Non-Specific Cathepsin B Inhibitor CA-074Me Inhibits Osteoclastogenesis via c-FOS Upregulation Blockade

INTRODUCTION:
Osteoclasts are a vital component of the skeletal system and play an intricate role in bone metabolism and matrix degradation. To date, multiple signaling events have been identified to positively or negatively regulate osteoclastogenesis and osteoclast activation. Tartrate-resistant acid phosphatase (TRAP) and cathepsin K are the two major enzymes responsible for the degradation of bone mineral and collagen matrices. Furthermore, cathepsin K knockout mice demonstrate severe osteoporosis due to a deficit in matrix degradation. Several studies have confirmed that cathepsin K is the predominant cysteine protease expressed in human osteoclast, however, cathepsins B, D and L are also detected in the rat osteoclasts. Cathepsin B is implicated a number of inflammatory diseases and pathological conditions, such as bronchitis, rheumatoid arthritis, acute pancreatitis and cancer progression.

CA-074Me-induced osteoclastogenesis follow a specific intracellular signaling cascade that ultimately leads to the transcription of several key factors such as cFOS and NFATc1. NFATc1 and cFOS deficiency leads to the development of severe osteopetrosis due to the signaling inhibition of osteoclastogenesis and matrix degradation. Recently, cFOS was identified as a key component of the AP-1 complex, which initiates transcription of NFATc1. The objective of this study is to determine the specificity of CA-074Me as a Cathepsin B inhibitor and elucidate its cellular target as a potent inhibitor of osteoclastogenesis.

METHODS:
Cell Culture: Bone marrow-derived monocytes (BMMs) were prepared from C57BL/6, NOD/ShiLtJ and NOD/CTSB-/- mice. Non-adherent bone marrow cells are cultured in Minimum Essential Medium (MEM) α Medium supplemented with 10 % fetal bovine serum, antibiotics and 30 ng/ml of 4-CBF-M-CSF to obtain only BMMs. BMMs were cultured in MEM supplemented only with M-CSF for 3 days, then new media containing 50 ng/ml of RANKL with or without CA-074Me (Enzo Life Sciences, Plymouth Meeting, PA), cathepsin B inhibitor, was added to induce osteoclastogenesis.

TRAP Staining: After 7 days of culturing, BMMs were fixed with a solution containing acetone and citrate, and TRAP staining was performed with a kit purchased from Sigma according to the manufacturer’s instruction. TRAP positive cells with three or more nuclei were counted as multinucleated osteoclasts under microscopy.

Reverse Transcription Realtime Polymerase Chain Reaction: Single stranded CDNA was synthesized from total RNA with the SuperScript III system (Invitrogen, Carlsbad, CA). RT-realtime PCR for each target was performed with LightCycler FastStar DNA Master SYBR Green I (Roche, Nutley, NJ) using the Realplex system (Eppendorf, Westbury, NY). Each gene was normalized with GAPDH.

Western blot analysis: Western blot was performed using standard methods. The nitrocellulose membrane was probed with NFATc1, cFOS and GAPDH antibodies followed by reaction with horseradish peroxidase conjugated secondary antibody.

Calvarial Osteolysis: RANKL (0.08 mg/kg) and Cathepsin B inhibitor CA-074Me (10 mg/kg or 50 mg/kg) were administered in sterile saline. The calvaria were excised, fixed in 4% formaldehyde for 24 hours, decalcified in 20% EDTA for 1 week and TRAP stained.

RESULTS:
Cathepsin B inhibitor CA-074Me inhibited RANKL-induced osteoclastogenesis in vitro in a dose-dependent manner as assessed by qualitative TRAP staining and quantitative relative TRAP5b mRNA expression (Figure 1a,b). Similar results were yielded in vivo (Figure 2). CA-074Me also reduced mRNA expression of MMP-9, cathepsin K (data not shown). Furthermore, there was notable difference in osteoclastogenesis or CA-074Me-induced osteoclast inhibition between C57BL/6 WT and NOD/ShiLtJ WT mice. Cathepsin B-/- mice did not exhibit reduced osteoclastogenesis in vitro (Figure 3a,b). There are marked osteoclasts in both NOD/ShiLtJ and CTSB-/- mice (Figure 3a). Furthermore, the relative TRAP5b expression was insignificant between the WT and CTSB-/- mice (figure 3b). Hence, Cathepsin B inhibitor, CA-074Me does not inhibit osteoclastogenesis via cathepsin B pathway and acts a non-specific inhibitor to modulate additional signaling pathways. We targeted other known signaling pathways and revealed that the upregulation of cFOS, a critical transcription factor, was inhibited (Figure 4). The western blot unequivocally demonstrates that cFOS and the subsequent activation of NFATc1 are inhibited by the addition of CA-074Me.

DISCUSSION:
CA-074 Me is a potent, irreversible, cell permeable cathepsin B inhibitor. Methyl ester is hydrolyzed by intracellular esterases releasing the active inhibitor. We discovered that CA-074Me inhibits RANKL-induced osteoclastogenesis in BMMs; however, its action is independent of cathepsin B. In other studies, the use of CA-074 (non-cell permeable inhibitor) is known as a potent and selective inhibitor of cathepsin B; however, in macrophages, the methyl-ester group generates a non-specific binding that leads to the inhibition of cFOS expression. cFOS is a major signaling target for osteoclast and this leads to NFATc1 inhibition, and ultimately inhibition of osteoclastogenesis.

SIGNIFICANCE:
The use of pharmaceutics to interrupt osteoclastogenesis can lead to a novel breakthrough in treating conditions such as osteoporosis. Since inhibition of most cathepsins does not create adverse events (due to the various isomers) CA-074Me can be a safer therapeutic option.

REFERENCES: