Development of a Fast Activating New Near Infrared-Labeled Agent for Detecting Cathepsin K Activity

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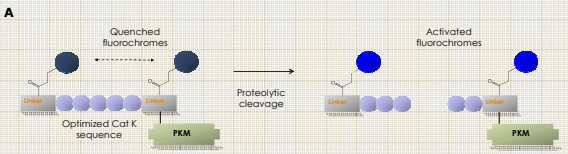
1 Abstract

Cathepsin K (Cat K), a lysosomal cysteine protease with strong collagenolytic activity, is expressed primarily 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 and clinical settings. Herein, we report the design, synthesis and evaluation of a fast-activating near-infrared fluorescence (NIRF) Cat K imaging agent. The agent, Cat K 680 FAST (PerkinElmer), was developed based on a human Cat K-cleavable sequence, a pair of self-quenching NIRF fluorochromes and a pharmacokinetic modifier to confer increased blood half-life. The agent is optically quenched in its native form, but upon cleavage by Cat K, it becomes highly fluorescent with EX/EM at 674/692 nm. In vitro selectivity was assessed by cleaving Cat K 680 FAST with a panel of enzymes and monitoring released fluorescence over time. The agent was preferentially cleaved by Cat K. Cell uptake assays showed that Cat K 680 FAST was activated by human synovial fibroblasts from rheumatoid arthritic patients and rabbit synoviocytes. In human osteoclasts on bone, Cat K 680 FAST localized to lysosomes and the resorption lacunae. The activation was significantly decreased in synovial fibroblasts treated with specific Cat K inhibitors.

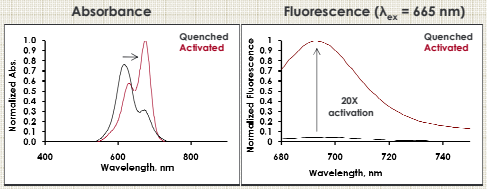
Cat K 680 FAST exhibited blood half-life of 25 minutes providing good distribution in target tissue, thus enabling a suitable window for *in vivo* imaging. These results highlight the potential of Cat K 680 FAST for detecting and monitoring Cat K activity in osteoporosis and arthritis, as well as in atherosclerosis, lung remodeling, obesity and cancer.

2 Description of the Agent

Schematic diagram of Cat K 680 FAST



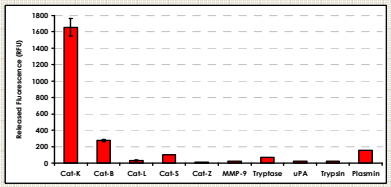
B **Absorption and Emission Spectra**



A. Cat K 680 FAST (PerkinElmer) comprises a Cat K-specific peptide sequence, flanked by two near-infrared (NIR) fluorochromes and a pharmacokinetic modifier (PKM) selected to provide optimal attributes for *in vivo* imaging. In its native state the agent is quenched and upon proteolysis of the substrate by Cat K, becomes highly fluorescent. The agent was characterized by LCMS, UV-VIS and fluorescence spectroscopy. **B.** A hypsochromic shift in the absorbance maxima is observed compared to the parent fluorophore for the quenched molecule. Absorbance and emission spectra of the autoquenched (black) and enzyme activated (red) forms are shown herein.

3 Biochemical Profile

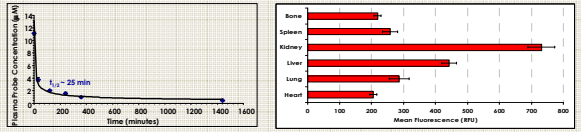
***In Vitro* Agent Activation**



Cat K 680 FAST (0.5 µM final concentration in the assay) was cleaved in the presence of 0.05-0.2 µM activated Cathepsins K, B, L, S, Z, MMP-9, tryptase, urokinase-type plasminogen activator (uPA), trypsin or plasmin. Reactions were carried out in optimal buffers, pH and temperature. Fluorescence was read using a fluorescence microplate reader 5 hrs after beginning the reaction. Shown is the fluorescence released after cleavage with the enzymes (after subtracting the background). Cat K 680 FAST is selectively cleaved by Cat K.

4 Pharmacokinetic and Biodistribution Profile

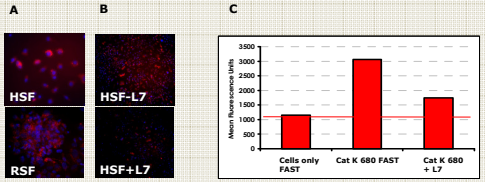
Pharmacokinetics **Biodistribution**



A. BALB/c mice were injected intravenously (i.v.) with Cat K 680 FAST (2 nmoles). Blood was drawn at various times, plasma obtained by centrifugation, and concentration of probe assessed after diluting plasma in DMSO 1:1 vol:vol (which causes the quenched agent to partially fluoresce) and using a calibration standard. Cat K 680 FAST exhibits a plasma half-life of ~ 25 min.

B. Mice were injected i.v. with Cat K 680 FAST (2 nmoles) and sacrificed 6 hrs later. Organs were excised and imaged using a reflectance imaging system. Regions of interest (ROI) were drawn around each organ and the mean fluorescence (Relative Fluorescence units, RFU) determined. Shown are Mean ± SEM.

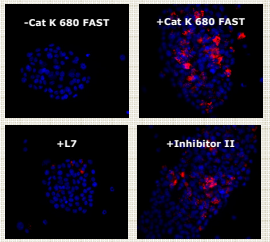
5 Activation of Cat K 680 FAST by Synovial Fibroblasts



A. Uptake and activation of Cat K 680 FAST by human synovial fibroblasts from a patient with rheumatoid arthritis (upper) and rabbit synoviocytes (HIG-62 cell line, lower panel). Cells were cultured in the presence of Cat K 680 FAST (1 µM) for 6 hrs. Red: Cat K 680 FAST, Blue: DAPI nuclear stain; final magnification 40x. **B.** Human synovial fibroblasts were pre-incubated in the absence (top) or presence (lower panel) of the specific Cathepsin K inhibitor L7 (200 nM) for 1 hr before addition of the agent. Cells were then cultured for an additional 6 hrs. Fluorescence microscopy clearly shows the inhibition of activation by the cathepsin K inhibitor. Final magnification 20x **C.** Flow cytometry of human synovial fibroblasts shows that the addition of the cathepsin K inhibitor resulted in a 70% inhibition of the uptake and activation of Cat K 680 FAST in cells.

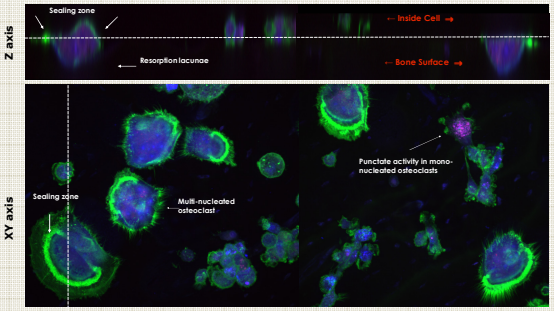
6 Activation of Cat K 680 FAST In Vitro

Bone marrow-derived RANKL-activated macrophages



Bone marrow cells were isolated from CD1 mice and cultured at 1 x 10^6 cells/well in aMEM with 10% FBS. Non-adherent cells were discarded after 3 days and adherent cells cultured for 5 days in the presence of mouse M-CSF (40 ng/ml) and human RANKL (100 ng/ml). On the 6th day, Cat K 680 FAST was added at a final concentration of 0.5 µM in the absence or presence of specific Cathepsin K inhibitors L7 (Merck) (C) or Inhibitor II (Calbiochem). After 4 hrs, cells were washed, cytopsun onto glass slides and visualized under fluorescence microscopy (Red: activated agent). Controls included cells with no agent. Cells were counterstained with DAPI nuclear stain (Blue). Final magnification 40x.

Human Osteoclasts



Human osteoclasts were cultured on bovine cortical bone for 7 days. Cat K 680 FAST (1 µM) was added for 24 hrs. Cells were visualized by confocal microscopy. Cathepsin K activity was detected in the resorption lacunae and intracellularly in both mononuclear and multinucleated cells (blue: activated Cat K 680 FAST). Acidic vesicles in lysozomes were stained in red using Lysotracker and osteoclast actin rings were visualized using FITC-phalloidin (green).

7 Summary

Cat K 680 FAST is a cathepsin K activatable agent that is optically silent upon injection and produces fluorescent signal after being cleaved by disease-related cathepsin K. Cathepsin K is highly expressed in osteoclasts, suggesting a specialized role in bone resorption, and also in synovial fibroblasts. It is found extracellularly in resorption lacunae and intracellularly in lysozomes. Besides application in bone disease such as osteolysis, bone metastasis, and osteoporosis, Cat K 680 FAST can also be used for a wide range of disease models, such as atherosclerosis, obesity, arthritis, lung fibrosis and diabetes.