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Introduction

When tumor cells home to bone they secrete factors that stimulate osteoclast formation, resulting in increased bone resorption. This increases the release of factors from bone matrix that stimulate the growth of tumor cells, leading to a vicious cycle characterized by extensive bone loss and enhanced tumor growth in bone.¹ Thus, factors that inhibit osteoclast differentiation or activity may have the potential to inhibit the vicious cycle. The RANKL inhibitor denosumab is approved for treatment of bone metastases from solid tumors, and the cathepsin K inhibitor odanacatib has been shown to suppress bone resorption in breast cancer patients with bone metastases.

Aim of the Study

Our goal was to optimize separate *in vitro* culture systems for determining osteoclast differentiation and activity, and validate denosumab and odanacatib as reference inhibitors of osteoclast differentiation and activity, respectively.

Materials and Methods

Cell culture

CD34⁺ human osteoclast precursor cells (Poietics® Human Osteoclast Precursors, Lonza)² were cultured on bovine bone slices for 7 days in the presence of M-CSF and RANKL,³ and allowed to differentiate into bone-resorbing osteoclasts. After completion of osteoclast differentiation at day 7, the culture medium was removed and new culture medium was added into the wells. The mature osteoclasts were then cultured for an additional 3 days, allowing them to resorb bone. In the osteoclast differentiation assay, denosumab (Prolia, Amgen) was added in the cultures at day 0, and the cultures were stopped at day 7. In the osteoclast activity assay, the cells were cultured without test compounds for 7 days, followed by medium change at day 7 and addition of odanacatib (ChemieTek). The cultures were stopped at day 10.

Endpoint measurements

In the osteoclast differentiation assay, tartrate-resistant acid phosphatase 5b activity (TRACP 5b, BoneTRAP® assay, IDS Ltd) was measured in the culture medium collected at day 7 as an index of the number of formed osteoclasts.^{3,4} In the osteoclast activity assay, C-terminal cross-linked telopeptides of type I collagen (CTX-I, CrossLaps® for culture ELISA, IDS Ltd) was measured in the culture medium collected at day 10 to quantitate bone resorption during days 7-10.

Statistical analysis

Statistical analysis was performed with statistical software R (version 3.1.2, www.r-project.org) using one-way ANOVA. Because statistically significant differences were observed ($p < 0.001$ between all groups) the results of all other groups were compared separately with the results of the baseline group using Tukey's HSD test.

Results / Osteoclast differentiation

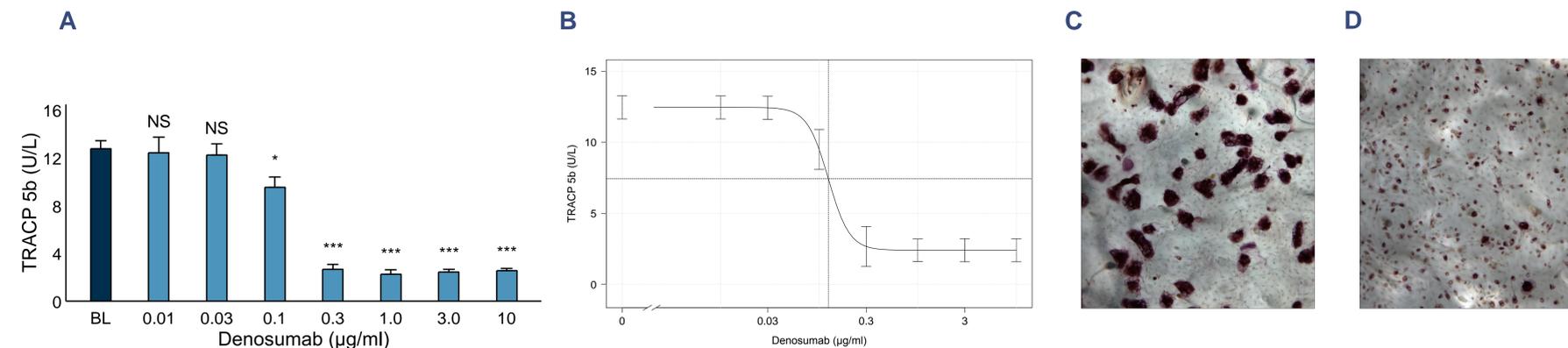


Figure 1: The effects of denosumab on differentiation of human osteoclasts *in vitro*. A) TRACP 5b activity (U/L) measured at day 7. NS = not significant, * $p < 0.05$, *** $p < 0.001$ compared to the baseline (BL) group; B) EC50 value determination (EC50 = 0.124 µg/ml); C) and D) Representative microscopic images of the effects of denosumab on formation of TRACP positive multinuclear osteoclasts at day 7, C) Baseline (no added compounds), D) Denosumab 0.3 µg/ml, magnification x 100. Denosumab blocks osteoclast differentiation at a stage where the mononuclear osteoclast precursor cells are TRACP positive, and the mononuclear osteoclast precursor cells do not secrete active TRACP 5b enzyme into the culture medium.

Results / Osteoclast activity

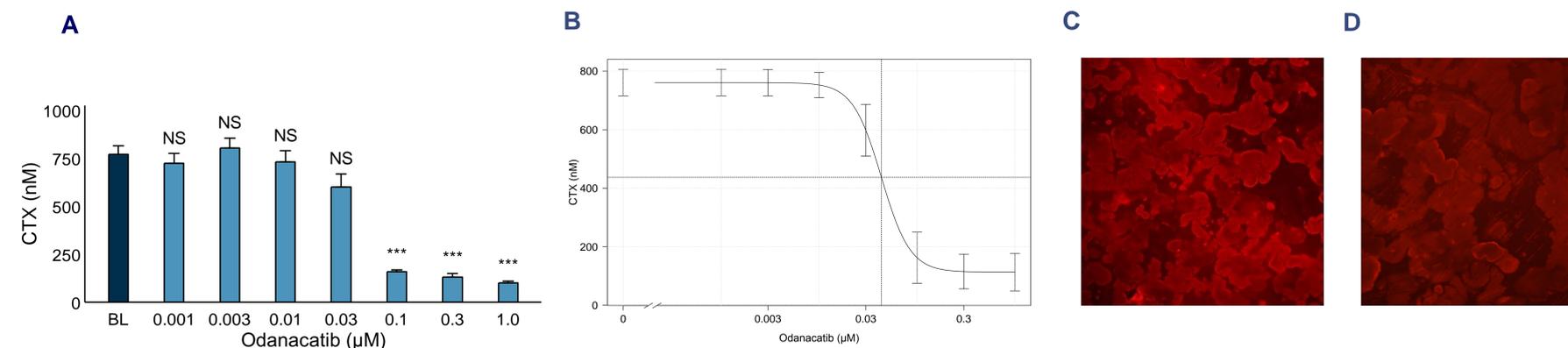


Figure 2: The effects of odanacatib on resorption activity of human osteoclasts *in vitro*. A) CTX values determined at the end of the resorption period at day 10. NS = not significant, *** $p < 0.001$ compared to the baseline (BL) group; B) EC50 value determination (EC50 = 0.0433 µM); C) and D) Representative microscopic images of the effects of odanacatib on resorption pit formation (TRITC-labeled wheat germ agglutinin lectin staining) at day 10, C) Baseline (no added compounds), D) Odanacatib 0.1 µM, magnification x 100. The strong inhibition of bone resorption by odanacatib is partly due to decreasing the depth of the formed resorption pits, which is not seen in the microscopic images, indicating that CTX measurements detect more reliably total effects on resorption.

Summary

- Denosumab and odanacatib showed strong concentration dependent inhibition of osteoclast differentiation and activity, respectively.
- EC50 value was 0.124 µg/ml for denosumab and 0.0433 µM for odanacatib.
- Denosumab and odanacatib are useful reference compounds in *in vitro* human osteoclast differentiation and activity assays, respectively.

Conclusions

We have validated denosumab and odanacatib as reference compounds of osteoclast differentiation and activity, respectively, in a human *in vitro* osteoclast culture system. The culture system is a clinically reliable tool for identifying new compounds affecting the vicious cycle of osteolytic bone metastases, and clarifying if these active compounds target directly osteoclast differentiation or activity

Acknowledgements

We thank Riikka Kytömaa, Jani Seppänen and Esa Alhoniemi (Avoltus) for skillful technical assistance.

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