Enoxacin Directly Inhibits Osteoclastogenesis without Inducing Apoptosis*5


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Enoxacin has been identified as a small molecule inhibitor of binding between the B2-subunit of vacuolar H+--ATPase (V-ATPase) and microfilaments. It inhibits bone resorption by calcitriol-stimulated mouse marrow cultures. We hypothesized that enoxacin acts directly and specifically on osteoclasts by disrupting the interaction between plasma membrane-directed V-ATPases, which contain the osteoclast-selective a3-subunit of V-ATPase, and microfilaments. Consistent with this hypothesis, enoxacin dose-dependently reduced the number of multinuclear cells expressing tartrate-resistant acid phosphatase (TRAP) activity produced by RANK-L-stimulated osteoclast precursors. Enoxacin (50 μM) did not induce apoptosis as measured by TUNEL and caspase-3 assays. V-ATPases containing the a3-subunit, but not the “housekeeping” a1-subunit, were isolated bound to actin. Treatment with enoxacin reduced the association of V-ATPase subunits with the detergent-insoluble cytoskeleton. Quantitative PCR revealed that enoxacin triggered significant reductions in several osteoclast-selective mRNAs, but levels of various osteoclast proteins were not reduced, as determined by quantitative immuno-blot. When their mRNA levels were reduced. Immunoblot demonstrated that proteolytic processing of TRAP5b and the cytoskeletal protein L-plastin was altered in cells treated with 50 μM enoxacin. Flow cytomtery revealed that enoxacin treatment favored the expression of high levels of DC-STAMP on the surface of osteoclasts. Our data show that enoxacin directly inhibits osteoclast formation without affecting cell viability by a novel mechanism that involves changes in posttranslational processing and trafficking of several proteins with known roles in osteoclast function. We propose that these effects are downstream to blocking the binding interaction between a3-containing V-ATPases and microfilaments.

Enoxacin is a fluoroquinolone antibiotic that has been used extensively in humans for the treatment of urinary tract infections and gonorrhea with minimal side effects (2). Recently, unexpected properties of enoxacin have come to light. Our group, making use of a rational reverse chemical genetic strategy, identified enoxacin as an inhibitor of vacuolar H+-ATPase (V-ATPase)3-microfilament binding and of osteoclast formation and bone resorption in cell culture (1). Concurrently, others have identified enoxacin in screens for small molecule stimulators of RNA interference and microRNA activity (3, 4). Very recently enoxacin was reported to be a “cancer-specific” inhibitor that blocks the growth and metastases of human colorectal cancers in a mouse model (5). Because of the therapeutic potential of enoxacin, it is vital to understand the mechanisms by which it selectively affects osteoclasts.

V-ATPases are essential “housekeeping” enzymes in all eukaryotic cells that are necessary for the acidification of compartments of the endocytic and phagocytic pathways (6, 7). Most cell types express only the low levels of V-ATPase required to carry out housekeeping functions, but some cell types also contain an additional subset of V-ATPases that plays...
a role in the unique functions of the cell. V-ATPases are composed of more than 10 subunits, and a number of these subunits have multiple isoforms. Housekeeping V-ATPases are composed of a specific subset of subunit isoforms, whereas non-housekeeping V-ATPases are marked by the inclusion of cell type-restricted isoforms of one or more of the subunits. These subpopulations are targeted and utilized differently than the housekeeping enzymes. Although it is well documented that particular isoforms of certain V-ATPase subunits are found in V-ATPases that are targeted to atypical cellular locations, the underlying mechanisms by which isoforms contribute to differential targeting and use of V-ATPases are not understood (7).

Osteoclasts are highly specialized cells that have the capacity to resorb bone or, perhaps more accurately, to invade mineralized tissue in a regulated manner (8). They express very high levels of a subpopulation of V-ATPases (a3- and d2-containing V-ATPases) that are targeted to the ruffled plasma membrane, a subdomain of the plasma membrane, when osteoclasts encounter bone (9–11). The targeting of V-ATPases to the ruffled plasma membrane is absolutely required for bone resorption (11). Mutations in the a3 and d2 isoforms of V-ATPase lead to osteopetrosis in mice (a3 and d2) and humans (a3) (11–13). Recently, interest has increased in osteoclast V-ATPase-directed therapeutic development because it has been demonstrated that the osteopetrotic pathology associated with the a3 and d2 mutations involves both reductions in bone resorption and increases in bone formation (13, 14). Because neither a3 nor d2 is expressed in osteoblasts (11, 13), it is thought that these mutations set up a situation where osteoclasts are rendered unable to resorb bone efficiently but continue to produce signals that recruit and activate osteoclasts to form bone. It has been proposed that osteoclast inhibitors that mimic the effects of these mutations might prove to be bone anabolic and therefore particularly useful for the treatment of osteoporosis (14). A recent mouse study directly supports this idea (15). Unfortunately, the nature of the unique activities conferred on V-ATPases by the presence of a3 or d2 has not been identified.

Cancer cells also express relatively high levels of V-ATPase, and some have high levels of the a3-subunit (16). V-ATPases have been shown to enter the plasma membrane of cancer cells, which is proposed to be vital for the survival and metastasis of cancer cells (17). A number of studies have demonstrated that cancer cells are sensitive to V-ATPase inhibitors (18–23), and very recently it was shown that cancer growth and metastasis could be blocked in mice by knocking down of the a3-subunit in B16 melanoma cells (24).

We have found that in osteoclasts, but not most other cell types, a high proportion of V-ATPases is bound to microfilaments (25). This interaction correlates with the activation state of osteoclasts and is mediated by a specific region of the B2-subunit that includes the “profilin-like domain,” so named because of its sequence and structural similarity to the actin-binding domain of mammalian profilin 1 (26–29). The total actin-binding domain region is composed of 44 amino acids (amino acids 29–73 in mammalian B2-subunits) (26). The profilin-like domain is smaller and is composed of amino acids 55–68 in B2. Small changes in the profilin-like domain of the B2-subunit disrupt actin binding activity without interfering with the capacity of the altered B2-subunit to contribute to the enzymatic activity of the multisubunit V-ATPase (30). B-subunits so altered are not targeted to the ruffled plasma membrane of osteoclasts (28).

Although the actin-binding site in the B-subunits (both the B1 and B2 isoforms bind actin (27)) is present in all mammalian V-ATPases, they are not recovered from most cells bound to microfilaments. In yeast, molecular genetic studies have demonstrated that actin binding activity is only important for the growth and survival of yeast in the presence of specific environmental stressors (30). These data suggest that actin binding activity may not be required for the function of housekeeping V-ATPases.

Together, these data led us to hypothesize that small molecules that bind to the profilin-like domain of subunit B2 and competitively interfere with its binding to microfilaments might represent a new class of osteoclast-selective anti-resorptive therapeutic agents. Computational chemistry techniques were used to conduct a virtual screen to identify small molecules predicted to bind the actin binding surface of the B2-subunit and thus block its interaction with microfilaments (1). From the candidates identified, we reported that the fluoroquinolone antibiotic enoxacin blocks the interaction between the recombinant B2-subunit and microfilaments in the test tube and also blocks osteoclast differentiation and bone resorption in mixed cell cultures (1). V-ATPase transport to ruffled plasma membranes of osteoclasts on bone slices in mixed cultures is also disrupted. Enoxacin does not prevent growth or mineralization by osteoblasts at concentrations where osteoclast activity is inhibited. In the current study, we tested the hypothesis that enoxacin acts directly and specifically on osteoclasts by disrupting the interaction between plasma membrane-directed V-ATPases, which contain the osteoclast-selective a3-subunit of V-ATPase, and microfilaments. Based on the phenotype of osteoclasts in which mutations of the a3- and d2-subunits of V-ATPase occur, we anticipated that enoxacin would not provoke apoptosis (11, 13). Because enoxacin inhibited cell fusion and expression of tartrate-resistant acid phosphatase (TRAP) activity, we suspected that the expression levels of certain proteins, including ADAM (a disintegrin and metalloproteinase) 8, ADAM12, and dendritic cell-specific transmembrane protein (DC-STAMP), which are linked to cell fusion, and TRAP5b, might be reduced.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—The polyclonal anti-E-, anti-a3-, and anti-B2-subunit antibodies were described previously (26, 31). The other antibodies used were obtained from the following suppliers as indicated: anti-TRAP antibody (BioLegend, San Diego, catalog No. 648401); anti-leukocyte (L)-plastin (also known as plastin-2; Abcam, Cambridge, MA, catalog No. ab83496); anti-cortactin (sc-25577), anti-DC-STAMP (sc-25579), anti-ADAM12 (sc-25579), anti-nuclear factor activated in T-cells c1 (NFAT-c1; sc7294), and anti-lamin A/C (sc-6215) (all from Santa Cruz Biotechnology, Santa Cruz, CA); anti-calcitonin receptor (Abbiotec, San Diego); anti-Src homology region 2 domain-containing phosphatase-1 (Shp1; Abcam, ab18708); and anti-cathepsin K (Santa Cruz Biotechnology,
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sc-6506). Unless otherwise noted, other antibodies and reagents were obtained from Sigma-Aldrich. Recombinant receptor activator of nuclear factor κB ligand (RANK-L) was produced in *Escherichia coli* as described previously (33) or obtained from Peprotech (Rocky Hill, NJ). Macrophage colony-stimulating factor (CSF-1) was obtained from Peprotech. Enoxacin was obtained from Sigma-Aldrich and dissolved in DMSO (1) or 0.1 M sodium hydroxide (32).

**Osteoclast Differentiation**—To grow primary osteoclasts, the femora and tibiae were removed and marrow was expelled from bones using a syringe with α-MEM complete medium (Sigma-Aldrich) (10% fetal bovine serum (Mediatech), 1% l-glutamine (Thermo Scientific), and 1% penicillin/streptomycin/amphotericin B (Fisher)). Cells were seeded in T75 flasks at a concentration of 1.5 × 10^6 cells/ml supplemented with 5 ng/ml recombinant murine CSF-1 (M-CSF; Peprotech) and allowed to culture for 24 h at 37 °C in 5% CO₂. Non-adherent cells were removed, and adherent cells (5.9 × 10^6 cells/ml) were seeded in standard 24-well plates or on UpCell tissue culture plastic (Nunc). All cultures were supplemented with 10 ng/ml CSF-1 and -5 (33) or 50 ng/ml recombinant murine RANK-L (Peprotech). Cells were treated with vehicle or enoxacin, as indicated, and cultured for 5 or 6 days with complete medium refreshed every 3 days.

Raw 264.7 cells were stimulated to differentiate into osteoclast-like cells with recombinant RANK-L as described previously (33). Raw 264.7 were seeded on 24-well plate at a density of 1.25 × 10⁵ cells/well or on 6-well plates at 1.8 × 10⁶ cells/well. These cells were cultured for 5 days with 5 ng/ml RANK-L and fed on day 3 in culture.

**TRAP Activity Assay**—TRAP activity was detected using the leukocyte acid phosphatase kit (Sigma-Aldrich, catalog No. 387A-KT) following the instructions from the manufacturer. Osteoclasts were detected as staining positive for TRAP activity. TRAP⁺ cells were counted and classified according to the number of nuclei present as mono- or multinuclear (2–10 nuclei) or as giant cells (more than 10 nuclei).

**Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) and Caspase-3 Assays**—After 5 days of cell culture with 50 μM enoxacin, the cells were rinsed twice with PBS and fixed for 2 min in 4% paraformaldehyde at room temperature. Apoptotic cells were detected by using an *in situ* apoptosis detection kit (Promega, G7132) according to the manufacturer's instructions.

To assay for caspase-3 activity cells were plated in 24-well plates at a density of 0.5 × 10⁴ cells/cm² and treated with 50 ng/RANK-L plus or minus 50 μM enoxacin for 24, 48, and, 72 h. Caspase-3 assays were performed following the manufacturer's instructions (catalog No. APT131, Millipore, Temecula, CA). At the end of each time point, the plate was centrifuged at 1200 rpm for 10 min. Cells were treated with 100 μl of chilled cell lysis buffer, and cell lysates were centrifuged at 1200 rpm for 10 min. Cell lysates were treated according to the manufacturer's recommendations and the colorimetric reaction was quantified using a BioTek KC4 spectrophotometer (Winooski, VT) at 405 nm.

**Immunoprecipitations**—Immunoprecipitations were performed as described previously (26, 34). Osteoclasts were generated from Raw 264.7 cells or primary osteoclasts. Cells were washed in PBS and solubilized in Triton X-100 buffer (1% Triton X-100, 20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 0.1% SDS, 10% glycerol, 5 mM sodium azide, and protease inhibitors). After centrifugation at 20,000 × g for 10 min to remove insoluble material, the extracts were incubated for 1 h at 4 °C with 1:200 μg of an anti-a3 or anti-a1 subunit polyclonal antibodies (31). Fifty microliters of protein A beads (Sigma) was added, and the mixture was incubated for 1 h at 4 °C with rocking. The protein A beads were collected by centrifugation at 10,000 × g for 15 s at 4 °C and washed three times with the Triton X-100 buffer. The wash buffers were removed by aspiration with a bent 23-gauge needle, and Laemmli sample buffer was added. The samples were heated to 85 °C for 10 min, cooled to room temperature, and centrifuged at 10,000 × g for 1 min, and the supernatants were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with antibodies as described in the legends to Fig. 1–5.

**Quantitative Immunoblotting**—Immunoblots were performed by standard procedures using the SuperSignal West-Dura chemiluminescence detection system (Thermo Scientific/Pierce). To determine the association of V-ATPase with the detergent-insoluble cytoskeletal fraction, blots of supernatants and pellets were obtained by extraction of osteoclasts with 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 0.5 mM ATP, 0.2 mM CaCl₂, and 0.2 mM dithiothreitol (Buffer F) plus 1% Triton X-100 and protease inhibitors with ultracentrifugation (100,000 × g for 45 min). The samples were heated at 85 °C for 10 min, cooled to room temperature, and centrifuged at 10,000 × g for 1 min, and the supernatants were applied to SDS-polyacylamide gels, transferred to nitrocellulose, and probed with antibodies as described in the legends to Fig. 3 and 4. Cell sample dilutions were applied to SDS-polyacylamide gel and stained with Coomassie Blue stain (Bio-Rad, catalog No. 161-0436) using staining levels of multiple proteins to establish equal loading between treatment groups. Blots were then performed to identify and put target proteins into the linear range of detection by immunoblot. Samples from enoxacin treatment were compared with vehicle controls to correlate changes in protein levels with dosages of enoxacin. Bands were quantitated by densitometry using an Alpha Innotech FluorChem 8000 (Alpha Innotech, San Leandro, CA).

**Real-time PCR**—Total RNA from Raw 264.7 cells was isolated with an RNAeasy mini kit (Qiagen, Tokyo) and real-time quantitative PCR (qPCR) was performed using the TaqMan One-Step RT-PCR master mix reagents kit and an ABI Prism 7700 HT detection system (Applied Biosystems, Foster City, CA) (33). Probes and primers for the mouse genes that encode the following: c-fos, c-jun, c-myc, bcl-2 (Mm00487425_m1), integrin β3 (Mm0043980_m1), nuclear factor activated in T-cells (Nfatc1; Mm00479445_m1), SFFV proviral integration 1 (Pu.1; Mm00488142_m1), and hypoxanthine phosphoribosyltransferase 1 (Hprt1; Mm00446968_m1) were obtained from...
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FIGURE 1. Enoxacin dose-dependently reduces the number of TRAP+ multinuclear osteoclast-like cells that form as the result of RANK-L stimulation of primary marrow and Raw 264.7 cells. A, primary mouse marrow cells were stimulated with 5 ng/ml recombinant RANK-L and 5 ng/ml CSF-1 for 5 days in the presence of vehicle (DMSO) or enoxacin at the micromolar concentrations indicated. At the end of 5 days, cells were fixed and stained for TRAP activity, and the number of mononuclear, multinuclear (2–10 nuclei), and giant cells (>10 nuclei) was counted. The average number of TRAP+ cells in each category in the vehicle-treated cultures was defined as 100%. The numbers in enoxacin-treated cultures are depicted as a percentage of the vehicle controls. The average numbers for the vehicle control cultures were 1299 for mononuclear cells, 845 for multinuclear cells, and 103 for giant cells. B, Raw 264.7 cells were stimulated with 5 ng/ml recombinant RANK-L for 5 days in the presence of vehicle (DMSO) or enoxacin at the micromolar concentrations indicated. At the end of 5 days, cells were fixed and stained for TRAP activity, and the number of mononuclear, multinuclear (2–10 nuclei), and giant cells (>10 nuclei) was counted. The average number of TRAP+ cells in each category in the vehicle-treated cultures was defined as 100%. The numbers in enoxacin-treated cultures are depicted as a percentage of the vehicle controls. The average numbers for the vehicle control cultures were 3810 for mononuclear cells, 1141 for multinuclear cells, and 244 for giant cells. One-way ANOVA was utilized to determine whether there were differences within groups, and Student’s t test was used to test for differences between specific treatment groups and the vehicle control. p < 0.05 was considered significant. C–J, representative photographs from the experiment documented in A–J: vehicle control; D, 10 μM enoxacin; E, 50 μM enoxacin; F, 100 μM enoxacin; G–J, representative photos from the experiment documented in B: G, vehicle control; H, 10 μM enoxacin; I, 50 μM enoxacin; J, 100 μM enoxacin. Scale bar = 50 μm.

Applied Biosciences. Raw 264.7 cells were treated as indicated in the legend to Fig. 4; RNA was isolated and assayed on day 5, and the level of each of the genes relative to HPRT1 was determined. The experiment was analyzed statistically using t tests of the ΔCt values. p < 0.05 was considered significant.

Flow Cytometry—Cells were allowed to dissociate from the bottom of UpCell coated plates (Thermo Scientific) at room temperature for 30–45 min. Suspended cells were washed with fluorescence-activated cell sorter (FACS) buffer (1× PBS, 5% FBS, and 0.372 g of EDTA) and treated with 1:200 dilutions of antibodies for 1 h at 4°C in the dark. Biotin-conjugated rat anti-mouse RANK (eBioscience) with Alexa Fluor 647-conjugated streptavidin (Invitrogen) and rabbit anti-mouse DC-STAMP (Invitrogen) with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen) were used in this study. Cells were acquired using a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FCS Express (De Novo Software).

Statistics—Counters were precalibrated for their ability to identify TRAP+ multinuclear cells and then blinded to treatment groups. Results are expressed as mean ± S.E. Samples were compared by one-way ANOVA and Student’s t test using the program GraphPad Prism 5 (GraphPad Software, La Jolla, CA). p values < 0.05 were considered significant.

RESULTS

Enoxacin Inhibits Differentiation of Primary Marrow Cells and Raw 264.7 Cells into Osteoclasts without Inducing Excess Apoptosis—Primary marrow-derived osteoclast precursors or Raw 264.7 cells were stimulated with recombinant RANK-L and M-CSF in 24-well plates and treated with vehicle or different concentrations of enoxacin. After 5 days, the cells were fixed and stained for TRAP activity and then examined for TRAP activity and fusion into multinuclear cells (both are characteristics of osteoclasts). A significant dose-dependent reduction in the number of TRAP-positive, multinucleated cells was detected in response to enoxacin. The IC50 value was the same for both primary osteoclasts (Fig. 1, A and C–J) and Raw 264.7 cells (Fig. 1, B and G–J).

To determine whether enoxacin had disrupted cell growth or survival, we counted the number of nuclei after 5 days in culture of the Raw 264.7 cells treated with RANK-L plus various concentrations of enoxacin. Although the phenotype of the cells
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was very different when they were treated with enoxacin, there were no significant differences in the number of nuclei at concentrations as high as 50 µM.

To test whether enoxacin induced apoptosis during the maturation process, Raw 264.7 cells were treated with either vehicle or 50 µM enoxacin and stimulated with RANK-L. TUNEL assays were performed. No significant difference was observed in the number of apoptotic cells that was attributable to enoxacin (Fig. 2, B–D). To confirm this result, we assayed RANK-L-stimulated Raw 264.7 cells treated with either vehicle or 50 µM enoxacin for caspase-3 activity (a marker of apoptosis (35)) at various time points after stimulation with RANK-L (Fig. 2E).

Enoxacin significantly reduced the level of caspase-3 activity at 72 h in this assay compared with RANK-L-stimulated cells treated with vehicle. Cells that were not stimulated with RANK-L displayed less caspase-3 activity, and this was not significantly affected by enoxacin.

V-ATPases That Bind Microfilaments in Osteoclasts Contain the a3-subunit but Not the a1-subunit—B2 is expressed in all cell types, but usually V-ATPases are not detected bound to microfilaments (25, 34). We hypothesized that the a-subunit isoform, a3, which is selectively expressed in osteoclasts and is plasma membrane-directed, might be preferentially associated with actin binding activity. To test this idea, primary mouse marrow osteoclasts were extracted with buffer F (20 mM Tris, pH 7.5, 100 mM NaCl, 0.5 mM MgCl2, 0.2 mM CaCl2, 0.2 mM ATP, and 0.2 mM DTT) plus 1% Triton X-100 and subjected to ultracentrifugation to pellet the detergent-insoluble cytoskeletal fraction. The proteins from the supernatant and pellet fractions were collected in equal volumes of SDS-PAGE sample buffer, loaded equivalently on gels, separated by SDS-PAGE, blotted to nitrocellulose, and probed with antibodies to a3 and a1 (Fig. 3A). 92 ± 4% (n = 3; by densitometry of blots) of a3 was found in the pellet, whereas only 7 ± 5% of a1 was present in the pellet. To confirm that a3 was present in the pellets because it was bound to F-actin, primary osteoclasts were homogenized with buffer F plus 1% Triton X-100 and spun at 5,000 × g to remove nuclei and large insoluble material. In preliminary experiments, we found that the amount of actin or V-ATPase in this pellet was less than 5% of the total. We then performed immunoprecipitations from the soluble fraction using either anti-a1 or anti-a3 antisera. Anti-a3 pulled down subunit E, a peripheral subunit of V-ATPase, demonstrating that a3 was assembled with peripheral subunits and with actin. Anti-a1 also pulled down subunit E, but actin was not detected in the a1 immunoprecipitations (Fig. 3B).

As a further control, F-actin was depolymerized in detergent extracts by dialyzing into 20 mM Tris, pH 7.5, 0.2 mM CaCl2, 0.2 mM ATP, and 0.2 mM DTT plus 50 µM latrunculin A (36) for 8 h at 4 °C and then subjecting the samples to ultracentrifugation. The amount of subunit a3 that pelleted from latrunculin-treated extracts (Lat.) was reduced to 24% of the untreated extracts (Ct.) (Fig. 3C) as measured by densitometry. Subunit a3, but not a1, was also associated with the detergent-insoluble cytoskeleton of Raw 264.7 cells (supplemental Fig. S1).

To determine whether enoxacin reduced the amount of V-ATPase associated with the cytoskeleton, we examined the level of B2-, E-, and a3-subunits associated with the detergent-insoluble cytoskeleton plus or minus 50 µM enoxacin for 5 days in both RANK-L-stimulated Raw 264.7 osteoclast-like cells and primary osteoclasts. There was no difference detected in the total level of any of the V-ATPase-subunits, but a shift was detected in the B2-, E-, and a3-subunits from the detergent-insoluble cytoskeleton to the soluble fraction (Fig. 3D and supplemental Fig. S2). This result is consistent with our finding that enoxacin blocks binding between recombinant B2-subunit and microfilaments (1). Although all three subunits assayed were shifted by enoxacin from the cytoskeletal fraction toward the supernatant, the shift was most profound with respect to the B2-subunit.

Enoxacin Reduced Levels of Several Osteoclast and V-ATPase-selective mRNAs, but Protein Levels Were Not Reduced—To examine the molecular mechanisms underlying the effects of enoxacin, we analyzed a number of V-ATPase and osteoclast-selective genes by qPCR (Fig. 4A). Raw 264.7 cells were cultured for 5 days with RANK-L and treated with vehicle or 50 µM enoxacin. We detected small, but significant reductions in the a3-, B2-, and E-subunits of V-ATPase, TRAP5b, L-plastin, c-Fos, and WASH. Larger reductions in the levels of β3 integrin, calcitonin K, and the calcitonin receptor were detected. As a reference, we observed that the expression level of TRAP5b in enoxacin-treated cells was reduced by 1.96-fold compared with control, whereas in RANK-L-stimulation increased its level was 350-fold compared with cells not stimulated with RANK-L. The levels of ADAM8 and PU.1 mRNA were not altered by enoxacin. The level of NFATC1 mRNA was significantly increased in enoxacin-treated cells. Despite the differences in mRNA levels, we were unable to detect any reductions in protein levels in total protein extracts separated by SDS-PAGE and analyzed by quantitative immunoblotting using actin as an internal standard (Fig. 4B and supplemental Fig. S3). These included the a3-, E-, and B2-subunits of V-ATPase, cortactin, DC-STAMP, calcitonin receptor, Shp1, and β3 integrin. Cathepsin K and NFAT-c1 displayed slight increases. In addition, we found that TRAP5b, and L-plastin showed staining patterns most consistent with altered proteolytic processing (Fig. 4, C and D). Dose-dependent reduction in the number of RANK-L-stimulated cells displaying TRAP activity is a characteristic effect of enoxacin, but surprisingly, enoxacin only slightly reduced the level of TRAP5b mRNA. TRAP5b is expressed as a 38-kDa latent pro-enzyme and is proteolytically activated to the active form, which is characterized in blots by a 16-kDa band (37). Anti-TRAP immunoblots displayed the expected 16-kDa band in vehicle controls, but a series of bands starting at 38 kDa, and very little at 16 kDa, was detected in the cultures treated with 50 µM enoxacin (Fig. 4C and supplemental Fig. S4).

Enoxacin was shown previously to inhibit actin ring formation (1). L-plastin, a protein tied to actin ring formation (38–41), was assayed for changes associated with enoxacin treatment. The anti-L-plastin antibody detected a dose-dependent shift from the expected 67-kDa band to a 57-kDa band (Fig. 4D). The amount of the 67-kDa band was reduced by 88% (determined by densitometry). Mass spectroscopy confirmed the presence of L-plastin at 57 kDa (data not shown). Because the anti-L-plastin antibody detects the C terminus, this shift may involve proteolytic cleavage of the N-terminal domain.
FIGURE 2. Enoxacin does not alter the number of viable nuclei or increase the levels of apoptosis as measured by TUNEL and caspase-3 assays. 

A, five randomly selected fields/well of a 24-well plate were counted and averaged. Four wells were counted per treatment group. One-way ANOVA was utilized to determine whether there were differences within groups, and this indicated that there were no differences between any of the groups. Enoxacin at a concentration of 100 μM appeared to reduce the nuclei number, although it did not reach significance. Because of this we focused our studies on the effects of 50 μM enoxacin, a concentration at which osteoclastogenesis and bone resorption (1) were strongly inhibited but numbers of nuclei were not affected. 

B, quantitative analysis of counts of the number of TUNEL-positive cells after 5 days in cultures treated with vehicle or 50 μM enoxacin. C and D, Raw 264.7 cells stimulated with RANK-L for 5 days and treated with vehicle (C) or 50 μM enoxacin (D). Apoptotic nuclei are dark. Arrows point the edges of giant cells in C and the edge of a multinuclear cell in D. Bar = 110 μm. 

E, quantitative analysis of caspase-3 activity of Raw 264.7 cells treated with vehicle, RANK-L (5 ng/ml), and enoxacin 50 μM as noted for the culture times indicated. Units are defined as the amount of enzyme that cleaves 1 nm colorimetric substrate/h. DMSO was used as the vehicle control. No statistically significant differences exist between vehicle and enoxacin treatment in unstimulated (−RANKL) cells. At 48 and 72 h, a significant increase in caspase-3 activity was detected in both treatment groups after stimulation with RANK-L. Enoxacin (ENX) significantly decreased the levels of caspase-3 activity at 72 h compared with control. *, p < 0.05 by one-way ANOVA. The data represent mean ± S.E. (n = 3).
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Enoxacin Stimulates an Increase in the Surface Expression of DC-STAMP and RANK—The seven-transmembrane region receptor DC-STAMP is preferentially expressed in osteoclasts after stimulation by RANK-L. Osteoclasts from DC-STAMP-deficient mice display reduced fusion but comparable TRAP activity, formation of ruffled plasma membranes, and actin rings, indicating that DC-STAMP is not involved in osteoclast activation except for cellular fusion (42). We examined DC-STAMP surface protein expression by immunoblot and FACS analysis. Osteoclasts were stimulated to differentiate for 6 days and then analyzed after 3 additional days of culture with 50 μM enoxacin or vehicle and continued stimulation with RANK-L. Immunoblots had indicated that enoxacin did not affect the overall expression of DC-STAMP in RANK-L-stimulated cells (Fig. 4B). Flow cytometry revealed that after treatment with enoxacin, a significantly higher portion of the cells expressed high levels of DC-STAMP on the cell surface (Fig. 5). Surprisingly, significantly more cells from enoxacin-treated cultures also expressed high levels of RANK on their surfaces (Fig. 5D). This is consistent with enoxacin altering the cellular vesicular trafficking pattern of DC-STAMP and RANK to promote the expression of higher levels of these proteins on the cell surface.

DISCUSSION

In this study, we have shown for the first time that enoxacin inhibits osteoclastogenesis directly with an IC₅₀ of about 10 μM without inducing apoptosis. We demonstrated that V-ATPases containing a3, but not a1, bind to the actin cytoskeleton, which provides a possible explanation of why blocking the interaction between the ubiquitous B2-subunit and microfilaments has cell type-selective consequences. This also provides a first example of an activity (interaction with microfilaments) that is associated with the presence of a specific isoform of the V-ATPase in a cell. Moreover, we directly demonstrated reductions in the relative amounts of V-ATPase subunits associated with the detergent-insoluble “cytoskeletal” fraction that were induced by enoxacin treatment. Although 50 μM enoxacin shifted most of the B2-subunit from the cytoskeletal fraction to the soluble fraction, a smaller proportion of the E-subunit and a3-subunit was solubilized by enoxacin. This indicates that a significant portion of these subunits is not assembled with B2 and that they may be associated with the cytoskeleton by an alternate linkage. One possibility is that these subunits are associated with the C-subunit, which is thought to bind microfilaments when V-ATPases are not fully assembled (43–45).

The protein levels of several V-ATPase subunits and a selection of other osteoclast proteins were not affected by enoxacin. The mRNA levels of a number of osteoclast-selective genes were significantly reduced in enoxacin-treated cells, but others were unchanged, and one, NFATc1, increased. In a number of cases we showed that changes in mRNA levels were not reflected by altered levels of the protein product. For example, although cathepsin K mRNA levels were very significantly reduced, the amount of cathepsin K protein recovered from cells was not reduced. Enoxacin triggered changes in osteoclasts including reduction in the proteolytic activation of TRAP5b, a shift in L-plastin from the 67- to 57-kDa form, and alterations in the transport of DC-STAMP and RANK to the osteoclast plasma membrane.
Changes in the sorting of DC-STAMP to the plasma membrane represent direct evidence that a vesicle sorting pathway in osteoclasts is perturbed by enoxacin. Because DC-STAMP is known to be involved in the fusion of osteoclast precursors (42, 46–48), the change could be linked functionally to the reduction in multinuclear and giant cells detected in enoxacin-treated cultures. Levels of cell surface DC-STAMP have been shown to gradually decline during osteoclastogenesis (49). These results indicate that enoxacin influences DC-STAMP toward a cell surface expression pattern of DC-STAMP associated with less mature osteoclasts.

The failure of TRAP5b to be activated by proteolytic cleavage could also be explained if the interaction between V-ATPase and microfilaments plays a role in directing vesicular traffic.
Acid cysteine proteinase activity has been implicated in the activation of TRAP, suggesting that failure of V-ATPases to be sorted to the same pathway as TRAP5b, and the consequent failure to acidify vesicles might disrupt the action of activating acid proteinases.

A change in L-plastin is consistent with a reduction in the formation of actin rings detected previously in response to enoxacin (1). L-plastin is an actin-cross-linking protein that has been shown to be present in the core of podosomes and in actin rings (50). Regulation of L-plastin levels has been tied to the capacity of osteoclasts to form actin rings and resorb bone (41). Our data suggest that L-plastin is likely cleaved proteolytically so that the N-terminal EF-hand domain is removed in response to enoxacin. The EF-hand binds calcium and mediates inhibition of binding of L-plastin to microfilaments in the presence of calcium (51–53).

The mRNA levels of several osteoclast- and V-ATPase-specific genes were significantly reduced in response to enoxacin. However, reductions in the mRNA levels did not correspond to significant differences in the protein levels. There are a variety of explanations for the observed disconnect including altered translation from mRNAs (54), altered life span of proteins (55) in enoxacin-treated cells and changes in the amount of a specific proteins being secreted. For example, cathepsin K and TRAP5b are both secreted. A reduction in the secretion of these enzymes triggered by enoxacin could lead to maintaining similar cellular levels compared with controls even if mRNA levels and/or protein translation are reduced.

Could the diverse effects we detected all be the result of blocking the interaction between V-ATPase and microfilaments? One explanation for the results relies in the suggestion made by Brown et al. (56) that V-ATPases may serve, like clathrin, caveolin, and coatamer protein complexes, to regulate vesicular trafficking. Brown and colleagues (57) propose that the recently identified interactions between V-ATPases and ARF6 and ARNO could work in coordination with the actin binding activity of B2 to modulate vesicular trafficking and cytoskeletal reorganization. This hypothesis implies that the V-ATPase/microfilament interaction could be a vital node integrating multiple regulatory pathways. This could explain the diverse, but selective, effects that enoxacin has on osteoclasts.

Very strong support for the notion that the V-ATPase/microfilament interaction can have a vital role in vesicular sorting has appeared recently (58). It was reported that the capacity of V-ATPase to bind microfilaments works in coordination with localized actin polymerization nucleated by actin-related protein 2/3 (ARP2/3)-WASH protein complex to sort V-ATPases from exocytic vesicles in Dictyostelium. Although this sorting was in the context of a constitutive exocytic pathway that allows the amoeba to rid itself of indigestible material, WASH and the actin-binding site in the B-subunit are both evolutionarily highly conserved (1, 58). It is plausible that this basic sorting mechanism has been adapted in mammalian cells, like osteoclasts, for cell type- and situation-specific functions. Efforts
are currently under way in our laboratory to test that idea in osteoclasts.

Enoxacin was recently identified as a stimulator of RNA interference and microRNA activity (3). We have reported that pefloxac in, which does not have microRNA stimulating activity (3) but does block the B2-microfilament binding interaction, has similar inhibitory effects on osteoclasts compared with enoxacin (1). We have also identified a non-fluoroquinolone inhibitor of the B2-microfilament interaction, which we refer to as Binhib16, with similar inhibitory effects on osteoclasts.4 Given these results, we have tentatively concluded that the effects on microRNAs are not primarily responsible for the ability of enoxacin to inhibit osteoclasts. However, the complexity of the response of osteoclasts to enoxacin, as well as the dearth of information regarding both V-ATPase trafficking and microRNA function in osteoclasts, currently makes a definitive conclusion impossible.

In summary, enoxacin directly inhibited osteoclasts without triggering apoptosis. It reduced the amount of the B2-subunit bound to the detergent-insoluble cytoskeletal fraction, altered the transport of DC-STAMP to the plasma membrane, and blocked proteolytic activation of TRAP5b from its latent proenzyme to the active form. Enoxacin triggered a reduction in the size of L-plastin, likely because of proteolytic cleavage of the N-terminal calcium-binding EF-hand domain. Enoxacin may be selectively active toward osteoclasts because only those V-ATPases that contain the a3 isoform of subunit a, which is selectively expressed in osteoclasts and required for osteoclast function, are detected bound to microfilaments in osteoclasts. We propose that enoxacin selectively blocks elements of the osteoclast differentiation and activation program that are downstream of the binding interaction among a3-containing V-ATPases and microfilaments, which is mediated by the actin-binding site on the B2-subunit.

This report shows clearly that enoxacin is a novel type of selective inhibitor of osteoclasts. Enoxacin, or other molecules with similar activity, may prove useful for the treatment of osteoporosis and other bone pathologies. Because enoxacin is a potent inhibitor of the growth and metastasis of cancers (5), and selectively inhibits osteoclasts (1), it is a particularly attractive candidate to test as a therapeutic agent for the treatment of bone cancer.

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Enoxacin Directly Inhibits Osteoclast Formation


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