Imaging of Cathepsin K activity in rodent models of bone turnover and soft tissue calcification

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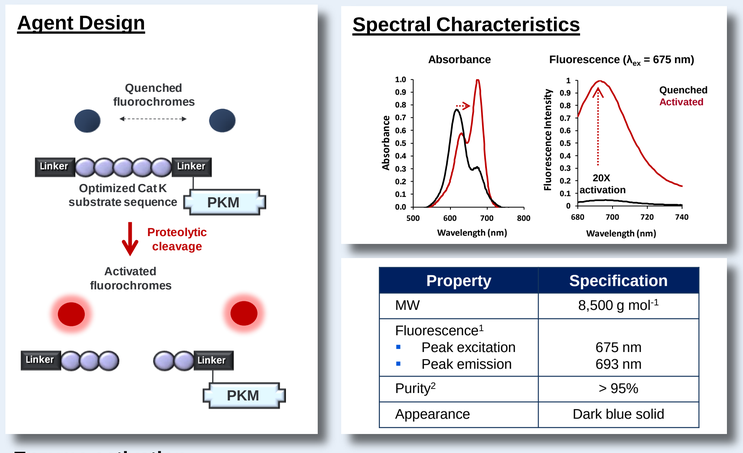
Abstract

Cathepsin K (Cat K), a lysosomal cysteine protease with strong collagenolytic activity, is expressed predominantly in osteoclasts, chondrocytes and synovial fibroblasts. Since Cat K is critically involved in bone resorption and collagen degradation, Cat K inhibitors are being evaluated in clinical trials for osteoporosis and the treatment of women with breast cancer and bone metastases. Clearly, a specific imaging agent allowing the detection, quantification and monitoring of Cat K activity in vivo would prove valuable in pre-clinical research. Herein, we report the use of a selective near-infrared (NIR) fluorescent Cat K imaging agent in a variety of in vivo preclinical applications. This agent was developed based on a human Cat K-cleavable sequence, and it is optically quenched in its native form but becomes highly fluorescent (EX/EM 675/693 nm) upon specific enzymatic cleavage. In arthritis and osteoporosis, osteoclasts are well characterized for their role in escalating bone turnover, leading to either immune-driven bone damage or non-inflammatory bone density decreases, respectively. BALB/c mice with moderately advanced anti-collagen antibody-induced arthritis (CAIA) showed 4-fold increases in Cat K-associated fluorescent signal in arthritic paws as compared to control paws, with predominant distribution of signal within the ankle region. To address non-inflammatory bone resorption, we used two different models of bone loss in the proximal tibia; rat ovariectomy (OVX) and vitamin D-induced bone loss in mice. Female rats were ovariectomized, or sham-ovariectomized, at 3 months of age and imaged on days 1, 9, 16, and 27 with a 750 nm NIR bone-turnover imaging agent (OsteoSense®) and the Cat K imaging agent by multiplex imaging. Throughout this time course, there was an apparent and quantifiable 3-fold increase in Cat K activity in the proximal tibias of OVX rats as compared to those of controls. In contrast, OsteoSense, which detects regions of both bone growth and bone loss, showed no clear differences between groups. Both agents, however, detected 2-5-fold increased signal in the proximal tibial regions of mice treated for 4 days with high-doses of vitamin D, supported by the significantly increased plasma levels of calcium in these mice. These two NIR agents also detected 5-8-fold fluorescent increases within the kidneys of treated mice, correlating with both ex vivo imaging of kidneys, fluorescence microscopy of kidney tissue sections, and assays for tissue calcium. Tissue calcification in the aortas of apoE deficient mice on high fat diet for 25 weeks was also explored, showing significant increases in Cat K agent fluorescence measured in living mice by non-invasive fluorescence molecular tomography (FMT®) imaging. Prophylactic treatment of these mice with ezetimibe blocked fluorescence increases in the aorta to the level of wild-type control mouse aortas, and these results correlated well with ex vivo tissue fluorescence and with staining by Oil Red O. In conclusion, the NIR Cat K activatable imaging agent is a useful tool for investigating both bone resorption and pathologic conditions involving soft tissue calcification.

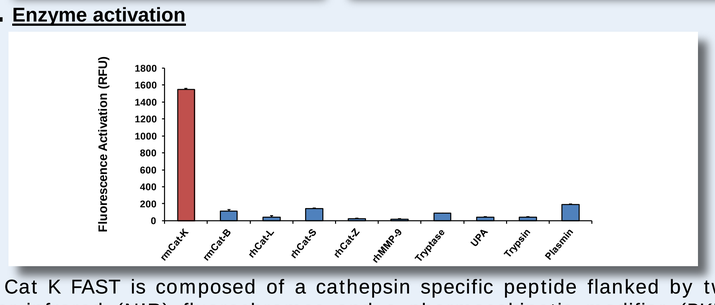
1 Cat K FAST™ 680 characterization

A. Structure and physicochemical properties



Property	Specification
MW	8,500 g mol^{-1}
Fluorescence[1] ▪ Peak excitation ▪ Peak emission	675 nm 693 nm
Purity[2]	> 95%
Appearance	Dark blue solid

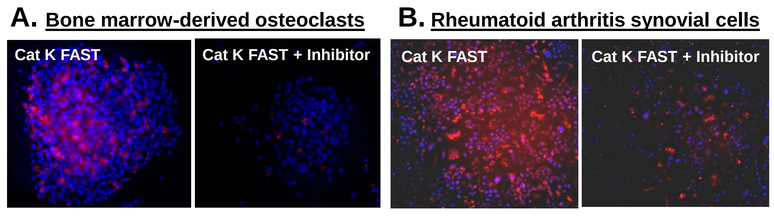
B. Enzyme activation



A. Cat K FAST is composed of a cathepsin specific peptide flanked by two near-infrared (NIR) fluorochromes and a pharmacokinetic modifier (PKM) for optimal in vivo imaging. Upon cathepsin K cleavage of the substrate sequence, the agent becomes highly fluorescent. **B.** Cat K FAST was incubated in the presence of a variety of activated cathepsins, MMPs, and other enzymes at optimal pH and temperature. Fluorescence was read at peak of activation (24h) using a fluorescence microplate reader and showed good selectivity for cat K.

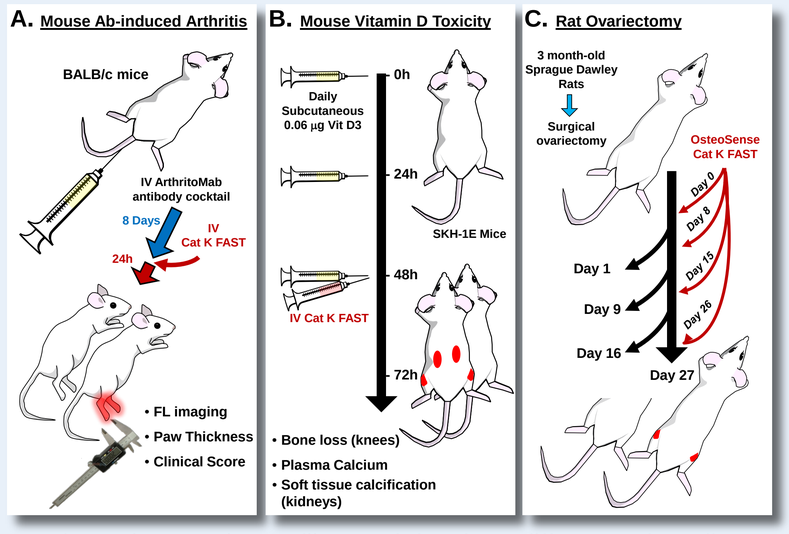
2 Cell Activity

A. Bone marrow-derived osteoclasts **B.** Rheumatoid arthritis synovial cells



A. Bone marrow cells were prepared from CD1 mice (Charles River Laboratories) according to previously-described methods. Briefly, bone marrow was flushed from mouse femurs, cultured for 3 days, and adherent cells were further cultured in the presence of recombinant mouse MCSF (40 ng/ml, Sigma, St Louis, MO) + human RANKL (100 ng/ml). **B.** Human primary synovial fibroblasts from rheumatoid arthitis patients were obtained from Asterand (Detroit, MI) and maintained in DMEM with 10% fetal bovine serum. For in vitro specificity assessment, cells were pre-incubated 1h with a selective Cat K inhibitor (200 nM) prior to addition of 0.5 uM Cat K FAST (Blue = DAPI nuclear stain; Red = Cat K FAST fluorescence).

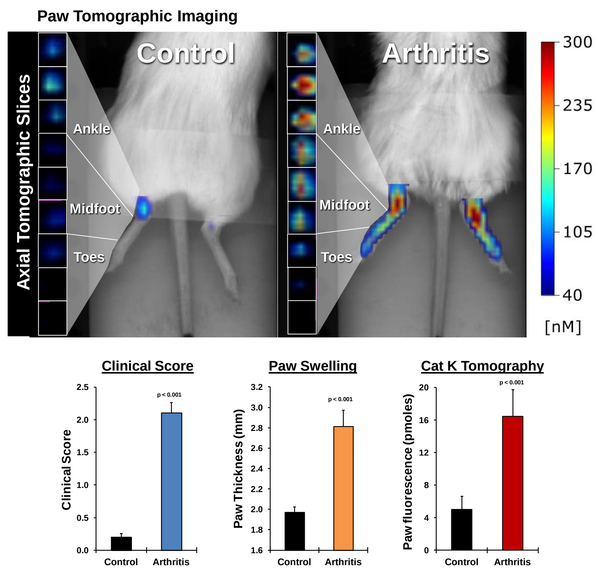
3 Animal Models of Bone Turnover



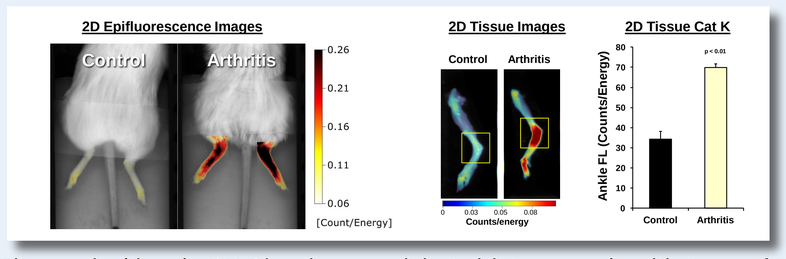
Experimental protocols are illustrated for three different rodent disease models; **A.** mouse antibody-induced arthritis, **B.** mouse vitamin D3-induced toxicity, and **C.** rat ovariectomy-induced osteoporosis. Tomographic (3D) paw, knee, and kidney imaging was performed on the FMT 2500 24h after Cat K 680 FAST and/or OsteoSense 750 injection.

4 Mouse Arthritis Imaging

A. Paw imaging and quantification

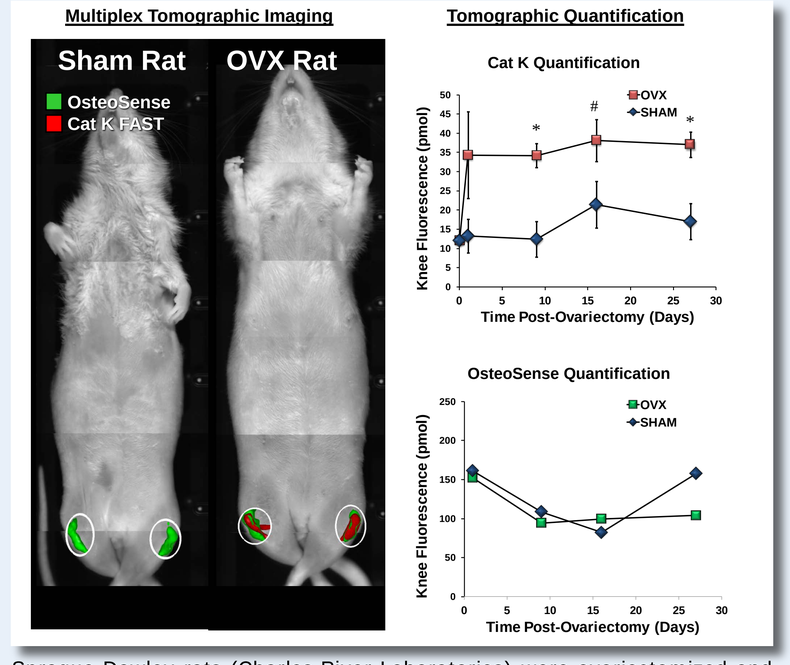


B. 2D epifluorescence and ex vivo tissue assessment



Five week-old male BALB/c mice were injected intravenously with 4 mg of an arthritigenic monoclonal antibody cocktail (ArthritoMab™ Antibody Cocktail, MD Biosciences, St. Paul, MN) followed 3 days later by 50 µg of lipopolysaccharide (LPS) injected intraperitoneally. **A.** Changes in disease-induced mice were determined on day 9 by FMT 2500 imaging, paw thickness (vernier caliper), clinical observation score, and quantification of Cat K FAST fluorescence from tomographic datasets. **B.** Tomographic imaging results were confirmed by in vivo and ex vivo 2D epifluorescence imaging.

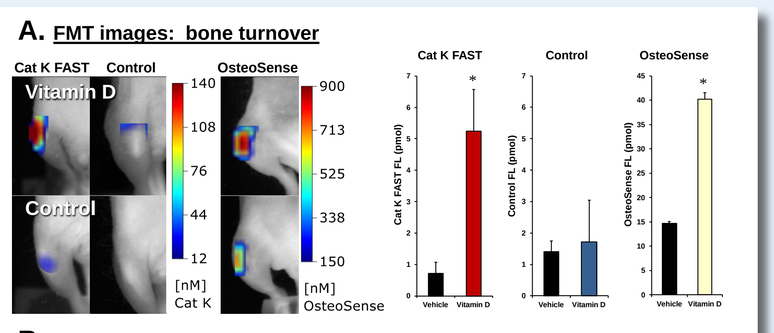
5 Rat Ovariectomy: Osteoporosis Imaging



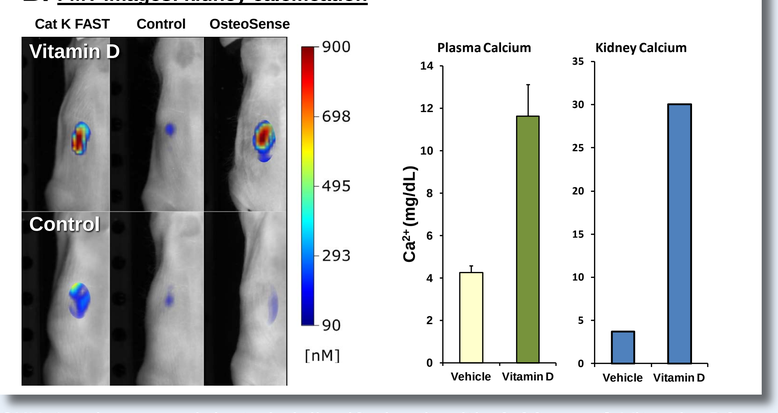
Sprague Dawley rats (Charles River Laboratories) were ovariectomized and depilated to allow imaging of the lower torso and legs by FMT 2500. Rats were injected intravenously with Cat K 680 FAST and OsteoSense 750EX for imaging 24h later. For subsequent time points, rats were pre-imaged and then re-injected with imaging agents for imaging 24h later. OsteoSense quantification was determined by subtraction of pre-image results. Graphs show no difference in OsteoSense incorporation into the knee region, however a significant increase in Cat K FAST signal was seen as early as one day post surgery. Symbols indicate statistical significance (*p < 0.01, # p < 0.05).

5 Vitamin D3 Toxicity

A. FMT images: bone turnover



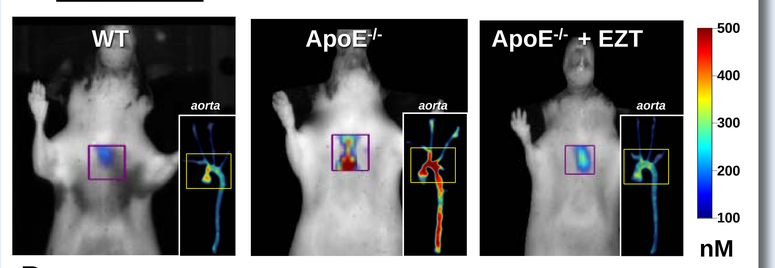
B. FMT images: kidney calcification



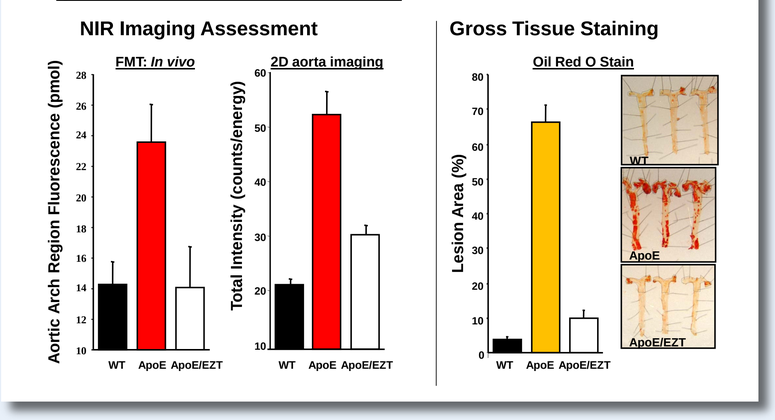
SKH-1 mice were injected daily (3 days) with 0.06 µg of Vitamin D3, a vitamin known to cause bone loss by increasing calcium mobilization and reabsorption. **A.** Mice showed clear changes in the knees as detected by both an increase in Cat K activity (Cat K FAST) and increased bone turnover (OsteoSense). A constitutively fluorescent, un-targeted control agent was used to confirm imaging specificity of Cat K FAST and OsteoSense. **B.** Soft tissue calcification was detected in the kidneys by calcium microplate assay, with higher levels than in plasma. Imaging with Cat K FAST and OsteoSense confirmed biological alterations in the kidneys consistent with calcification.

6 Cat K Imaging in Atherosclerosis

A. Imaging results



B. Quantification and ex vivo validation



ApoE-/- mice (The Jackson Labs) were maintained on high fat diet +/- 0.005% (wt/wt) Ezetimibe (EZT). As negative controls, age-matched wild type mice were maintained on normal chow. After 25 weeks, mice were injected IV with Cat K FAST and imaged tomographically by FMT 2500 to quantify fluorescence changes in the aorta. **A.** Fluorescence 3D images of representative mice showing upper heart region fluorescence (*i.e.* capturing aortic root and arch), with insets showing 2D images of excised aortas. **B.** Quantification of heart region signal and corroboration of results with ex vivo Oil Red O staining of dissected aortic tissues. Data shows excellent inhibition of disease by ezetimibe, which can be readily detected and quantified by Cat K FAST.

Summary

We have developed a near infrared fluorescent cell labeling agent, Cat K FAST 680, that can specifically detect changes in Cat K activity associated with osteoclast-mediated bone resorption. The data highlights the utility of this agent in three different models of bone resorption as well as in two models known to develop soft tissue calcification. Noninvasive fluorescence tomography by FMT 2500 allowed robust quantification of tissue changes in living animals. As bone turnover is of critical importance in many diseases and conditions, Cat K FAST provides an important imaging tool to study bone biology in vivo.

7 References

1. Kozloff KM *et al*. Non-invasive optical detection of cathepsin K-mediated fluorescence reveals osteoclast activity in vitro and in vivo. Bone. 2009; 44: 190-198.
2. Jaffer AF *et al*. Optical visualization of Cathepsin K activity in atherosclerosis with a novel, protease-activatable fluorescence sensor. Circulation. 2007; 15: 2292-2298.

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