In vitro effects G-protein coupled oestrogen receptor (GPER) agonist and antagonist on osteoclastogenesis and resorption in RAW 264.7 murine macrophages

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Introduction

Bone is a metabolically active tissue that continuously undergoes restructuring in a process known as bone remodelling. This process involves mainly osteoclasts and osteoblasts. Osteoclasts are cells that form from mononucleated haematopoietic stem cells. They are responsible for the resorption of bone. Differentiation of haematopoietic cells to multinucleated bone resorbing osteoclasts is facilitated by binding of receptor activator of nuclear factor κB ligand (RANKL) to receptor activator of nuclear factor κ B (RANK). Osteoblasts form from mesenchymal stem cells. The differentiation of mesenchymal cells to bone forming osteoblasts is stimulated through the action of core-binding factor α 1 (Cbfa1). Bone formation and resorption are uncoupled and coupled as the bone structure requires. When there is an imbalance in the rate for resorption and formation, a pathology is the result. Osteoporosis arises from an increase in number of osteoclasts that resorb bone quicker than it is replaced by osteoblasts.

G-protein coupled oestrogen receptor (GPER) is a receptor that binds oestrogen and is believed to mediate the non-genomic effects that oestrogen. It is known that oestrogens are responsible for mediating bone activity by stimulating osteoblast and inhibiting osteoclasts. The role that GPER plays in oestrogen signalling is unclear, therefore this study aims to investigate the *in vitro* effects of GPER agonist (G1) and GPER antagonist (G15) on osteoclasts.

Methods

- RAW 264.7 murine cells were seeded into a 96-well pate at a density of 5000 cells/well and G1 and G15 were added at 10^{-10} – 10^{-5} M concentrations.
- A resazurin assay was done to determine cell viability. This was in the absence of RANKL
- Western blotting was performed to determine GPER expression in RAW 264.7 macrophages.
- Tartrate-resistant acid phosphatase (TRAP) staining was used to observe the effects of G1 and G15 on osteoclast differentiation. Cells were exposed and re-exposed to RANKL (15ng/ml) on Day 1 and Day 3 to stimulate osteoclast differentiation.
- Termination and staining occurred of Day 5 and TRAP positive osteoclasts (containing 3 or more nuclei) were counted.

Results

- Cell viability was not affected by G1 and G15 at any of the concentrations tested.
- Western blotting showed that undifferentiated RAW 264.7 macrophages expressed high levels of GPER
- TRAP staining showed a that G1 at concentration 10^{-6} M significantly decreased osteoclast formation in G1. G15 showed no effect on osteoclast formation at any of the concentrations tested.

RAW 264.7 Cell Viability

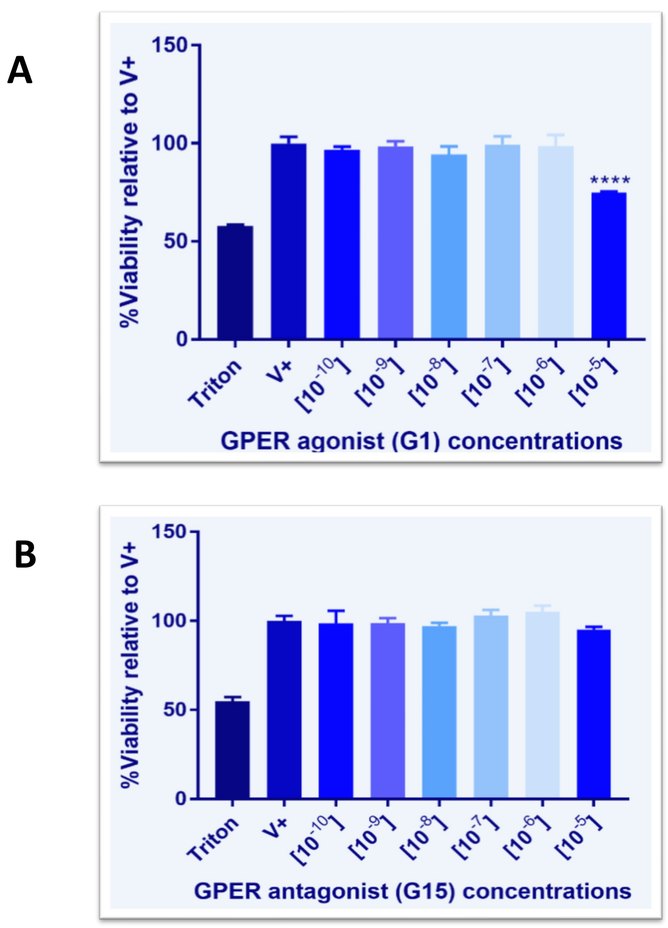

Figure 1: Cell viability of RAW 264.7 murine macrophages
A. RAW 264.7 cells were seeded at 5 000 cells/well in a 96-well plate on Day 1 and exposed to G1 (10^{-10}–10^{-5} M) on Day 3.
B. The graph of G15 (10^{-10}-10^{-5} M). Cell viability was determined by a resazurin assay. The assay showed that cell viability is not affected by G1 and G15. ***p<0.001 vs V+. V+ = vehicle control

Protein expression

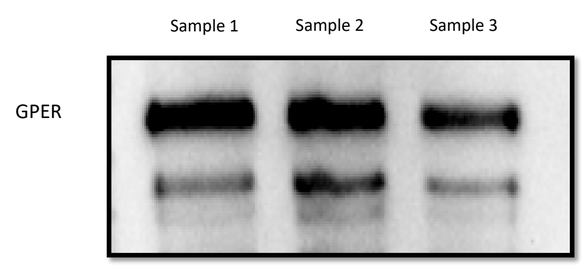

Figure 2: Expression of GPER in undifferentiated RAW 264.7 murine macrophages
Samples from three different passages were taken. The cells were seeded at a density of 500 000 cells/well in a 6-well plate. GPER is expressed in undifferentiated cells and bands are visible at 20 and 30 seconds of exposure to UV light in the BioRad ChemiDoc Imaging System. The molecular weight is approximately 42kDa.

Osteoclast Formation in differentiated RAW 264.7 cells

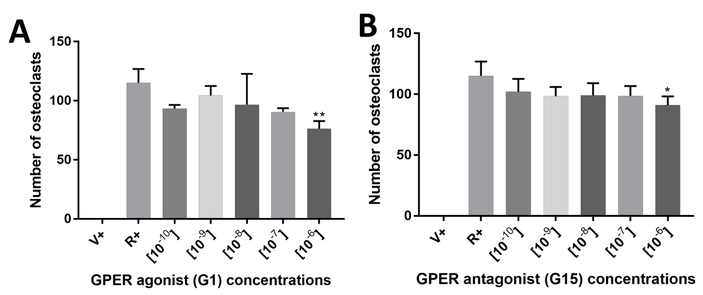

Figure 3: Number of osteoclasts after differentiation of RAW 264.7 murine macrophages
A. The graph shows the number of osteoclasts at varying concentrations of G1.
B. The graph shows the number of osteoclasts at varying concentrations of G15. *p<0.05 vs R+, **p<0.01 vs R+, (RANKL) R+ is the positive control.

Osteoclast Formation in differentiated RAW 264.7 cells

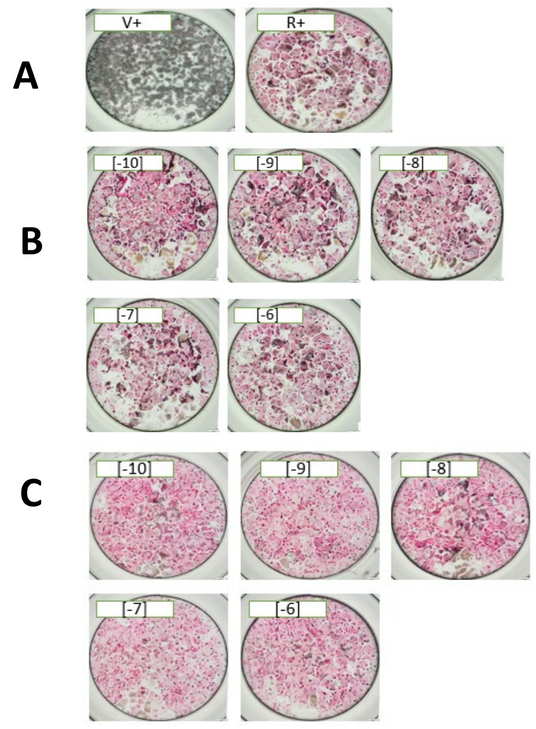

Figure 4: TRAP staining shows differentiation of RAW 264.7 murine macrophages into multinucleated osteoclasts.
A. Vehicle control (V+) and RANKL (R+)
B. The effects of GPER agonist (G1) at different concentrations (10^{-10}–10^{-6} M) are shown.
C. The effects of GPER antagonist (G15) at different concentrations (10^{-10}–10^{-6} M) shown.
These concentrations were achieved by serial dilution of each of the compounds from a G1 and G15 stock solutions with concentration 10mM respectively.

Discussion and conclusion

- The study demonstrates that the activation of GPER may inhibit osteoclast formation through mechanisms that are still unclear.
- TRAP staining has shown that G1 decreases osteoclast formation at 10^{-6} M.
- Western blot will be conducted in an effort to establish whether GPER activation modulates mitogen activated protein kinase (MAPK) pathways. These pathways are vital to RANKL-mediated osteoclast formation.

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