

## GluCEST imaging of Tumor Protease Activity

Mohammad Haris<sup>1,2</sup>, Anup Singh<sup>1</sup>, Imran Mohammed<sup>3</sup>, Ranjit Ittyerah<sup>4</sup>, Kavindra Nath<sup>4</sup>, Ravi Prakash R Nanga<sup>1</sup>, Catherine Debrosse<sup>1</sup>, Feliks Kogan<sup>1</sup>, Kejia Cai<sup>1,5</sup>, Harish Poptani<sup>4</sup>, Damodar Reddy<sup>1</sup>, Hari Hariharan<sup>1</sup>, and Ravinder Reddy<sup>1</sup>

<sup>1</sup>CMROI, Radiology, University of Pennsylvania, Philadelphia, Pennsylvania, United States, <sup>2</sup>Research Branch, Sidra Medical and Research Center, Doha, Qatar,

<sup>3</sup>Department of Pharmacology and Institute for Translational Medicine and Therapeutics, University of Pennsylvania, Philadelphia, Pennsylvania, United States,

<sup>4</sup>Molecular Imaging, Radiology, University of Pennsylvania, Philadelphia, Pennsylvania, United States, <sup>5</sup>CMRR 3T Research Program, University of Illinois at Chicago, Chicago, IL, United States

**Introduction:** Tumors have been shown to exhibit elevated proteases in incipient stages, which are thought to play a crucial role in tumor angiogenesis, invasion and metastasis. Cathepsins, a cysteine family proteases, are lysosomal proteases involved in cellular protein degradation and are over expressed in many tumor cells as well as host cells associated with the tumor<sup>1,2</sup>. Out of eleven human cysteine cathepsins, cathepsin B (Ctb) and cathepsin L (Ctl) are primarily involved in protein degradation within the lysosome<sup>3</sup>. The wide availability of cathepsins on tumor cells makes them attractive targets for tumor detection as well as for studying tumor evolution under therapy. However, despite tremendous significance, it is challenging to accomplish noninvasive detection of protease activity *in vivo*. Only near infrared fluorescence (NIRF) optical imaging has been shown to detect Ctb activity in tumors *in vivo*<sup>4,6</sup>. In spite of its high sensitivity, NIRF suffers from poor depth penetration (few mm) and cannot be translatable into a 3D imaging tool either in large animal models or humans. Here, we propose a noninvasive magnetic resonance imaging (MRI) method that is capable of measuring expression of cathepsins in tumors *in vivo*. Briefly, we exploit the breakdown of L-poly glutamate (L-PG), a nontoxic polymer that can be readily injected into animal models upto several mM without causing any adverse reactions<sup>6,7</sup>, by using it as a chemical exchange saturation transfer (CEST) agent. Owing to very slow exchange rates of amide protons on L-PG, the L-PG in its native form does not exhibit appreciable CEST under physiological conditions. However, once it is degraded by the lysosomal enzymes such as Ctb or Ctl, degradation products involve glutamate moieties and smaller glutamate peptides, which readily exhibit CEST effect from their amine protons<sup>8</sup>. Determination of relationship between the protease expression and L-PG degradation via CEST will lead to a noninvasive MRI method for quantifying tumor protease expression with high sensitivity.

### Materials and Methods:

**Phantom Experiment:** Solutions of 20 mg/ml L-PG (Sigma Aldrich) were prepared in phosphate buffered saline (PBS) with and without addition of 0.5mg/ml Ctb enzyme in 10 mm NMR tubes. In addition, 10 mM glutamate solution was also prepared in PBS. The CEST imaging of these phantoms were performed at room temperature on a 7T whole body MRI scanner (Siemens Medical Systems, Malvern, PA, USA). CEST images were collected at peak  $B_{1rms}$  of 250 Hz and 1 s saturation duration for the frequencies (0.2 ppm between  $\pm 3.6$  ppm) from water resonance.  $B_1$  and  $B_0$  maps were also obtained to correct for any inhomogeneities using the previously described method<sup>8</sup>. Water suppressed <sup>1</sup>H MRS was also obtained from these phantoms. **Cell culture experiments:** Rat gliosarcoma 9L cells growing in log phase were trypsinized and washed with sterile PBS and transferred to 0.5ml microfuge tube. Six tubes with 10 million 9L cells were obtained for CEST imaging. These six tubes were categorized in two groups. In the first group (n=3) 0.1ml of normal saline was added, while in the 2nd group (n=3) 0.1ml of L-PG prepared in PBS was added as such that it maintains a total concentration of 20 mg/ml L-PG. CEST imaging was performed on a 9.4T horizontal bore small animal MR scanner (Varian, Palo Alto, CA) using  $B_{1rms}$  of 250 Hz and 1 s saturation duration. **Western blotting:** Western blotting for Ctb and Ctl was performed from 9L cells lysates. **In vivo Study:** Six Syngeneic female Fisher rats (F344/NCR, four-six weeks old) weighing 130–150 g were used to generate intracranial tumors with 9L cells<sup>9</sup>. After three weeks of cell implantation MR imaging was performed on the 9.4T animal MR scanner using a 35-mm diameter quadrature proton coil (m2m Imaging Corp., Cleveland, OH). CEST imaging was performed ( $B_{1rms}$ =250 Hz and 1s saturation duration) before and after injection of L-PG (160 mg/kg) through rat tail vein. At the end of the study the rats were euthanized by CO<sub>2</sub> asphyxiation and brain tissue was collected for immunohistochemical studies. The total imaging time was around 30 min. The Institutional Animal Care and Use Committees of the University of Pennsylvania approved experimental protocols. **Immunohistochemistry:**

immunohistochemistry of Ctb was performed on a 6  $\mu$ m paraffin embedded brain tissue section using the standard protocol. **Image Processing:** All image processing and data analysis were performed using software routines written in MATLAB (version 7.5, R2007b). The GluCEST contrast map was generated using the equation<sup>9</sup> - GluCEST (%) = 100 \* [( $S_{-ve}$  -  $S_{+ve}$ )/ $S_{-ve}$ ], where  $S_{-ve}$  and  $S_{+ve}$  are the  $B_0$  corrected MR signals at -3ppm and +3 ppm respectively. The GluCEST contrast was further corrected for any  $B_1$  inhomogeneity<sup>8</sup>.

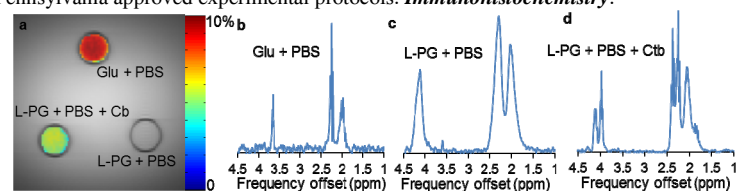
### Results and Discussion:

The cleavage of L-PG by Ctb resulted in CEST contrast at 3ppm as well as splitting of 2.3 and 4.1ppm resonances (Fig 1). No CEST contrast from L-PG owing to the slow exchange of amide protons present on L-PG (Fig 1a & Fig 2c). Expression of mature Ctb in 9L cells responsible for cleavage of L-PG. This degradation exposed significant number of labile -NH<sub>2</sub> protons, which can be easily detected using CEST. We suggest that *in vivo* Ctb enzyme cleaves L-PG more rapidly into its monomer i.e glutamate than that of phantom and cells line, this could be due to continuous expression of Ctb in tumor. Once validated, the technology will have numerous applications in cancer research including differentiation of radiation necrosis from tumor growth as well longitudinal monitoring of cancer therapies. Furthermore, in the long term, it is envisaged that, the proposed technology has potential to monitor L-PG conjugated drug delivery and therapeutic response of tumors.

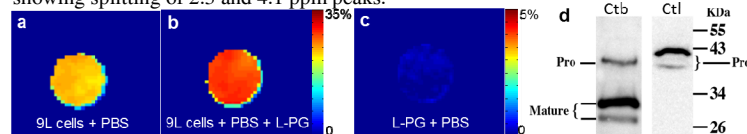
**Funding:** This project was supported by the National Institute of Biomedical Imaging and Bioengineering of the National Institutes of Health through Grant Number P41-EB015893, P41-EB015893S1.

**Acknowledgement:** We acknowledge the Small Animal Imaging Facility at the University of Pennsylvania for supporting the work at 9.4 T MRI facility.

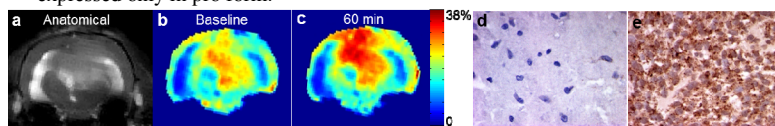
**References:** 1. Jedeszko et al. *BiolChem* 385;1017-27, 2004., 2. Mohamed et al. *Nat Rev Cancer*. 6;764-75, 2006., 3. Keppler et al. *Enzyme Protein* 49:94-105, 1996., 4. Blum et al. *Nat ChemBiol* 3;668-77, 2007., 5. Weissleder et al. *Nat Biotechnol* 17;375-8, 1999., 6. Melancon et al. 24;1217-24, 2007., 7. Li C., *Adv Drug Deliv Rev* 54;695-713, 2002., 8. Cai et al. *Nat Med* 18;302-6, 2012., 9. Kim et al. *NMR Biomed* 21;208-16, 2008.



**Figure 1. a.** CEST contrast map at 3ppm downfield to the bulk water from glutamate, L-PG and L-PG with Ctb. Glutamate (Glu) shows a strong CEST contrast. No CEST contrast was observed from L-PG alone. After addition of Ctb an appreciable CEST contrast was observed, this is due to the degradation of L-PG in to smaller fragments by Ctb. **b.** <sup>1</sup>H MR spectrum from Glu shows multiple peaks related to aliphatic protons at ~2, 2.3 and 3.75 ppm. **c.** Spectrum from L-PG shows 3 peaks respectively at ~2, 2.3 and 4.1 ppm. **d.** Phantom with L-PG and Ctb is showing splitting of 2.3 and 4.1 ppm peaks.



**Figure 2. a.** 9L cancer cells without any L-PG. **b.** same cell line (as in a) in the presence of L-PG (20mg/ml) for 1 hour. GluCEST is elevated by ~17% of the baseline. **c.** L-PG alone in the saline does not show any appreciable GluCEST effect. **d.** Western blot analysis of tumor cells lysates show both mature and pro form of Ctb while the Ctl expressed only in pro form.



**Figure 3: a & b.** Base line anatomical and GluCEST map from a rat brain tumor. **c.** At 1 hour post intra-venous injection of L-PG (160mg/kg) ~32 % increase in CEST contrast at 3ppm downfield to the bulk water was observed in the tumor region. Cathepsin b (Ctb) staining from normal appearing brain region (**d**) and tumor region (**e**) (40 x). Tumor cells show strong staining for Ctb.