Glycosphingolipid synthesis inhibitors prevent osteoclast activation and myeloma bone disease

Ersek et al.

Supplemental Materials and Methods

Reagents

All media components were purchased from Life Technologies Ltd (Paisley, UK). Antibody against MAP Kinases: ERK1/2, p38, JNK, phospho-ERK1/2, phospho-p38, phospho-JNK, TRAF6, c-Src, and GM1 were purchased from Cell Signaling Technology (Beverly, MA, USA) and recombinant IGF-1 from Peprotech (London, UK).

Cell lines

The RAW 264.7 murine macrophage cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). RAW 264.7 cells were cultured in RPMI-1640 containing 10% FBS. 5TGM1-GFP cell line was obtained from Dr Claire Edwards, Botnar Research Centre, NDORMS, University of Oxford, with the permission of Dr Kay Oyajobi, University of Texas Health Science Center, San Antonio. 5TGM1-GFP cells were cultured in RPMI-1640 containing 10% FBS, 1% nonessential amino acids and 1% sodium pyruvate.

Western Blot analysis

Antibodies used; anti-ERK (sc153) from Santa Cruz Biotechnology (Santa Cruz, CA), anti-p38 (SAK7 in house), anti-JNK (S9252), phospho- ERK (S9101) and phospho-p38 MAPK (S9215) from Cell Signaling, (Hitchen, UK), phospho-JNK (700031) from Life Technologies (Paisley, UK) and β-tubulin (T6199) from Sigma (Dorset UK). Cells were lysed in 1X gel sample buffer and sonicated for 2 rounds of 6 pulses of 2 seconds. Cell extracts were

resolved by SDS-PAGE and transferred to polyvinyl difluoride (PVDF) membranes (Millipore, USA) which were blocked for 1 hr with blocking buffer (5% w/v fat-free milk, 0.1% v/v Tween-20 in TBS) followed by 1 hr incubation with the antibodies, diluted 1:2000-5000) in blocking buffer. HRP-conjugated anti-mouse IgG or anti-rabbit IgG (GE Healthcare Amersham, UK) were used as secondary antibodies at a dilution of 1:4000. Bound antibody was detected using the ECL plus (GE Healthcare Amersham, UK) and visualised using Hyperfilm MP (GE Healthcare, Amersham, UK). Blots were stripped with Restore Plus (Pierce, Thermo Fisher Scientific, Rockford , USA).

Lipid raft isolation

Fifty million RAW 264.7 cells were cultured with 50ng/ml RANKL (Peprotech) or 50ng/ml RANKL plus 500µM NB-DNJ for 3 days. Lipid raft as low density, detergent resistant membrane fraction was isolated by discontinuous sucrose gradient ultracentrifugation. A total of 9 fractions (1ml each) were collected from the top to the bottom of the gradients. Cell surface ganglioside GM1 was labelled with HRP conjugated cholera toxin B subunit for 1 h at 4°C. Cells were then lysed and lipid rafts isolated using the caveolae/raft isolation kit (Sigma-Aldrich, Dorset, UK). Fractions 2 to 5 were positive for GM1, thus contained lipid rafts. The non-raft fractions were 7 to 9.

Phalloidin staining

Cells were washed with PBS, fixed with 4% paraformadehyde in PBS for 20 min and permeabilised with 0.1 % Triton X-100 for 5 min. OC actin rings were stained using Alexa886-phalloidin (Molecular Probes, Oregon, USA) (1:50 dilution) for 20 min. Cells were mounted with mounting medium (Vector laboratories, Inc.). Actin rings were visualized

fluorescently using Nikon Eclipse TE2000-U microscope. Mature OC were defined as actin ring positive and undisrupted.

ELISA

RatLaps EIA kit for determining CTX was obtained from IDS (Immunodiagnostics Systems Ltd., Boldon, UK). ELISA assay were performed according to the manufacturer's instructions.

Monocyte elutriation

Peripheral blood mononuclear cells were isolated by centrifugation over Lympholyte-H (Cedarlane Laboratories Ltd, Emergo Europe, Zierikzee, Netherlands) density gradient solution and then subjected to centrifugal elutriation in a Beckman JE6 elutriator (Beckman Coulter High Wycombe, UK) at 2500rpm. Centrifugal elutriation separates cells by size subjecting them to centrifugal force that is countered by continuously flowing media as described previously (1, 2). Monocyte purity was assessed by FACS with monocyte fractions containing >90% of CD14+ cells.

Cell viability assays

Cells cultured with different doses (0 μM, 5μM, 50 μM and 500 μM) of NB-DNJ for 24 h or 72 h were evaluated by MTT assay (Sigma-Aldrich, Dorset,UK) according to the manufacturer's instructions. Optical density (OD) of each well was determined at wavelength 540 nm using a Multiskan Biochromatic™ plate reader and Ascent™ 2.4.2 software (Labsystems, Helsinki, Finland). Relative cell viability (percent of control) was calculated using the equation: (mean OD of treated cells/mean OD of control cells) ×100; while cell

inhibition rate was calculated with the equation: 100% x (OD control - OD treatment)/OD control.

Flow cytometry analysis of myeloma tumor burden

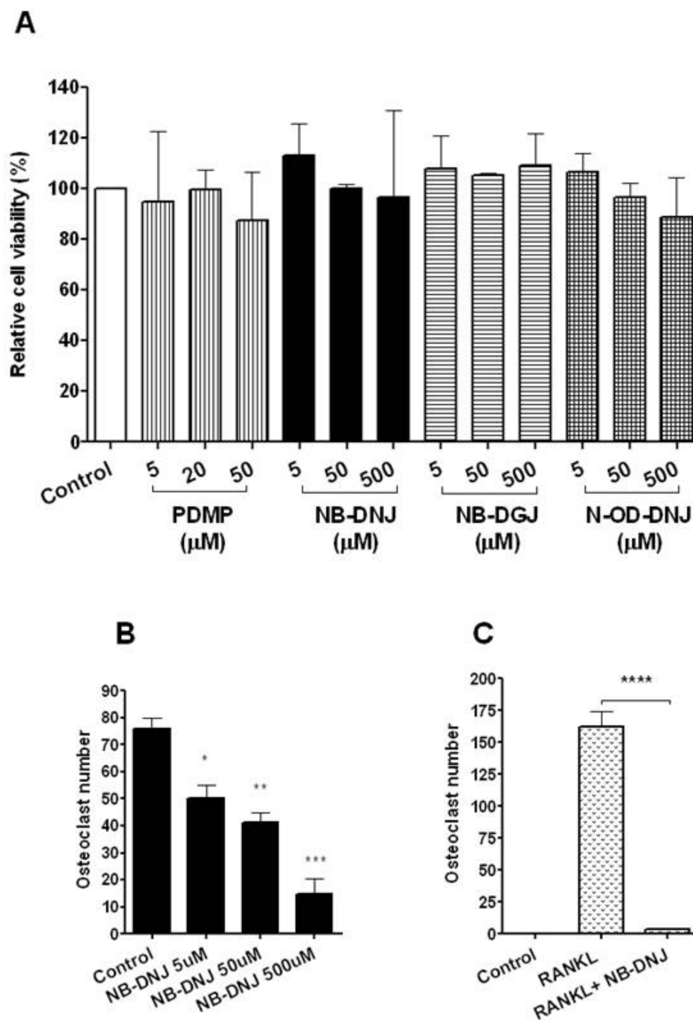
Bone marrow respectively spleen cells from 5GTM1-GFP 1x10^6 cells transplanted mice followed by PBS or NB-DNJ treatment were incubated with PE-conjugated anti-CD45 (BD, Oxford, UK) antibody for 20 mins at 4˚C. The cells were then washed twice with PBS prior to acquisition using a BD ™ LSR II Flow Cytometer (BD Biosciences). Cells that expressed CD45 hematopoietic marker as well as GFP were identified as murine myeloma 5GTM1-GFP cells. Subsequent analysis was carried out using FlowJo software (Treestar).

Supplemental Table 1. HPLC profiling of GSL from healthy bone marrow-derived mononuclear cells

GSL	BM1	BM2
Lactosylceramide	81	81
Hex_3/Gb3	0	0
$HexHexNAcHex_2$/GA1	0	0
GM3	0.9	0.7
GM2	0	0
GM1a	0	0
Gb4	0.3	0.2
PG	8.3	8.6
SPG	1.0	0.9
$NeuAc_2LacNAc_2Hex_2$/DSPG	2.2	2.7
GT3	0.9	1.0
GQ1b	0.5	0.6

PG, SPG and DSPG are paragloboside, sialylparagloboside and disialylparagloboside

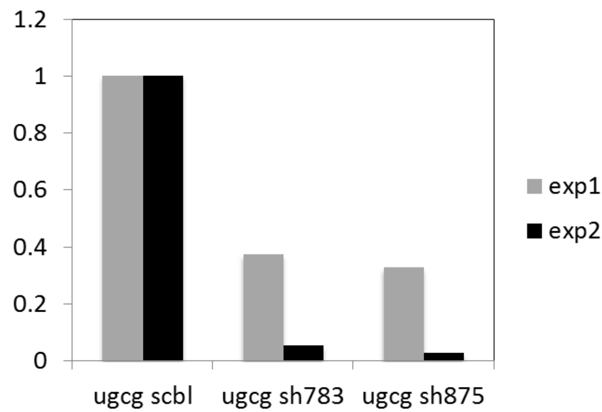
Supplemental Figure 1.

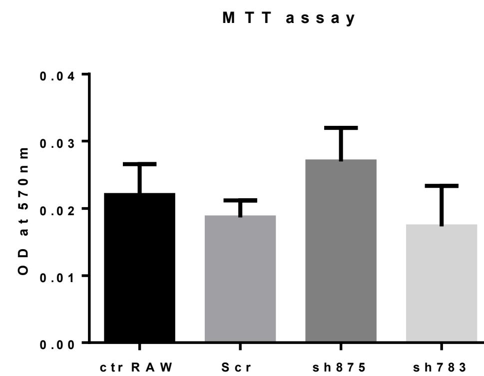


Supplemental Figure 1. GSL inhibitors have no effect on cell viability but inhibit osteoclastogenesis. (A) Mouse BM cells were cultured in the presence of 25 ng/ml M-CSF with 50 ng/ml RANKL (Control) and with or without PDMP (5, 20 or 50 μM), NB-DNJ, NB-DGJ, or N-OD-DNJ (5, 50 or 500 μM) in 96-well plates for 3 days and relative cell viability was evaluated by MTT assay **(B)** Human monocytes purified by elutriation were cultured at 10^5cells/well in 96-well plates in the presence of 25 ng/ml M-CSF for 48 h, followed by the addition of only 5 ng/ml RANKL (control) with or without NB-DNJ (5, 50 or 500 μM) for 5 more days. Cultures on plastic plates were fixed, stained for TRAP and OC were counted. **(C)** Differentiation of RAW264.7 murine macrophage cell line to OC in the presence of RANKL 50ng/ml was effectively inhibited by NB-DNJ. (n=3) Error bars correspond to standard error of the mean (SEM), n=3/group in all cases (*p<0.05, **p<0.01, ***p<0.001 versus control (**B**); versus RANKL treatment (**C**)).

Supplemental Figure 2.

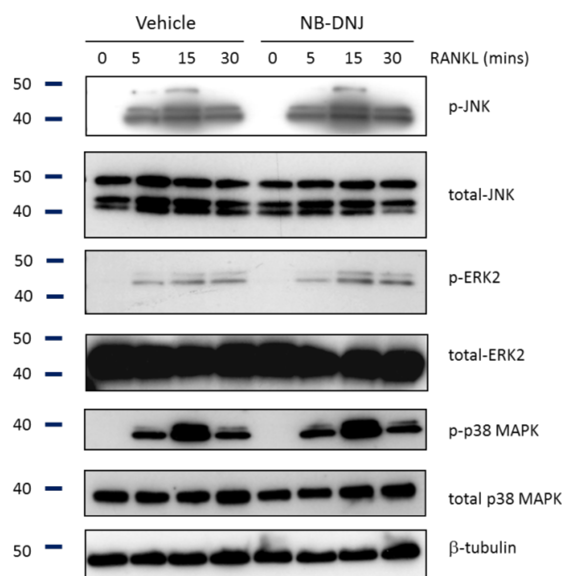
A **B**

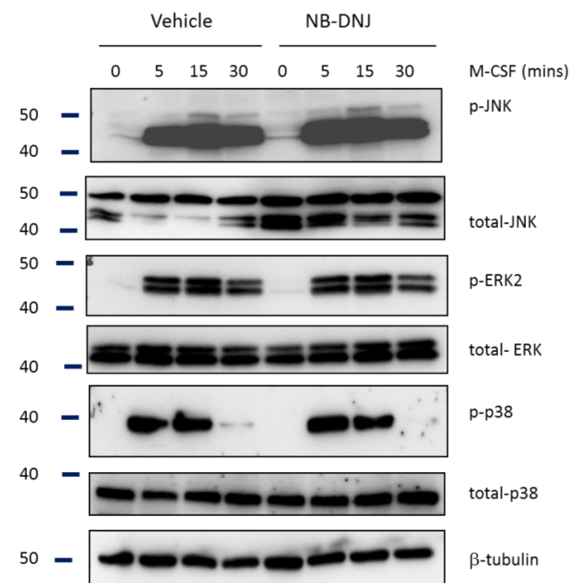




Supplemental Figure 2. Transfection with shRNA knocksdown Ugcg gene expression but does not affect RAW264.7 viability. Murine RAW264.7 cells were transfected with Ugcg sh783, sh875, or scrambled shRNA, or left untransfected. After 72 hours in 96-well plates, cells were either (A) lysed for RT-PCR analysis of ugcg gene expression relative to shRNA scrambled control (data from 2 individual experiments are shown), or (B) relative cell viability was evaluated by MTT assay (n=3).

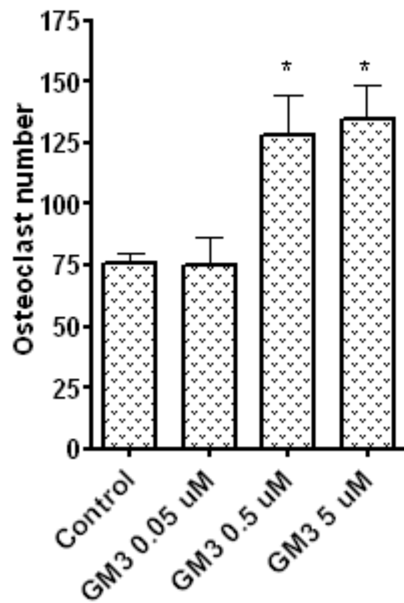
Supplemental Figure 3





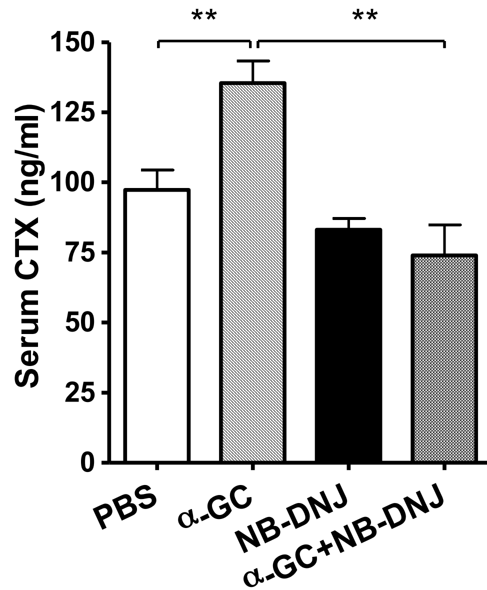
Supplemental Figure 3. MAPK activation is not affteced by NB-DNJ following RANKL or M-CSF treatment. RAW264.7 cells were starved in 0.5% serum medium overnight then treated with (A) RANKL +/- NB-DNJ or (B) M-CSF +/- NB-DNJ for the time points indicated and immunoblotted with α-pERK1/2, α-pp38, α-pJNK Ab. Membranes were stripped and reblotted with α-ERK, α-p38, α-JNK Ab. β-tubulin was used as a loading control.

Supplemental Figure 4



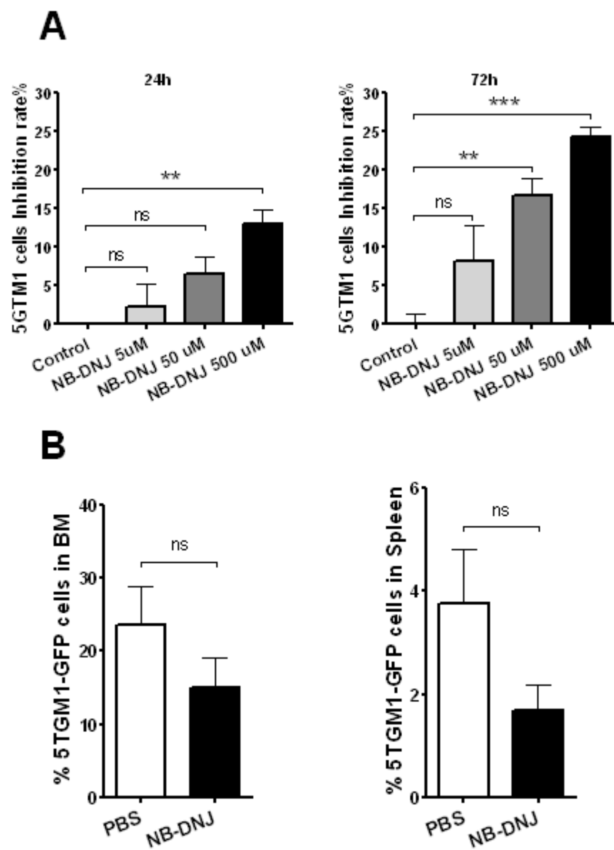
Supplemental Figure 4. GM3 promotes osteoclastogenesis in the presence of RANKL with human precursors. (**A**) Highly purified human monocytes obtained by PBMC elutriation were cultured at 10^5cells/well in 96-well plates in the presence of 25 ng/ml M-CSF for 48 h, followed by the addition of only 5ng/ml RANKL with or without GM3 (0.05, 0.5, 5 μM) for 5 more days. Cultures on plastic plates were fixed and stained for TRAP. TRAP positive multi-nuclear (>3 nuclei) OC were counted. (n=3) Error bars correspond to standard error of the mean (SEM). n=3/group in all cases (*p<0.05, **p<0.01, ***p<0.001 versus control).

Supplemental Figure 5.



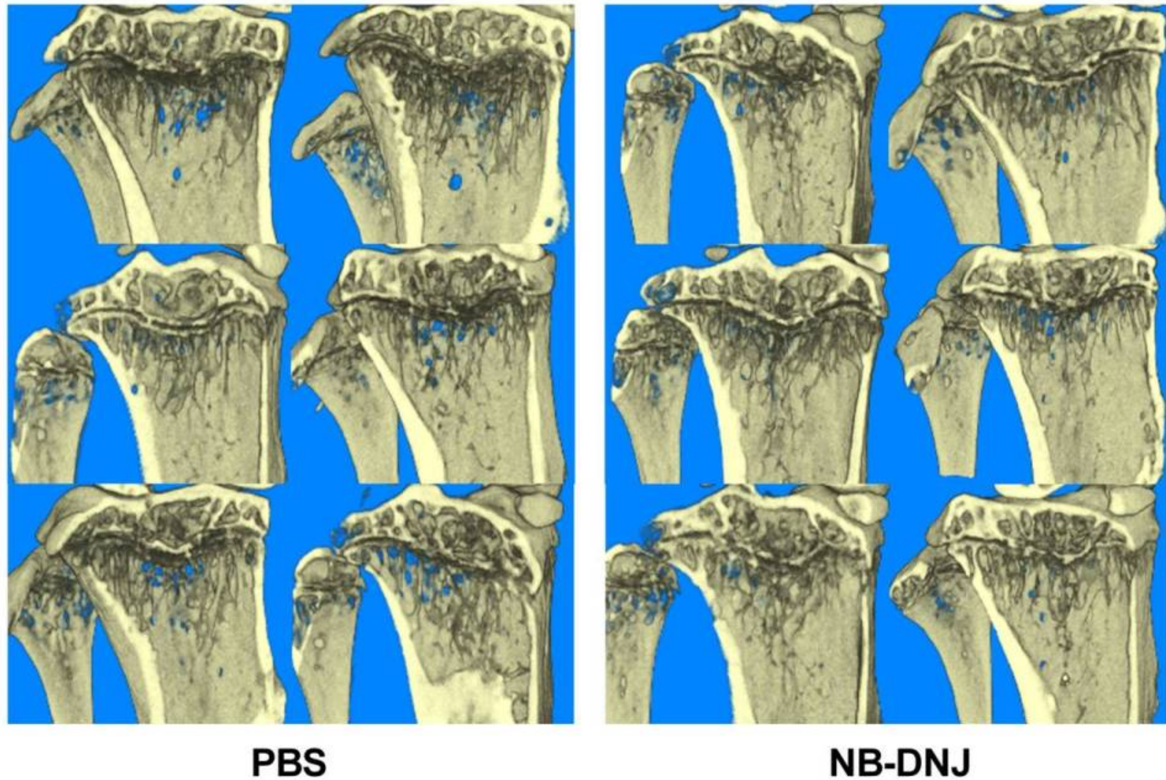
Supplemental Figure 5. NB-DNJ inhibits exogenous alpha-galactosylceramide mediated OC activation. Serum CTX levels in mice that were treated with NB-DNJ or PBS for a total of 6 days and also in PBS- or NB-DNJ-treated mice that received a single injection of alpha-galactosylceramide (α-GC) (2µg/mouse i.p) on day 3. CTX levels were assayed by ELISA on day 7 (n=6). Error bars correspond to standard error of the mean (SEM). n=3/group in all cases (*p<0.05, **p<0.01, ***p<0.001 versus control).

Supplemental Figure 6.



Supplemental Figure 6. NB-DNJ inhibits 5GTM1 murine myeloma cells viability in vitro and in vivo. (**A**) 5TGM1-GFP murine myeloma cells plated at a density of 5x10^4 cells /well in 96 well plates with or without NB-DNJ (5, 50 or 500 µM) were evaluated at 24 h and 72 h by MTT assay and cell inhibition rate was calculated Error bars correspond to standard error of the mean (SEM) (n=3; *p<0.05, **p<0.01, ***p<0.001 versus control). (**B**) Flow-cytometer analysis of 5TGM1-GFP murine multiple myeloma cell tumor burden in PBS and NB-DNJ treated mouse BM and spleen (n=6; 1 tailed Student`s t test).

Supplemental Figure 7.



Supplemental Figure 7. NB-DNJ effectively inhibits 5TGM1 myeloma cells caused bone lesions in C57BL/KaLwRijHsd mice. Longitudinal cross-section of diseased and PBS (left panel) or NB-DNJ (right panel) treated mouse tibiae evaluated by micro-CT reconstruction. All the experimental mice tibiae are shown (n=6).

Supplemental Reference list

1. Chiu KM, McPherson LH, Harris JE, Braun DP. The separation of cytotoxic human peripheral blood monocytes into high and low phagocytic subsets by centrifugal elutriation. Journal of Leukocyte Biology1984 December 1, 1984;36(6):729-37.
2. Willson RA, Liem HH, Miyai K, Muller-Eberhard U. Heterogeneous distribution of drug metabolism in elutriated rat hepatocytes. Biochemical Pharmacology1985;34(9):1463-70.