Potent and Selective Cathepsin L Inhibitors Do Not Inhibit Human Osteoclast Resorption in Vitro*

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Cathepsins K and L are related cysteine proteases that have been proposed to play important roles in osteoclast-mediated bone resorption. To further examine the putative role of cathepsin L in bone resorption, we have evaluated selective and potent inhibitors of human cathepsin L and cathepsin K in an in vitro assay of human osteoclastic resorption and an in situ assay of osteoclast activity. The potently selective cathepsin L inhibitors (Ki = 0.0099, 0.034, and 0.27 nM) were inactive in both the in situ cytotoxic assay (IC50 > 1 μM) and the osteoclast-mediated bone resorption assay (IC50 > 300 nM). Conversely, the cathepsin K selective inhibitor was potently active in both the cytotoxic assay (IC50 = 63 nM) and resorption (IC50 = 71 nM) assays. A recently reported dipeptide aldehyde with activity against cathepsins L (Ki = 0.052 nM) and K (Ki = 1.57 nM) was also active in both assays (IC50 = 110 and 115 nM, respectively). These data confirm that cathepsin K and not cathepsin L is the major protease responsible for human osteoclastic bone resorption.

Osteoclasts are multinucleated cells of hematopoietic origin that are responsible for resorbing bone (1, 2). Following tight attachment to the bone surface, osteoclasts begin the resorptive process by secreting protons into the extracellular space (3), which results in the removal of bone mineral and the exposure of the underlying matrix. It also provides optimal conditions for the activity of osteoclast-derived proteases that ultimately solubilize these matrix components, which include type I collagen, osteopontin, osteonectin, and other components. The identification of the key enzyme(s) responsible for this protein matrix degradation is essential for understanding the mechanisms of bone metabolism and for the design of inhibitors of the resorptive process for the intervention of metabolic bone diseases such as osteoporosis.

Numerous reports have suggested that cathepsin L and/or cathepsin K may be the major proteases involved in this process (4–6). However, recent studies have indicated that mRNA for cathepsin L is undetectable in human osteoclasts (5), and the cathepsin L null mouse does not show a bone-related phenotype (7). In contrast, it has been reported that the knockout of cathepsin K results in osteopetrosis that is characterized by osteosclerosis (8, 9). Both microcomputed tomography and histomorphometry indicate an increase in trabecular number and in trabecular and cortical thickening in the knockout animals when compared with their wild-type littermates (9). Furthermore, this mutation leads to the elimination of all osteoclast-related cathepsin activity and ultimately to a reduction in osteoclast-mediated matrix degradation.

The importance of cathepsin K in the resorptive process is further supported by a rare skeletal disorder in humans called pyknomysostosis, which occurs as a result of mutations in the cathepsin K gene, resulting in elimination of cathepsin K activity (10). The disorder is characterized by a reduction in the rate of bone turnover, which leads to poor quality dense bone that is predisposed to fracture (11, 12). Other skeletal manifestations of the disorder include a dysplasia in the bones of the face and clavicle, abnormal tooth eruption, and a lack of closure in the cranial sutures.

Several reports claim that potent and selective inhibitors of cathepsin L are able to inhibit bone resorption both in vitro and in vivo (4, 6). Similarly, several studies suggest that small molecule inhibitors of cathepsin K are also capable of inhibiting in vitro and in vivo bone resorption (13, 14). In this study, we address this discrepancy and determine the relative importance of cathepsins L and K in human osteoclast-mediated bone resorption. In the course of a program directed at specific inhibition of cathepsin K, we have discovered several inhibitors that are selective for this enzyme, but we also have found selective inhibitors of cathepsin L. We also have studied the inhibitor of Woo et al. (4) that we find to be a potent inhibitor of both cathepsins L and K. Nonelective inhibitors from our inhibitor collection have also been studied. Our data suggest that cathepsin K and not cathepsin L is the major protease responsible for human osteoclastic bone resorption.

EXPERIMENTAL PROCEDURES

Enzyme Assays—Inhibition of human cathepsin K activity was measured as previously described (13). Inhibition of cathepsin L was measured under identical experimental conditions using the substrate Z-Phe-Arg-aminomethylcoumarin. As appropriate, linear portions of the initial velocity data from product progress curves were analyzed to generate steady-state constants. A standard curve with AMC was used in the conversion of fluorescence to molar units.

Tissue Processing—Human osteoclastoma tissue (Jefferson Hospital, Philadelphia, PA) was obtained (with informed consent) at the time of surgery and frozen as described previously (15). Cryostat sections (7 μm) were cut on a Hacker cryocut (Hacker Instruments, Inc., Fairfield, NJ) equipped with a finely polished tungsten-tipped steel knife and flash-dried onto glass 4-well slides.

In Situ Cytochemical Cathepsin Activity Assay—Cryostat sections of human osteoclastoma were assayed for cathepsin activity using a slightly modified version of the azo-coupling procedure described by...
Each section was incubated in the presence or absence of inhibitors (0.01–1 μM) for 10 min at 37 °C in 150 μl of the following reaction medium: 20% polypeptide (Sigma) in 0.1 M phosphate buffer, pH 5.5, containing 2.5 mM EDTA and 7 mM substrate Ac-Leu-Arg-4-methoxy-β-naphthylamide. The medium was removed, and the sections were postcoupled with 0.25 mg/ml Fast Blue B Base (Sigma) for 10 min at room temperature. The sections were then rinsed in phosphate-buffered saline and finally incubated for 10 min at room temperature with 100 mM CuSO4. The reaction product was measured on a per cell basis (× 40 objective at 550 nm) in a minimum of 10 osteoclasts in duplicate sections of osteoclastoma using a Vickers M85 scanning and integrating microdensitometer as described previously. Results are presented as IC50 for individual inhibitors as calculated from the resulting dose response curves.

In Vitro Human Osteoclast Resorption Assay—Human osteoclasts were isolated as previously described (17) and were used in an in vitro bone resorption assay (18). Isolated osteoclast-enriched cell preparations were seeded onto bovine cortical bone slices in the presence of compound (0.001–3 mM) or vehicle (dimethyl sulfoxide) for 48 h at 37 °C. Compounds were not tested at concentrations higher than 3 mM, because the vehicle is inhibitory at high concentrations. The culture supernatants were harvested, and the levels of the C-terminal peptide of the α1 chain of human type I collagen were quantified with a biochemical readout of resorption using a second generation one-step enzyme-linked immunosorbent assay (Osteometer Biotech A/S, Herlev, Denmark). This is a modification of the original competitive assay described by Foged et al. (19). The results are expressed as percent inhibition of resorption. The IC50 values were determined from the resultant dose response curves.

Compounds—The compounds were synthesized in the Department of Medicinal Chemistry (SmithKline Beecham). Details of the design and synthesis of these compounds will be reported elsewhere. SB 412515, a potent cathepsin L and cathepsin K inhibitor, was originally synthesized and described by Woo et al. (4). SB 290190, a potent and selective cathepsin K inhibitor, is described by Veber et al. (20).

### RESULTS

**Enzyme Inhibitory Activity**—The human cathepsin L inhibitors (SB 468420, SB 468432, and SB 468433 in Table I) used in this study are very potent inhibitors of this enzyme ($K_i = 0.0099 ± 0.0003, 0.033 ± 0.006, and 0.272 ± 0.026$ nM, respectively) and demonstrate impressive selectivity compared with the closely related cysteine protease, human cathepsin K (66- to >50,000-fold selectivity). The dipeptide aldehyde SB 412515 is also a potent cathepsin L inhibitor ($K_i = 0.052 ± 0.002$ nM), but it also shows activity against cathepsin K ($K_i = 1.57 ± 0.367$ nM, Table I). This compound was originally described as a potent cathepsin L inhibitor (4), but no data were presented showing its activity against human cathepsin K. In contrast, SB 290190 is a potent inhibitor of cathepsin K ($K_i = 0.050 ± 0.050$ nM) and shows ~72-fold selectivity over cathepsin L ($K_i = 3.58$ nM, Table I). The data in Table I are presented as $K_i ± S.D.$

**Activity of the Inhibitors in an in Situ Cytochemical Assay of Human Cathepsin Activity and an In Vitro Human Osteoclast Resorption Assay**—To determine whether the cathepsin L selective inhibitors could inhibit native osteoclast cathepsins within whole tissue sections of human osteoclastoma, the compounds were evaluated in an in situ cytochemical assay using the cathepsin K/L substrate, Ac-Leu-Arg-4M-β-naphthylamide. Using this assay none of the cathepsin L selective compounds at concentrations of up to 1 μM inhibited enzyme activity (Table II). The selective cathepsin L inhibitors also failed to

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**TABLE I**

| Compound | Cathepsin L, $K_i$ (nM) | Cathepsin K, $K_i$ (nM) |
|----------|-------------------------|-------------------------|
| SB 468430 | 0.0099 (+/- 0.0003)      | 503 (+/- 45.0)          |
| SB 468432 | 0.073 (+/- 0.006)        | 420 (+/- 43.6)          |
| SB 468433 | 0.27 (+/- 0.026)         | 18 (+/- 4.6)            |
| SB 412515 | 0.052 (+/- 0.002)        | 1.57 (+/- 0.367)        |
| SB 290190 | 3.58 (+/- 0.003)         | 0.030 (+/- 0.003)       |

**TABLE II**

| Compound | in situ cytochemical assay $K_{IC50}$ (nM) | Human osteoclast resorption assay $K_{IC50}$ (nM) |
|----------|------------------------------------------|------------------------------------------|
| SB 468430 | >1000                                    | >300                                     |
| SB 468432 | >1000                                    | >300                                     |
| SB 468433 | >1000                                    | >300                                     |
| SB 290190 | 110 (+/- 5)                              | 71 (+/- 25)                              |
| SB 412515 | 63 (+/- 4)                               | 115 (+/- 40)                             |

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1 Dodds, R. A., James, I. E., Rieman, D., Ahern, R., Hwang, S. M., Connor, J. R., Thompson, S. D., Veber, D. F., Drake, F. H., Holmes, S., Lark, M. W., and Gowen, M. (2001) *J. Bone Miner. Res.* 16, 478–486.
decrease bone resorption at all concentrations tested (0.001–300 nM, Table II), even though their potency against cathepsin L ranged from $K_i = 0.097$ nM to $K_i = 1.7$ nM (Table I). In contrast, SB 290190, a potent and selective cathepsin K inhibitor ($K_i = 0.050 \pm 0.005$ nM), showed potent inhibition of osteoclast cathepsin activity in the cytochemical assay (Fig. 1, IC$_{50} = 63 \pm 4$ nM), and this activity translated into potent inhibition of bone resorptive activity in the in vitro resorption assay (Fig. 1, IC$_{50} = 71 \pm 25$ nM, Table II, and Fig. 2). Significantly, SB 412515, a compound that had been reported previously as a selective inhibitor of cathepsin L (4), also demonstrated potent activity in both the in situ cytochemical assay (IC$_{50} = 110 \pm 5$ nM) and the in vitro resorption assay (IC$_{50} = 115 \pm 40$ nM). Subsequent measurement of the enzyme inhibitory activity of this compound revealed that it is a potent inhibitor of both human cathepsins K and L. Together these data indicate that inhibition of cathepsin K is required for the inhibition of bone resorption in vitro. Potent inhibition of cathepsin L without cathepsin K inhibition has no effect on this process.

**DISCUSSION**

Conflicting data suggest that cathepsin L may play a pivotal role in human osteoclast-mediated resorption. This study was designed to determine whether potent and selective inhibitors of this protease can indeed inhibit the resorptive process and to contrast them with potent and selective cathepsin K inhibitors. The data presented here show a lack of inhibition of both human osteoclast cathepsin activity and resorption by these selective cathepsin L inhibitors. In contrast, the compounds that show potent inhibition of cathepsin K (with some cathepsin L inhibitory activity) potently inhibit cathepsin activity and resorption in human osteoclasts. Taken together, these data indicate that cathepsin K is the single most important protease in human osteoclast-mediated bone resorption and that cathepsin L is not likely to play a role in this process.

The pivotal role for cathepsin K in osteoclast-mediated bone matrix degradation has been described using multiple approaches. Northern blot (16, 21) and in situ hybridization (5) studies have demonstrated that cathepsin K mRNA is abundant in osteoclasts. Immunocytochemical studies, using an anti-cathepsin K antibody, indicate that the enzyme is present in osteoclasts opposed to the bone surface (5). Furthermore, the enzyme is activated as the cells approach bone and distributed in a polarized fashion to the surface of the cell that interacts with the bone surface. In contrast, in situ hybridization studies have shown that mRNA for cathepsins L, B, and S is unde-
It has been reported previously by Woo et al. (4) that the dipeptidyl aldehyde SB 412515 was a potent cathepsin K inhibitor that could inhibit both in vitro and in vivo bone resorption. Although this report describes the compound as a more potent inhibitor for cathepsin L than other cysteine proteases, such as cathepsin B and calpain II, its inhibition of cathepsin K was not reported. Similarly, Yasuma et al. (6) reports the preparation of a series of peptide aldehyde derivatives that demonstrated potent activity against cathepsin L; data were not reported for cathepsin K inhibition. One of these compounds inhibited bone resorption in vitro and prevented bone loss in the ovariec-tomized mouse (6). Our enzyme activity studies revealed that SB 412515 had potent activity against human cathepsin K, as well as cathepsin L, and the compound from the Yasuma study (6) is a potent inhibitor of both human and mouse cathepsin K (data not shown). The lack of activity of the cathepsin L selective compounds confirm that it is the potent cathepsin K activity of SB 412515 and SB 290190 that explains their potent antiresorptive activity.

In conclusion, we demonstrate using two distinct in vitro assays of human osteoclast function that cathepsin L selective inhibitors are unable to inhibit human osteoclast cathepsin activity, and this results in a lack of inhibition of osteoclast-mediated bone resorption. In contrast, potent inhibitors of cathepsin K reproducibly inhibited osteoclast function thus providing unequivocal evidence that it is cathepsin K and not cathepsin L that plays the pivotal role in osteoclast-mediated matrix degradation.

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fig. 2 SB 412515, a potent dual cathepsin L and cathepsin K inhibitor, restricts in vitro human osteoclast-mediated resorption and in situ cathepsin activity. The compound was tested in the human osteoclast resorption (n = 4) (A) and cathepsin cytochemical assays (n = 4) (B). The compound was active in both assays and showed similar potency. The data are presented as percent inhibition ± S.E.
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