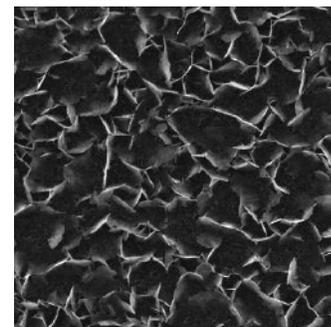


Drug Screening Using Corning® Osteo Assay Surface

Customer Application Note



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Introduction

The healthy skeleton is a dynamic framework of bone that is constantly remodeled. Without remodeling, bone accumulates, microfractures and loses its mechanical strength. The process of remodeling is mediated primarily by the concerted action of two specialized cell types, bone-resorbing osteoclasts and bone-depositing osteoblasts. In healthy bone, a finely tuned balance between resorption and deposition is achieved, but menopause and disease can shift this balance in favor of resorption. The result is pathological bone loss, as is seen in osteoporosis, inflammatory arthritis, periodontitis and some metastatic tumors. These diseases erode the quality of life in millions and result in tens of billions of dollars in increased healthcare costs. Consequently, there has been a remarkable research interest in the pathophysiology and molecular cell biology of bone remodeling, and especially in the development of new antiresorptive therapeutics.

In vitro, osteoclast resorption is studied by plating the cells on thin, polished slices of dentin obtained from teeth or tusks. Dentin slices are traditionally the “gold standard” for assaying osteoclast resorption, because of their close resemblance to bone matrix, while offering a microscopically uniform surface. The acquisition of dentin is limiting, its preparation is extraordinarily labor intensive, and sample handling is tedious. Therefore, for high-throughput screening applications, dentin is not cost effective.

Corning Life Sciences has recently met the need for a reliable, cost-effective, bone-like mineral surface, for both routine resorption assays and high-throughput screening, with its new Corning Osteo Assay Surface multiple well plates. The wells of these plates are coated with a proprietary hydroxyapatite mineral surface that is highly uniform and microscopically fine-grained. These plates offer an excellent alternative to dentin for the study of both osteoclast and osteoblast function. The surface allows for easy quantification without affecting cell differentiation and function.

We have used Corning Osteo Assay Surface plates for secondary screening of potential antiresorptives identified in a molecular-interaction primary screen. This approach allows rapid, cost-effective elimination of compounds that are toxic, affect osteoclast differentiation, or do not inhibit resorption at a reasonable EC₅₀, prior to committing to expensive animal testing. This technical report will cover materials and procedures that were used for *in vitro* screening of osteoclast function, tartrate-resistant acid phosphatase (TRAP) staining of osteoclasts, von Kossa staining of the mineral surface for increased contrast in pit identification, and the use of NIH ImageJ for quantification of resorption pit images.

Materials and Procedures

Osteoclast Cell Culture for Pit Formation

RAW 264.7 cells, a mouse macrophage cell line, were differentiated into osteoclasts in the presence of RANKL (GST-soluble recombinant murine RANKL fusion protein). These cells were maintained in high-glucose DMEM (Sigma-Aldrich, Cat. No. D5796) in tissue culture treated polystyrene 75 cm² flasks at 37°C in a humidified 5% CO₂ incubator. Typically, cells of low passage and 50 to 70% confluence were washed with DPBS after removing spent DMEM, and were removed from the plastic by scraping. Cells were centrifuged at 300 x g for 5 minutes and were resuspended in 4 mL of α -MEM (without ribonucleosides or deoxyribonucleosides; Invitrogen, Cat. No. 12571-063), containing fetal bovine serum (FBS) and antibiotics, which was pre-warmed to 37°C. Cell density was determined by counting with a hemacytometer or Coulter® counter calibrated for RAW 264.7 cells.

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For 96 well plates, the number of cells seeded was 5,000 per well. Cells were diluted to 5×10^4 /mL in the complete medium and 100 μ L of the cell suspension was added to each well. Plates were incubated for 2 hours to allow for cell attachment.

The effects of various antiresorptive lead candidates on the ability of RAW 264.7 to differentiate, and then resorb mineral, was measured. Typically, serial dilutions of drug compounds were tested on cells being differentiated *in situ* with RANKL. For RAW 264.7 cells, media controls and serial

drug dilutions in medium all contained 100 ng/mL of RANKL and these conditions were maintained to the end of the experiment. Drug-free and vehicle controls were always included.

The total volume of medium per well in the 96 well plates was 200 μ L. Experimental media were prepared as 2X concentrates with respect to the drugs being tested. 100 μ L volume was added per well to the plates, after the 2 hours of cell attachment incubation. The final RANKL concentration was 50 ng/mL and drug concentrations were 1X. The plates

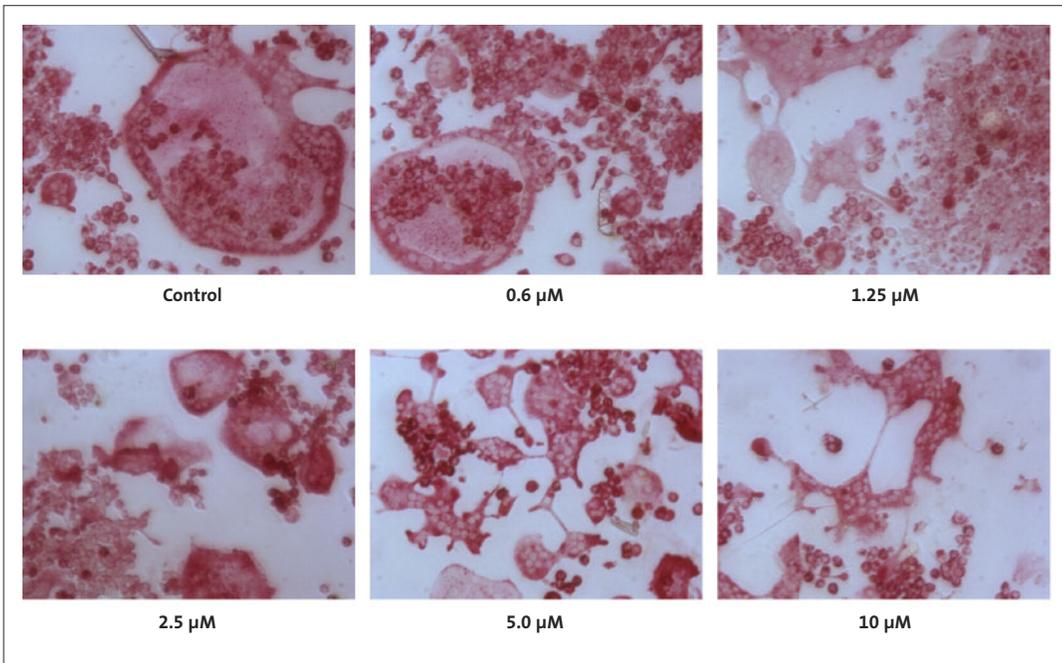


Figure 1. Dose-dependent drug response as measured by TRAP staining of osteoclasts on Corning Osteo Assay Surface.

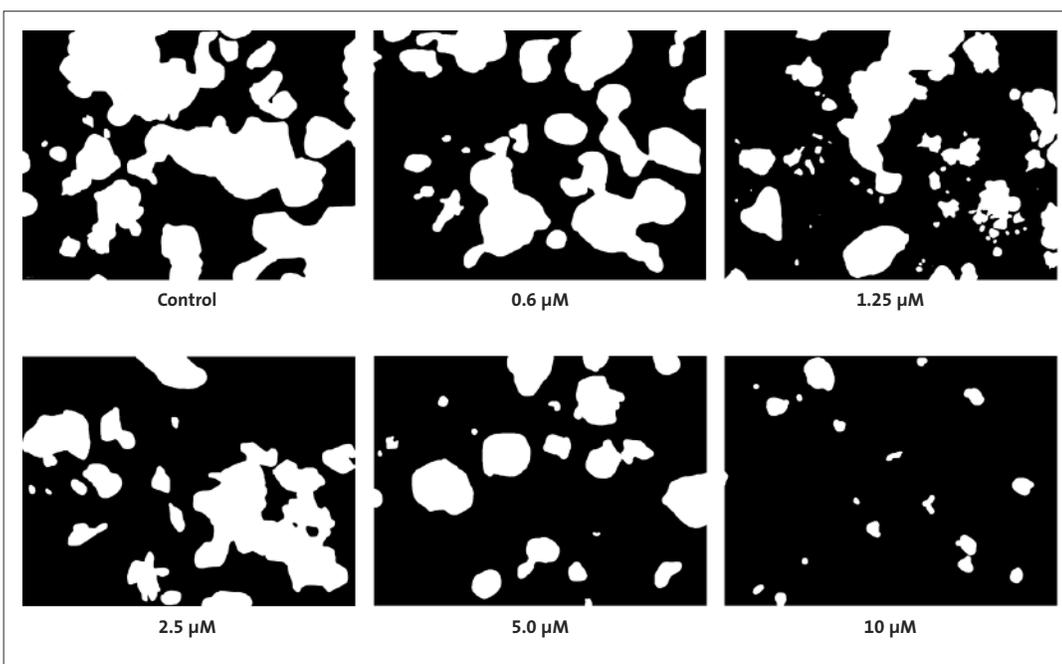


Figure 2. Von Kossa staining for image enhancement on Corning Osteo Assay Surface.

were incubated for another 72 hours, then observed under the microscope. Complete 1X media were exchanged and the plates were incubated for a further 48 hours.

On day 5, all media were aspirated and plates were stained for TRAP to visualize osteoclasts (Figure 1). Alternatively, plates were bleached and contrast enhanced to assess resorbed surface area (Figure 2).

If storage was required, bleached plates were stored with 200 μ L of water per well (aspirated for imaging) at 4°C. It was important to store the bleached plates in water to avoid cracking of the dry mineral surface. TRAP stained plates were stored in DPBS at 4°C. If salts crystallized and precipitated during storage, the plates were rinsed with distilled water to dislodge and dissolve the crystals, and the buffer was then replaced.

Staining and Image Analysis

For TRAP staining, TRAP Buffer (pH 5.0) was prepared fresh for use by mixing 50 mL acetate buffer (35.2 mL 0.2M sodium acetate and 14.8 mL 0.2 M acetic acid), 10 mL 0.3M sodium tartrate, 1 mL 10 mg/mL naphthol AS-MX phosphate disodium salt (Sigma-Aldrich, Cat. No. N-5000), 0.10 mL Triton® X-100 and 38.9 mL distilled water. TRAP stain was prepared fresh for use by dissolving 0.3 mg Fast Red Violet LB salt (Sigma-Aldrich, Cat. No. F-3381) per mL of TRAP buffer at 37°C. For TRAP staining, medium was aspirated from cells and cells were washed with PBS. Cells were fixed with 200 μ L/well formalin (Sigma-Aldrich; Cat. No. HT501128) for 15 minutes at 37°C, then washed three times with PBS at 37°C. Cells were incubated in TRAP stain for 5 to 10 minutes at 37°C. TRAP stain was aspirated and cells were washed with Ca⁺⁺/Mg⁺⁺-free PBS and were stored in the same buffer at 4°C.

The modified von Kossa staining protocol was used to improve the contrast for resorbed pit image analysis and quantification. For brightfield visualization, von Kossa staining greatly increases the image contrast, facilitating imaging and quantification of resorption pits (see Figure 2). For von Kossa staining, 100 μ L of 5% (w/v) aqueous silver nitrate solution was added to each of the bleached wells of a 96 well plate. Plates were incubated for 30 minutes at room temperature in the dark (covered with foil). The silver nitrate solution was discarded into a hazardous waste container and the plates were then soaked in distilled water for 5 minutes. The water was discarded into a hazardous waste container. The mineral surface appears yellow after this step. The ionic silver (I) was reduced to metallic silver, developing a dark color, by adding 100 μ L of 5% sodium carbonate (w/v in commercial buffered formalin) and incubated for 4 minutes at room temperature. The carbonate/formalin solution was discarded into a hazardous waste container and the plates were dried at 50°C for 1 hour.

For microscopy, the multiple well plate was oriented with the bottom of the wells closest to the objective lens of an inverted microscope. Digital images were captured using resident image capture software. NIH ImageJ software was

Table 1. Resorbed Surface Area Analysis on Corning Osteo Assay Surface

Test Compound (μ M)	Resorbed Surface (Percent Area)
Control	46.36
0.60	33.95
1.25	27.04
2.50	26.60
5.00	17.95
10.00	3.83

used for image analysis (downloaded from the NIH web site, <http://rsbweb.nih.gov/ij/>).

Results and Discussion

TRAP staining was performed as described in the Methods above, to determine the effect of an antiresorptive lead compound on RAW 264.7 cell differentiation. Wells were observed using brightfield microscopy, and TRAP positive multinucleated osteoclasts were scored for a drug concentrations range (0.6 μ M to 10 μ M). In the example shown in Figure 1, no effect on differentiation was seen at the EC₅₀ of the test compound (1.25 μ M), although some inhibition was evident at higher concentrations.

Plates to be quantified for resorption were first bleached with a 1:4 dilution of commercial household bleach in distilled water. Von Kossa staining was performed, as detailed above. Results for brightfield microscopy are shown here (Figure 2). The white areas of the image are the pits resorbed by osteoclasts derived from RAW 264.7 cells; the silver-stained mineral is black. Percentages of surface resorbed by differentiated osteoclasts at various drug concentrations were quantified and are shown in Table 1.

A serial dilution of test compound shows an EC₅₀ of 1.2 μ M. At this concentration, growth and differentiation of RAW 264.7 number cells were unaffected, suggesting that this compound is a potential lead candidate for further drug development. The imaging and image analysis can be automated, providing an efficient, high-throughput screening tool. The uniformity of the mineral surface of the Corning® Osteo Assay Surface plates is far superior to other artificial surfaces on the market and tested to date, allowing unprecedented precision in quantitative resorption assays.

Conclusion

The objective of this study was to screen chemical libraries for potential novel antiresorptive therapeutics, using defined molecular targets. Lead candidates identified using *in vitro* assays must at some point be validated *in vivo*; however, if hundreds of candidates are available, a simple, high-throughput intermediate or secondary assay needs to be performed to choose only the best possible candidates before proceeding to costly animal studies.

Prior to the availability of the Corning® Osteo Assay Surface plates, alternative artificial mineral coated plates and dentin slices were used. The former lacked uniformity and were prohibitively expensive, while the latter had the serious procurement and handling issues mentioned in the introduction. Neither choice was suitable for high-throughput formats.

The present results show that the osteoclast model cell line, RAW 264.7, could differentiate without difficulty on Corning Osteo Assay Surface plates, and effects on differentiation and mineral resorption were observed in the presence of test compounds. In addition, pit formation was analyzed with an easily automated microscopic approach. While studies on bone-like matrices such as dentin will remain desirable as a final analytical tool, and animal studies are a requirement for drug development, using Corning Osteo Assay Surface

plates in an intermediate cellular assay allows high-throughput secondary screening to isolate the most valuable lead candidates.

These plates provide an ideal assay surface for high-throughput drug screening, or for basic research in bone biology.

Further Reading

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Bradley, E.W. and M.J. Oursler. Osteoclast culture and resorption assays. (2008) *Met Molec Biol* 455, 19-35.

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