Efficacy of a Cathepsin K Inhibitor in a Preclinical Model for Prevention and Treatment of Breast Cancer Bone Metastasis

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Running Title: Cathepsin K inhibitor for metastatic bone disease

Keywords: Cathepsin K inhibitor, osteoclast, metastatic bone disease, osteolysis, breast cancer.

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Funding Statement - This study was funded by Merck & Co., Whitehouse Station, NJ, USA
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**Potential Conflict of Interests**

LTD, GAW, PL, RO and MP are employees or former employees of Merck & Co. Inc., the study sponsor, and may own stock/stock options in this company. RO is currently an employee of Inception Sciences Canada Inc. and may own stock/stock options in this company.
**List of Abbreviations**

BrCa: Breast cancer; BV: Bone volume; BV/TV: Bone volume / Tissue volume; CatK: Cathepsin K; DAB: 3,3’-diaminobenzidine; H&E: hemotoxylin and eosin; p.o., per os (oral dosing); i.p: intraperitoneal dosing; b.i.d.: twice-a-day; LV.BMD: lumbar spine bone mineral density; mAb: monoclonal antibody; MBD: Metastatic bone disease; OVX: Ovariectomized; PRC: Piedmont Research Center; RT: Reverse transcription; SRE: Skeletal related events; TMA: Tissue microarray; TNM: grading for primary tumor (T), lymph node involvement (N) and distal metastasis (M); TV: Tissue volume; uNTX: urinary N-terminal telopeptide of collagen; VOI: Volume of interest; L-235: L-006235; ZOL: Zoledronic acid
Abstract

Cathepsin K (CatK) is essential for osteoclast mediated bone resorption. CatK expression is also detected in breast cancer (BrCa) cells that metastasize to bone. Here, the CatK inhibitor L-235 dosed in prevention (10, 30 and 100mg/kg, p.o., b.i.d.) or treatment regimen (30mg/kg) was compared to the bisphosphonate zoledronic acid (ZOL, 7.5μg/kg/wk, s.c.) in the intratibial injection model of MDA-MB-231 breast carcinoma in nude rats. Progression of osteolysis, skeletal tumor burden and local metastasis were evaluated by radiography through 42 days and ex vivo μCT and histology. Immunohistochemistry and RT-PCR confirmed the increases in CatK protein and mRNA levels in human BrCa primary and metastatic tumors. In the experimental model of BrCa bone metastasis, L-235 dosed in preventive mode resulted in a dose-related reduction of osteolysis of 72, 75, and 87% respectively, compared to ZOL by 86% vs. intact. Similarly, L-235 significantly reduced intratibial tumor volume by 29, 40 and 63% respectively, compared to 56% by ZOL vs. vehicle. Efficacy of L-235 and ZOL on reduction of osteolytic lesions and tumor burden were comparable in treatment vs. preventive regimens. All L-235 doses inhibited cortical disruption and extraskeletal tumor growth to a level comparable with ZOL. Assessment of local metastasis demonstrated that treatment with the CatKi was more effective than ZOL in reducing BrCa invasion. These data support the role of CatK in BrCa skeletal growth and metastasis and CatK inhibitors may represent a novel oral therapy for treatment of metastatic breast cancer.
Introduction

The most common metastatic site for breast carcinoma is bone (1,2). The development of metastatic bone disease (MBD) in breast cancer (BrCa) is associated with morbidity and skeletal related events (SREs), including pathological fractures, spinal cord compression, hypercalcemia and bone pain (2). Established MBD is considered an incurable disease and the current therapeutic options include palliation and prevention of SREs. MBD is predominantly osteolytic in BrCa, but it is also infrequently associated with osteoblastic lesions (3). Bone-residing tumor cells produce parathyroid hormone-related protein (PTHrP), multiple interleukins (IL-6, IL-8, IL-11), cytokines (TNF and M-CSF) and prostaglandins, all of which stimulate osteoclastogenesis. Osteoclast (OC) bone resorption, in turn, releases local growth factors (TGFβ, IGFs, FGFs, PDGFs, and BMPs) to promote proliferation of tumor cells. This produces a self-sustaining cycle that eventually leads to bone destruction (3).

Bisphosphonates (BPs) are the current standard of care for the treatment of BrCa patients with skeletal metastases (4). BPs disrupt the destructive cycle in the bone microenvironment primarily by blocking bone resorption, leading to reduction of the release of local growth factors resulting in retardation of tumor growth and protection against osteolysis. Clinical trials with the nitrogen-containing BPs (pamidronate, zoledronate or ibandronate), predominantly as intravenous therapies, demonstrated their efficacy in reduction of SREs, morbidity rate and prolonging the time to first SREs. While potential direct anti-tumor effects of the BPs have been proposed, recent large clinical trials have demonstrated this class of antiresorptives does not provide life-prolonging benefit to the patients with advanced cancer (5-7). Furthermore, the intravenous BPs are often associated with adverse experiences such as renal toxicity and acute phase reactions (8). Hence,
there is a need for alternative bone agents with a better tolerability profile and perhaps greater efficacy in reduction of SREs and disease reoccurrence.

Cathepsin K (CatK), a lysosomal cysteine protease, is expressed predominantly in OCs (9). CatK degrades type I collagen by cleaving the triple helical domains at multiple sites and directly releasing the telopeptides (10). Modulation of CatK activity by small molecule inhibitors reduces bone resorption activity of OCs in vitro and in vivo (11,12). In preclinical models of estrogen-deficiency induced bone loss, multiple CatK inhibitors have been demonstrated to effectively prevent loss of bone mineral density, increase cortical thickness and improve bone strength in ovariectomized (OVX) rodents, rabbits and monkeys (11). Expression of CatK mRNA or protein has been reported in breast and prostate cancers (13,14). Le Gall et al previously reported the expression of CatK in breast tumors and its expression is further increased in BrCa cells that metastasize to bone (15). In that study, a preclinical model of skeletal metastasis utilized the intratibially implanted CatK expressing human BT474 BrCa cells in nude mice, and intraperitoneal dosing of the CatK inhibitor (CatKi) AFG-495 was shown to reduce osteolytic lesions and skeletal tumor burden (15).

The selective CatKi odanacatib (ODN) is currently in large phase III fracture prevention trials as an orally active 50mg once-weekly drug for the treatment of postmenopausal women and men with osteoporosis (12). Recently, ODN was also evaluated in a small clinical study involving breast cancer patients with established metastatic bone disease, who received either ODN (5mg, once-daily) or zoledronic acid (ZOL, a single intravenous infusion of 4mg) for 4 weeks of treatment. ODN suppressed the bone resorption marker, urinary N-telopeptide similarly to ZOL (16). This result demonstrated efficacy of ODN as an effective inhibitor of bone resorption in this
patient population, suggesting that the CatK inhibitors offer a potentially important, novel oral therapeutic approach for the treatment of MBD.

Because of the species differences between the rat/mouse CatK (87-88% homology) and human CatK enzyme, ODN displays a low potency against the rodent enzyme. To further our understanding on the potential benefits and mechanisms of the CatK inhibitors compared to the bisphosphonates for the treatment of MBD, we selected L-006235 (L-235) as a proof-of-concept CatKi for evaluation in various rodent models of metastatic disease. The reversible CatKi, L-235, is structurally related to ODN (17,18). This compound inhibits human CatK with a Ki of 0.25 nM, and is >4000-fold selective against other human cathepsins L, B and S (17). However, unlike ODN, L-235 shows relatively good potency against mouse (IC₅₀=20nM) and rat (IC₅₀=12nM) CatK activity (19,20). Efficacy of L-235 as a bone resorption inhibitor has been well established in rabbits, it inhibits rabbit CatK (IC₅₀=0.5nM) and bone resorption in vitro by rabbit OCs (IC₅₀=5nM) (17). When dosed orally once-daily for 27 weeks in ovariectomized- rabbits, L-235 at 10mg/kg prevented estrogen deficiency-induced osteopenia (21). Hence, the aim of this study was to evaluate the efficacy of L-235 as a preclinical proof-of-concept CatKi, compared to ZOL in the prevention and treatment of osteolysis and skeletal tumor progression and local metastasis in the intratibial injection of the BrCa MDA-MB-231 cells in nu/nu rats.
Material and Methods

Breast Cancer Tissue Specimens -- Human breast tumor RNA matched pairs and tissue microarray (TMA) slides containing malignant and non-malignant breast tumor tissue (n=88) were obtained from AccuMax Array (ISU Abxis Co., Ltd., Seoul, Korea). The breast tumor RNA pairs included 12 primary tumors (n=12) and 4 pairs of primary/metastatic tumors. Each pair consisted of primary breast tumor and corresponding adjacent normal tissue; the metastasis tumor pairs consisted of primary tumor and corresponding metastatic tissue. Total RNA in each pair was obtained from the same donor. Donors were diagnosed with invasive ductal carcinoma with poor to moderate differentiation. The tissue microarray slides were constructed from tissue donors and included at least two cores (1mm in diameter) per patient. Samples were reviewed by the vendor’s pathologist for classification regarding the histopathology, the class, lymph node involvement and the grade of the tumor. Samples were categorized based on TNM grading of: the size of primary tumor (T), degree of regional lymph node involvement (N) and presence of distal metastasis (M).

Ethics approval was not obtained for the use of the array from a commercial source. ISU Abxis provided documentation that the vendor obtained informed consent from all donors prior to tissue collection and confirmed that the patient’s personal information is kept strictly confidential according to their privacy policies. We only received blinded pathological information including age, sex, histological diagnosis and TNM stage to increase effectiveness of experiments.

Reverse transcription and PCR – Reverse transcription (RT) reactions were carried out in MicroAmp reaction tubes using TaqMan reagents and containing 250 ng of total RNA in 50μL of 1× TaqMan RT buffer, 5.5mM MgCl₂, 500μM of each dNTP, 2.5μM of oligo-d(T)16 primers, 2.5μM of random hexamers, 0.4U/μL RNase inhibitor and 1.25U/μL MultiScribe Reverse
Transcriptase, at 25°C for 10 min, 48°C for 30 min and 95°C for 5 min. Primers and fluorogenic probes for CatK were designed using Primer Express v.1.0 (Applied Biosystems, CA) using the forward primer (5′-CTGGCTATGAACCACCTGGG-3′), reverse primer (5′-TGCGGGAATGAGCAGGGG-3′) and fluorogenic probe (5′-AAGAGGTGGTTCAGAAGATGACTGGACTCAAAAGT-3′). GAPDH primers and fluorogenic probes were from Applied Biosystems with the fluorescent reporter dye FAM (6-carboxy-fluorescein) at the 5′-end and the quencher dye TAMRA (6-carboxytetramethyl-rhodamine) at the 3′-end. Real-time PCR (50 μL) included 10 μL of RT product (50 ng total RNA), 100 nM forward-primer, 100 nM reverse-primer, 200 nM probe and 1× Universal Master Mix (Applied Biosystems) for 2 min at 50°C, 10 min at 95°C followed by 40 cycles at 95°C for 15 sec, 60°C for 1 min. All reactions were performed in ABI Prism 7700 Sequence Detection System. Expression levels of mRNA were expressed relative to GAPDH.

**Immunohistochemistry** -- The TMA slides were deparaffinized with xylene, rehydrated with ethanol and incubated in 0.3% H₂O₂ in PBS. Tissue sections were probed with anti-human CatK mAb (Oncogene, CA) overnight at 4°C. After washing with PBS containing 0.03% Tween-20 (PBS-T), sections were incubated with secondary antibody followed by washing with PBS-T. Immunohistochemical reaction color was developed with 3,3′-diaminobenzidine (DAB) substrate (DAB substrate kit, Vector Laboratories). Slides were washed and counterstained with hematoxylin. The percent of tumors positive for CatK was then assessed within each group according to the TNM grade. To detect the injected MDA-MB-231 cells, sections of decalcified tibia were deparaffinated and treated with 0.5% pepsin in 5 mM HCl for 30 min at 37°C and washed in PBS-T, followed by incubation with 0.3% H₂O₂ in methanol. Slides were blocked with goat serum followed by biotin-avidin blocking reagent. Tissue was then incubated with biotinylated
mouse-anti-human cytokeratin AE1/AE3 (Dako, Cat# M3515), and then avidin-HRP conjugate. Positive reaction was visualized with DAB as described above. OCs in the same sections were localized by staining the tissues for tartrate resistant acid phosphatase (TRAP) activity.

**Animal model of intratibial tumorigenesis** -- The in life portion of the studies were conducted at Piedmont Research Center (PRC, Morrisville, NC). PRC complies with the recommendations of the Guide for Care and Use of Laboratory Animals with respect to restraint, husbandry, surgical procedure, feed, and fluid regulation. All protocols and procedures were approved by the PRC Institutional Animal Care and Use Committee (IACUC). These studies were monitored on a routine basis by the attending veterinarian to ensure continued compliance with the proposed protocols.

**Drugs** - Structure, potency and selectivity profile of the cathepsin K inhibitor L-006,235 has previously been reported (17). L-235 [N-(1-((cyanomethyl)carbamoyl)cyclohexyl)-4-(2-(4-methylpiperazin-1-yl)thiazol-4-yl)benzamide] and zoledronic acid [1-hydroxy-2-(1H-imidazole-1-yl)ethylidene-bisphosphonic acid] were synthesized by Merck Res. Labs. according to previously reports (17,22).

**In vitro tumor cell proliferation and invasion assays** – Human MDA-MB-231 breast carcinoma cells (ATCC, Manassas, VA) were maintained in RPMI 1640 medium containing 10% heat-inactivated FBS, 2mM glutamine, 100U/ml penicillin-G sodium, 100μg/ml streptomycin sulfate, 0.25μg/ml amphotericin B and 25μg/ml gentamicin at 37°C and 5% CO₂. The cell proliferation and invasion assays were done essentially as previously described (15).
**Intra-tibial injection of breast cancer cells** - Female nude rats (rnu/rnu, Harlan Indianapolis, IN) were 6 weeks of age with a body weight range 127 – 160g on day 0 of the study. For implantation, MDA-MB-231 cells (1.2x10⁶ cells/ml) were harvested during log phase growth and suspended in PBS. Animals were sedated with acepromazine maleate (2.5mg/kg, i.p.) and anesthetized with isoflurane/O₂ during the engraftment procedure. The tumor cell suspension was injected with a 25-gauge needle and at a 45° angle, from distal to proximal, through the medial aspect of the tibia. The animals received 6x10⁵ cells bilaterally on day 0. After tumor cell implantation, animals were randomized into 5 groups, and dosed with: vehicle of 0.5% carboxymethylcellulose / 1% fructose (Sigma-Aldrich, St. Louis, MO); L-006235 (L-235) in the same carboxy-methycellulose vehicle at 10, 30 or 100mg/kg, p.o., twice-daily; or with zoledronic acid (ZOL) in phosphate buffered saline at 7.5µg/kg/week, s.c., 3x per week, starting on day 1 (prevention regimen) or on day 7 (treatment regimen) up to day 42 (n=6/group). To determine drug exposure levels, plasma was obtained from L-235 treated rats at 1, 2, 4, 8 and 24 hrs after the final dose and analyzed from drug concentration. After intratibial injection of the cells, body weights were monitored on a daily basis for the first week, and then switched to weekly basis until the study ended. Animals were sacrificed on day 42 and the hind limbs were harvested and fixed in 10% buffered formalin for histological analyses and μCT imaging.

To monitor osteolysis on days 0, 7, 14, 21, 28 and 35, the rats were sedated, anesthetized and subjected to digital x-ray imaging of the mid region of the hindlimbs using a Faxitron MX-20 (Field Emission Co.) with a 3sec exposure time on calibrated settings (~25 kV). A fixed square 100x100 pixel region of interest (ROI) within the proximal tibial image was processed for pixel intensity on a scale of 0-255 using Image Tool Processing Kit 3.0 plug-in filters. The average pixel intensity was computed over the entire ROI for each tibia image.
Analyses of tumor progression and osteolysis

**Micro-computed tomography (µCT)** - Bones were scanned in a SCANCO µCT-40 (Scanco Medical AG, Brüttisellen, Switzerland) with a 20µm isotropic voxel size (cone beam X-ray 55kV, 72µa, 1024 samples, 250 projections/180°, 60ms integration time) for determining bone volume fraction. To evaluate osteolytic lesions, the bone volume was determined for all samples from the tibial plateau distally to the junction with the fibula. Volume of interest (VOI) was selected by drawing contours to include the tibia, but exclude the fibula. Bone volume (BV) was determined from the VOI; however, actual tissue volume (TV) could not be determined in many samples due to severe loss of cortical bone. Hence, TV of the same region was determined in age matched intact animals (n=8) and the average TV was used to calculate BV/TV for all samples. To estimate tumor volume from µCT images from sagittal plane, contours were drawn every 5–15 planes and "morphed" through the entire sample to obtain a 3-D volume representing space occupied by tumor within the confines of the resorbed cortical bone. The bone component was subtracted from this volume to yield an estimate of actual space occupied by the tumor cells.

**Histology and Histomorphometry** - Bones were decalcified with 0.5M EDTA and embedded in paraffin. Sagittal planes of the entire length of the rat tibia were collected as serial sections at 5µm thickness and 19-20mm in the mean length and subjected to either hemotoxylin and eosin (H&E) staining or immunohistochemical evaluation.

Tumor areas in the tibial sections included the areas of "solid" tumor devoid of trabecular bone, and "infiltrated" tumor areas where tumor cells invaded to the surrounding bone marrow space (23). The sagittal H&E stained sections were obtained as close to the central tibial plateau as possible. Images of the slides were captured using a 1.25X objective on an Olympus IX-51
microscope equipped with a Spot Slider digital camera (Diagnostic Instruments, Sterling Heights, MI) and saved as JPEG files. From these images, areas of tumor and bone were evaluated using Image Pro imaging software (Media Cybernetics, Silver Spring, MD). Areas for "solid" and "infiltrated" tumor were drawn manually. Extraskeletal tumor area outside of the bone cortical envelope was also contoured.

To evaluate the effect of ZOL and the CatKi on cortical bone, the degree of cortical disruption was evaluated in H&E stained sagittal sections by manually tracing the gaps or disrupted lengths of cortical bone of the proximal tibia (10mm length from the growth plate) using ImagePro software. Cortical disruption (%) was determined from the ratio of lengths of cortical gaps to total length of two cortical surfaces.

To determine distal metastases within tibial marrow and away from the original injected site, the slides were scanned on a Hewlett Packard Scanjet 8200 flatbed scanner (Hewlett Packard, Houston, TX) and imported into Image Pro. Distal tumor tissue was identified along the marrow of the tibial shaft. Matching the microscopic observation with the scanned image, the distance from the growth plate to the most distal tumor tissue was measured using the Image Pro software.

Statistical Analysis – All data were analyzed with StatView software (version 5.0; SAS Institute, Inc.). Statistical analyses were carried out by unpaired Student’s t-test or ANOVA, followed by a Fisher’s protected least significant difference test. P<0.05 were considered statistically significant. The differences between the average pixel intensity from Faxitron images were determined by ANOVA with Tukey’s multiple comparison test for analysis of means.
Results

Expression of Cathepsin K in Breast Cancer

The BrCa tissue microarray (TMA) slides contained malignant and non-malignant breast tumor samples from 88 patients diagnosed with breast ductal carcinoma. The tissue array grading included breast biopsies from: 49 patients with primary breast tumor who had no regional lymph node metastasis and no distal metastasis (N=0, M=0), 27 patients with metastasis in 1 or more regional lymph nodes without distal metastasis (N≥1, M=0), and 12 patients with lymph node involvement as well as presence of distal metastasis (M>0). As shown in Figure 1, high CatK expression was specifically detected in the epithelial cells of breast ductal carcinoma, while the surrounding basal cells showed little to no expression of this protease (Fig. 1A). At high magnification, CatK was localized in the cytoplasm and with punctate appearance (Fig. 1A-d). Approximately 40% of breast tumor tissue biopsies surveyed were positive for CatK expression (42-48%) regardless of TMN grade in BrCa severity (Table 1).

We also correlated CatK mRNA levels in human metastatic ductal carcinoma (Fig. 1B). Note, each matched pair was from the same donor. The primary pairs (n=12) consisted of primary breast tumor and adjacent normal tissue while the metastatic pairs (n=4) consisted of primary breast tumor and corresponding metastatic tissue. CatK expression was frequently upregulated (7 of 12 samples) in primary tumors versus the respective adjacent normal tissue (Fig. 1B-a). Primary tumor tissue demonstrated a 55% increase (p<0.05) in CatK expression as compared to normal adjacent tissue (Fig 1B-a). CatK mRNA level was more dramatically increased when comparing primary tumor to that in metastatic tissue (Fig. 1B, b). CatK mRNA in metastatic tissue was elevated ~3-fold (p<0.05) as compared to its matched primary tumor in all 4 donors (Fig 1B-b).
Cathepsin K inhibitor reduced osteolysis in an experimental model of human BrCa metastasis

We conducted two different studies that included dose ranging of L-235, comparing to ZOL, in prevention (dosing began day-1) or treatment mode (dosing started day-7 post-injection). The structure of L-235 is shown in Figure 2A. L-235 orally dosed at 10, 30 and 100mg/kg twice daily provided mean plasma exposure levels of 10.4, 35.6 and 166.2\(\mu\text{M}\cdot\text{hr}\), respectively. ZOL was subcutaneously dosed at 7.5\(\mu\text{g/kg}\) weekly. For both studies, there were no treatment-related adverse effects observed. There were no differences in body weights of animals treated with either L-235 or ZOL as compared to vehicle treated controls over the study duration of 42-days (data not shown).

Progression of osteolytic lesions was followed by Faxitron analysis and quantified every week up to day-35 as bone density (mean pixel intensity). In the prevention regimen, bone density measurements showed significant osteolysis in vehicle controls at day-7, -21 and -35 post-injection (Fig. 2B-a), while treatment with ZOL and L-235 protected against focal osteolysis (Fig. 2B-b&c). From density measurements within a defined ROI in the proximal tibia, the vehicle controls showed -3.3% change from baseline intensity measured at day 0. L-235, 10, 30 and 100mg/kg, bid, resulted in -2.5, 6.5 and 9.5% change in bone density at day-35 vs. baseline. ZOL at 7.5 \(\mu\text{g/kg}\) (s.c., weekly) showed comparable efficacy to L-235 100mg/kg (p.o., b.i.d.) with a 9.5% increase in bone pixel density \(p<0.05\). In the treatment regimen, vehicle-treated animals showed -5.9% change of bone density vs. baseline, while L-235 30mg/kg and ZOL increased bone density 1.9 and 7.3% respectively.

To obtain a more sensitive measurement of osteolysis, bone volume/total tissue volume (BV/TV) was evaluated from proximal tibia post-necropsy (day-42) by \textit{ex-vivo} \(\mu\text{CT}\). Note, BV/TV (%) is expressed relative to that of age matched intact nude rats. In the prevention regimen (Fig.
2C, a), BV/TV of vehicle-treated proximal tibia was 61%, while the ZOL-treated tibia was 86% of intact controls. L-235 dose dependently reduced osteolysis, with BV/TV of 72, 75 and 87% of intact controls, respectively. Similar results were obtained in the treatment experiment (Fig. 2C-b), where osteolysis was significantly inhibited by either ZOL or L-235 30mg/kg, resulting in BV/TV of 101% and 80% respectively, significantly above vehicle.

**Characterization of tumor growth post-intratibial injection**

To evaluate the effects of the CatKi and ZOL on tumor progression, sagittal sections of the tibia from vehicle were subjected to immunohistochemical staining for cytokeratin to visualize the BrCa tumor cells (Fig. 3A-a). The tibial medullary space contained areas of normal bone marrow and intratibial solid tumor mass originating from the injection site of MDA-MB231 cells, and led to aggressive trabecular osteolysis. Extraskeletal tumor mass was also detected outside of the cortical envelope and in contact with the surrounding soft tissue. Abundant TRAP-positive OCs were recruited to endosteal (Fig. 3A-b) and periosteal (Fig. 3A-c) cortical surfaces, as well as trabecular surfaces (data not shown) in proximity to tumor cells. In addition, cytokeratin positive cells were also detected in the bone marrow space distant from the solid tumor mass. We characterized the positive cellular mass as “solid” and “infiltrated” tumor and micrometastasis. Solid tumor was characterized by a solid mass of tumor cells with no bone within the mass, while the “infiltrated” tumor area represented BrCa cells growing amongst the trabecular bone and into the surrounding bone marrow space (Fig. 3A-d). It should be noted that the infiltrated tumor area included some degree of host fibroblastic tissue as demonstrated by cytokeratin staining. Interestingly, small discrete micrometastases were frequently detected toward the distal tibia, a site distant from the
original injection site (Fig. 3A-e). Using the growth plate as a consistent landmark, we suggest that the distance to the micrometastasis from the landmark site represented the invasive activity of MDA-MB-231 BrCa tumor cells.

To further characterize the observations in cytokeratin-stained sections, measurements on tumor areas were determined from the H&E stained tibial sections from vehicle, ZOL and L-235 treated rats as described in Figure 3B (a-c). We examined treatment-related effects on BrCa tumor growth within the tibia at higher magnification (Fig. 3B, d-f). Tumor growth was extensively detected in the vehicle treated tibia, even at the site distal from the injected site (Fig. 3B-d, same region as the white box in Fig. 3B-a). ZOL treatment resulted in protection of trabecular and cortical structures in the BrCa cell injected tibia (Fig. 3B-b); however, tumor (T) cell growth were found to infiltrate among the trabeculae (Fig. 3B-e, same region as the white box in Fig. 3B-b). L-235 treatment protected both trabecular and cortical structures, and significantly reduced BrCa tumor cell infiltration even at a site proximal to the injected site (Fig. 3B-f, same region as the white box in Fig. 3B-c). The parameters described in the following sections, were determined using image analyses, including the intratibial solid tumor area, extraskeletal tumor area, infiltrated tumor area and distance to micrometastasis.

**Cathepsin K inhibitor protected against cortical disruption**

The data on cortical integrity is expressed here as the percent of the combined length of the eroded cortex for the anterior and posterior cortical surfaces to the total cortical length. When the tibial cortical envelope was completely disrupted (Fig. 3B-a), the cortical length was estimated by image analysis (Fig. 3B-a; dash lines). In the prevention study, ~70% of the cortical bone was eroded in untreated animals. L-235 dose-dependently preserved cortical bone and reduced cortical
disruption to 36, 17 and 11% respectively, versus vehicle, while ZOL resulted in 25% cortical disruption (p<0.05; Fig. 4a). Similar results were seen in the treatment regimen where L-235 30mg/kg and ZOL showed 31 and 24% cortical disruption, respectively, compared to vehicle that showed 58% cortical disruption (p<0.05; Fig. 4b). The effects of L-235 versus ZOL on the preservation of cortical bone correlated well with the reduction of tumor burden as described in the section below.

**Cathepsin K inhibitor reduced skeletal BrCa tumor burden**

*Reduction of intratibial BrCa tumor*

Extensive tumor growth was evident in the vehicle group in both the prevention (Fig. 3B-a; Fig. 5A-a) and treatment protocol (Fig. 5A-b). Treatment with L-235 (up to 10μM) did not change the rate of MDA-MB231 cell proliferation *in vitro* (data not shown). On the other hand, histological examination of the *in vivo* study clearly showed that the intratibial tumor burden was significantly reduced by treatment with ZOL (Fig. 3B-b) or L-235 100mg/kg dose (Fig 3B-c). In the prevention protocol, the vehicle group showed solid tumor area of 24±1.2mm² (Fig. 5A-a). While ZOL potently reduced solid tumor area by more than 85% vs. vehicle, the CatKi at 30 and 100mg/kg markedly reduced solid tumor area by 70-75% vs. vehicle. Similarly, in treatment mode, efficacy of L-235 30mg/kg was comparable to ZOL in reducing solid tumor burden (Fig. 5A-b). Intratibial solid tumor volume was also determined from the μCT-based 3-D reconstructed images of the osteolytic void volume (Fig. 5B). In the prevention study, tumor volume was reduced by treatment with L-235 at 10, 30 and 100mg/kg doses by 29, 40 and 63%, respectively, as compared to ZOL that reduced tumor volume by 56% (Fig. 5B-a). Similar responses were observed in the
treatment experiment where L-235 30mg/kg and ZOL resulted in 37% and 72% reduction of tumor volume, respectively (Fig. 5B-b).

**Reduction of extraskeletal BrCa tumor**

Tumor area outside of periosteal surface of the proximal tibia was also measured from the H&E sagittal sections. In the case that the tibial cortical envelope was completely disrupted (Fig. 3B-a), tumor area was estimated based on the predicted cortical outlines (Fig. 3B-a; dash lines). All treatments significantly reduced tumor area outside of the cortical bone envelope (Fig. 6A). In the prevention study, the CatKi at all doses significantly reduced tumor burden in soft tissue by 60 – 80% vs. vehicle, while ZOL resulted in a 68% reduction (Fig. 6A-a). Similar results were seen in the treatment study where L-235 at 30mg/kg and ZOL reduced tumor area outside of bone by 73% (p<0.05) and 62% (NS), respectively, versus vehicle (Fig 6A-b).

**Cathepsin K inhibitor reduced BrCa local metastasis**

**Infiltrated tumor**

Efficacy of L-235 in inhibiting the BrCa MDA-MB-231 cell invasion *in vitro* was evaluated essentially as previously described (15). This CatKi potently inhibited the BrCa matrigel invasion with an IC$_{50}$ of 3.2 nM (data not shown) as compared to its activity in blocking osteoclastic bone resorption (IC$_{50} = 5$ nM) (17). Therefore, the ability of the intratibially injected MDA-MB-231 cells to invade to sites distant from the original injected site was characterized *in vivo* by immunohistochemically staining the sections from injected tibia for cytokeratin, a marker of the BrCa cells (Fig 3A-d&e). ZOL markedly reduced solid tumor area, and protected trabecular and cortical bone. However, closer examination of the ZOL-treated tibia revealed that BrCa cells
infiltrated the bone marrow among the preserved trabeculae. Small tumors could be detected further down the tibial shaft (Fig. 3B-b). In contrast, the L-235-treated tibia showed high density of trabecular spicules associated with normal bone marrow integrity (Fig 3B-c). The “infiltrated” tumor area was determined by measuring the total tumor-containing trabecular area and subtracting out the trabecular bone area. In the prevention protocol (Fig. 6B, a), the majority of tumor burden in vehicle-treated animals was solid tumor (24.1±1.2mm²) as shown above, with very little infiltrated tumor (7.2±1.6mm²), resulting in overall intratibial tumor burden of 31.3±1.4mm². ZOL drastically reduced solid tumor area to 2.6±1.1mm²; but the infiltrated area was 18.3±2.6mm² resulting in a tumor burden of 20.9±2.0mm² within the medullary space. Meanwhile, L-235 at all doses markedly reduced infiltrated tumor area (Fig. 6B-a). L-235 100mg/kg reduced solid and infiltrated tumor areas to 6.5±2.8 and 1.9±0.7mm² respectively, resulting in an overall tumor burden of 8.4±3.1mm² in tibial bone marrow space. In the treatment experiment, neither ZOL nor L-235 treatment was as effective at reducing tumor cells infiltrated into the trabecular bone as compared to that in the preventive regimen (Fig. 6B-b).

Local Metastasis

Based on our examination of the tibial sections stained with cytokeratin, small discrete tumor areas were detected toward the distal tibial end. Since bone marrow is known for high vascularization activity, we believe these small islands of BrCa cells represent local metastatic activity, and thus the distance from growth plate to the most distal site of observable micromass of tumor was determined to assess treatment-related effects on local metastasis. In the prevention protocol (Fig. 6C-a), L-235 at 30 and 100mg/kg doses significantly reduced this metastatic distance by 38 and 51 % respectively vs. vehicle (p<0.05). ZOL and the lowest dose of the CatKi did not appear to show significant effect on this parameter. On the other hand, in the treatment protocol,
both L-235 30mg/kg and ZOL reduced BrCa tumor metastasis by 42 and 47%, respectively, vs. vehicle (Fig. 6C-b, p<0.05).
Discussion

The pivotal role of CatK in OC bone resorption has been well established by both genetic and pharmacological evidence in animals and humans (11,12). CatK-deficient mice develop osteopetrosis associated with impaired OC function in degrading bone matrix. Loss-of-function mutations in the CatK gene lead to pycnodysostosis, a rare autosomal recessive disorder associated with bone sclerosis in humans (11,12). In a small clinical study with 43 BrCa women with bone metastasis, the CatKi ODN dosed as 5mg daily was recently demonstrated to be well tolerated and to suppress the bone resorption marker uNTX similarly to the clinical dose of ZOL at 4mg, i.v. after 4 weeks of treatment (16). Bone destruction associated with skeletal metastasis is known to be mediated by OC bone resorption (2) and the clinical finding with ODN in BrCa patients supports the notion that CatK inhibition may be a novel therapeutic approach for treating bone metastasis. However, there is still very limited understanding on whether the orally active CatK inhibitor(s) could be safe, efficacious and readily differentiated from the current injectable agents such as the bisphosphonates for the chronic treatment of metastatic bone disease. Hence, studies in animals have been thus conducted to further validate CatK as a target for the development of an oral inhibitor of osteoclastic bone resorption and for exploring its potential direct effects on tumor cell metastasis.

Previously, treatment with the CatKi AFG-495 was reported to significantly reduce cancer-induced osteolysis and skeletal tumor burden in an intratibial injection with human BT474 BrCa cells expressing CatK into nude mice (24). AFG-495 was reported to inhibit human CatK with an IC$_{50}$ of 3–6 nM and an excellent selectivity profile versus human cathepsins L and S; however, its potency and selectivity profiles against rodent cathepsins were not available (25). Because of species differences between the binding sites of human versus rodent CatK, there are usually
significant reductions in potency against the rodent CatK with the inhibitors developed against the human enzyme. Even if the human osteoporosis dose of AFG-495 has not been determined, it was dosed via i.p. injection twice daily in order to achieve sufficient high drug exposure in mice (15). Interestingly in that study, AFG-495 at 50mg/kg, i.p., twice daily for 39-days reduced tumor burden by 62%, whereas a single high bolus subcutaneous injection of ZOL 100μg/kg did not inhibit skeletal tumor burden (15). Of note, Le Gall et al also confirmed that AFG-495 did not inhibit subcutaneous growth of BrCa B02 tumor xenografts in nude mice at a dosing regimen that was demonstrated to inhibit skeletal tumor burden, demonstrating that CatK inhibition does not affect BrCa cell proliferation (15,24).

Here, we evaluated the CatKi L-235 orally at 10, 30 and 100mg/kg twice daily for 42 days in an intratibial injection of the BrCa MDA-MB-231 cells in nude rats. Efficacy of L-235 was evaluated in this preclinical model of bone metastasis in both prevention and treatment modes in comparison to ZOL. L-235 is selective against other human cathepsins (17) and retains good selectivity (>30-fold) toward rat CatB, CatL and CatS (data not shown). Similar to AFG-495, L-235 exhibits lysosomotropic properties; that is, these compounds accumulate within acidic subcellular organelles such as lysosomes or the resorption lacunae. This feature may compromise selectivity of the CatKi in vivo by inhibiting other lysosomal cathepsins at the doses used in this study (19,20). Several cysteine cathepsins, particularly CatB, have been shown to be causally involved in the migration and invasion of tumor cells (26,27); the lysosomotropic property of L-235 may in part contribute to its anti-invasive activity in this model of bone metastasis as discussed below. Despite the above drawback of L-235, this inhibitor showed potency equivalent to our clinical candidate ODN against the human CatK (IC$_{50}$ of ~ 0.2 nM) (18), but has a ~50-fold shift in potency against the rat enzyme while ODN is not active in rodent. Because the effective dose of
ODN at 5mg p.o., daily provided mean plasma concentration of 512 ± 203 nM in BrCa patients (16) and due to the relatively short half-life of L-235 in rodent, we thus selected L-235 at 10, 30, and 100mg/kg twice-daily, which provided the mean plasma concentrations of 10.4, 35.6 and 166.2 μM•hr, respectively in the rats. At this range of drug exposures, L-235 should display full CatK enzyme inhibition activity for studying the differential biological effects of inhibiting human CatK on MDA-MB-231 mediated cell invasion as well as blocking rat CatK activity of host osteoclastic bone resorption.

Interestingly, L-235 at the selected doses appeared to be a potent inhibitor of cortical disruption. While the lowest dose of L-235 at 10mg/kg showed partial protection of bone volume fraction and reduction on intratibial tumor growth, this dose showed substantial inhibition of cortical disruption in the same group. This observation is aligned with previous findings on the favorable efficacy of CatK inhibitors on enhancing cortical bone formation while reducing endocortical and intracortical bone remodeling (28). This unique mechanism of CatK inhibition on cortical protection is also highly correlated with the ability of CatKi to reduce extraskeletal tumor growth, suggesting that the cortical envelope may serve as a barrier to prevent the BrCa tumor overgrowth into the extramedullary soft tissue in this model of bone metastasis.

The function of CatK in BrCa is presently unknown. Besides its expression in OCs, there is limited evidence that CatK is also expressed in human breast carcinomas (14,15). In addition, BrCa cells residing in bone metastases overproduce CatK relative to the expression levels in primary tumors and soft tissue metastases (15). These findings suggest that cancer cells metastasizing to bone express bone-related genes to adapt and thrive in the bone microenvironment (29). We confirmed high expression of CatK in primary BrCa tumors and its upregulation in metastatic tumors as previously reported (15). To further examine the role of CatK in BrCa metastasis, Le
Gall et al demonstrated that the CatKi, AFG-495, did not directly inhibit BrCa cell proliferation while reduced matrigel cell invasion \textit{in vitro}. However, these authors did not further examine the effects of CatK inhibition on the local tumor metastasis \textit{in vivo}.

Secretion of the active CatK enzyme from macrophages and prostate cancer cells has been demonstrated (13,26). This pool of secreted enzyme could potentially participate in local invasion of tumor cells by mediating the extracellular or intracellular degradation or both of matrix proteins. In the experimental model of human BrCa bone metastasis in nude rats, we speculate that the ability of tumor cells to infiltrate into the surrounding bone marrow and among the trabecular spicules represented the local invasive activity of the BrCa cells. While ZOL showed no effect, even the low dose of L-235 at 10mg/kg significantly reduced the area of infiltrated tumor when dosed in prevention mode. This finding was supported by the direct action of this CatKi in inhibiting the human BrCa MDA-MB-231 cell matrigel invasion assay \textit{in vitro}. Curiously, this anti-invasive activity of the CatKi appeared to be reduced when dosed in treatment mode, although the effect of CatKi was still differentiated from ZOL. As an exploratory approach, we confirmed the potential of injected human tumor cells in the proximal tibia to develop micrometastasis at the distal end of the rat tibia. Consistent with the effects of CatKi in reduction of local tumor infiltration, L-235 treatment significantly reduced the incidence of distant metastasis of human BrCa cells.

In summary, we confirmed the high expression of CatK in primary and metastatic BrCa tumors and demonstrated that an orally active CatKi in both prevention and treatment modes protected against tumor-induced osteolytic lesions and cortical disruption, and reduced skeletal tumor burden in an experimental model of BrCa bone metastasis. The potential of the CatKi to reduce local tumor invasion implicated an upside potential of the mechanism of CatK inhibition over the standard antiresorptives such as the bisphosphonates, for the long-term care of BrCa.
patients with established bone metastasis. Additional studies on the molecular and cellular changes within the skeletal environment in the L-235-treated intratibial injected MDA-MB-231 model of MBD would be subjects of future investigation. Taken together, our results from this study support the role of CatK in BrCa skeletal metastasis and CatK inhibitors may represent a novel oral therapy for treatment of metastatic breast cancer.
Acknowledgments

We thank the staff at Piedmont Research Center, Morrisville, NC for carrying out the in-life portion of the studies; and the following Merck & Co., Inc, employees, G. Neusch and A. Cheema for their excellent technical support to the early phase of study execution.
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**Table 1**

Correlation of cathepsin K expression to histological grading of a breast tumor tissue microarray.

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Figure Legends

**Figure 1. Expression of cathepsin K in breast tumors.** (A) Localization of CatK in breast carcinoma by immunohistochemistry. Representative images of primary breast tumors (a) a negative control with BrCa tumor section without anti-CatK antibody; (b) CatK expression was highly induced in breast ductal epithelial cells as compared to adjacent stromal cells from a patient diagnosed with high grade invasive ductal carcinoma, and (c) another patient with infiltrated breast carcinoma. (d) The same image of the inset in (c, dashed box), is displayed at higher magnification demonstrating intense and punctated intracellular CatK expression. Bars, (a-c) 100μm and (d) 10μm. (B) CatK mRNA determined by RT-PCR elevated in BrCa tissue RNA extracted from: (a) primary tumors compared to adjacent normal tissues (n=12 matched pairs), and (b) metastatic tissues versus primary tumors from the same donor (n=4 matched pairs). *p<0.05 vs. matched pair controls.

**Figure 2. Treatment with the CatKi or zoledronic acid prevented osteolysis and reduced cortical disruption induced by BrCa cells in nude rats.** (A) Structure of L-235. (B) Human MDA-MB-231 BrCa cells were injected in the tibial bone marrow cavity of nude rats. Animals were treated 1 day after intratibial injection of BrCa cells with: (a) vehicle (Veh); (b) ZOL 7.5μg/kg/wk, s.c.; or (c) L-235 30mg/kg, p.o., bid. *In vivo* Faxitron monitoring weekly up to day-35 showed progression of osteolytic lesions in Veh. *Ex vivo* µCT imaging of the same tibia harvested at day 42. (C) Bone volume fraction (BV/TV, %) of the proximal tibia expressed relative to total tissue volume (TV) of the same region from the age-matched intact nude rats. L-
Cathepsin K inhibitor for metastatic bone disease

235 (10, 30 and 100 mg/kg, p.o., bid) and ZOL (7.5μg/kg/wk, s.c.) were dosed in (a) prevention and (b) treatment mode post-cell injection vs. Veh. **p<0.01; ***p<0.001 vs. Veh.

Figure 3. Histological characterization of breast cancer tumor growth in the human MDA-MB-231 intratibial injection model of bone metastasis in nude rats. (A) A representative image of a sagittal view of a tibia engrafted with MDA-MB-231 BrCa cells co-stained for TRAP(+) OCs (red, arrows) and for tumor cells using anti-cytokeratin 7 Ab (brown). The regions of interest included: (a&b) intratibial solid tumor area; b: enlarged view of the black dashed lined inset. (a&c) extraskeletal solid tumor area outside of the cortical envelope; c: same as black solid lined inset. (a&d) Area of tumor cells infiltrated in between trabecular bone spicules; d: same as white dashed lined inset. (a&e) Distance of tumor cells metastasized from the implantation site toward distal tibial end; e: same as white solid lined inset. Magnification, (a) 0.6X; (b&c) 10X; (d&e) 20X. (B) Representative images of the H&E stained sections of tibia engrafted with BrCa cells at day 42: (a&d). In the Veh-treated tibia, injected BrCa cells developed into solid tumor mass in the marrow cavity and extraskeletal outgrowth. Regions of disrupted cortical bone (dashed lines) are shown. d: same as inset in a; (b&e) ZOL (7.5μg/kg/wk, s.c.) reduced loss of cortical and trabecular bone structures, and decreased areas of infiltrated tumor cells, e: same as inset in b; (c&f) L-235 (100mg/kg, p.o., b.i.d.) treated tibia showed normal morphologies of bone marrow, trabecular and cortical bone, f: same as inset in c. Magnification, (a-c) 1X; (d-f) 4X or bar, 500μm. * the injected site; T, tumor; Ct, cortical; Tb, trabecular bone; BM, bone marrow.

Figure 4. Treatment with the CatKi L-235 compared to Zoledronic acid inhibited cortical disruption in the experimental model of BrCa bone metastasis. Cortical disruption was
measured from H&E stained sections as the percent of cortical surface completely eroded through from the estimated total length of both cortical envelopes. (a) L-235 at 30mg/kg, p.o., b.i.d. preserved cortical bone in the prevention regimen compared to ZOL (7.5μg/kg/wk, s.c.); (b) L-235 at 30mg/kg, p.o., b.i.d. showed comparable efficacy as ZOL (7.5μg/kg/wk, s.c.) in the treatment regimen.

Figure 5. Treatment with the CatKi or zoledronic acid inhibited intratibial human BrCa tumor growth in nude rats. (A) Solid tumor area was manually contoured from histological images of sagittal tibial sections stained with H&E. L-235 (10, 30 and 100 mg/kg, p.o., b.i.d.) and ZOL (7.5 μg/kg/wk, s.c.) versus Veh were dosed in (a) prevention, (b) and treatment mode. (B) Tumor volume was estimated from μCT-based volume as the “non-bone” osteolytic regions within the proximal tibia from the same studies. With the same dosages as described in (A), L-235 and ZOL versus Veh were dosed in (a) prevention, (b) and treatment mode. *p<0.05, **p<0.01, ***p<0.001 vs. Veh.

Figure 6. Treatment with the CatKi reduced local BrCa invasiveness in the experimental model of human BrCa bone metastasis in nude rats. (A) Area of solid tumor developed outside of the tibial cortical envelope, surrounding the injected site was manually contoured from H&E stained histological tibial images of nude rats dosed with Veh, L-235 (10, 30 and 100 mg/kg, p.o., b.i.d.) and ZOL (7.5 μg/kg/wk, s.c.) in (a) prevention and (b) treatment mode. (B) Area of infiltrated tumor cells detected in bone marrow space and amongst the trabecular networks was measured from H&E stained sections obtained from tibia of animals dosed with Veh, L-235 and
ZOL at the doses as indicated, in (a) prevention and (b) treatment mode. (C) The longest distance from the growth plate to detected BrCa micrometastasis was determined as described in Methods. The mean distances of micrometastasis were determined from tibial of nude rats dosed with Veh, L-235 and ZOL at the doses indicated, in (a) prevention and (b) treatment mode. *p<0.05, **p<0.01, ***p<0.001 vs. Veh.
Figure 1 – Duong et al 2014

A

B

Expression relative to GAPDH

0.00 0.05 0.10 0.15 0.20 0.25

Normal (N=12) Primary (N=12)

Expression relative to GAPDH

0.00 0.02 0.04 0.06 0.08 0.10

Primary (N=4) Metastatic (N=4)
Figure 2 – Duong et al 2014

A

L-006,235

C

a

B

D7 D21 D35 D42

Veh ZOL L-235

10 30 100 mg/kg (bid)

0 20 40 60 80 120

BV/TV (%)

**p<0.01; ***p<0.001 vs. Veh

a b c
Figure 4 – Duong et al 2014

a

Disr.S/Ct.S (%)

Veh ZOL L-235

10 30 100 mg/kg (bid)

b

Disr.S/Ct.S (%)

Veh ZOL L-235

30 mg/kg (bid)

*p<0.05; **p<0.01; ***p<0.001 vs. Veh
Figure 5 – Duong et al 2014

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*p<0.05; **p<0.01; ***p<0.001 vs. Veh
Figure 6 – Duong et al 2014

A

![Graph A](image)

Tumor Area (mm²)

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B

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Inf. Tumor Area (mm²)

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C

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Distance (mm)

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*p<0.05; **p<0.01; ***p<0.001 vs. Veh; § p=0.533 vs. ZOL
Molecular Cancer Therapeutics

Efficacy of a Cathepsin K Inhibitor in a Preclinical Model for Prevention and Treatment of Breast Cancer Bone Metastasis

Le T. Duong, Gregg A Wesolowski, Patrick Leung, et al.

Mol Cancer Ther Published OnlineFirst September 23, 2014.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-14-0253

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