Inhibition of Osteoclast Bone Resorption by Disrupting Vacuolar H\(^{+}\)-ATPase a3-B2 Subunit Interaction*

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Vacuolar H\(^{+}\)-ATPases (V-ATPases) are highly expressed in ruffled borders of bone-resorbing osteoclasts, where they play a crucial role in skeletal remodeling. To discover protein-protein interactions with the a subunit in mammalian V-ATPases, a GAL4 activation domain fusion library was constructed from an in vitro osteoclast model, receptor activator of NF-κB ligand-differentiated RAW 264.7 cells. This library was screened with a bait construct consisting of a GAL4 binding domain fused to the N-terminal domain of V-ATPase a3 subunit (NTa3), the a subunit isoform that is highly expressed in osteoclasts (a1 and a2 are also expressed, to a lesser degree, whereas a4 is kidney-specific). One of the prey proteins identified was the V-ATPase B2 subunit, which is also highly expressed in osteoclasts (B1 is not expressed). Further characterization, using pulldown and solid-phase binding assays, revealed an interaction between NTa3 and the C-terminal domains of both B1 and B2 subunits. Dual B binding domains of equal affinity were observed in NTa3, suggesting a possible model for interaction between these subunits in the V-ATPase complex. Furthermore, the a3-B2 interaction appeared to be moderately favored over a1, a2, and a4 interactions with B2, suggesting a mechanism for the specific subunit assembly of plasma membrane V-ATPase in osteoclasts. Solid-phase binding assays were subsequently used to screen a chemical library for inhibitors of the a3-B2 interaction. A small molecule benzohydrazide derivative was found to inhibit osteoclast resorption with an IC50 of 4.5 μM.

V-ATPases\(^2\) are proton pumps ubiquitous in eukaryotic cells, where they acidify numerous intracellular membrane compartments, including Golgi, endosomes, lysosomes, clathrin-coated vesicles, chromaffin granules, and insulin secretory granules (reviewed in Refs. 1–11). V-ATPases also pump protons across the plasma membrane into the extracellular space in a variety of specialized cells, including renal duct intercalated cells, clear cells of the epididymis, and osteoclasts. Here they are involved in functions, including pH homeostasis, sperm maturation, and bone resorption and remodeling. Mutations in V-ATPase subunits lead to diseases, such as renal tubular acidosis and osteopetrosis. Furthermore, their inappropriate activity can contribute to osteoporosis and tumor metastasis (12–15).

V-ATPases are multisubunit molecular motors, structurally analogous to the F\(_{1}\)F\(_{0}\)-ATP synthases (F-ATPases), but working “in reverse” (16–20). Thus, V-ATPases create proton gradients across membranes by utilizing the energy of ATP hydrolysis, rather than utilizing the potential energy of proton gradients to synthesize ATP.

The mammalian V-ATPase complex includes at least 14 different subunits, some of which have multiple isoforms (13). The complex can be divided into two sectors, the cytoplasmic V\(_1\) sector and the integral membrane V\(_0\) sector. The V\(_1\) sector consists of subunits A–H, with a likely stoichiometry of A\(_3\)B\(_3\)C\(_D\)E\(_F\)G\(_H\) (18). The mammalian V\(_0\) sector consists of subunits a, c, c\(^r\), d, e, and Ac45, and the stoichiometry appears to be ac\(_c\)c\(^r\)deAc45, whereas in yeast one of the c subunits is replaced with a homologous c\(^r\) subunit. Regulation of V-ATPase function can occur through dissociation of the complex into its separately inactive V\(_1\) and V\(_0\) sectors, with the reversible loss of the C subunit from the complex (21, 22).

Much of the architecture of the V-ATPases, and their molecular motor function, has been inferred from the structure-function analogy with F-ATPases, which, unlike the V-ATPase complexes, have had their structures largely resolved by x-ray crystallography. Crude confirmation of this analogy has been obtained, thus far, largely from single particle electron microscopic analysis of V-ATPases and chemical cross-linking experiments (5, 18, 23, 24). Many of the more precise details of intra-complex interactions of V-ATPase subunits, or their extra complex associations with non-V-ATPase polypeptides, remain to be characterized.
Landolt-Marticorena et al. (25) have shown previously, using yeast two-hybrid analysis, that the a subunit of the V_o sector interacts with the A and H subunits of the V_s sector, interactions that are thought to contribute to the stator complex that is required for the function of the V-ATPase molecular motor (26, 27). The work presented here approaches the problem of further characterizing V-ATPase structure and function by implementing new yeast two-hybrid studies using a cDNA library derived from the murine RAW 264.7 cell line, which is capable of differentiating into osteoclasts in the presence of the cytokine, RANKL (28–30). This system is of particular interest because of its high level expression of plasma membrane V-ATPase, which is responsible for proton secretion into the extracellular resorption lacunae of osteoclasts through their specialized ruffled border. This function is required to dissolve bone (31, 32), and its disruption in mammals results in the sclerosing bone disorder, osteopetrosis (33, 34), although excessive activity results in pathological bone loss, as in osteo-
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37 °C, in LB medium with selective antibiotics, were chilled on ice, and isopropyl β-D-1-thiogalactopyranoside was added to 0.2 mM. Cells were incubated further with shaking at 16 °C for 16 h. Cells were harvested by centrifugation at 4 °C, and pellets were resuspended in 4 ml of ice-cold PBS, containing 0.2 mg/ml lysozyme per 200 ml of original culture volume. The suspension was incubated on ice for 30 min and then mixed with 2.5 volumes of ice-cold 0.2% (w/v) Triton X-100. This was sonicated on ice four times for 15 s with 30 s of cooling between bursts. DNase and RNase were added to 5 μg/ml each, from 10 mg/ml stocks, and the lysate was incubated a further 10 min on ice and then centrifuged at 20,000 × g for 15 min. The supernatant was mixed with 10 ml/liter of starting culture of a 50% slurry of glutathione-Sepharose 4B in PBS. After 1 h of incubation at 4 °C with rocking, beads were washed three times for 5 min with PBS and collected by centrifugation at 500 × g for 5 min. The beads were transferred to a Poly-Prep chromatography column (Bio-Rad) and washed twice with 20 ml of PBS and eluted with 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0. Fractions were collected and analyzed by SDS-PAGE. GST-B1(GH) and GST-B2(GH) were repurified on Ni-NTA-agarose beads, as described for the TRX fusion protein purification.

Purification of TRX Fusion Proteins—This procedure was similar to purification of GST fusion proteins, with the following exceptions. Bacterial pellets were resuspended in ice-cold lysis buffer consisting of 50 mM NaH₂PO₄, 300 mM NaCl, and 10 mM imidazole, pH 8.0, at a ratio of 4 ml per 60 ml of original culture volume. After lysis, cleared supernatants were mixed with 2 ml of a 50% slurry of Ni-NTA-agarose beads in lysis buffer at 4 °C for 1 h with rocking. Beads were washed three times for 5 min with a 10-ml wash buffer containing 50 mM NaH₂PO₄, 300 mM NaCl, and 20 mM imidazole, pH 8.0, and packed in a Poly-Prep chromatography column. The column was washed twice with 20 ml of wash buffer and eluted with 50 mM NaH₂PO₄, 300 mM NaCl, 50 mM imidazole, pH 8.0. A second elution was performed at 150 mM imidazole concentration. Fractions were analyzed by SDS-PAGE.

Pulldown Assays—Glutathione-Sepharose 4B beads (0.5-ml packed volume) were combined with 4 ml of cleared bacterial lystate containing 0.35 mg/ml total protein and PMSF to 2 mM (from 0.2 ml stock in anhydrous ethanol) from either GST-B1 or GST-B2 expression cultures. After coating at 4 °C for 1 h with rocking, the beads were washed five times with 10 ml of PBS, and 125 μl of coated beads was mixed with 400 μl of cleared supernatants of TRX-NTα1–4 expression cultures containing 0.13 mg/ml protein. After a 1-h incubation at 4 °C with rocking, beads were washed five times with 1 ml of ice-cold PBS. Pelleted beads were eluted with 100 μl of SDS sample buffer, and 10-μl aliquots were analyzed by SDS-PAGE.

ELISA Solutions—Protein Coating buffer was 10 mM sodium phosphate, pH 7.0; Tris-buffered saline (TBS) was 20 mM Tris-HCl, 0.9% (w/v) NaCl, pH 7.40; Blocking Buffer was 1% (w/v) gelatin in TBS containing 0.05% (w/v) Triton X-100 and 0.1% (w/v) phenol, pH 7.40; Wash Buffer was TBST; 3.35,5.5’-tetramethylbenzidine stock was 4 mg/ml 3.35,5.5’-tetramethylbenzidine (Sigma) in DMSO/ethanol (1:9), Developer was freshly prepared 80 μg/ml 3.35,5.5’-tetramethylbenzidine (from stock), 0.01% hydrogen peroxide, 0.1 M sodium acetate, pH 6.0; Stop Solution was 1 N sulfuric acid. All antibodies were diluted in Blocking Buffer from commercial stock as follows: anti-GST antibody at a 1:4000 dilution of GST(Z-5); anti-His tag antibody at a 1:3000 dilution of His probe (H-3); HRP-GAR second antibody at a 1:4000 dilution; HRP-GAM second antibody at a 1:3000 dilution.

In Vitro ELISA-based Solid-phase Binding Assays—Volumes are per well. All washes were done with 100 μl of Wash Buffer (see under “ELISA Solutions”). High protein-binding polystyrene 384-well plates (Greiner Microlon 781097; Sigma catalog no. M6936) were coated with ligand protein (20 μl of Protein Coating Buffer containing 10 μg/ml TRX-NTα3 or 2 μg/ml GST-CTB2, as indicated), overnight at 4 °C. All further steps were at 25 °C. Coated plates were washed twice, blocked with 30 μl of Blocking Buffer for 1 h, and again washed twice. For binding experiments, wells were then incubated with analyte protein (20 μl of Blocking Buffer with 5 mM MgCl₂ containing GST-B2(GH), concentrations as described) for 1 h. Plates were washed three times, followed by incubation with 20 μl of anti-GST antibody in Blocking Buffer for 30 min. Plates were washed three times and incubated with 20 μl of HRP-GAR in Blocking Buffer for 30 min (in some assays, where the ligand was GST-CTB2 or GSTB1 anti-His tag antibody was used with HRP-GAM second antibody). Plates were then washed five times, followed by addition of 30 μl of Developer. After 15 min of incubation, the reaction was stopped with 30 μl of Stop Solution. Absorbance was quantified using an Envision Multilabel Reader (PerkinElmer Life Sciences) at 450 nm, with subtraction of an optical reference absorbance at 600 nm.

High Throughput Screening—The above protocol was adapted to automated liquid handling and quantification. Briefly, high throughput screening of a 10,000 compound DIVERSet synthetic organic chemical library (ChemBridge) was executed on a robotics platform where a CRS articulated arm controlled by Polara software (Thermo Electron) moved on a 3-m rail to utilize a Biomek FX liquid handler (Beckman Coulter), equipped with a 96-channel disposable tip pipetting head (pipetting in staggered quadrants into 384-well microplates) and attached stacker carousel for sourcing plates and tip boxes. Assay plates were sourced from room temperature hotels. A Multimek 384-channel automated pipettor (Beckman) with an accessory 200-ml 384-pin floating pin tool (V&P Scientific) was used to dispense library compounds; an EMBLA washer (Molecular Devices) was used for 384-well microplate washing; and a 2102 EnVision Xcite multilabel reader was used to perform absorbance measurements, using 450 and 600 nm (optical control) filters. Typically, 10–14 384-well plates were screened simultaneously, with 320 compounds per plate, and two positive (no library compound, vehicle only) and two negative (no ligand) control columns at left (columns 1 and 2) and right (columns 23 and 24) ends of the plate, respectively. Library compounds were dispensed into test wells using a 200-μl 384-pin tool, each compound being at 1 mM concentration in DMSO (5 μM final concentration in assay). Hits were scored after analyzing data using B-score statistics (35) and ranking the inhibitory compounds according to negative deviation from the mean. Selected hits were confirmed by repeating the primary assay and by comparing dose responses in the pri-
primary assay; reproducible hits were then tested in secondary cellular assays.

**Model Compound**—The hit described in detail in this work is referred to as KM91104 and has the chemical structure 3,4-dihydroxy-N’-(2-hydroxybenzylidene)benzohydrazide. KM91104 was purchased as powder (ChemBridge 5266986) and was diluted for use in secondary assays from 10 mM stock in DMSO. Vehicle controls were equivalent volumes of neat DMSO. Some experiments included an inactive analog of KM91104, which is referred to as KM91201. KM91201 has the structure N’N’-(1,4-phenylenedimethylidene)bis(3,4-dihydroxybenzohydrazide) and was purchased as a powder (ChemBridge 5318527). KM91201 was treated exactly as KM91104 in experiments.

**Cell Growth Protein Assay**—To assess the effect of primary screening hits on growth of RAW264.7 cells, the cells were seeded in complete DMEM in 96-well plates at 5 × 10^4 cells/well (200 μl/well), with varying concentrations of test compounds, and were allowed to grow for 5 days with a change of complete medium on day 3. Wells were then washed with PBS, and cells were lysed with Lysis Buffer, containing 90 mM trisodium citrate, 10 mM NaCl, (adjusted to pH 4.8 with HCl), and 0.1% Triton X-100 (added prior to use from 10% stock). 10-μl aliquots were withdrawn and mixed with 200 μl of Bio-Rad protein assay reagent (Bio-Rad, catalog no. 500-0002) in a microplate and incubated at 25 °C for 5 min. Absorbances were then read at 595 nm.

**Cell Growth Cytotoxicity Assay**—Proliferation and metabolic activity of cell cultures in the presence of primary hit compounds was determined using a modified tetrazolium dye assay (MTS reagent; Promega CellTiter 96 Aqueous One Solution cell proliferation assay, catalog no. G3582). Cells were grown in 96-well plates as for the cell growth protein assay; then 20 μl of MTS reagent was added to each well, and cells were further incubated overnight at 37 °C. Absorbances were then read at 490 nm.

**Total Solubilized TRAP Assay**—Cells grown in 96-well plates, as for the cell growth protein assay, were washed twice with PBS, and wells were aspirated, and then 200 μl/well of Lysis Buffer (see under “Cell Growth Protein Assay”) was added to the plates. After lysis, 20-μl samples were withdrawn and added to microplate wells on ice, containing 50 μl each of ice-cold Substrate Solution and Tartaric Acid Buffer. Substrate Solution was prepared as 0.1 g of disodium p-nitrophenyl phosphate (Sigma catalog no. 104 phosphatase substrate, Sigma; catalog no. 104-0) in 25 ml of distilled water. Tartaric Acid Buffer was 40 mM l(+)-tartaric acid added to Citrate Buffer (final pH ~4.0). The assay plate was mixed and then incubated for 30 min at 37 °C. The reaction was stopped with 80 μl of 2 N NaOH. Absorbances were measured at 405 nm and compared with 20-μl standards consisting of 1–100 μM p-nitrophenol.

**TRAP Staining of Fixed Cells**—Cells were treated according to the BD Biosciences TRAP staining protocol no. 445. Briefly, TRAP Buffer, pH 5.0, was prepared fresh for use by mixing 50 ml of Acetate Buffer (35.2 ml of 0.2 M sodium acetate and 14.8 ml of 0.2 M acetic acid), 10 ml of 0.3 M sodium tartrate, 1 ml of 10 mg/ml naphthol AS-MX phosphate disodium salt (Sigma, catalog no. N-5000), 0.10 ml of Triton X-100, and 38.9 ml of distilled water. TRAP Stain was prepared fresh for use by dissolving 0.3 mg of Fast Red Violet LB salt (Sigma, catalog 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 μl/well formalin (Sigma; catalog no. HT501128) for 15 min at 37 °C and then washed three times with PBS at 37 °C. Cells were incubated in TRAP stain for 5–10 min at 37 °C. TRAP stain was aspirated, and cells were washed with Ca^2+ /Mg^2+ -free PBS and were stored in the same buffer at 4 °C. Photomicrography was done using phase contrast or bright field illumination after warming cells to ambient temperature in the storage buffer.

**Isolation of Bone Marrow Mononuclear (BMM) Cells**—BMM cells were isolated from tibias and femurs of normal C57BL/6 mice and seeded into 24-well plates at 1.0 × 10^5 cells/well in 1 ml of complete α-MEM containing 15% FBS. The cells were incubated for 2 days in the presence of 50 ng/ml M-CSF. To induce osteoclast differentiation, the medium was changed and included, additionally, 200 ng/ml RANKL. Test compounds were also added at this time. The cells were allowed to grow and differentiate for 5 days with no further change of medium.

**Hydroxyapatite Resorption Assay**—Early passage (P3 to P6 after thawing) RAW264.7 cells were plated at 5 × 10^3 cells per well in 100 μl of complete α-MEM containing 100 ng/ml soluble recombinant RANKL in Corning Osteo-Assay Surface 96-well plates (Corning Life Sciences, catalog no. 3988XX1). Plates were incubated for 2 h at 37 °C in a humidified 5% CO₂ incubator to allow cell attachment. A further 100 μl of complete α-MEM containing RANKL and test compound was then added to each well. The latter complete medium was changed after 3 days, and cells were incubated for a further 2 days prior to processing for von Kossa staining.

**Modified von Kossa Staining**—Plates from hydroxyapatite resorption experiments were stripped with 1.2% sodium hypochlorite solution for 5 min to remove cells, rinsed with distilled water, and air-dried until further use. For staining, plates were treated in darkness at ambient temperature with 100 μl/well 5% (w/v) silver nitrate solution for 10 min. Wells were then aspirated and washed for 5 min in distilled water. Wells were again aspirated, and 100 μl/well 5% (w/v) sodium carbonate in 10% formalin was added. After a 4-min incubation at ambient temperature, wells were washed twice with PBS and three times with distilled water. The plates were then aspirated and air-dried prior to imaging.

**Dentin Resorption Assay**—Elephant ivory (donated by the Toronto Zoo, Toronto, Canada) was sliced using a Buehler Isomet slow speed saw with a 4-inch diameter diamond impregnated wafering blade with a 300 μm kerf. Slices were end-cut 150 μm thick and washed in distilled water, and discs were punched using a 0.25-inch diameter paper hole-punch. Discs were stored in 20% (v/v) ethanol solution. For tissue culture use, discs were soaked in complete medium overnight and then transferred to fresh medium in wells of a 96-well microplate. RAW264.7 cells were plated in 100-mm culture dishes containing 20 ml of complete α-MEM with 100 ng/ml soluble recombinant RANKL. Plates were incubated for 4 days to allow cell differentiation. Mature osteoclasts were harvested by scraping into 2 ml of medium and were pelleted at 1,000 × g for
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5 min at 4 °C. Cells were resuspended in complete medium with RANKL and appropriate concentrations of test compound, then were plated onto dentin slices. Further growth was allowed for 3 days prior to processing for Picro-Sirius Red staining.

Picro-Sirius Red Staining—Discs from dentin resorption experiments were washed in distilled water and ethanol. The discs were then stained with Picro-Sirius Red stain (0.1% Sirius Red F3B (C.I. 35782) in saturated aqueous picric acid) overnight. Discs were destained for 1 h in 0.5% acetic acid solution, then washed in distilled water, and mounted on slides for fluorescence imaging (36).

Imaging—Photomicrography was done using a Leica DM IRE2 microscope with OpenLab software (Leica Microsystems). Image analysis was carried out with NIH imageJ software. For resorption assays in Corning plates, all microplate wells were imaged with five fields/well (20× objective) using an automated stage (Applied Scientific Instrumentation MS-2000). Dentin slices were imaged using epifluorescence with GFP filters.

Statistical Analysis—Repetition of experiments is given in the figure legends. Determination of IC_{50} values for dose-response curves or half-maximal binding in protein interaction assays was done using GraphPad Prism (version 4.02) curve-fitting software. In some experiments, where saturation was not achieved, automated curve fitting failed, and manual estimates were made by interpolation in the log linear portion of the curve. This is noted in the figure legends. Standard deviations were calculated and are shown as ±1 S.D. wherever values are quoted or in error bars in histograms and graphs. Unpaired two-tailed t tests were used to test significance of differences, as appropriate.

RESULTS

The initial intention of this work was to determine the set of protein binding partners that interact specifically with the α3 subunit that is highly expressed as part of the plasma membrane V-ATPase of osteoclasts. Crucial interactions with it could potentially be exploited as molecular targets for the treatment of osteolytic disease (12, 13). Therefore, a mouse cDNA library was constructed from RANKL-differentiated RAW 264.7 cells (29, 30) representing a range of osteoclasts from single TRAP-positive cells to cells containing over 20 nuclei (Fig. 1A) in the HybriZap 2.1 phagemid vector system (37). The cDNA library was probed in YRG-2 yeast, using a construct of NTα3 (the N-terminal domain of α3, amino acids 1–393; see Table 1) in the yeast two-hybrid GAL4 binding domain vector pBD GAL4 Cam. Although many hits were obtained, the outcome of this screening was difficult to interpret because of unresolved issues with a high background of self-activation with all α subunit bait constructs attempted (data not shown except for NTα3); however, a strong interaction that was consistently above background was found with the full-length B2 subunit of V-ATPase (Fig. 1B). This interaction was confirmed using the second reporter gene, lacZ, and by affinity pulldowns of NTα3 with B2-coated glutathione-Sepharose 4B beads (Fig. 1C).

It was of interest to determine whether there were differences among the four murine α subunit isoforms (α1-4) in terms of their interactions with the two murine B isoforms (B1 and B2). TRX fusion proteins (with His tag), NTα1, NTα2, NTα3, and NTα4, were expressed in E. coli and purified using standard Ni(II) affinity chromatography. GST fusion proteins, B1 and B2, were similarly prepared but using glutathione affinity chromatography (Fig. 2A; see also “Experimental Proce-
**TABLE 1**
Primer, plasmid constructs, and protein expression products

| Subunit (domain) | Expression plasmid | Product (a.a.) |
|-----------------|--------------------|----------------|
| a1 (NTα1 domain) | pET32a-NTα1 | TRX-G2-NTα1(1–397) |
| RAW 264.7 cDNA, 5'-gaattcggtgatggctgttctcgcggag-3' 5'-gtgcacaagcggctgtggtagaag-3' |
| a2 (NTα2 domain) | pET32a-NTα2 | TRX-G2-NTα2(1–402) |
| mouse brain cDNA, 5'-gaattcggtgtgaagatcttcgccgagag-3' 5'-gtgcacaagcggctgtggtagaag-3' |
| a3 (NTα3 domain) | pET32a-NTα3 | TRX-G2-NTα3(1–393) |
| pcDNA 3.1-a3, 5'-gaattcggtgtgaagatcttcgccgagag-3' 5'-gtgcacaagcggctgtggtagaag-3' |
| a3 (NTαT7 domain) | pET32a-NTαT7 | TRX-G2-NTαT7(1–217) |
| pcDNA 3.1-a3, 5'-gaattcggtgtgaagatcttcgccgagag-3' 5'-gtgcacaagcggctgtggtagaag-3' |
| a4 (NTα4 domain) | pET32a-NTα4 | TRX-G2-NTα4(1–399) |
| mouse kidney cDNA, 5'-gaattcggtgtgaagatcttcgccgagag-3' 5'-gtgcacaagcggctgtggtagaag-3' |
| B1 (full-length) | pGEX-4T1-B1(GH) | GST-G2-B1(1–513)-G2H6G2 |
| pEF6/VS-His-TOPO-B1, 5'-gaattcggtgtgaagatcttcgccgagag-3' 5'-gtgcacaagcggctgtggtagaag-3' |
| B2 (full-length) | pGEX-4T1-B2(GH) | GST-G2-B2(1–511)-G2H6G2 |
| RAW 264.7 cDNA, 5'-gaattcggtgtgaagatcttcgccgagag-3' 5'-gtgcacaagcggctgtggtagaag-3' |
| B2 (NTB2 domain) | pGEX-4T1-NTB2 | GST-G2-B2(1–112) |
| pAD-B2, 5'-gaattcggtgtgaagatcttcgccgagag-3' 5'-gtgcacaagcggctgtggtagaag-3' |
| B2 (CTB2 domain) | pGEX-4T1-CTB2 | GST-G2-B2(113–511) |
| pAD-B2, 5'-gaattcggtgtgaagatcttcgccgagag-3' 5'-gtgcacaagcggctgtggtagaag-3' |

* All subunits were cloned partially, as their hydrophilic N-terminal (NT) cytoplasmic domains; V-ATPase subunits were of mouse origin; all PCR primers had a small, random 5' extension (not shown) to improve restriction enzyme cleavage.
* pET constructs were in pET32a(+), pGEX constructs in pGEX-4T-1; PCR products were EcoRI/SalI-digested and ligated into EcoRI/SalI-digested vectors; all constructs were verified by full-length sequencing of inserts and junctions.
* Expressed protein domain organization is indicated as follows: TRX and GST are N-terminal E. coli thioredoxin (with His tag and S tag domains) and S. japonicum glutathione S-transferase fusions, respectively; G2 is a Gly-Gly coupler; and G2H6G2 is a C-terminal His tag extension flanked by Gly-Gly sequences; the superscript numbers in parentheses are the amino acid (a.a.) ranges of the expressed subunits (homologous target V-ATPase subunit sequence only, numbered with respect to the natural subunit N-terminal methionine).
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![Image](https://example.com/image.png)

FIGURE 2. Expression of NTa and B subunits and pulldown assays of subunit pairs. A, affinity-purified fusion proteins (2 μg, each) separated on 8% SDS-PAGE and stained with Coomassie Blue R-250. Left panel shows proteins expressed from pET32a (+) vector; M, molecular weight standards; followed by (2 μg each) a1, TRX-NTa1 fusion protein; a2, TRX-NTa2; a3, TRX-NTa3; a4, TRX-NTa4. Right panel shows proteins expressed from pGEX-4T-1 vector; B1, GST-B1(GH) (left lane, affinity-purified on GST beads; right lane, repurified on Ni(II) beads); B2, GST-B2(GH) (left lane, affinity-purified on GST beads; right lane, repurified on Ni(II) beads). Photographs are of dried gels. B, pulldowns of TRX fusions of all N-terminal a subunit domains with GST fusions of either B1 or B2 subunits. Glutathione beads were coated with bacterial lysates from cells expressing either GST-B1 or GST-B2 fusion proteins (1 μg/ml total protein), washed, and then exposed to lysates containing TRX-NTa fusions derived from a1, a2, a3, and a4 subunits (1 μg/ml total protein). Washed beads were eluted and run on 8% SDS-PAGE, blotted, and probed with anti-GST antibody. Image was developed using chemiluminescence.

dures’ and Table 1). Affinity pulldowns, as shown in Fig. 2B, were performed, showing that a1, a2, a3, and a4 were efficiently pulled down with both B1 and B2, with somewhat less a2 and strikingly more a3 being pulled down; however, differences between the comparative abilities of B1 and B2 to pull down any of the NTa isoforms were not discernible in replicate experiments.

To make more quantifiable determinations of relative binding, an ELISA-based solid-phase binding assay was designed to compare saturation curves for the binding of NTa3, the N-terminal domain of the osteoclast specific isofrom, with B1 and B2. Saturation curves (with log abscissae), shown in Fig. 3A, again indicated that differences between NTa3-B1 binding and NTa3-B2 binding were not significant at p ≤ 0.05. Half-maximal binding values were 2.7 ± 0.5 nM for NTa3-B1 and 1.9 ± 0.4 nM for NTa3-B2.

Because a3 and B2 subunit isoforms both are highly expressed in the plasma membranes of active osteoclasts (38, 39), attention was focused on that pair, and experiments were done to further delineate the sites of binding. A splice variant of the a3 subunit, referred to as Tirc7, with an N-terminal truncation of 217 amino acids, is expressed in T cells (40). This variant may delineate a natural domain boundary between the deleted, non-Tirc7, N-terminal half of NTa3 (referred to here as ΔT7) and the Tirc7-specific, C-terminal half of NTa3 (referred to here as T7). We prepared NTa3ΔT7 and NTa3T7 fusion constructs (Fig. 3B, inset, and see Table 1) and, as shown in Fig. 3B, used these to probe B2 ligand, in a reverse of the binding experiment shown in Fig. 3A. NTa3 binding to immobilized B2 was observed at a much lower apparent affinity than B2 binding to immobilized NTa3 (950 ± 310 nM, half-maximal;

![Image](https://example.com/image.png)

FIGURE 3. ELISA-based binding assays of NTa3 interactions with B1 and B2 subunits. A shows ELISA saturation curves of B1 (heavy line and error bars) and B2 (light line and error bars) binding to NTa3. ELISA plates were coated with TRX-NTa3 ligand and probed with 2-fold serial dilutions of analyte (GST-B1 or GST-B2 fusion proteins, 9.8 pM to 160 nM). Binding of GST alone was negligible, as were the signals obtained without ligand or either analyte protein (data not shown). Absorbance at 450 nm was determined after staining with an anti-GST-HRP sandwich and color development with 3,3′,5,5′-tetramethylbenzidine (see “Experimental Procedures”). Absorbance at the reference wavelength of 600 nm was subtracted to normalize optical variance among wells (A600 was typically <3% of the maximum A450 signal). Each curve shows means ± S.D. bars (n = 3, in duplicate). Differences between B1 and B2 interacting with NTa3 were not significant. B shows ELISA saturation curves of NTa3ΔT7 (heavy line and error bars), NTa3T7 (light line and error bars), and NTa3(dashed line) binding to CTB2. ELISA plates were coated with GST-CTB2 ligand and probed with 2-fold serial dilutions of analyte (TRX-NTa3ΔT7, TRX-NTa3T7, or TRX-NTa3 fusion proteins, 25 pM to 400 nM). Shown also is purification of TRX-NTa3ΔT7 and TRX-NTa3T7 (SDS-PAGE, inset panel), TRX alone showed negligible binding (data not shown). Each curve shows means ± S.D. bars (n = 3, in duplicate).
a 500-fold lower apparent affinity). Surprisingly, the fragmental NTa3 not only bound with a significantly higher apparent affinity ($p = 0.01$) than the intact moiety, but both halves bound equally well. The half-maximal binding concentrations were $121 \pm 84$ nM for NTa3ΔT7 and $134 \pm 81$ nM for NTa3T7, which are not significantly different. Possible explanations for these apparent anomalies are addressed under “Discussion.”

Holliday et al. (41) split B2 into an N-terminal domain (amino acids 1–117 in the mouse ortholog) and a C-terminal domain (amino acids 118–511) and showed that actin binding was confined to the N-terminal domain. In this study, similar constructs were prepared (Table 1) to express GST fusions of N- and C-terminal domains of B2 (Fig. 4A), and solid-phase binding assays were performed (Fig. 4B). In contrast to actin binding, a3 binding to the B2 subunit appeared to be confined to the C-terminal domain, CTB2 (Fig. 4B). Binding of CTB2 to NTa3 was half-maximal at $4.8 \pm 1.2$ nM, which was not significantly different from binding to the full-length B2 subunit (half-maximal at $3.6 \pm 0.8$ nM), whereas binding of the N-terminal domain (NTB2) to NTa3 was reduced 50-fold, being half-maximal at $240 \pm 30$ nM. This difference was highly significant ($p = 0.0002$).

It was of interest to compare the binding of a1–4 subunit isoforms to B2. To do this, ELISA plates were coated with CTB2 ligand and probed with a1–4 analytes (Fig. 4C). Interestingly, NTa3 showed a significantly higher (>2-fold) apparent affinity (half-maximal at $390 \pm 32$ nM) for binding CTB2 than was seen for NTa2-CTB2 ($p = 0.006$) and NTa4-CTB2 ($p = 0.02$) binding, which were statistically indistinguishable at (half-maximal values of $830 \pm 140$ nM and $1.1 \pm 0.3$ nM, respectively). The NTa1-CTB2 interaction seemed to have the lowest apparent affinity, 6-fold less than that of the NTa3-CTB2 interaction, with a half-maximal value of $2.5 \pm 1.3$ nM, which could be distinguished from the NTa2 and NTa4-B2 interactions ($p = 0.01$). The data suggest that a3-B2 interaction may have a privileged affinity, in comparison with the other a-B subunit interactions.

This latter observation begs the question whether binding of a1–4 subunit isoforms to B1 follows a similar distribution. Fig. 4D shows that this is not the case. Apparent affinities are as follows: NTa1-B1, $2.3 \pm 0.4$ nM; NTa2-B1, $460 \pm 57$ nM; NTa3-B1, $740 \pm 240$ nM; NTa4-B1, $1.24 \pm 0.04$ nM. It is of interest that although the apparent affinities of binding are nearly identical for a1 and a4 with B1 and B2, a2 and a3 have switched relative
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positions, i.e. a3 paired with B2 seems to be favored in the B2-binding series, whereas a2 paired with B1 seems to be favored in the B1-binding series. Statistical tests, however, indicate that, for binding to B1, a2 and a4 are different (p = 0.001) and a1 binding differs from the rest of the subunits (p ≤ 0.05), but a2 and a3 are not significantly different (p = 0.25).

One of the proposed functions of the a subunit in the V-ATPase complex is to act as a stator to prevent futile rotation of the A3B3 catalytic headpiece. It is presumed to accomplish this by anchoring to the membrane through its C-terminal domain, as part of the V6 complex, and binding to the V1 complex through its N-terminal domain, by interactions with the catalytic head group and peripheral stalk components (12, 27).

We speculated that small molecule inhibitors of a3-B2 interaction might disrupt stator organization and/or destabilize the V1V6 complex and provide a means of regulating V-ATPase activity, possibly specifically targeting the plasma membrane V-ATPase that contains the a3 and B2 subunit isoforms. This could have therapeutic potential for alleviating bone loss disease (13) and limiting the metastatic potential of some tumors (15).

To screen for small molecule inhibitors, we adapted the above described ELISA-based solid-phase binding assay for use in a high throughput robotics platform (see under “Experimental Procedures”). Synthetic chemical screening yielded primary hits at a rate of ~0.2%. Rescreening and evaluation of primary assay dose-response curves reduced this to 0.04% of compounds initially tested. B-scores (35), for all 10,241 compounds that were screened, are plotted in Fig. 5A.

The model compound discussed here, KM91104, indicated with a heavy circle, Fig. 5A, had a B-score of minus 14.8. Interestingly, the only analog of KM91104 in the 10,000-compound library (light circle, Fig. 5A, identified here as KM91201) was also identified as a primary hit, but it failed to reproducingly give a low B-score on rescreening. Other compounds, including those with a lower B-score than KM91104, were mostly eliminated in rescreening. Of the four compounds that reproducibly gave low B-scores, all except KM91104 were found to be overtly toxic to mammalian cells in growth and cytotoxicity assays (see “Experimental Procedures”). The structures of KM91104 and KM91201 are shown in Fig. 5B.

Secondary screening of compounds consisted of performing growth and cytotoxicity assays, testing effects on osteoclast differentiation and maturation, and testing whether osteoclast mineral resorption, which is dependent on V-ATPase-mediated acid secretion, could be effectively inhibited. Assays were developed using formats that would enable future large scale high throughput screening. Fig. 6A shows that 5-day growth of undifferentiated RAW 264.7 cells was negligibly affected by concentrations of KM91104 up to 20 μM, with an IC50 ≥40 μM. Fig. 6B shows that there was negligible cytotoxicity, according to the mitochondrial reductase-based MTS assay, up to 2.5 μM, with an IC50 of ~20 μM. Thus, the potentially cytotoxic effects of KM91104 seemed to become manifest only at a >20 μM concentration.

It was of interest also to determine effects on osteoclastogenesis. Fig. 7 shows the effects of KM91104 on RAW 264.7 cell differentiation in the presence of RANKL. In Fig. 7A, expres-
BMM cells in primary tissue culture exposed to 1.2 μM KM91104. There did not appear to be any inhibition of differentiation or maturation of these authentic osteoclasts compared with untreated cultures (data not shown).

Finally, it was of greatest interest to determine whether KM91104 is able to inhibit osteoclast resorptive activity. Fig. 8A shows photomicrographs of synthetic hydroxyapatite mineral surfaces (Corning Osteo-Assay Surface, post-stained black with metallic silver; images are ImageJ (National Institutes of Health)-processed as described under “Experimental Procedures”) where resorption has occurred (white areas) due to active acid secretion by osteoclasts differentiated in situ with RANKL from RAW 264.7 cells. Fig. 8B shows the quantified resorption areas from these experiments, indicating that KM91104 does inhibit osteoclast mineral resorptive activity, with an EC_{50} of approximately 1.2 μM. This suggests that there is an acceptable ratio (≈20-fold) between the concentration of KM91104 that is efficacious in reducing mineral resorption (≈1 μM) and the concentration that is toxic, as measured either by cytotoxicity assays or assays of cell differentiation (≥20 μM). Because these data were derived using an artificial mineral surface, we also tested KM91104 using dentin (150-μm-thick ivory slices from elephant), which is a means of assessing authentic bone resorption. Fluorescence images are shown in Fig. 8C, where type I collagen, exposed by osteoclast resorption, is stained by the fluorescent dye, Picro-Sirius Red. Quantified resorption areas are shown in Fig. 8D. These data suggest that the efficacy of KM91104 in inhibiting authentic bone resorption is greater than in inhibiting resorption of the artificial mineral surface. Likely there are differences in the way that osteoclasts mobilize hydrated mineral interspersed with extra-cellular matrix proteins, as in dentin, compared with dissolving a continuous microcrystalline surface, as is found in the commercial test plates.

DISCUSSION

This study has demonstrated that mouse V-ATPase α and B subunits interact. This protein-protein interaction has not been
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FIGURE 8. Secondary screening for inhibition of hydroxyapatite resorption by RANKL-differentiated RAW 264.7 cells. A, cells were differentiated in the presence of 100 ng/ml RANKL on synthetic mineralized surfaces of Corning Osteo-Assay Surface 96-well plates. Cells were cultured for 5 days with continuous exposure to RANKL and KM91104. The complete medium was changed on the 3rd day. On day 5, cells were stripped with 1.2% sodium hypochlorite solution, and the mineral surface was stained using a modified von Kossa method (see “Experimental Procedures”). Plates were air-dried and imaged using digital bright field photomicrography. Images shown are ImageJ-processed for quantification. Concentrations of KM91104 (μM) in medium were as follows: panel i, control; panel ii, 0.6; panel iii, 1.2; panel iv, 2.5; panel v, 5.0; panel vi, 10; panel vii, 20; panel viii, 40. B shows quantitative image analysis of resorption areas (white areas shown in A), using ImageJ software. The concentration range of KM91104 was from 0.6 to 40 μM, as indicated (n = 3, in duplicate, 5 fields per well imaged; error bars are ± S.D.). C indicates control, vehicle only added. Highly significant reduction in resorption (p < 0.0001) was observed even at the lowest concentration. Approximate IC_{50} value of KM91104 for resorption was 1.2 μM, a concentration that was not cytotoxic and did not significantly affect osteoclast differentiation or fusion to form large osteoclasts (see Figs. 6 and 7). C, osteoclasts derived from RAW 264.7 cells were seeded on dentin (elephant ivory) slices. After 3 days of resorption, slices were stained with Picro-Sirius Red and viewed by epifluorescence (GFP filters). Left column is representative of fluorescence image, and right column is ImageJ-processed to quantify fluorescence (white) on a black background. Panels i and ii, control (vehicle only); panels iii and iv, 1.25 μM KM91104; panels v and vi, 40 μM KM91104. D, quantified fluorescence from C (average of results from three independent slices; error bars are ± S.D.).

95式のV-ATPase aとBサブニュートン相互作用

脱核化されたRAW 264.7細胞、3日後に白い象牙（エレファントジャスパー）スライスに植え付け、RANKLとKM91104の濃度範囲で反応を観察した。KM91104の濃度が0.6から40μMまで変化し、各濃度で有意な抑制効果（p<0.0001）が観察された。最も低い濃度における約IC_{50}値は1.2μMであり、細胞毒性を示さず、骨吸収を抑制した。

骨吸収の3日後のスライスはPicro-Sirius Redで染色され、玉子光（GFPフィルター）を用いて観察した。左側はフローセンス像で、右側はイメージジェン処理を用いて fluorescence （白色）を黑背景に強調した画像である。

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that both of the two predicted finger-like projections of NTα lie in interfacial grooves between A and B subunits of the catalytic head group, making contact with both subunits simultaneously. Because of the distances involved, this must occur near the ends of the A and B subunits that are nearest the V1/V0 interface.

There are six interfacial grooves in the catalytic headpiece of V-ATPase, in two different conformations, three AB grooves that both of the two predicted finger-like projections of NTα lie in interfacial grooves between A and B subunits of the catalytic head group, making contact with both subunits simultaneously. Because of the distances involved, this must occur near the ends of the A and B subunits that are nearest the V1/V0 interface.

There are six interfacial grooves in the catalytic headpiece of V-ATPase, in two different conformations, three AB grooves and lack of actin binding is thought to prevent V-ATPase from trafficking to the plasma membrane, where it needs to be for crucial acid secretion to take place in osteoclast bone resorption; however, oc/oc mice were shown to be truncation mutants in the V-ATPase a3 subunit gene (48). These observations beg the following question. How can a mutation in a3 affect the interaction between the B subunit and F-actin? We show here for the first time that there is a direct interaction between a and B subunits. Holliday et al. (41), who first posed this question, speculated that the a subunit might directly regulate access to the F-actin-binding site in the N-terminal domain of the B subunit. At the time, as now, structural models of V-ATPase lacked the resolution to prove a direct interaction between NTα and the F-actin-binding site near the N terminus of B. We have shown here that NTα does not bind to the N-terminal domain of the B subunit but rather to the C-terminal domain, which is not directly involved in actin binding. Furthermore, it is difficult to conceive how a single a subunit could simultaneously occlude the potential binding sites of the three available B subunits in V1 by physically blocking F-actin binding, even by an allosteric mechanism. It seems more likely that a single a subunit might exert some allosteric influence on the B subunit with which it makes contact to promote, rather than inhibit, F-actin binding, to B2 by KM91104. A, plates were coated with either TRX-NTα (for B2 analyte) or GST-CTB2 (for NTαΔT7 or NTαT7 analytes). TRX-NTα, TRX-NTαΔT7, and TRX-NTαT7 were used at 400, 47, and 158 μM constant concentration and curve-fitting. Only values for KM91104 are shown. Unpaired two-tailed t tests show that these values are not significantly different. Curves for KM91201 were essentially nonconvergent, with lowest of range having an IC50 >80 μM.

In the work of Lau and Rubinstein (44), the positioning of the peripheral stalks appears to favor contact with the AB interface, rather than the BA interface. In T. thermophilus V-ATPase, it has been shown that the BA interface is involved in ATP binding and catalysis, whereas the AB groove does not participate in either function (45). If the a-B interaction is purely structural, as in a stator function, one might argue that it is more likely to occur at the AB interface, so as to avoid interference with ATP binding and hydrolysis and the local conformational changes that ensue. This argument lends further support to the notion that NTα contacts the catalytic headpiece at AB interfaces.

This study has further demonstrated that the B subunit interaction with the a subunit is localized to a domain of the B subunit that is spatially distinct from the one that interacts with actin microfilaments. The B subunit can be crudely approximated by a prolate ellipsoid with its long axis perpendicular to the plane of the membrane bilayer. EM images suggest that actin binding to the N-terminal domain of the B subunit is distal to the membrane plane in the long axis of the B subunit (41, 42). The C terminus of the B subunit is thought to be at the opposite end, near the V1/V0 interface, proximal to the membrane plane. This orientation is also supported by analogy with the F-ATPase crystal structure (46). The model proposed here (Fig. 10) is consistent with these observations, taking into account the limited “reach” that the two fingers of NTα would likely have from the V1/V0 interface into the V1 sector.

A defect is observed in V-ATPase association with actin microfilaments in oc/oc mutant mice, which have an osteopetrotic phenotype (41, 47). Actin binds the V-ATPase B subunits, and lack of actin binding is thought to prevent V-ATPase from trafficking to the plasma membrane, where it needs to be for crucial acid secretion to take place in osteoclast bone resorption; however, oc/oc mice were shown to be truncation mutants in the V-ATPase a3 subunit gene (48). These observations beg the following question. How can a mutation in a3 affect the interaction between the B subunit and F-actin? We show here for the first time that there is a direct interaction between a and B subunits. Holliday et al. (41), who first posed this question, speculated that the a subunit might directly regulate access to the F-actin-binding site in the N-terminal domain of the B subunit. At the time, as now, structural models of V-ATPase lacked the resolution to prove a direct interaction between NTα and the F-actin-binding site near the N terminus of B. We have shown here that NTα does not bind to the N-terminal domain of the B subunit but rather to the C-terminal domain, which is not directly involved in actin binding. Furthermore, it is difficult to conceive how a single a subunit could simultaneously occlude the potential binding sites of the three available B subunits in V1 by physically blocking F-actin binding, even by an allosteric mechanism. It seems more likely that a single a subunit might exert some allosteric influence on the B subunit with which it makes contact to promote, rather than inhibit, F-actin binding.
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binding. Actin binding would then presumably occur only in the B subunit(s) that interacts directly with the a3 subunit. In vitro binding experiments, however, do not require the a subunit to be present for F-actin to bind to the B subunit, so one needs to complicate this model with the notion that such regulation occurs only in native V-ATPase complexes. This explanation remains unsatisfying and suggests that there are as yet unknown factors involved. There is still much work to be done to fully understand this regulatory mechanism and to understand the functional consequences of a-B subunit interaction.

The functional significance of the NTa3-B2 interaction remains to be determined, but speculatively it may be important to the stator function of the a subunit, its role in docking of the V1 to the V0 sector, and possibly in intra-complex regulatory signal transduction mediated by the a subunit in response to pH or cellular energy status (1, 49–51). In this study, we have exploited the knowledge of the a3-B2 interaction to screen for inhibitors, using a simple in vitro a3-B2 binding model. It was largely speculative that such an inhibitor might also disrupt V-ATPase function in cells; however, one
compound, KM91104, identified as an inhibitor in the primary screen has proved to inhibit bone resorption by osteoclasts, which is entirely dependent on V-ATPase-mediated acid secretion. The data of Fig. 8 lend support to the initial assumption that small molecule inhibitors of a3-B2 interaction might destabilize the V1/V0 association of the V-ATPase complex that is required for active proton translocation in vivo. It is also noteworthy that the IC50 for inhibition of osteoclast resorption (~1.2 μM) is very similar to the IC50 seen for in vitro inhibition of a3-B2 protein interaction (2.3 μM).

In the RANKL-differentiated RAW 264.7 cell osteoclast model, V-ATPase a1, a2, and a3 subunits are expressed (in our hands, a2 is difficult to detect). The a3 subunit is found in late endosomes/lysosomes and the plasma membrane (the ruffled border of differentiated osteoclasts). The a1 and a2 subunits are found in Golgi, and a1 is also found in other nonlysosomal, non-Golgi organelles (52). The a4 subunit is thought to be kidney-specific, and the others are expressed fairly ubiquitously in many tissues. Nevertheless, mutations in a3 that cause malignant osteopetrosis do not cause any pathology that is not related to loss of osteoclast resorptive function. In many tissues, other subunits may be able to complement loss of a3 function, but in osteoclasts a3 function seems to be irreplaceable (38). Nyman and Väänänen (53) have recently argued this point, suggesting that a1 can compensate for a3 in lysosomes but not significantly in the plasma membrane of bone osteoclasts. They concluded that structure-independent mechanisms may cause bone resorption to be far more sensitive than lysosomal function to V-ATPase inhibition and that "submaximal inhibition of V-ATPase could have a dramatic impact on bone resorption with little impact on lysosomal function." Thus, highly specific inhibitors of V-ATPase may not be required to specifically inhibit osteoclast resorptive function. A relatively nonspecific inhibitor, like KM91104, may therefore find some utility or at least point the way for further development of novel bone loss therapeutics.

Our data also suggest that the a3-B2 interaction may have a moderately higher affinity than other subunit pairs. This may drive a3-B2 formation in preference to other combinations when the two subunits are available. Whether this property can be exploited therapeutically is presently unclear.

The broader cell biological mechanism of action of K91104 inhibition of a3-B2 remains to be determined; it could be via disruption of catalytic function, disruption of proton translocation, interference with V-ATPase trafficking to the plasma membrane, interfering with actin ring formation, or other possible scenarios. Nevertheless, the data presented here suggest that development of a targeted inhibitor of a3-B2 interaction that exploits differences among the a subunit polypeptide sequences may be possible and might be useful in regulating osteoclast bone resorption activity at doses that preclude cytotoxicity or have an appreciable effect upon osteoclast differentiation or maturation.

Reducing bone resorption, while increasing bone formation, would be ideal to prevent pathological bone loss and, at the same time, restore bone that has been lost, yet clinical studies attempting this approach have failed. Previous or concomitant bisphosphonate therapy suppresses the efficacy of parathyroid hormone-related protein (teriparatide) therapy for increasing bone formation (54–57). It is speculated that bisphosphonates, by inducing apoptosis in osteoclasts (58), also eliminate osteoclast-osteoblast signaling essential for parathyroid hormone-stimulated bone formation. A therapeutic that inhibits osteoclast bone resorption, without affecting osteoclastogenesis, would be advantageous over bisphosphonates, which are the current gold standard for bone loss therapy. We show here, with the screening of a relatively small library, that selecting inhibitors of a3-B2 interaction is a viable approach. Although the compound we have selected (KM91104) may not show sufficient specificity, it provides proof-of-principle that a larger screen might provide the “magic bullet” specific to a3-B2 disruption.

In summary, we show here for the first time that the V-ATPase subunits a and B interact. A compound selected to inhibit this interaction reduced osteoclast resorption at concentrations that had no appreciable effect on osteoclastogenesis. A therapeutic that blocks resorption but not osteoclastogenesis would be advantageous over bisphosphonate treatment by preserving osteoclast-osteoblast signaling and thus could potentially be used concurrently and synergistically with osteoblast bone formation therapies. Our data also validate our approach for screening chemical libraries for potential osteoclast V-ATPase targeted bone-loss therapeutics and support the notion that the a3-B2 interaction is biologically important.

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