bone volume in aged C57BL6 mice. Moreover, there is no difference of BV/TV between aged mice with or without injection of 1million 5TGM1-GFP MM cells. As the BV/TV was extremely low in the aged group (BV/TV% value near 0) (which means there was almost no mineralized tissue), thus it was not surprising that the presence of MM did not further exacerbate the condition. In addition, there was no difference in BV/TV between 80-week-old aged mice with MM and 80-week-old aged mice with MM+Elig. Taken together, there was no mineralized bone tissue detected by micro-CT in aged mice (80 weeks old) and therefore eliglustat can't restore the bone volume in aged mice by inhibiting the OCs (Figure 6.11).

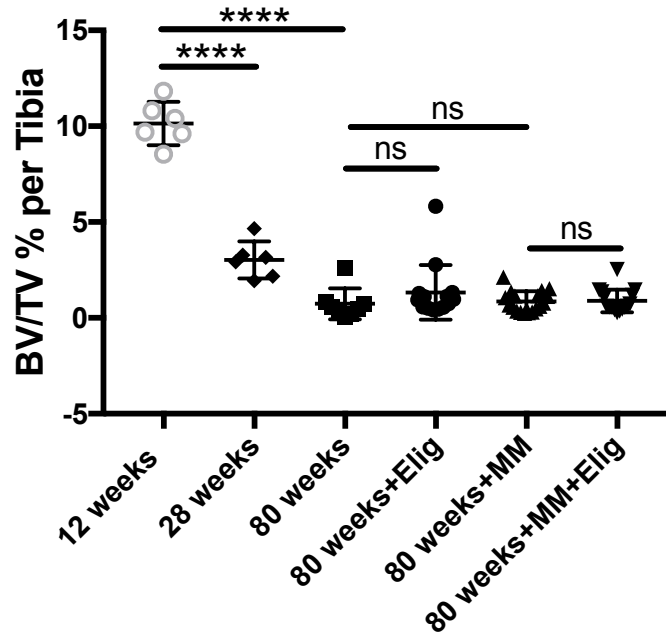


Figure 6.11 Eliglustat did not improve BV/TV in aged mice and aged mice with MM.

6 groups of female C57BL6 mice were used in this experiment; 12 weeks control, 28 weeks, 80 weeks (aged group), aged group with either eliglustat (Elig) or with MM, and aged group with both eliglustat and MM. Eliglustat was given at 150mg/kg/day for 28 days and 5TGM1-GFP MM cells were transplanted at 1×10^6 cell/mouse. Error bars correspond to SEM. **** $P < 0.0001$, ns means non-significant. Statistical analysis was performed using One-way ANOVA.

6.2.4 Eliglustat suppresses SRBC induced GC population *in vivo*

It has been reported that eliglustat can decrease the possibility of Gaucher's Disease patients progressing to MM disease and it is confirmed that eliglustat has no direct anti-MM effect on its own in the murine model. However, MM cells are regarded as abnormal PCs, the above findings exclude the possibility that eliglustat directly affect abnormal PCs *in vivo*, but it still remains possible eliglustat may affect the cells differentiate into PC. In this case, it could be that eliglustat affects the GC, the place

where PC comes from, thus decreasing the risk of generating excessive abnormal PCs such as MM cell.

To address this hypothesis, sheep red blood cell (SRBC) is use to boost T cell dependent B cell activation, which can greatly increase GC population in the murine spleen. To evaluate if eliglustat affects GC and PC from SRBC treated C57BL6 mice, eliglustat is administrated 7 days before the SRBC injection until the end of the experiment and the mice are culled 7 days after SRBC injection (Figure 6.12).



Figure 6.12 Schematic of the experimental design used to test if eliglustat affects GC and PC in SRBC treated mice. C57BL6 mice are administrated with eliglustat at 150 mg/kg/day since day 0. On day 7, SRBC is injected to mice via *i.p.* to boost the GC. On day 14, all mice were culled to evaluate the GC and PC population.

After the mice were sacrificed on day 14, spleen was collected from each mouse for flow cytometry analysis. The gating strategy of GC and PC for the SRBC group and Elig+SRBC group is shown as follows. In short, the GC population is commonly regarded as (B220⁺ CD138⁻ IgD⁻ Fas⁺ GL7⁺) and PC population is defined as (B220^{low} CD138⁺) (Figure 6.13).

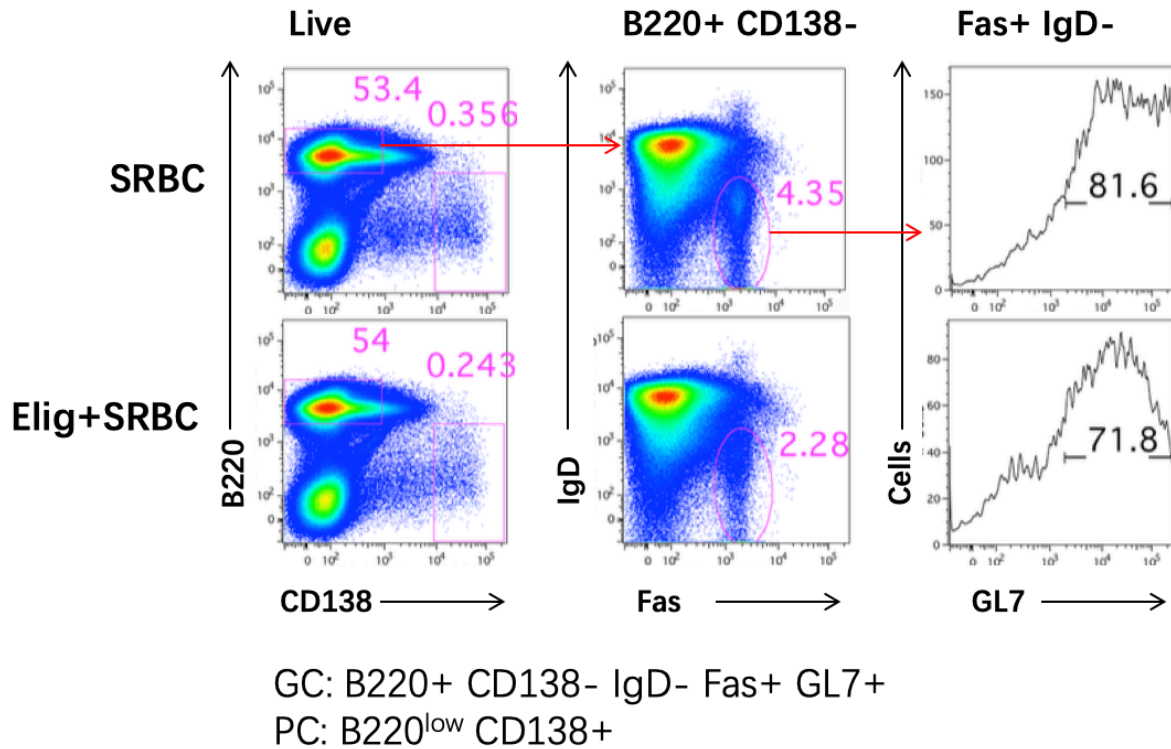


Figure 6.13 Gating strategy of GC and PC for SRBC group and Elig+SRBC group. GC population is regarded as (B220+ CD138- IgD- Fas+ GL7+) and PC population is defined as (B220^{low} CD138+).

For the spleen, the overall B220 positive B cell population did not significantly alter between the SRBC group and Elig+SRBC group, which indicates any potential cell population from this gate was purely attribute to the sub-population alteration only (Figure 6.14A). According to the gating strategy shown above (Figure 6.14), the GC B cell was further identified based on B220 positive population and there was a significant increase of GC observed in Elig+SRBC group as compared with SRBC group (Figure 6.14B). This finding indicates eliglustat has the ability to suppress GC in spleen. Moreover, spleen PC population was gated as described above (Figure 6.14). It was shown PC population in spleen also encounters a relatively low

percentage after treated with eliglustat which tells PC population may potentially affected by GC population (Figure 6.14C).

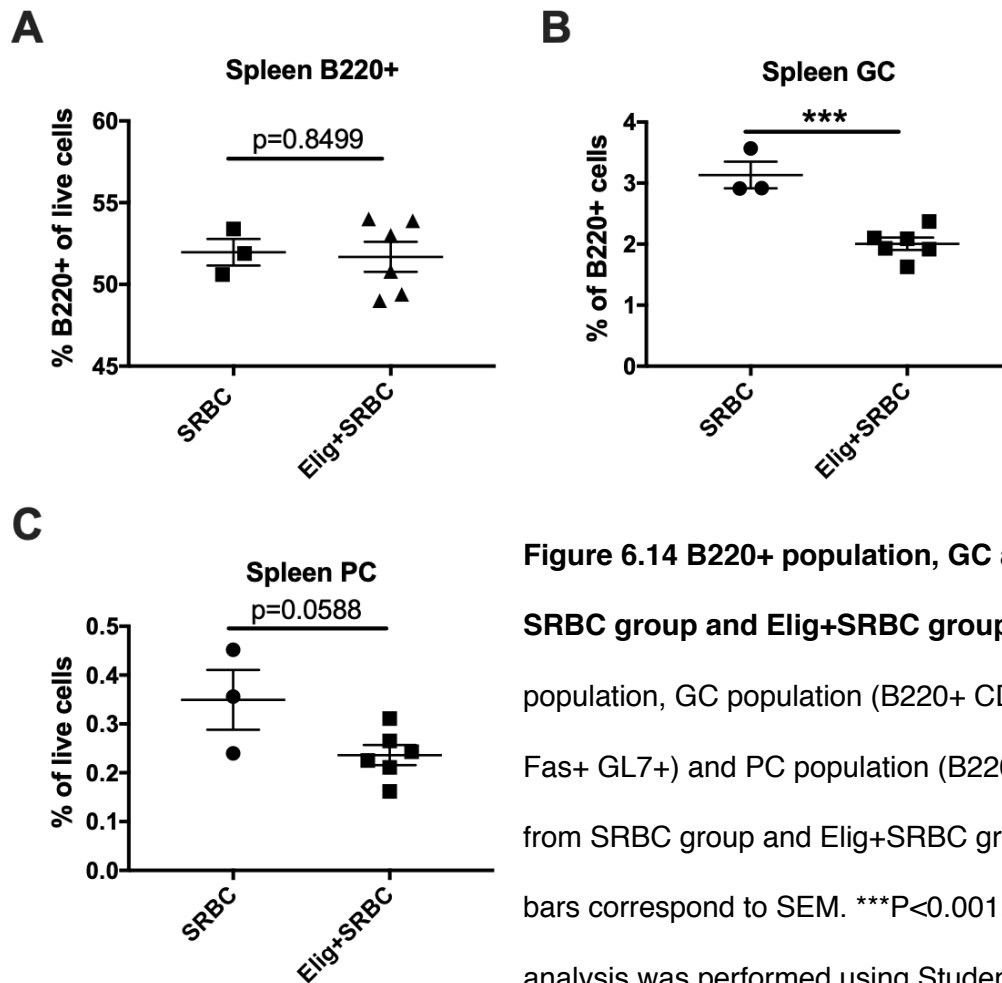


Figure 6.14 B220+ population, GC and PC for SRBC group and Elig+SRBC group. B220+ population, GC population (B220+ CD138- IgD- Fas+ GL7+) and PC population (B220low CD138+) from SRBC group and Elig+SRBC group. Error bars correspond to SEM. *** $P<0.001$. Statistical analysis was performed using Student *t* test.

Next, the same experiment was repeated with 2 more groups including naïve control group and eliglustat without SRBC group. All the sample process procedures and reagents used were the same as previous experiment. According to the data, there was no difference between SRBC group and Elig+SRBC group for B220 positive population (Figure 6.15A). SRBC did significantly boost the GC population in spleen while eliglustat can suppress such boost significantly, which was consistent with previous findings (Figure 6.15B). Moreover, the pattern of PC population shifts in Elig+SRBC group compared with SRBC group was also similar as previous

experiment (Figure 6.15C). In short, it was confirmed eliglustat was capable of suppressing GC population in spleen and may potentially affect the PC population as well.

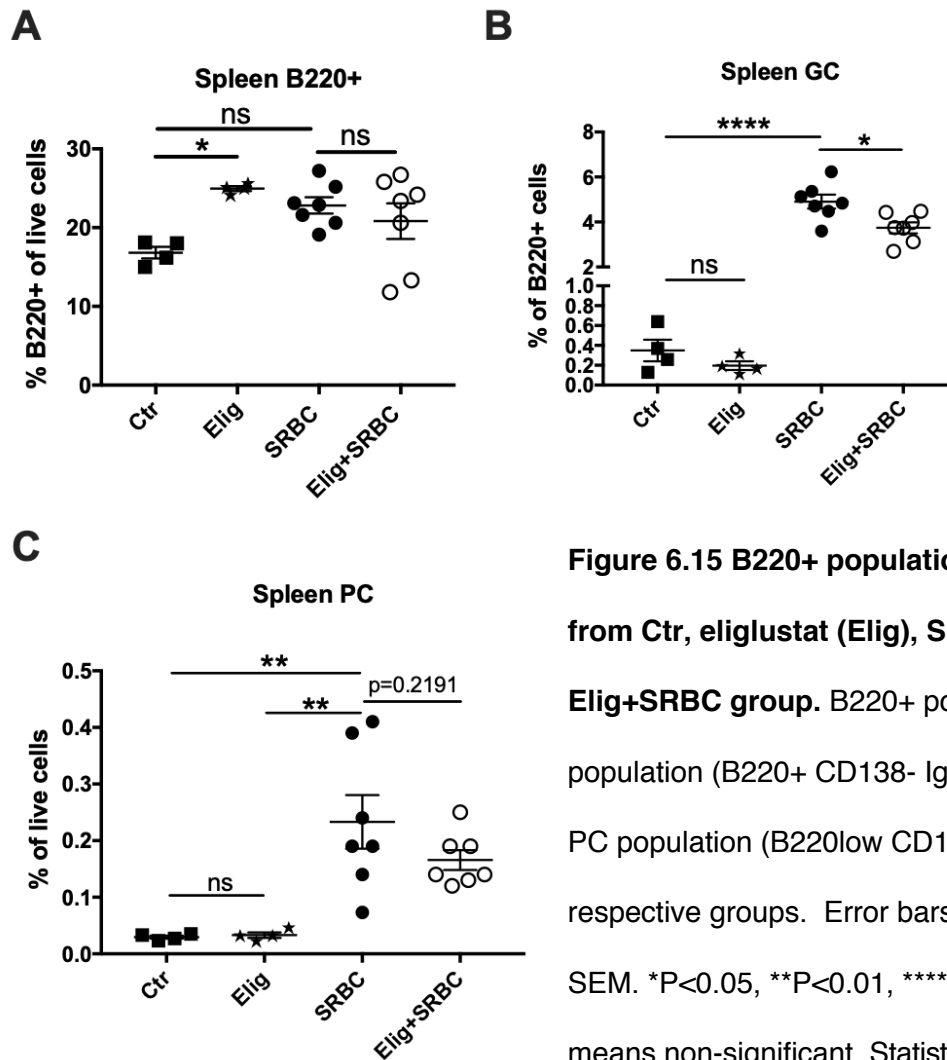


Figure 6.15 B220+ population, GC and PC

from Ctr, eliglustat (Elig), SRBC and

Elig+SRBC group. B220+ population, GC

population (B220+ CD138- IgD- Fas+ GL7+) and

PC population (B220low CD138+) from

respective groups. Error bars correspond to

SEM. *P<0.05, **P<0.01, ****P<0.0001. ns

means non-significant. Statistical analysis was

performed using one-way ANOVA.

Chapter 6. Eliglustat Combination Strategies and Translational Applications

6.3 Discussion

6.3.1 Does eliglustat inhibits OCs via a different mechanism from ZA?

As shown in section 6.2.1, eliglustat in combination with ZA significantly improved bone quality compared to either treatment alone. It remains unknown whether eliglustat exerts its effect via the same pathway as ZA or via a novel mechanism. To address this question, three aspects need to be considered including the chemical property and mechanism of action of each reagent, the actual effect on OCs, the dosage limitation and adverse effects.

ZA and eliglustat have different chemical structures and belong to different drug categories^{131,256}, which makes them likely to act via different mechanisms. For example, ZA is a member of the BPs, which bind to hydroxyapatite. ZA alters enzyme farnesyl diphosphate synthase and thus leads to OC apoptosis via the mevalonate pathway¹⁵⁵⁻¹⁵⁷. However, eliglustat is a GSL inhibitor and appears to inhibit OCs via NF- κ B pathway as the TRAF3 level was maintained by eliglustat (Figure 5.2 and 5.5).

Second, different from ZA which causes OC apoptosis¹⁵⁵⁻¹⁵⁷, eliglustat seems to have nothing to do with OC viability even at the highest dosage based on *in vitro* study (at the same concentration 50 μ M, eliglustat is sufficient to inhibit MM cell viability) (Figure 4.2). Thus, eliglustat inhibits OCs mainly through inhibition of OC formation instead of interfering with OC viability.

Thirdly, both ZA (0.01 mg/kg)¹⁹⁵ and eliglustat (150 mg/kg/day)¹⁹⁸ were administrated at a high dosage, a dosage which both agents probably reached their maximum effects. Eliglustat single use showed similar effects as ZA single use based on micro-CT

parameters. As ZA combined with eliglustat has a further improvement in ameliorating bone quality than the single use of each agent, it is likely ZA and eliglustat may come to effect via different mechanisms. Furthermore, histomorphometry findings suggest that eliglustat may suppress OC surface area better than ZA, which could be another indication that eliglustat differs from ZA in the mechanism of suppressing OC.

In summation, it is likely that eliglustat comes to effect via different mechanisms from ZA and may reveal a potential clinical strategy that is better than current clinical treatments; such finding may accelerate eliglustat's translational use into the clinic.

6.3.2 Does eliglustat have an anti-MM effect?

As the bone lesion phenotype is based on the murine MM model and it is reported that eliglustat may suppress the development of MM from GD^{197,213,214}, thus it is necessary to investigate whether eliglustat has a direct anti-MM effect. While it still seems an enigma if eliglustat has actual anti-MM effect based on the results to date.

According to the *in vitro* data, eliglustat affects murine MM cell viability (Figure 4.5) while the same dosage cannot inhibit OC viability (Figure 4.2). This finding gives a hint that eliglustat may have potential anti-MM function. While above finding seems not applicable in the murine MM model as eliglustat ameliorates bone quality without decreasing MM tumour burden (Figure 4.16). Lastly, the combination therapy of eliglustat with a less optimal dosage of BTZ appears to have better outcome than BTZ alone, which indicates eliglustat may assist BTZ to exert anti-MM effect (Section 6.2.2).

In some cases, it is recognized that the antiresorptive drugs (for example, BPs) not only ameliorate bone quality but also decrease the MM tumour burden as the vicious cycle is disrupted^{143,257}. While eliglustat seems different to BPs in inhibiting MM even though eliglustat is quite similar to BPs in antiresorptive performance. Combined with the *in vitro* data, this could possibly be due to the limitation of *in vivo* model. As the 5TGM1 cell is a rapid and vigorous MM cell type which makes the murine model quite sick within 23 days, while it may take a longer time for eliglustat to disrupt the vicious cycle and then inhibit MM tumour burden²⁰². Another possibility based on the Elig+BTZ experiment could be that eliglustat may assist BTZ to exert BTZ anti-MM effect while eliglustat has no anti-MM effect on its own. However, this explanation seems arguable as the inhibition effect is only observable from BM (the place where MM cell most distributed) flow cytometry result and there is no statistical difference in spleen and serum parameters.

In short, what can be conclude here is there is no evidence to support eliglustat has direct anti-MM effect *in vivo*. Further investigation may be conducted by adjusting combination dosage, MM cell type and the duration of the animal model.

6.3.3 Does eliglustat suppress GC fully, demonstrating the relationship between Gaucher's Disease and MM?

It is reported lipid disorders like Gaucher's disease and obesity are linked with MM as Gaucher's disease patients have a remarkable high risk of MM^{198,214}.

Glucocerebrosidase (GBA), the counterpart enzyme has opposite function to GCSs, is normally responsible for turning GSLs back to ceramide. Researchers have tried to knockout *GBA* in murine model and found this causes an increase of the GC B cell¹⁹⁸. Furthermore, lyso-glucosylceramide (LGL1), a lysolipid specifically increased in GD patients, is administrated to GBA KO mice and a further increase of spleen GC B cell and BM PC is observed. In addition, the monoclonal gammopathy and M spikes (clonal immunoglobulin) are also observed¹⁹⁸. These findings indicate high GSL accumulation level may cause MGUS or a pre-MM condition and the administration of eliglustat can reverse such situation as judging from their M spike intensity result¹⁹⁸. While what they didn't answer is whether eliglustat has a direct effect in inhibiting GC, the place where MM cells originates.

In my project, from an immunological point of view, SRBC is used to boost the GC population and a significant inhibition of GC B cells is observed after eliglustat treatment is observed. As MM is a post-GC disease, which is spontaneously generated in patients, the above finding directly suggests eliglustat is capable of suppression of a specific cell population, which may contribute to MM development, which is a further step and breakthrough based on previous findings. Moreover, another group of researchers have reported eliglustat may decrease the GD associated B cell malignancy¹⁹⁷ and my results explain an underlying mechanism from the physiological perspective. In short, the finding that eliglustat suppresses GC has expanded the potential translational use of eliglustat in preventing B cell lymphoma and other immune diseases related with B cell abnormalities.

Chapter 7. General Discussion

7.1 Glycosphingolipids and MM tumour burden

Work from our lab by Ersek *et al.* previously reported that inhibition of MM-associated GSL (specifically, GM3 and GM2, the two prevailing GSLs in MM cells) by the GSL inhibitor, miglustat, inhibits OC activation in MM bearing mice¹⁵. However, miglustat did not suppress murine MM tumour burden *in vivo* despite the abundance of MM-associated GSLs, including GM3 and GM2, expressed by the human MM cells. Similarly, in this project, eliglustat was unable to inhibit 5TGM1-GFP induced MM burden. However, eliglustat was very effective at inhibiting osteolysis and OCs in MM disease.

The lack of effect on tumour burden could be due to two reasons. Firstly, the duration of 5TGM1-GFP MM model is quite short, hence eliglustat and other GSL inhibitors¹⁵ may not have time to fully exert a direct anti-MM effect. However, this hypothesis needs to be further studied as GM3 is the dominant GSL in MM cells. GM3 is known to promote osteoclastogenesis¹⁵. Therefore, as long as GM3 is inhibited by eliglustat, MM cells could be the cell type that are directly being influenced leading to a reduction in OCs. While the vicious cycle would suggest that osteoclast inhibition should lead to an indirect reduction in tumour burden, the aggressive nature of the model may prevent this from being observed. Indeed, it is not unprecedented to demonstrate an effect on bone disease with no effect tumour burden in murine models of myeloma^{195,258}.

Secondly, it was shown that the GM3 and GM2 are human MM-associated GSL and expressed in primary human MM cells and human MM cell lines¹⁵. However, GM3 or

GM2 may not be prevailing in murine MM cells including 5TGM1-GFP, or, all GSLs' level in murine MM cells could be similar as other cell types with murine origin. To address the above possibilities, NOD-scid IL2r^gnull (NSG) mice^{259,260} inoculated with human MM cells should be treated with eliglustat to see if the MM burden is alleviated. In addition, 5T2MM murine MM cell^{202,261} could be used in the murine model as MM disease caused by this cell type can last for 8-12 weeks, which could be sufficient for eliglustat fully impact the tumour and microenvironment.

7.2 Specificity of eliglustat for Osteoclasts

From the data described in this thesis, eliglustat specifically inhibits OCs in murine models. eliglustat was found to inhibit OCs, MM cells and OBs *in vitro* in a dose-dependent manner. However, eliglustat was unable to inhibit OBs or MM cells *in vivo*, emphasising a specificity for OCs in the bone. As the *in vitro* culture system is quite different from the *in vivo* system, the data from the *in vivo* system should be more persuasive. In addition, I demonstrated that both myeloid cells (including monocytes) and OC progenitors were not affected *in vivo*, which indicates eliglustat primarily effects OC formation.

There are a small number of papers describing lipid organization²⁶²⁻²⁶⁴ during OC formation process, however none of them investigate GSLs. To gather further insight as to why eliglustat primarily affects OCs, further work should be conducted to see whether the GSL profile of the OC cell membrane is changed during OC formation process using lipidomics.

7.3 Does eliglustat maintain OC TRAF3 levels by disrupting cell membrane lipid organisation?

Lipid rafts are organized microdomains that consist of GSLs (sphingolipids and glycolipids), cholesterol, saturated phospholipids and lipidated proteins in the plasma membrane of mammalian cells²³⁶. Therefore, lipid rafts are important for signalling transduction. eliglustat is known to inhibit GSL formation by inhibiting GCS^{265,266}. It was shown that eliglustat leads to TRAF3 protein accumulation during OC formation without increasing its mRNA levels, which could be due to alterations in the cell membrane lipid organisation/composition.

RANKL binds to RANK receptor to initiate TRAF3 degradation, which in turn stimulates NF- κ B signalling to promote OC formation. Ha *et al* showed that membrane lipid raft microdomains are vital for RANKL-induced OC differentiation and function²²². By inhibiting the synthesis of GSL, eliglustat may disrupt the lipid composition/order of the cell membrane that will interfere with the transduction of RANK signalling²²², and therefore TRAF3 degradation is not initiated.

Signal transduction in both canonical and non-canonical NF- κ B pathways involves the binding of RANKL to RANK receptor on the cell surface followed by downstream signalling. Eliglustat may change the membrane lipid organisation (particularly GSL and consequent lipid ordering) altering the lipid environment where RANK is anchored. This may lead to inhibition of RANK/RANKL signalling transduction, resulting in the failure to degrade TRAF3. To address the lipid environment in the membranes, the overall lipid organisation was evaluated by spectral imaging after NR12S staining of

the membrane²³². The result indicates eliglustat fluidized the plasma membrane and disrupted lipid ordering.

In addition, further experiments can be conducted. For example, to determine how eliglustat changes the plasma membrane composition, the plasma membrane vesicles derived from OC can be analysed using shot-gun lipidomics²⁶⁷. Moreover, the organization of RANK receptors with respect to the lipid composition and organisation can be imaged by super-resolution stimulated emission depletion (STED) microscopy and combined STED- (fluorescence correlation spectroscopy) FCS methodology²³². These technologies may reveal the nanoscale protein organisation (e.g., nanoclustering) and nano-environment of the proteins. Finally, the composition of the cell membrane of the OC could be analysed using lipidomics in order to obtain more understanding about eliglustat.

Importantly, as GSLs are derived from ceramide, it would be possible that ceramide is increased and thus affect OC formation²⁶⁸. In D-PDMP treated murine BM derived OC, ceramide level did increased, however, adding extra C2-ceramide (5 μ M) did not change the TRAP+ multi/mono nucleus cells, indicating ceramide level may not affect the OCs²¹¹. In addition, N-(5-adamantane-1-yl-methoxy-pentyl)-deoxynojirimycin (AMP-DNM), a GCS inhibitor and iminosugar, did not increase the level of ceramide in HepG2 cells at different time point up to 72 hours²⁶⁹, which indicates that ceramide is metabolized via other pathways and may not accumulated. Therefore, eliglustat inhibition of OC may have less to do with ceramide, however this would need to be confirmed in osteoclasts.

7.4 Does eliglustat maintain OC TRAF3 level by inhibiting autophagy?

As eliglustat prevented TRAF3 degradation in OC without changing its mRNA levels and as TRAF3 in OCs is degraded via autophagy^{231,270} therefore TRAF3 could be maintained by eliglustat as an autophagy inhibitor.

GSL are lipids present not only in the cell membrane but also in the membranes of intracellular organelles²⁷¹. For example, GD3, a member of GSL, is an important component of autophagosomal or autolysosomal membranes, thus central for the initiation of autophagosomal/ lysosomal biogenesis²³⁵. In addition, it was proposed that other lipids including phospholipids and sphingolipids may regulate autophagy²⁷²⁻²⁷⁴. Therefore, eliglustat may inhibit autophagy by blocking TRAF3 degradation.

It has been reported that RANKL induces TRAF3 degradation via the lysosome/autophagy system⁷¹. My show that the lysosomal inhibitor BafA inhibits TRAF3 degradation. Moreover, eliglustat was found to be an autophagy inhibitor according to the protein expression levels of LC3 and p62. Therefore, the inhibition of GSL by eliglustat may directly disrupt the autophagosomal flux, so TRAF3 protein levels are maintained.

To further determine if eliglustat affects the lysosomes in OC, the lysosome numbers and pH can be evaluated (for example, acidic organelles can be visualized by LysoTracker, pH measured by LysoSensor). Lysosome protein levels (CTSK, Lamp1, Lamp2) and GSL distribution in autolysosomal membrane can be determined using confocal imaging and electron microscopy. Then, the GSL contribution to

autophagosomal membranes could be determined, via lipidomics of gradient-purified autophagosomes and colocalization of GSL with LC3 can be imaged (DQ Green BSA, confocal microscopy). Finally, experiments can be conducted to exclude that eliglustat affects the degradation of TRAF3 by the proteasome via ubiquitination⁷¹.

7.5 Therapeutic prospects

In this project, eliglustat successfully ameliorated bone quality in different murine MM bone disease within a short time period. Moreover, eliglustat was found to increase the therapeutic effect when combined with ZA and possibly BTZ, indicates eliglustat can be translationally used in clinic for MM disease and its bone complication.

Focusing on the role of eliglustat in MM bone disease and its mechanism regulating TRAF3 and autophagy, this project has a tremendous potential for the translational use of eliglustat as a therapeutic in bone loss diseases and B cell malignancy.

First, many diseases involve bone loss such as post-menopausal osteoporosis, breast cancer and prostate cancer metastasis to bone, osteomyelitis and arthritis. Eliglustat was proven to ameliorate bone quality both under healthy conditions and in the MM disease state. Therefore, eliglustat, a clinically approved drug, could be repurposed for patients who suffer from various bone loss diseases in the case which ZA is not suitable, or they are no longer able to take BP.

TRAF3, a tumour suppressor, is mutated in various human B cell cancer such as MM²⁷⁵ and diffuse large B-cell lymphoma (DLBCL)^{276,277}. As TRAF3 is maintained by

eliglustat, and, TRAF3 suppresses GC (where MM cells originate from) as well as stops GD progress to MM¹⁹⁷, therefore eliglustat may have the ability to suppress development and/or progression of some B cell malignancies. While our experiments found no evidence for anti-tumour responses, they were not designed to address this question, and the use of eliglustat in a preventative setting would be of interest.

7.6 Conclusions

MM, a terminally differentiated plasma cell malignancy accompanied with typical bone destructions, brings great suffering to the patients including bone pain and fractures. As MM bone disease is caused by overactivated bone resorbing OCs, hence the development of compounds that can inhibit OC formation or function is a strong clinical therapeutic strategy. GSLs are constituents of the mammalian cell membrane and their alteration is linked with diseases including GD. Eliglustat, a novel small molecule compound, was designed to target GSL which subsequently resolve GD.

In this DPhil project, different murine MM models were assessed prior to testing eliglustat in the C57BL/KaLwRij MM model and C57BL/6 HFD MM model, which both have bone lesion development as a consequence of increased tumour burden. After confirming that the bone quality in diseased group was improved, mechanistic studies of eliglustat were conducted that will underpin future studies in this area. The findings are summarized as a diagram in Figure 7.1.

Bone quality is significantly compromised in MM murine models; this can be ameliorated by eliglustat treatment. By inhibiting bone resorbing OC formation,

eliglustat prevents further bone loss *in vivo* without affecting OC progenitors, OBs, MM cells, HSCs or cells from myeloid lineage. Myeloma-bearing mice with diet-induced obesity have a different lipid metabolism from healthy mice due to excessive adipose tissue. Eliglustat successfully improved bone quality in MM mice administered with HFD with negligible side effects, indicating that eliglustat has specific target in lipid metabolism and is tolerable under different physiological conditions. In addition, ZA, the current first-line bisphosphonate for MM bone disease, has an additive effect in improving bone quality when combined with eliglustat *in vivo*. Importantly, for the first time, this project revealed that eliglustat suppressed GC formation significantly, which may resolve the question as to how eliglustat prevents the progression of GD to MM.

TRAF3, a negative regulator of OC formation, was maintained by eliglustat. The intact membrane lipid order is essential for NF- κ B signalling transduction, followed by TRAF3 degradation and OC formation. Eliglustat was found to decrease cholesterol or increase the unsaturated lipids in the plasma membrane, which fluidizes the membrane and interrupts OC formation signalling transduction. Moreover, eliglustat was proven to be an autophagy inhibitor that can maintain TRAF3 protein level and hence inhibit OC formation.

Therefore, this project identified a novel small molecule inhibitor that maintains bone quality by preventing the degradation of TRAF3 in OC.

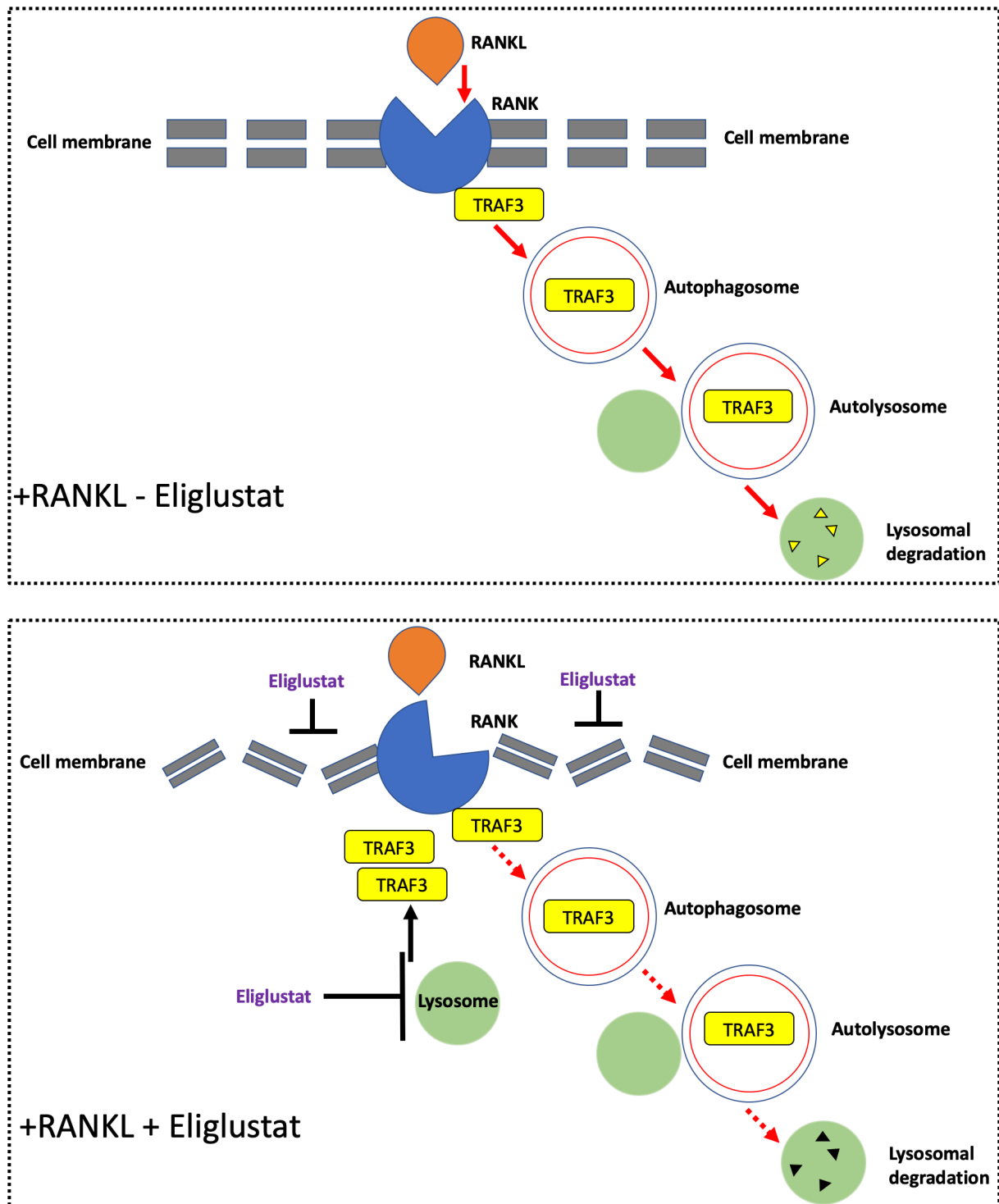


Figure 7.1 Proposed working model of how eliglustat maintains TRAF3 levels in

OCs to inhibit OC formation. Eliglustat is a small molecule that prevents

RANKL/RANK-initiated TRAF3 degradation signalling by disrupting cell membrane lipid orders and inhibiting autophagy. Therefore, TRAF3 negatively regulates OC formation signalling.

Bibliography

- 1 Hadjidakis, D. J. & Androulakis, I. Bone remodeling. *Ann N Y Acad Sci* **1092**, 385-396, doi:10.1196/annals.1365.035 (2006).
- 2 Sims, N. A. & Gooi, J. H. Bone remodeling: Multiple cellular interactions required for coupling of bone formation and resorption. *Semin Cell Dev Biol* **19**, 444-451, doi:10.1016/j.semcdb.2008.07.016 (2008).
- 3 Matsuo, K. & Irie, N. Osteoclast-osteoblast communication. *Arch Biochem Biophys* **473**, 201-209, doi:10.1016/j.abb.2008.03.027 (2008).
- 4 Boyce, B. F. Advances in the regulation of osteoclasts and osteoclast functions. *J Dent Res* **92**, 860-867, doi:10.1177/0022034513500306 (2013).
- 5 Li, Z., Kong, K. & Qi, W. Osteoclast and its roles in calcium metabolism and bone development and remodeling. *Biochem Biophys Res Commun* **343**, 345-350, doi:10.1016/j.bbrc.2006.02.147 (2006).
- 6 Lakkakorpi, P. T., Horton, M. A., Helfrich, M. H., Karhukorpi, E. K. & Vaananen, H. K. Vitronectin receptor has a role in bone resorption but does not mediate tight sealing zone attachment of osteoclasts to the bone surface. *J Cell Biol* **115**, 1179-1186, doi:10.1083/jcb.115.4.1179 (1991).
- 7 Saltel, F., Destaing, O., Bard, F., Eichert, D. & Jurdic, P. Apatite-mediated actin dynamics in resorbing osteoclasts. *Mol Biol Cell* **15**, 5231-5241, doi:10.1091/mbc.e04-06-0522 (2004).
- 8 Luxenburg, C. *et al.* The architecture of the adhesive apparatus of cultured osteoclasts: from podosome formation to sealing zone assembly. *PLoS One* **2**, e179, doi:10.1371/journal.pone.0000179 (2007).
- 9 Chabadel, A. *et al.* CD44 and beta3 integrin organize two functionally distinct actin-based domains in osteoclasts. *Mol Biol Cell* **18**, 4899-4910, doi:10.1091/mbc.e07-04-0378 (2007).
- 10 Vaananen, H. K. & Horton, M. The osteoclast clear zone is a specialized cell-extracellular matrix adhesion structure. *J Cell Sci* **108 (Pt 8)**, 2729-2732 (1995).
- 11 Mulari, M., Vaaraniemi, J. & Vaananen, H. K. Intracellular membrane trafficking in bone resorbing osteoclasts. *Microsc Res Tech* **61**, 496-503, doi:10.1002/jemt.10371 (2003).
- 12 Arana-Chavez, V. E. & Bradaschia-Correa, V. Clastic cells: mineralized tissue resorption in health and disease. *Int J Biochem Cell Biol* **41**, 446-450, doi:10.1016/j.biocel.2008.09.007 (2009).
- 13 Florencio-Silva, R., Sasso, G. R., Sasso-Cerri, E., Simoes, M. J. & Cerri, P. S. Biology of Bone Tissue: Structure, Function, and Factors That Influence Bone Cells. *Biomed Res Int* **2015**, 421746, doi:10.1155/2015/421746 (2015).
- 14 Schlesinger, P. H., Blair, H. C., Teitelbaum, S. L. & Edwards, J. C. Characterization of the osteoclast ruffled border chloride channel and its role in bone resorption. *J Biol Chem* **272**, 18636-18643, doi:10.1074/jbc.272.30.18636 (1997).
- 15 Ersek, A. *et al.* Glycosphingolipid synthesis inhibition limits osteoclast activation and myeloma bone disease. *J Clin Invest* **125**, 2279-2292, doi:10.1172/JCI59987 (2015).
- 16 Teitelbaum, S. L. Osteoclasts: what do they do and how do they do it? *Am J Pathol* **170**, 427-435, doi:10.2353/ajpath.2007.060834 (2007).

- 17 Qin, A. *et al.* V-ATPases in osteoclasts: structure, function and potential inhibitors of bone resorption. *Int J Biochem Cell Biol* **44**, 1422-1435, doi:10.1016/j.biocel.2012.05.014 (2012).
- 18 Witwicka, H. *et al.* Studies of OC-STAMP in Osteoclast Fusion: A New Knockout Mouse Model, Rescue of Cell Fusion, and Transmembrane Topology. *PLoS One* **10**, e0128275, doi:10.1371/journal.pone.0128275 (2015).
- 19 Hartgers, F. C. *et al.* Genomic organization, chromosomal localization, and 5' upstream region of the human DC-STAMP gene. *Immunogenetics* **53**, 145-149, doi:10.1007/s002510100302 (2001).
- 20 Kukita, T. *et al.* RANKL-induced DC-STAMP is essential for osteoclastogenesis. *J Exp Med* **200**, 941-946, doi:10.1084/jem.20040518 (2004).
- 21 Yagi, M. *et al.* DC-STAMP is essential for cell-cell fusion in osteoclasts and foreign body giant cells. *J Exp Med* **202**, 345-351, doi:10.1084/jem.20050645 (2005).
- 22 Miyamoto, H. *et al.* Osteoclast stimulatory transmembrane protein and dendritic cell-specific transmembrane protein cooperatively modulate cell-cell fusion to form osteoclasts and foreign body giant cells. *J Bone Miner Res* **27**, 1289-1297, doi:10.1002/jbmr.1575 (2012).
- 23 Yang, M. *et al.* Osteoclast stimulatory transmembrane protein (OC-STAMP), a novel protein induced by RANKL that promotes osteoclast differentiation. *J Cell Physiol* **215**, 497-505, doi:10.1002/jcp.21331 (2008).
- 24 Khan, U. A., Hashimi, S. M., Bakr, M. M., Forwood, M. R. & Morrison, N. A. Foreign body giant cells and osteoclasts are TRAP positive, have podosome-belts and both require OC-STAMP for cell fusion. *J Cell Biochem* **114**, 1772-1778, doi:10.1002/jcb.24518 (2013).
- 25 Iwasaki, R. *et al.* Cell fusion in osteoclasts plays a critical role in controlling bone mass and osteoblastic activity. *Biochem Biophys Res Commun* **377**, 899-904, doi:10.1016/j.bbrc.2008.10.076 (2008).
- 26 Xing, L., Xiu, Y. & Boyce, B. F. Osteoclast fusion and regulation by RANKL-dependent and independent factors. *World J Orthop* **3**, 212-222, doi:10.5312/wjo.v3.i12.212 (2012).
- 27 Vignery, A. Macrophage fusion: molecular mechanisms. *Methods Mol Biol* **475**, 149-161, doi:10.1007/978-1-59745-250-2_9 (2008).
- 28 Helming, L. & Gordon, S. Molecular mediators of macrophage fusion. *Trends Cell Biol* **19**, 514-522, doi:10.1016/j.tcb.2009.07.005 (2009).
- 29 Capulli, M., Paone, R. & Rucci, N. Osteoblast and osteocyte: games without frontiers. *Arch Biochem Biophys* **561**, 3-12, doi:10.1016/j.abb.2014.05.003 (2014).
- 30 Grigoriadis, A. E., Heersche, J. N. & Aubin, J. E. Differentiation of muscle, fat, cartilage, and bone from progenitor cells present in a bone-derived clonal cell population: effect of dexamethasone. *J Cell Biol* **106**, 2139-2151, doi:10.1083/jcb.106.6.2139 (1988).
- 31 Ducy, P., Zhang, R., Geoffroy, V., Ridall, A. L. & Karsenty, G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell* **89**, 747-754, doi:10.1016/s0092-8674(00)80257-3 (1997).
- 32 Komori, T. *et al.* Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* **89**, 755-764, doi:10.1016/s0092-8674(00)80258-5 (1997).

- 33 Fakhry, M., Hamade, E., Badran, B., Buchet, R. & Magne, D. Molecular mechanisms of mesenchymal stem cell differentiation towards osteoblasts. *World J Stem Cells* **5**, 136-148, doi:10.4252/wjsc.v5.i4.136 (2013).
- 34 Nakashima, K. *et al.* The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* **108**, 17-29, doi:10.1016/s0092-8674(01)00622-5 (2002).
- 35 Glass, D. A., 2nd *et al.* Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. *Dev Cell* **8**, 751-764, doi:10.1016/j.devcel.2005.02.017 (2005).
- 36 Damoulis, P. D. & Hauschka, P. V. Nitric oxide acts in conjunction with proinflammatory cytokines to promote cell death in osteoblasts. *J Bone Miner Res* **12**, 412-422, doi:10.1359/jbmr.1997.12.3.412 (1997).
- 37 Jilka, R. L., Weinstein, R. S., Bellido, T., Parfitt, A. M. & Manolagas, S. C. Osteoblast programmed cell death (apoptosis): modulation by growth factors and cytokines. *J Bone Miner Res* **13**, 793-802, doi:10.1359/jbmr.1998.13.5.793 (1998).
- 38 Teitelbaum, S. L. Bone resorption by osteoclasts. *Science* **289**, 1504-1508, doi:10.1126/science.289.5484.1504 (2000).
- 39 Boyce, B. F., Hughes, D. E., Wright, K. R., Xing, L. & Dai, A. Recent advances in bone biology provide insight into the pathogenesis of bone diseases. *Lab Invest* **79**, 83-94 (1999).
- 40 Yoshida, H. *et al.* The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature* **345**, 442-444, doi:10.1038/345442a0 (1990).
- 41 Richards, J. B., Zheng, H. F. & Spector, T. D. Genetics of osteoporosis from genome-wide association studies: advances and challenges. *Nat Rev Genet* **13**, 576-588, doi:10.1038/nrg3228 (2012).
- 42 Sodek, J. & McKee, M. D. Molecular and cellular biology of alveolar bone. *Periodontol 2000* **24**, 99-126 (2000).
- 43 Deng, L. *et al.* Activation of the I κ B kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell* **103**, 351-361, doi:10.1016/s0092-8674(00)00126-4 (2000).
- 44 Miyamoto, T. The dendritic cell-specific transmembrane protein DC-STAMP is essential for osteoclast fusion and osteoclast bone-resorbing activity. *Mod Rheumatol* **16**, 341-342, doi:10.1007/s10165-006-0524-0 (2006).
- 45 Kobayashi, Y., Udagawa, N. & Takahashi, N. Action of RANKL and OPG for osteoclastogenesis. *Crit Rev Eukaryot Gene Expr* **19**, 61-72 (2009).
- 46 Matsumoto, M. *et al.* Essential role of p38 mitogen-activated protein kinase in cathepsin K gene expression during osteoclastogenesis through association of NFATc1 and PU.1. *J Biol Chem* **279**, 45969-45979, doi:10.1074/jbc.M408795200 (2004).
- 47 Yasuda, H. *et al.* Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci U S A* **95**, 3597-3602, doi:10.1073/pnas.95.7.3597 (1998).
- 48 Boyce, B. F. & Xing, L. Functions of RANKL/RANK/OPG in bone modeling and remodeling. *Arch Biochem Biophys* **473**, 139-146, doi:10.1016/j.abb.2008.03.018 (2008).

- 49 Hofbauer, L. C., Neubauer, A. & Heufelder, A. E. Receptor activator of nuclear factor-kappaB ligand and osteoprotegerin: potential implications for the pathogenesis and treatment of malignant bone diseases. *Cancer* **92**, 460-470, doi:10.1002/1097-0142(20010801)92:3<460::aid-cnrcr1344>3.0.co;2-d (2001).
- 50 Hayden, M. S. & Ghosh, S. Shared principles in NF-kappaB signaling. *Cell* **132**, 344-362, doi:10.1016/j.cell.2008.01.020 (2008).
- 51 Vallabhapurapu, S. & Karin, M. Regulation and function of NF-kappaB transcription factors in the immune system. *Annu Rev Immunol* **27**, 693-733, doi:10.1146/annurev.immunol.021908.132641 (2009).
- 52 Liu, T., Zhang, L., Joo, D. & Sun, S. C. NF-kappaB signaling in inflammation. *Signal Transduct Target Ther* **2**, doi:10.1038/sigtrans.2017.23 (2017).
- 53 Sun, S. C. The non-canonical NF-kappaB pathway in immunity and inflammation. *Nat Rev Immunol* **17**, 545-558, doi:10.1038/nri.2017.52 (2017).
- 54 Boyce, B. F. Advances in osteoclast biology reveal potential new drug targets and new roles for osteoclasts. *J Bone Miner Res* **28**, 711-722, doi:10.1002/jbmr.1885 (2013).
- 55 Xing, L. *et al.* NF-kappaB p50 and p52 expression is not required for RANK-expressing osteoclast progenitor formation but is essential for RANK- and cytokine-mediated osteoclastogenesis. *J Bone Miner Res* **17**, 1200-1210, doi:10.1359/jbmr.2002.17.7.1200 (2002).
- 56 Xing, L. *et al.* Expression of either NF-kappaB p50 or p52 in osteoclast precursors is required for IL-1-induced bone resorption. *J Bone Miner Res* **18**, 260-269, doi:10.1359/jbmr.2003.18.2.260 (2003).
- 57 Boyce, B. F., Xiu, Y., Li, J., Xing, L. & Yao, Z. NF-kappaB-Mediated Regulation of Osteoclastogenesis. *Endocrinol Metab (Seoul)* **30**, 35-44, doi:10.3803/EnM.2015.30.1.35 (2015).
- 58 Liu, S. & Chen, Z. J. Expanding role of ubiquitination in NF-kappaB signaling. *Cell Res* **21**, 6-21, doi:10.1038/cr.2010.170 (2011).
- 59 Hu, H. & Sun, S. C. Ubiquitin signaling in immune responses. *Cell Res* **26**, 457-483, doi:10.1038/cr.2016.40 (2016).
- 60 Sun, S. C., Chang, J. H. & Jin, J. Regulation of nuclear factor-kappaB in autoimmunity. *Trends Immunol* **34**, 282-289, doi:10.1016/j.it.2013.01.004 (2013).
- 61 Hayden, M. S. & Ghosh, S. NF-kappaB, the first quarter-century: remarkable progress and outstanding questions. *Genes Dev* **26**, 203-234, doi:10.1101/gad.183434.111 (2012).
- 62 Sun, S. C. The noncanonical NF-kappaB pathway. *Immunol Rev* **246**, 125-140, doi:10.1111/j.1600-065X.2011.01088.x (2012).
- 63 Xiao, G., Harhaj, E. W. & Sun, S. C. NF-kappaB-inducing kinase regulates the processing of NF-kappaB2 p100. *Mol Cell* **7**, 401-409, doi:10.1016/s1097-2765(01)00187-3 (2001).
- 64 Senftleben, U. *et al.* Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. *Science* **293**, 1495-1499, doi:10.1126/science.1062677 (2001).
- 65 Liao, G., Zhang, M., Harhaj, E. W. & Sun, S. C. Regulation of the NF-kappaB-inducing kinase by tumor necrosis factor receptor-associated factor 3-induced degradation. *J Biol Chem* **279**, 26243-26250, doi:10.1074/jbc.M403286200 (2004).

- 66 Shi, J. H. & Sun, S. C. Tumor Necrosis Factor Receptor-Associated Factor Regulation of Nuclear Factor kappaB and Mitogen-Activated Protein Kinase Pathways. *Front Immunol* **9**, 1849, doi:10.3389/fimmu.2018.01849 (2018).
- 67 Ha, H., Han, D. & Choi, Y. TRAF-mediated TNFR-family signaling. *Curr Protoc Immunol* **Chapter 11**, Unit11 19D, doi:10.1002/0471142735.im1109ds87 (2009).
- 68 Xie, P. TRAF molecules in cell signaling and in human diseases. *J Mol Signal* **8**, 7, doi:10.1186/1750-2187-8-7 (2013).
- 69 Deshaies, R. J. & Joazeiro, C. A. RING domain E3 ubiquitin ligases. *Annu Rev Biochem* **78**, 399-434, doi:10.1146/annurev.biochem.78.101807.093809 (2009).
- 70 Armstrong, A. P. *et al.* A RANK/TRAF6-dependent signal transduction pathway is essential for osteoclast cytoskeletal organization and resorptive function. *J Biol Chem* **277**, 44347-44356, doi:10.1074/jbc.M202009200 (2002).
- 71 Xiu, Y. *et al.* Chloroquine reduces osteoclastogenesis in murine osteoporosis by preventing TRAF3 degradation. *J Clin Invest* **124**, 297-310, doi:10.1172/JCI66947 (2014).
- 72 Wong, B. R. *et al.* TRANCE, a TNF family member, activates Akt/PKB through a signaling complex involving TRAF6 and c-Src. *Mol Cell* **4**, 1041-1049, doi:10.1016/s1097-2765(00)80232-4 (1999).
- 73 Wong, B. R. *et al.* The TRAF family of signal transducers mediates NF-kappaB activation by the TRANCE receptor. *J Biol Chem* **273**, 28355-28359, doi:10.1074/jbc.273.43.28355 (1998).
- 74 Galibert, L., Tometsko, M. E., Anderson, D. M., Cosman, D. & Dougall, W. C. The involvement of multiple tumor necrosis factor receptor (TNFR)-associated factors in the signaling mechanisms of receptor activator of NF-kappaB, a member of the TNFR superfamily. *J Biol Chem* **273**, 34120-34127, doi:10.1074/jbc.273.51.34120 (1998).
- 75 Gohda, J. *et al.* RANK-mediated amplification of TRAF6 signaling leads to NFATc1 induction during osteoclastogenesis. *EMBO J* **24**, 790-799, doi:10.1038/sj.emboj.7600564 (2005).
- 76 Takayanagi, H. *et al.* Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. *Dev Cell* **3**, 889-901 (2002).
- 77 Lomaga, M. A. *et al.* TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. *Genes Dev* **13**, 1015-1024, doi:10.1101/gad.13.8.1015 (1999).
- 78 Kadono, Y. *et al.* Strength of TRAF6 signalling determines osteoclastogenesis. *EMBO Rep* **6**, 171-176, doi:10.1038/sj.embor.7400345 (2005).
- 79 Jin, W. *et al.* Deubiquitinating enzyme CYLD negatively regulates RANK signaling and osteoclastogenesis in mice. *J Clin Invest* **118**, 1858-1866, doi:10.1172/JCI34257 (2008).
- 80 Bai, S. *et al.* FHL2 inhibits the activated osteoclast in a TRAF6-dependent manner. *J Clin Invest* **115**, 2742-2751, doi:10.1172/JCI24921 (2005).
- 81 Bai, S., Zha, J., Zhao, H., Ross, F. P. & Teitelbaum, S. L. Tumor necrosis factor receptor-associated factor 6 is an intranuclear transcriptional coactivator in osteoclasts. *J Biol Chem* **283**, 30861-30867, doi:10.1074/jbc.M802525200 (2008).
- 82 Kim, N. *et al.* Osteoclast differentiation independent of the TRANCE-RANK-TRAF6 axis. *J Exp Med* **202**, 589-595, doi:10.1084/jem.20050978 (2005).

- 83 Zhang, Y. H., Heulsmann, A., Tondravi, M. M., Mukherjee, A. & Abu-Amer, Y. Tumor necrosis factor- α (TNF) stimulates RANKL-induced osteoclastogenesis via coupling of TNF type 1 receptor and RANK signaling pathways. *J Biol Chem* **276**, 563-568, doi:10.1074/jbc.M008198200 (2001).
- 84 Hacker, H., Tseng, P. H. & Karin, M. Expanding TRAF function: TRAF3 as a tri-faced immune regulator. *Nat Rev Immunol* **11**, 457-468, doi:10.1038/nri2998 (2011).
- 85 Yang, C. *et al.* NIK stabilization in osteoclasts results in osteoporosis and enhanced inflammatory osteolysis. *PLoS One* **5**, e15383, doi:10.1371/journal.pone.0015383 (2010).
- 86 Yao, Z., Xing, L. & Boyce, B. F. NF- κ B p100 limits TNF-induced bone resorption in mice by a TRAF3-dependent mechanism. *J Clin Invest* **119**, 3024-3034, doi:10.1172/JCI38716 (2009).
- 87 Bista, P. *et al.* TRAF3 controls activation of the canonical and alternative NF κ B by the lymphotoxin beta receptor. *J Biol Chem* **285**, 12971-12978, doi:10.1074/jbc.M109.076091 (2010).
- 88 Zarnegar, B., Yamazaki, S., He, J. Q. & Cheng, G. Control of canonical NF- κ B activation through the NIK-IKK complex pathway. *Proc Natl Acad Sci U S A* **105**, 3503-3508, doi:10.1073/pnas.0707959105 (2008).
- 89 Lalani, A. I. *et al.* Myeloid cell TRAF3 regulates immune responses and inhibits inflammation and tumor development in mice. *J Immunol* **194**, 334-348, doi:10.4049/jimmunol.1401548 (2015).
- 90 Levine, B. & Kroemer, G. Autophagy in the pathogenesis of disease. *Cell* **132**, 27-42, doi:10.1016/j.cell.2007.12.018 (2008).
- 91 Mizushima, N. Autophagy: process and function. *Genes Dev* **21**, 2861-2873, doi:10.1101/gad.1599207 (2007).
- 92 Mizushima, N., Levine, B., Cuervo, A. M. & Klionsky, D. J. Autophagy fights disease through cellular self-digestion. *Nature* **451**, 1069-1075, doi:10.1038/nature06639 (2008).
- 93 Pierrefite-Carle, V., Santucci-Darmanin, S., Breuil, V., Camuzard, O. & Carle, G. F. Autophagy in bone: Self-eating to stay in balance. *Ageing Res Rev* **24**, 206-217, doi:10.1016/j.arr.2015.08.004 (2015).
- 94 Hosokawa, N. *et al.* Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. *Mol Biol Cell* **20**, 1981-1991, doi:10.1091/mbc.E08-12-1248 (2009).
- 95 Hosokawa, N. *et al.* Atg101, a novel mammalian autophagy protein interacting with Atg13. *Autophagy* **5**, 973-979, doi:10.4161/auto.5.7.9296 (2009).
- 96 Mercer, C. A., Kaliappan, A. & Dennis, P. B. A novel, human Atg13 binding protein, Atg101, interacts with ULK1 and is essential for macroautophagy. *Autophagy* **5**, 649-662, doi:10.4161/auto.5.5.8249 (2009).
- 97 Ganley, I. G. *et al.* ULK1.ATG13.FIP200 complex mediates mTOR signaling and is essential for autophagy. *J Biol Chem* **284**, 12297-12305, doi:10.1074/jbc.M900573200 (2009).
- 98 Jung, C. H. *et al.* ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. *Mol Biol Cell* **20**, 1992-2003, doi:10.1091/mbc.E08-12-1249 (2009).

- 99 Polson, H. E. *et al.* Mammalian Atg18 (WIPI2) localizes to omegasome-anchored phagophores and positively regulates LC3 lipidation. *Autophagy* **6**, 506-522, doi:10.4161/auto.6.4.11863 (2010).
- 100 Mari, M. *et al.* An Atg9-containing compartment that functions in the early steps of autophagosome biogenesis. *J Cell Biol* **190**, 1005-1022, doi:10.1083/jcb.200912089 (2010).
- 101 Orsi, A. *et al.* Dynamic and transient interactions of Atg9 with autophagosomes, but not membrane integration, are required for autophagy. *Mol Biol Cell* **23**, 1860-1873, doi:10.1091/mbc.E11-09-0746 (2012).
- 102 Yamamoto, H. *et al.* Atg9 vesicles are an important membrane source during early steps of autophagosome formation. *J Cell Biol* **198**, 219-233, doi:10.1083/jcb.201202061 (2012).
- 103 Yang, Z. & Klionsky, D. J. Mammalian autophagy: core molecular machinery and signaling regulation. *Curr Opin Cell Biol* **22**, 124-131, doi:10.1016/j.ceb.2009.11.014 (2010).
- 104 Shpilka, T., Mizushima, N. & Elazar, Z. Ubiquitin-like proteins and autophagy at a glance. *J Cell Sci* **125**, 2343-2348, doi:10.1242/jcs.093757 (2012).
- 105 Klionsky, D. J. *et al.* Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* **12**, 1-222, doi:10.1080/15548627.2015.1100356 (2016).
- 106 Larsen, K. B. *et al.* A reporter cell system to monitor autophagy based on p62/SQSTM1. *Autophagy* **6**, 784-793, doi:10.4161/auto.6.6.12510 (2010).
- 107 Lin, N. Y. *et al.* Inactivation of autophagy ameliorates glucocorticoid-induced and ovariectomy-induced bone loss. *Ann Rheum Dis* **75**, 1203-1210, doi:10.1136/annrheumdis-2015-207240 (2016).
- 108 Kim, M. S. *et al.* MCP-1-induced human osteoclast-like cells are tartrate-resistant acid phosphatase, NFATc1, and calcitonin receptor-positive but require receptor activator of NFkappaB ligand for bone resorption. *J Biol Chem* **281**, 1274-1285, doi:10.1074/jbc.M510156200 (2006).
- 109 Kim, M. S., Magno, C. L., Day, C. J. & Morrison, N. A. Induction of chemokines and chemokine receptors CCR2b and CCR4 in authentic human osteoclasts differentiated with RANKL and osteoclast like cells differentiated by MCP-1 and RANTES. *J Cell Biochem* **97**, 512-518, doi:10.1002/jcb.20649 (2006).
- 110 Zhou, L. *et al.* Monocyte chemoattractant protein-1 induces a novel transcription factor that causes cardiac myocyte apoptosis and ventricular dysfunction. *Circ Res* **98**, 1177-1185, doi:10.1161/01.RES.0000220106.64661.71 (2006).
- 111 Wang, K., Niu, J., Kim, H. & Kolattukudy, P. E. Osteoclast precursor differentiation by MCP-1 via oxidative stress, endoplasmic reticulum stress, and autophagy. *J Mol Cell Biol* **3**, 360-368, doi:10.1093/jmcb/mjr021 (2011).
- 112 Chung, Y. H. *et al.* Beclin-1 is required for RANKL-induced osteoclast differentiation. *J Cell Physiol* **229**, 1963-1971, doi:10.1002/jcp.24646 (2014).
- 113 Zhao, Y. *et al.* Autophagy regulates hypoxia-induced osteoclastogenesis through the HIF-1alpha/BNIP3 signaling pathway. *J Cell Physiol* **227**, 639-648, doi:10.1002/jcp.22768 (2012).
- 114 Sambandam, Y. *et al.* Microgravity control of autophagy modulates osteoclastogenesis. *Bone* **61**, 125-131, doi:10.1016/j.bone.2014.01.004 (2014).

- 115 DeSelm, C. J. *et al.* Autophagy proteins regulate the secretory component of osteoclastic bone resorption. *Dev Cell* **21**, 966-974, doi:10.1016/j.devcel.2011.08.016 (2011).
- 116 Hocking, L. J., Whitehouse, C. & Helfrich, M. H. Autophagy: a new player in skeletal maintenance? *J Bone Miner Res* **27**, 1439-1447, doi:10.1002/jbmr.1668 (2012).
- 117 Chung, Y. H. *et al.* Microtubule-associated protein light chain 3 regulates Cdc42-dependent actin ring formation in osteoclast. *Int J Biochem Cell Biol* **44**, 989-997, doi:10.1016/j.biocel.2012.03.007 (2012).
- 118 Yamamoto, A. *et al.* Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. *Cell Struct Funct* **23**, 33-42, doi:10.1247/csf.23.33 (1998).
- 119 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, doi:10.1002/jcb.25442 (2016).
- 120 Neutsky-Wulff, A. V. *et al.* Alterations in osteoclast function and phenotype induced by different inhibitors of bone resorption--implications for osteoclast quality. *BMC Musculoskelet Disord* **11**, 109, doi:10.1186/1471-2474-11-109 (2010).
- 121 Merrill, A. H., Jr. *et al.* Sphingolipids--the enigmatic lipid class: biochemistry, physiology, and pathophysiology. *Toxicol Appl Pharmacol* **142**, 208-225, doi:10.1006/taap.1996.8029 (1997).
- 122 Kolter, T., Proia, R. L. & Sandhoff, K. Combinatorial ganglioside biosynthesis. *J Biol Chem* **277**, 25859-25862, doi:10.1074/jbc.R200001200 (2002).
- 123 Ichikawa, S. & Hirabayashi, Y. Glucosylceramide synthase and glycosphingolipid synthesis. *Trends Cell Biol* **8**, 198-202 (1998).
- 124 Fyrst, H. & Saba, J. D. An update on sphingosine-1-phosphate and other sphingolipid mediators. *Nat Chem Biol* **6**, 489-497, doi:10.1038/nchembio.392 (2010).
- 125 Nomura, T. *et al.* Purification, cDNA cloning, and expression of UDP-Gal: glucosylceramide beta-1,4-galactosyltransferase from rat brain. *J Biol Chem* **273**, 13570-13577, doi:10.1074/jbc.273.22.13570 (1998).
- 126 Huwiler, A., Kolter, T., Pfeilschifter, J. & Sandhoff, K. Physiology and pathophysiology of sphingolipid metabolism and signaling. *Biochim Biophys Acta* **1485**, 63-99, doi:10.1016/s1388-1981(00)00042-1 (2000).
- 127 Tettamanti, G. Ganglioside/glycosphingolipid turnover: new concepts. *Glycoconj J* **20**, 301-317, doi:10.1023/B:GLYC.0000033627.02765.cc (2004).
- 128 Tettamanti, G., Bassi, R., Viani, P. & Riboni, L. Salvage pathways in glycosphingolipid metabolism. *Biochimie* **85**, 423-437, doi:10.1016/s0300-9084(03)00047-6 (2003).
- 129 Sandhoff, K. & Kolter, T. Biosynthesis and degradation of mammalian glycosphingolipids. *Philos Trans R Soc Lond B Biol Sci* **358**, 847-861, doi:10.1098/rstb.2003.1265 (2003).
- 130 Kolter, T. A view on sphingolipids and disease. *Chem Phys Lipids* **164**, 590-606, doi:10.1016/j.chemphyslip.2011.04.013 (2011).
- 131 Poole, R. M. Eliglustat: first global approval. *Drugs* **74**, 1829-1836, doi:10.1007/s40265-014-0296-3 (2014).
- 132 Giussani, P., Tringali, C., Riboni, L., Viani, P. & Venerando, B. Sphingolipids: key regulators of apoptosis and pivotal players in cancer drug resistance. *Int J Mol Sci* **15**, 4356-4392, doi:10.3390/ijms15034356 (2014).

- 133 Abian, O. *et al.* 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, doi:10.1021/mp200313e (2011).
- 134 Cox, T. M. *et al.* The role of the iminosugar N-butyldeoxynojirimycin (miglustat) in the management of type I (non-neuronopathic) Gaucher disease: a position statement. *J Inherit Metab Dis* **26**, 513-526, doi:10.1023/a:1025902113005 (2003).
- 135 Rosenbloom, B. E., Becker, P. & Weinreb, N. Multiple myeloma and Gaucher genes. *Genet Med* **11**, 134, doi:10.1097/GIM.0b013e318195e2b6 (2009).
- 136 Siegel, R. L., Miller, K. D. & Jemal, A. Cancer statistics, 2016. *CA Cancer J Clin* **66**, 7-30, doi:10.3322/caac.21332 (2016).
- 137 Rajkumar, S. V. *et al.* International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. *Lancet Oncol* **15**, e538-548, doi:10.1016/S1470-2045(14)70442-5 (2014).
- 138 Greipp, P. R. *et al.* International staging system for multiple myeloma. *J Clin Oncol* **23**, 3412-3420, doi:10.1200/JCO.2005.04.242 (2005).
- 139 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, doi:10.1182/blood-2003-02-0380 (2003).
- 140 Yin, T. & Li, L. The stem cell niches in bone. *J Clin Invest* **116**, 1195-1201, doi:10.1172/JCI28568 (2006).
- 141 Kawano, Y. *et al.* Targeting the bone marrow microenvironment in multiple myeloma. *Immunol Rev* **263**, 160-172, doi:10.1111/imr.12233 (2015).
- 142 Quail, D. F. & Joyce, J. A. Microenvironmental regulation of tumor progression and metastasis. *Nat Med* **19**, 1423-1437, doi:10.1038/nm.3394 (2013).
- 143 Abe, M., Hiura, K., Ozaki, S., Kido, S. & Matsumoto, T. Vicious cycle between myeloma cell binding to bone marrow stromal cells via VLA-4-VCAM-1 adhesion and macrophage inflammatory protein-1alpha and MIP-1beta production. *J Bone Miner Metab* **27**, 16-23, doi:10.1007/s00774-008-0012-z (2009).
- 144 Podar, K., Chauhan, D. & Anderson, K. C. Bone marrow microenvironment and the identification of new targets for myeloma therapy. *Leukemia* **23**, 10-24, doi:10.1038/leu.2008.259 (2009).
- 145 Raje, N. S., Bhatta, S. & Terpos, E. Role of the RANK/RANKL Pathway in Multiple Myeloma. *Clin Cancer Res* **25**, 12-20, doi:10.1158/1078-0432.CCR-18-1537 (2019).
- 146 Fowler, J. A., Edwards, C. M. & Croucher, P. I. Tumor-host cell interactions in the bone disease of myeloma. *Bone* **48**, 121-128, doi:10.1016/j.bone.2010.06.029 (2011).
- 147 Fowler, J. A. *et al.* Host-derived adiponectin is tumor-suppressive and a novel therapeutic target for multiple myeloma and the associated bone disease. *Blood* **118**, 5872-5882, doi:10.1182/blood-2011-01-330407 (2011).
- 148 Nelson, W. G., DeWeese, T. L. & DeMarzo, A. M. The diet, prostate inflammation, and the development of prostate cancer. *Cancer Metastasis Rev* **21**, 3-16 (2002).
- 149 Taylor, M. D., Mellert, T. K., Parmentier, J. L. & Eddy, L. J. Pharmacological protection of reoxygenation damage to in vitro brain slice tissue. *Brain Res* **347**, 268-273, doi:10.1016/0006-8993(85)90186-6 (1985).

- 150 Cao, J. J., Sun, L. & Gao, H. Diet-induced obesity alters bone remodeling leading to decreased femoral trabecular bone mass in mice. *Ann N Y Acad Sci* **1192**, 292-297, doi:10.1111/j.1749-6632.2009.05252.x (2010).
- 151 Lwin, S. T., Olechnowicz, S. W., Fowler, J. A. & Edwards, C. M. Diet-induced obesity promotes a myeloma-like condition in vivo. *Leukemia* **29**, 507-510, doi:10.1038/leu.2014.295 (2015).
- 152 Terkeltaub, R. A. Inorganic pyrophosphate generation and disposition in pathophysiology. *Am J Physiol Cell Physiol* **281**, C1-C11, doi:10.1152/ajpcell.2001.281.1.C1 (2001).
- 153 Schenk, R., Merz, W. A., Muhlbauer, R., Russell, R. G. & Fleisch, H. Effect of ethane-1-hydroxy-1,1-diphosphonate (EHDP) and dichloromethylene diphosphonate (Cl 2 MDP) on the calcification and resorption of cartilage and bone in the tibial epiphysis and metaphysis of rats. *Calcif Tissue Res* **11**, 196-214 (1973).
- 154 McCloskey, E. V., Dunn, J. A., Kanis, J. A., MacLennan, I. C. & Drayson, M. T. Long-term follow-up of a prospective, double-blind, placebo-controlled randomized trial of clodronate in multiple myeloma. *Br J Haematol* **113**, 1035-1043, doi:10.1046/j.1365-2141.2001.02851.x (2001).
- 155 Hameed, A., Brady, J. J., Dowling, P., Clynes, M. & O'Gorman, P. Bone disease in multiple myeloma: pathophysiology and management. *Cancer Growth Metastasis* **7**, 33-42, doi:10.4137/CGM.S16817 (2014).
- 156 Dhodapkar, M. V. *et al.* Anti-myeloma activity of pamidronate in vivo. *Br J Haematol* **103**, 530-532, doi:10.1046/j.1365-2141.1998.00976.x (1998).
- 157 Berenson, J. R. *et al.* Long-term pamidronate treatment of advanced multiple myeloma patients reduces skeletal events. Myeloma Aredia Study Group. *J Clin Oncol* **16**, 593-602, doi:10.1200/JCO.1998.16.2.593 (1998).
- 158 Russell, R. G. Pharmacological diversity among drugs that inhibit bone resorption. *Curr Opin Pharmacol* **22**, 115-130, doi:10.1016/j.coph.2015.05.005 (2015).
- 159 Morgan, G. J. *et al.* Long-term follow-up of MRC Myeloma IX trial: Survival outcomes with bisphosphonate and thalidomide treatment. *Clin Cancer Res* **19**, 6030-6038, doi:10.1158/1078-0432.CCR-12-3211 (2013).
- 160 Modi, N. D. & Lentzsch, S. Bisphosphonates as antimyeloma drugs. *Leukemia* **26**, 589-594, doi:10.1038/leu.2011.282 (2012).
- 161 Sanfilippo, K. M. *et al.* Comparative effectiveness on survival of zoledronic acid versus pamidronate in multiple myeloma. *Leuk Lymphoma* **56**, 615-621, doi:10.3109/10428194.2014.924117 (2015).
- 162 Berenson, J. R. *et al.* Zoledronic acid reduces skeletal-related events in patients with osteolytic metastases. *Cancer* **91**, 1191-1200, doi:10.1002/1097-0142(20010401)91:7<1191::aid-cnrcr1119>3.0.co;2-0 (2001).
- 163 Badros, A. *et al.* Natural history of osteonecrosis of the jaw in patients with multiple myeloma. *J Clin Oncol* **26**, 5904-5909, doi:10.1200/JCO.2008.16.9300 (2008).
- 164 Kennel, K. A. & Drake, M. T. Adverse effects of bisphosphonates: implications for osteoporosis management. *Mayo Clin Proc* **84**, 632-637; quiz 638, doi:10.1016/S0025-6196(11)60752-0 (2009).
- 165 Sierra, F. *et al.* [Effect of pamidronate infusion time on renal function in patients with multiple myeloma]. *Farm Hosp* **34**, 23-26, doi:10.1016/j.farma.2009.09.003 (2010).

- 166 Sterz, J. *et al.* The potential of proteasome inhibitors in cancer therapy. *Expert Opin Investig Drugs* **17**, 879-895, doi:10.1517/13543784.17.6.879 (2008).
- 167 Maki, C. G., Huibregtse, J. M. & Howley, P. M. In vivo ubiquitination and proteasome-mediated degradation of p53(1). *Cancer Res* **56**, 2649-2654 (1996).
- 168 Stancovski, I., Gonen, H., Orian, A., Schwartz, A. L. & Ciechanover, A. Degradation of the proto-oncogene product c-Fos by the ubiquitin proteolytic system in vivo and in vitro: identification and characterization of the conjugating enzymes. *Mol Cell Biol* **15**, 7106-7116, doi:10.1128/mcb.15.12.7106 (1995).
- 169 Marshansky, V. *et al.* Proteasomes modulate balance among proapoptotic and antiapoptotic Bcl-2 family members and compromise functioning of the electron transport chain in leukemic cells. *J Immunol* **166**, 3130-3142, doi:10.4049/jimmunol.166.5.3130 (2001).
- 170 Traenckner, E. B., Wilk, S. & Baeuerle, P. A. A proteasome inhibitor prevents activation of NF-kappa B and stabilizes a newly phosphorylated form of I kappa B-alpha that is still bound to NF-kappa B. *EMBO J* **13**, 5433-5441 (1994).
- 171 Strauss, S. J. *et al.* The proteasome inhibitor bortezomib acts independently of p53 and induces cell death via apoptosis and mitotic catastrophe in B-cell lymphoma cell lines. *Cancer Res* **67**, 2783-2790, doi:10.1158/0008-5472.CAN-06-3254 (2007).
- 172 Hideshima, T. *et al.* The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. *Cancer Res* **61**, 3071-3076 (2001).
- 173 Pekol, T. *et al.* Human metabolism of the proteasome inhibitor bortezomib: identification of circulating metabolites. *Drug Metab Dispos* **33**, 771-777, doi:10.1124/dmd.104.002956 (2005).
- 174 von Metzler, I. *et al.* Bortezomib inhibits human osteoclastogenesis. *Leukemia* **21**, 2025-2034, doi:10.1038/sj.leu.2404806 (2007).
- 175 Pennisi, A. *et al.* The proteasome inhibitor, bortezomib suppresses primary myeloma and stimulates bone formation in myelomatous and nonmyelomatous bones in vivo. *Am J Hematol* **84**, 6-14, doi:10.1002/ajh.21310 (2009).
- 176 Curran, M. P. & McKeage, K. Bortezomib: a review of its use in patients with multiple myeloma. *Drugs* **69**, 859-888, doi:10.2165/00003495-200969070-00006 (2009).
- 177 Terpos, E., Dimopoulos, M. A. & Sezer, O. The effect of novel anti-myeloma agents on bone metabolism of patients with multiple myeloma. *Leukemia* **21**, 1875-1884, doi:10.1038/sj.leu.2404843 (2007).
- 178 Heider, U. *et al.* Bortezomib increases osteoblast activity in myeloma patients irrespective of response to treatment. *Eur J Haematol* **77**, 233-238, doi:10.1111/j.1600-0609.2006.00692.x (2006).
- 179 Zangari, M. *et al.* Response to bortezomib is associated to osteoblastic activation in patients with multiple myeloma. *Br J Haematol* **131**, 71-73, doi:10.1111/j.1365-2141.2005.05733.x (2005).
- 180 Terpos, E., Sezer, O., Croucher, P. & Dimopoulos, M. A. Myeloma bone disease and proteasome inhibition therapies. *Blood* **110**, 1098-1104, doi:10.1182/blood-2007-03-067710 (2007).
- 181 Adams, J. The proteasome: a suitable antineoplastic target. *Nat Rev Cancer* **4**, 349-360, doi:10.1038/nrc1361 (2004).

- 182 Chen, D., Frezza, M., Schmitt, S., Kanwar, J. & Dou, Q. P. Bortezomib as the first proteasome inhibitor anticancer drug: current status and future perspectives. *Curr Cancer Drug Targets* **11**, 239-253 (2011).
- 183 Skrott, Z. & Cvek, B. Linking the activity of bortezomib in multiple myeloma and autoimmune diseases. *Crit Rev Oncol Hematol* **92**, 61-70, doi:10.1016/j.critrevonc.2014.05.003 (2014).
- 184 Schmidt, M. & Finley, D. Regulation of proteasome activity in health and disease. *Biochim Biophys Acta* **1843**, 13-25, doi:10.1016/j.bbamcr.2013.08.012 (2014).
- 185 Moreau, P. *et al.* Proteasome inhibitors in multiple myeloma: 10 years later. *Blood* **120**, 947-959, doi:10.1182/blood-2012-04-403733 (2012).
- 186 Dick, L. R. & Fleming, P. E. Building on bortezomib: second-generation proteasome inhibitors as anti-cancer therapy. *Drug Discov Today* **15**, 243-249, doi:10.1016/j.drudis.2010.01.008 (2010).
- 187 Buac, D. *et al.* From bortezomib to other inhibitors of the proteasome and beyond. *Curr Pharm Des* **19**, 4025-4038, doi:10.2174/1381612811319220012 (2013).
- 188 Smith, A. J. *et al.* Noxa/Bcl-2 protein interactions contribute to bortezomib resistance in human lymphoid cells. *J Biol Chem* **286**, 17682-17692, doi:10.1074/jbc.M110.189092 (2011).
- 189 Yang, D. T., Young, K. H., Kahl, B. S., Markovina, S. & Miyamoto, S. Prevalence of bortezomib-resistant constitutive NF-kappaB activity in mantle cell lymphoma. *Mol Cancer* **7**, 40, doi:10.1186/1476-4598-7-40 (2008).
- 190 Oerlemans, R. *et al.* Molecular basis of bortezomib resistance: proteasome subunit beta5 (PSMB5) gene mutation and overexpression of PSMB5 protein. *Blood* **112**, 2489-2499, doi:10.1182/blood-2007-08-104950 (2008).
- 191 Lu, S. *et al.* Point mutation of the proteasome beta5 subunit gene is an important mechanism of bortezomib resistance in bortezomib-selected variants of Jurkat T cell lymphoblastic lymphoma/leukemia line. *J Pharmacol Exp Ther* **326**, 423-431, doi:10.1124/jpet.108.138131 (2008).
- 192 Miller, C. P., Singh, M. M., Rivera-Del Valle, N., Manton, C. A. & Chandra, J. Therapeutic strategies to enhance the anticancer efficacy of histone deacetylase inhibitors. *J Biomed Biotechnol* **2011**, 514261, doi:10.1155/2011/514261 (2011).
- 193 McConkey, D. J. & Zhu, K. Mechanisms of proteasome inhibitor action and resistance in cancer. *Drug Resist Updat* **11**, 164-179, doi:10.1016/j.drug.2008.08.002 (2008).
- 194 Ersek, A. *et al.* Strain dependent differences in glucocorticoid-induced bone loss between C57BL/6J and CD-1 mice. *Sci Rep* **6**, 36513, doi:10.1038/srep36513 (2016).
- 195 McDonald, M. M. *et al.* Inhibiting the osteocyte-specific protein sclerostin increases bone mass and fracture resistance in multiple myeloma. *Blood* **129**, 3452-3464, doi:10.1182/blood-2017-03-773341 (2017).
- 196 Muchtar, E. *et al.* Bone mineral density utilization in patients with newly diagnosed multiple myeloma. *Hematol Oncol* **35**, 703-710, doi:10.1002/hon.2303 (2017).
- 197 Pavlova, E. V. *et al.* Inhibition of UDP-glucosylceramide synthase in mice prevents Gaucher disease-associated B-cell malignancy. *J Pathol* **235**, 113-124, doi:10.1002/path.4452 (2015).
- 198 Nair, S. *et al.* Clonal Immunoglobulin against Lysolipids in the Origin of Myeloma. *N Engl J Med* **374**, 555-561, doi:10.1056/NEJMoa1508808 (2016).
- 199 Mikosch, P. & Hughes, D. An overview on bone manifestations in Gaucher disease. *Wien Med Wochenschr* **160**, 609-624, doi:10.1007/s10354-010-0841-y (2010).

- 200 Radl, J., Hollander, C. F., van den Berg, P. & de Glopper, E. Idiopathic paraproteinaemia. I. Studies in an animal model--the ageing C57BL/KaLwRij mouse. *Clin Exp Immunol* **33**, 395-402 (1978).
- 201 Amend, S. R. *et al.* Whole Genome Sequence of Multiple Myeloma-Prone C57BL/KaLwRij Mouse Strain Suggests the Origin of Disease Involves Multiple Cell Types. *PLoS One* **10**, e0127828, doi:10.1371/journal.pone.0127828 (2015).
- 202 Lwin, S. T., Edwards, C. M. & Silbermann, R. Preclinical animal models of multiple myeloma. *Bonekey Rep* **5**, 772, doi:10.1038/bonekey.2015.142 (2016).
- 203 Matthews, G. M. *et al.* Preclinical screening of histone deacetylase inhibitors combined with ABT-737, rhTRAIL/MD5-1 or 5-azacytidine using syngeneic Vk*MYC multiple myeloma. *Cell Death Dis* **4**, e798, doi:10.1038/cddis.2013.306 (2013).
- 204 Chesi, M. *et al.* Drug response in a genetically engineered mouse model of multiple myeloma is predictive of clinical efficacy. *Blood* **120**, 376-385, doi:10.1182/blood-2012-02-412783 (2012).
- 205 Fowler, J. A., Mundy, G. R., Lwin, S. T. & Edwards, C. M. Bone marrow stromal cells create a permissive microenvironment for myeloma development: a new stromal role for Wnt inhibitor Dkk1. *Cancer Res* **72**, 2183-2189, doi:10.1158/0008-5472.CAN-11-2067 (2012).
- 206 Glick, A. F. *et al.* Age matters: Young T lymphocytes offer better protection from myeloma proliferation. *Immun Ageing* **10**, 5, doi:10.1186/1742-4933-10-5 (2013).
- 207 Halade, G. V., Rahman, M. M., Williams, P. J. & Fernandes, G. High fat diet-induced animal model of age-associated obesity and osteoporosis. *J Nutr Biochem* **21**, 1162-1169, doi:10.1016/j.jnutbio.2009.10.002 (2010).
- 208 Scheller, E. L. *et al.* Changes in Skeletal Integrity and Marrow Adiposity during High-Fat Diet and after Weight Loss. *Front Endocrinol (Lausanne)* **7**, 102, doi:10.3389/fendo.2016.00102 (2016).
- 209 Fujita, Y. & Maki, K. High-fat diet-induced obesity triggers alveolar bone loss and spontaneous periodontal disease in growing mice. *BMC Obes* **3**, 1, doi:10.1186/s40608-016-0082-8 (2015).
- 210 Fehrendt, H. *et al.* Negative influence of a long-term high-fat diet on murine bone architecture. *Int J Endocrinol* **2014**, 318924, doi:10.1155/2014/318924 (2014).
- 211 Iwamoto, T. *et al.* Lactosylceramide is essential for the osteoclastogenesis mediated by macrophage-colony-stimulating factor and receptor activator of nuclear factor-kappa B ligand. *J Biol Chem* **276**, 46031-46038, doi:10.1074/jbc.M104464200 (2001).
- 212 Bennett, L. L. & Turcotte, K. Eliglustat tartrate for the treatment of adults with type 1 Gaucher disease. *Drug Des Devel Ther* **9**, 4639-4647, doi:10.2147/DDDT.S77760 (2015).
- 213 Pavlova, E. V. *et al.* B cell lymphoma and myeloma in murine Gaucher's disease. *J Pathol* **231**, 88-97, doi:10.1002/path.4227 (2013).
- 214 Mistry, P. K., Taddei, T., vom Dahl, S. & Rosenbloom, B. E. Gaucher disease and malignancy: a model for cancer pathogenesis in an inborn error of metabolism. *Crit Rev Oncog* **18**, 235-246 (2013).
- 215 Kyle, R. A. *et al.* Review of 1027 patients with newly diagnosed multiple myeloma. *Mayo Clin Proc* **78**, 21-33, doi:10.4065/78.1.21 (2003).
- 216 Jacquin, C., Gran, D. E., Lee, S. K., Lorenzo, J. A. & Aguila, H. L. Identification of multiple osteoclast precursor populations in murine bone marrow. *J Bone Miner Res* **21**, 67-77, doi:10.1359/JBMR.051007 (2006).

- 217 Hey, Y. Y., Quah, B. & O'Neill, H. C. Antigen presenting capacity of murine splenic myeloid cells. *BMC Immunol* **18**, 4, doi:10.1186/s12865-016-0186-4 (2017).
- 218 Nestorowa, S. *et al.* A single-cell resolution map of mouse hematopoietic stem and progenitor cell differentiation. *Blood* **128**, e20-31, doi:10.1182/blood-2016-05-716480 (2016).
- 219 Schnaar, R. L. & Kinoshita, T. in *Essentials of Glycobiology* (eds rd *et al.*) 125-135 (2015).
- 220 Ersek, A., Karadimitris, A. & Horwood, N. J. Effect of glycosphingolipids on osteoclastogenesis and osteolytic bone diseases. *Front Endocrinol (Lausanne)* **3**, 106, doi:10.3389/fendo.2012.00106 (2012).
- 221 Croucher, P. I. *et al.* Zoledronic acid treatment of 5T2MM-bearing mice inhibits the development of myeloma bone disease: evidence for decreased osteolysis, tumor burden and angiogenesis, and increased survival. *J Bone Miner Res* **18**, 482-492, doi:10.1359/jbmr.2003.18.3.482 (2003).
- 222 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, doi:10.1074/jbc.M212626200 (2003).
- 223 Yao, Z. *et al.* RANKL cytokine enhances TNF-induced osteoclastogenesis independently of TNF receptor associated factor (TRAF) 6 by degrading TRAF3 in osteoclast precursors. *J Biol Chem* **292**, 10169-10179, doi:10.1074/jbc.M116.771816 (2017).
- 224 Pamarthy, S., Kulshrestha, A., Katara, G. K. & Beaman, K. D. The curious case of vacuolar ATPase: regulation of signaling pathways. *Mol Cancer* **17**, 41, doi:10.1186/s12943-018-0811-3 (2018).
- 225 Shacka, J. J. *et al.* Bafilomycin A1 inhibits chloroquine-induced death of cerebellar granule neurons. *Mol Pharmacol* **69**, 1125-1136, doi:10.1124/mol.105.018408 (2006).
- 226 Shacka, J. J., Klocke, B. J. & Roth, K. A. Autophagy, bafilomycin and cell death: the "a-B-cs" of plecomacrolide-induced neuroprotection. *Autophagy* **2**, 228-230, doi:10.4161/auto.2703 (2006).
- 227 Zhang, H. *et al.* Polyamines Control eIF5A Hypusination, TFEB Translation, and Autophagy to Reverse B Cell Senescence. *Mol Cell*, doi:10.1016/j.molcel.2019.08.005 (2019).
- 228 Zhang, H. *et al.* Polyamines Control eIF5A Hypusination, TFEB Translation, and Autophagy to Reverse B Cell Senescence. *Mol Cell* **76**, 110-125 e119, doi:10.1016/j.molcel.2019.08.005 (2019).
- 229 Ganef, C. *et al.* Induction of the alternative NF-kappaB pathway by lymphotoxin alphabeta (LTalphabeta) relies on internalization of LTbeta receptor. *Mol Cell Biol* **31**, 4319-4334, doi:10.1128/MCB.05033-11 (2011).
- 230 Wei, J. *et al.* Protective role of endogenous gangliosides for lysosomal pathology in a cellular model of synucleinopathies. *Am J Pathol* **174**, 1891-1909, doi:10.2353/ajpath.2009.080680 (2009).
- 231 Boyce, B. F., Li, J., Xing, L. & Yao, Z. Bone Remodeling and the Role of TRAF3 in Osteoclastic Bone Resorption. *Front Immunol* **9**, 2263, doi:10.3389/fimmu.2018.02263 (2018).

- 232 Sezgin, E. *et al.* Polarity-Sensitive Probes for Superresolution Stimulated Emission Depletion Microscopy. *Biophys J* **113**, 1321-1330, doi:10.1016/j.bpj.2017.06.050 (2017).
- 233 Wei, J. *et al.* Gangliosides' protection against lysosomal pathology of synucleinopathies. *Autophagy* **5**, 860-861, doi:10.4161/auto.8825 (2009).
- 234 Shen, W. *et al.* Inhibition of glucosylceramide synthase stimulates autophagy flux in neurons. *J Neurochem* **129**, 884-894, doi:10.1111/jnc.12672 (2014).
- 235 Matarrese, P. *et al.* Evidence for the involvement of GD3 ganglioside in autophagosome formation and maturation. *Autophagy* **10**, 750-765, doi:10.4161/auto.27959 (2014).
- 236 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, doi:10.1038/nrm.2017.16 (2017).
- 237 Meckes, D. G., Jr., Menaker, N. F. & 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, doi:10.1128/JVI.02519-12 (2013).
- 238 Higuchi, M., Izumi, K. M. & 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, doi:10.1073/pnas.081075298 (2001).
- 239 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, doi:10.1002/ijc.20503 (2005).
- 240 Kim, Y. H., Baek, N. S., Han, Y. H., Chung, M. A. & Jung, S. D. Enhancement of neuronal cell adhesion by covalent binding of poly-D-lysine. *J Neurosci Methods* **202**, 38-44, doi:10.1016/j.jneumeth.2011.08.036 (2011).
- 241 Nilforoushan, D., Gramoun, A., Glogauer, M. & Manolson, M. F. Nitric oxide enhances osteoclastogenesis possibly by mediating cell fusion. *Nitric Oxide* **21**, 27-36, doi:10.1016/j.niox.2009.04.002 (2009).
- 242 Titze, I. R., Klemuk, S. A. & Lu, X. Adhesion of a monolayer of fibroblast cells to fibronectin under sonic vibrations in a bioreactor. *Ann Otol Rhinol Laryngol* **121**, 364-374, doi:10.1177/000348941212100602 (2012).
- 243 Tonge, D. A. *et al.* Fibronectin supports neurite outgrowth and axonal regeneration of adult brain neurons in vitro. *Brain Res* **1453**, 8-16, doi:10.1016/j.brainres.2012.03.024 (2012).
- 244 Cornelissen, C. G. *et al.* Fibronectin coating of oxygenator membranes enhances endothelial cell attachment. *Biomed Eng Online* **12**, 7, doi:10.1186/1475-925X-12-7 (2013).
- 245 Fuller, K., Ross, J. L., Szewczyk, K. A., Moss, R. & Chambers, T. J. Bone is not essential for osteoclast activation. *PLoS One* **5**, doi:10.1371/journal.pone.0012837 (2010).
- 246 Black, D. M. *et al.* The effects of parathyroid hormone and alendronate alone or in combination in postmenopausal osteoporosis. *N Engl J Med* **349**, 1207-1215, doi:10.1056/NEJMoa031975 (2003).
- 247 Kanis, J. A., Melton, L. J., 3rd, Christiansen, C., Johnston, C. C. & Khaltaev, N. The diagnosis of osteoporosis. *J Bone Miner Res* **9**, 1137-1141, doi:10.1002/jbmr.5650090802 (1994).

- 248 Compston, J. *et al.* UK clinical guideline for the prevention and treatment of osteoporosis. *Arch Osteoporos* **12**, 43, doi:10.1007/s11657-017-0324-5 (2017).
- 249 Svedbom, A. *et al.* Osteoporosis in the European Union: a compendium of country-specific reports. *Arch Osteoporos* **8**, 137, doi:10.1007/s11657-013-0137-0 (2013).
- 250 Morrison, S. J. & Scadden, D. T. The bone marrow niche for haematopoietic stem cells. *Nature* **505**, 327-334, doi:10.1038/nature12984 (2014).
- 251 McHeyzer-Williams, M., Okitsu, S., Wang, N. & McHeyzer-Williams, L. Molecular programming of B cell memory. *Nat Rev Immunol* **12**, 24-34, doi:10.1038/nri3128 (2011).
- 252 Nutt, S. L. & Tarlinton, D. M. Germinal center B and follicular helper T cells: siblings, cousins or just good friends? *Nat Immunol* **12**, 472-477 (2011).
- 253 Nutt, S. L., Hodgkin, P. D., Tarlinton, D. M. & Corcoran, L. M. The generation of antibody-secreting plasma cells. *Nat Rev Immunol* **15**, 160-171, doi:10.1038/nri3795 (2015).
- 254 LeBlanc, R. *et al.* Proteasome inhibitor PS-341 inhibits human myeloma cell growth in vivo and prolongs survival in a murine model. *Cancer Res* **62**, 4996-5000 (2002).
- 255 Jilka, R. L. The relevance of mouse models for investigating age-related bone loss in humans. *J Gerontol A Biol Sci Med Sci* **68**, 1209-1217, doi:10.1093/gerona/glt046 (2013).
- 256 Berenson, J. R. Advances in the biology and treatment of myeloma bone disease. *Am J Health Syst Pharm* **58 Suppl 3**, S16-20, doi:10.1093/ajhp/58.suppl_3.S16 (2001).
- 257 Guenther, A. *et al.* The bisphosphonate zoledronic acid has antimyeloma activity in vivo by inhibition of protein prenylation. *Int J Cancer* **126**, 239-246, doi:10.1002/ijc.24758 (2010).
- 258 Heath, D. J. *et al.* Inhibiting Dickkopf-1 (Dkk1) removes suppression of bone formation and prevents the development of osteolytic bone disease in multiple myeloma. *J Bone Miner Res* **24**, 425-436, doi:10.1359/jbmr.081104 (2009).
- 259 Piddock, R. E. *et al.* PI3Kdelta and PI3Kgamma isoforms have distinct functions in regulating pro-tumoural signalling in the multiple myeloma microenvironment. *Blood Cancer J* **7**, e539, doi:10.1038/bcj.2017.16 (2017).
- 260 Marlein, C. R. *et al.* CD38-Driven Mitochondrial Trafficking Promotes Bioenergetic Plasticity in Multiple Myeloma. *Cancer Res* **79**, 2285-2297, doi:10.1158/0008-5472.CAN-18-0773 (2019).
- 261 Libouban, H. The use of animal models in multiple myeloma. *Morphologie* **99**, 63-72, doi:10.1016/j.morpho.2015.01.003 (2015).
- 262 Oikawa, T., Kuroda, Y. & Matsuo, K. Regulation of osteoclasts by membrane-derived lipid mediators. *Cell Mol Life Sci* **70**, 3341-3353, doi:10.1007/s00018-012-1238-4 (2013).
- 263 Irie, A., Yamamoto, K., Miki, Y. & Murakami, M. Phosphatidylethanolamine dynamics are required for osteoclast fusion. *Sci Rep* **7**, 46715, doi:10.1038/srep46715 (2017).
- 264 Tintut, Y., Morony, S. & Demer, L. L. Hyperlipidemia promotes osteoclastic potential of bone marrow cells ex vivo. *Arterioscler Thromb Vasc Biol* **24**, e6-10, doi:10.1161/01.ATV.0000112023.62695.7f (2004).
- 265 Shayman, J. A. ELIGLUSTAT TARTRATE: Glucosylceramide Synthase Inhibitor Treatment of Type 1 Gaucher Disease. *Drugs Future* **35**, 613-620 (2010).

- 266 Larsen, S. D. *et al.* Property-based design of a glucosylceramide synthase inhibitor that reduces glucosylceramide in the brain. *J Lipid Res* **53**, 282-291, doi:10.1194/jlr.M021261 (2012).
- 267 Sezgin, E. *et al.* Elucidating membrane structure and protein behavior using giant plasma membrane vesicles. *Nat Protoc* **7**, 1042-1051, doi:10.1038/nprot.2012.059 (2012).
- 268 Takeda, H. *et al.* Sphingomyelinase and ceramide inhibit formation of F-actin ring in and bone resorption by rabbit mature osteoclasts. *FEBS Lett* **422**, 255-258, doi:10.1016/s0014-5793(98)00005-2 (1998).
- 269 Bijl, N. *et al.* The glucosylceramide synthase inhibitor N-(5-adamantane-1-yl-methoxy-pentyl)-deoxynojirimycin induces sterol regulatory element-binding protein-regulated gene expression and cholesterol synthesis in HepG2 cells. *J Pharmacol Exp Ther* **326**, 849-855, doi:10.1124/jpet.108.139394 (2008).
- 270 Newman, A. C., Kemp, A. J., Drabsch, Y., Behrends, C. & Wilkinson, S. Autophagy acts through TRAF3 and RELB to regulate gene expression via antagonism of SMAD proteins. *Nat Commun* **8**, 1537, doi:10.1038/s41467-017-00859-z (2017).
- 271 Harvald, E. B., Olsen, A. S. & Faergeman, N. J. Autophagy in the light of sphingolipid metabolism. *Apoptosis* **20**, 658-670, doi:10.1007/s10495-015-1108-2 (2015).
- 272 Dall'Armi, C., Devereaux, K. A. & Di Paolo, G. The role of lipids in the control of autophagy. *Curr Biol* **23**, R33-45, doi:10.1016/j.cub.2012.10.041 (2013).
- 273 Knaevelsrud, H. & Simonsen, A. Lipids in autophagy: constituents, signaling molecules and cargo with relevance to disease. *Biochim Biophys Acta* **1821**, 1133-1145, doi:10.1016/j.bbalip.2012.01.001 (2012).
- 274 Carlsson, S. R. & Simonsen, A. Membrane dynamics in autophagosome biogenesis. *J Cell Sci* **128**, 193-205, doi:10.1242/jcs.141036 (2015).
- 275 Zapata, J. M. *et al.* Lymphocyte-specific TRAF3 transgenic mice have enhanced humoral responses and develop plasmacytosis, autoimmunity, inflammation, and cancer. *Blood* **113**, 4595-4603, doi:10.1182/blood-2008-07-165456 (2009).
- 276 Bushell, K. R. *et al.* Genetic inactivation of TRAF3 in canine and human B-cell lymphoma. *Blood* **125**, 999-1005, doi:10.1182/blood-2014-10-602714 (2015).
- 277 Perez-Chacon, G. *et al.* Dysregulated TRAF3 and BCL2 Expression Promotes Multiple Classes of Mature Non-hodgkin B Cell Lymphoma in Mice. *Front Immunol* **9**, 3114, doi:10.3389/fimmu.2018.03114 (2018).