Near-Infrared Quantitative Fluorescence Imaging of Renin Activity in Kidney Tissue

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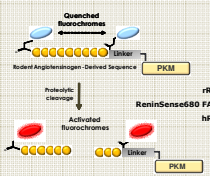
1 Abstract

The Renin-Angiotensin System (RAS) is well studied for its regulation of blood pressure and fluid homeostasis. Renin, the rate-limiting enzyme of RAS, cleaves angiotensinogen to form angiotensin I (Ang-I). Ang-I is further cleaved by angiotensin converting enzyme, to produce the main effector of RAS, angiotensin II (Ang-II). Ang-II induces increases in blood pressure and fluid retention in the kidneys and stimulates cardiac hypertrophy. Abnormal RAS function has been linked to hypertension and cardiovascular disease. Our objective was to synthesize peptide substrates containing two internally quenched near infrared (NIR) fluorochromes, which are selectively activated by renin cleavage, providing an agent to non-invasively image and quantify the signal of renin activity in the kidneys by Fluorescence Molecular Tomography (FMT 2500, PerkinElmer). To address selectivity of activation, the agents were tested for activation by a protease panel containing renin from various species, cathepsins (D and G) and human neutrophil elastase. Based on its activation profile, rodent renin specific constructs were selected for further ex vivo and in vivo studies. Renin activity in kidneys was induced in mice by short-term low salt diet combined with diuretic treatment. This treatment resulted in upregulation of renin activity which was quantified by measuring the increase in the fluorescence signal in the kidneys and plasma samples as compared to the controls using FRI tissue imaging and FMT imaging of live animals. These studies illustrate the potential of NIR fluorescence imaging to non-invasively quantify renin activity in mice. This approach could provide new opportunities to assess disorders linked to altered renin/RAS activity and to closely monitor the efficacy of therapeutic treatments.

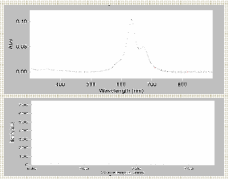
2 Description of the Imaging Agents

A **Schematic diagram of Renin Agents**



rRS680: rodent seq. 1 (no PKM)
ReninSense680 FAST: rodent seq. 2 **(with PKM)**
hRS680: human seq. (no PKM)

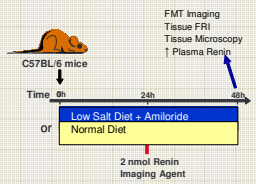
B **Absorption and Emission Spectra**



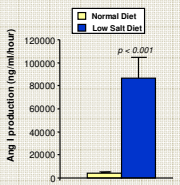
Over 25 renin agents were produced using rodent and human angiotensinogen-derived peptide sequences and the NIR fluorophore VivoTag-S 680 (PerkinElmer). Selected constructs were derivatized with pharmacokinetic modifiers (PKMs). A. Based on SAR data we identified as the most promising compounds: rat renin substrate (rRS)680 – rodent angiotensinogen-derived sequence 1 (no PKM); ReninSense680 FAST (PerkinElmer) - rodent angiotensinogen-derived sequence 2 conjugated with PKM and human renin substrate (hRS)680 –human angiotensinogen-derived sequence (no PKM). B. Upon protease cleavage of the substrate sequence, the internally quenched agents become highly fluorescent. The agents were characterized by LC-MS, UV-VIS and fluorescence spectroscopy. Absorbance and emission spectra of the autoquenched (grey) and enzyme activated (red) forms of rRS680 are shown as examples.

3 Description of the Mouse Model of Renin Upregulation

A **Induction of *In Vivo* Renin Activity**



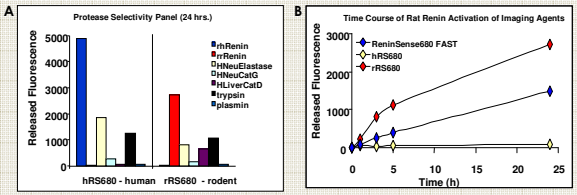
B **Plasma Renin Activity**



A. Kidney renin activity in C57BL/6 mice was increased experimentally by feeding mice a low salt diet (0.02% sodium) and including a diuretic (amiloride - 0.1 mg/5ml or ~5 mg/kg/day) in their drinking water for 48h. B. Plasma renin upregulation (an indicator of increased kidney renin) was confirmed by a competition-based enzyme immunoassay to detect Angiotensin I resulting from Angiotensinogen cleavage (Analytical Biochemistry 388 (2009) 134-139).

4 Biochemical Profile of Renin Imaging Agents

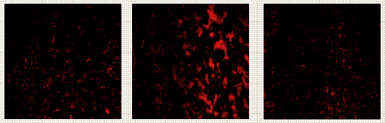
***In Vitro* Activation Profile of Human and Rodent Renin Agents**



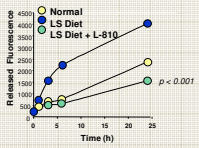
A. Activatable agents (1 mM final concentration in the assay) derived from human and rat angiotensinogen peptide sequences were cleaved in the presence of 0.1 mM recombinant human renin (rhRenin), recombinant rat renin (rrRenin), human neutrophil elastase (HNeu Elastase), human neutrophil cathepsin G (HNeu CatG), human liver cathepsin D (HLiver Cat D), trypsin or plasmin. Reactions were carried out in optimal buffers, pH and temperature. Fluorescence was measured using a fluorescence microplate fluorimeter at 24 hrs after beginning the reaction. B. Only the rodent renin agents rRS680 and ReninSense680 FAST were cleaved by the recombinant rat renin (rrRenin).

5 *In Vivo* Renin Inhibitor Confirms Specific Renin Upregulation in Kidney Tissue and Plasma of Mice on Low Salt Diet

A **Mouse Kidney Fluorescence Microscopy**



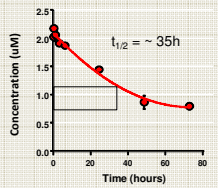
B **Plasma Renin Assay**



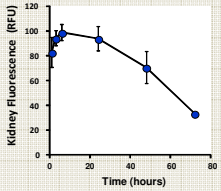
C57BL/6 mice were fed with a normal or sodium deficient diet + amiloride. Rodent renin inhibitor L-810 (30 mg/kg) was administered by oral gavage in two doses (1st dose: 8 h after start of diet and 2nd dose: 24 h after start of diet). After 24h of diet, kidneys (A) and plasma samples (B) were collected for assessment of renin activity using rRS680. Plasma renin activity was assessed as described in Fig 4. Kidney renin was assessed in situ by incubating kidney slices (10 µm thick) with 1 µM rRS680 at 37°C for 3h. Fluorescent microscopy images of kidney cortical regions were captured with an acquisition time of 440 ms.

6 ReninSense680 FAST Plasma and Kidney Tissue Pharmacokinetics of Activation

A **Plasma PK**



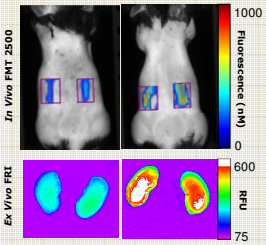
B **Kinetics of Kidney Signal**



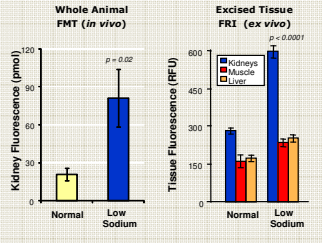
Normal C57BL/6 mice were injected i.v. with 2 nmol of ReninSense680 FAST for assessment of the kinetics of activation in (A) plasma samples and (B) kidney tissue at the times indicated. Plasma levels were assessed using an ELISA kit to detect the PEG moiety on the agent (Epitomics, Burlingame CA). Kidney tissue renin was assessed ex vivo by fluorescence reflectance imaging of intact, excised kidneys.

7 Non-Invasive Imaging of Renin Upregulation in Mouse Kidneys

A **Imaging of Kidneys**



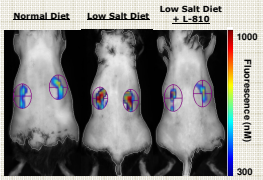
B **Quantification of Kidney Signal**



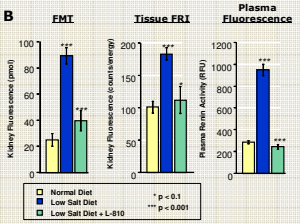
24h after starting C57BL/6 mice on normal or low salt diet, **ReninSense680 FAST** was injected intravenously (2 nmol/mouse). Animals were imaged 24h post probe injection and then kidneys were removed and imaged. **A.** Images of representative mice imaged by FMT (upper panels) and excised kidneys imaged by FRI (lower panels). **B.** Measurement of kidney region fluorescence by FMT (left panel) and average fluorescence intensity of removed kidneys (right panel). Data represents a composite of 2 representative studies.

8 Validation of Selective Renin Activation in Kidneys using Renin Inhibitor L-810

A



B



C57BL/6 mice were placed on normal or low salt diet/amiloride, and the rodent renin inhibitor L-810 (30 mg/kg) was administered to one group of low salt diet/amiloride mice by oral gavage 8 h, 22h, and 30h after the start of diet. At 24 h after start of diet, ReninSense680 FAST was injected intravenously (2 nmol/mouse), and mice were imaged non-invasively 24h later by FMT 2500 to generate tomographic kidney region images. Further assessment of the fluorescence of excised kidney and collected plasma samples was assessed as a verification of FMT imaging results. **A.** Tomographic near infrared images showing kidney region fluorescence. **B.** Quantification of FMT tomography, tissue fluorescence by FRI, and plasma fluorescence by microplate fluorimeter. These results show that ReninSense680 FAST can detect and quantify changes in kidney renin activity *in vivo* with results that correspond to renin activity changes within the plasma and excised kidneys.

9 Summary

We have developed a novel FAST ("Fluorescent Activatable Sensor Technology") *in vivo* imaging agent based on an angiotensinogen substrate sequences that is selectively cleaved and activated by rodent renin. ReninSense680 FAST, a PKM-modified agent suitable for in vivo administration, distributes to the kidney, is activated in a model of renin upregulation, and provides a tool for *in vivo* imaging and quantification of the changes in renin activity in kidneys and plasma. This study illustrates the potential of NIR fluorescence to monitor abnormal renin-angiotensin system (RAS) function and the efficacy of therapeutic treatment in disorders such as hypertension and cardiovascular disease.

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