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 preclinical 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 (680/693 nm) upon specific enzymatic cleavage. In arthritis and osteoporosis, osteoclasts are well characterized for their role in escalating arthritic or osteoporotic conditions, leading to bone damage or non-inflammatory bone density decreases, respectively. BALB/c mice with moderately advanced anti-collagen antibody-induced arthritis (CollaIgM AsS) were used to confirm imaging specificity of Cat K FAST. In aortic calcification, Cat K activity associated with osteoclast-mediated bone resorption was observed. To address non-inflammatory bone resorption, we used two different models of bone loss in the proximal tibia; rat aorta (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. Through this time course, there was an apparent and quantifiable 3-fold increase in Cat K activity in the proximal tibial 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 and these results correlated well with ex vivo imaging of kidneys, fluorescence microscopy of kidney tissue sections, and assays for tissue calcification in the aorta of aper deficient mice on high fat diet for 25 weeks was also explored, showing significant increases in Cat K activity fluorescence measured in living mice by non-invasive fluorescence molecular tomography (FMT®) imaging. Prophylactic mouse vitamin D3- hydroxylation administration increased bone turnover, with higher levels than in plasma. Imaging with Cat K FAST and OsteoSense confirmed biological alterations in the kidneys consistent with calcification.

1 Cat K FAST™ 680 Characterization

A. Structure and physicochemical properties

- **Agent Design**
  - **Spectral Characteristics**
    - Absorbance
      - 600 nm
    - Emission
      - 680 nm
  - **MW 8,500 g mol⁻¹
  - **pKₐ 5.2
  - **pIC₅₀ 10⁻⁴ M
  - **IC₅₀ 10⁻⁴ M
  - **Exposure activity
    - **Cat K FAST** is composed of a cathepsin specific peptide flank 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. **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 excitation (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

3 Animal Models of Bone Turnover

A. Mouse Anti-Inflammatory Arthritis
B. Mouse Vitamin D Toxicity
C. Cat Ovariectomy

4 Mouse Arthritis Imaging

A. Paw imaging and quantification
B. 2D epifluorescence and ex vivo tissue assessment

5 Rat Ovariectomy: Osteoporosis Imaging

A. Multimodality Imaging
B. Tomographic Quantification

6 Cat K Imaging in Atherosclerosis

A. Imaging results
B. Quantification and ex vivo validation

7 References


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.