Resonance frequency-shifting nitroxide for probing proteolytic activity *in vivo* using the Overhauser-enhanced MRI technique

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Purpose: Molecular imaging of proteolysis is a challenging field of research. As compared to more sensitive imaging techniques, MRI needs new probe-design for high and protease-specific biological contrast. Here, the low MRI sensitivity was unveiled using Overhauser-enhanced MRI. The OMRI approach[1], based on the Overhauser effect between unpaired electrons of a nitroxide species and surrounding water protons (^{1}H) after electron spin saturation, enables high NMR signal enhancement, thus revealing the presence of unpaired electrons in MR images, even *in vivo*[2]. The novelty of our study was to validate *in vivo* a line-shifting nitroxide probe-design, synthesized chemically with an acetyl functional group which can be hydrolyzed enzymatically to give a hydroxyl functional group. Upon hydrolysis, their nitrogen and phosphorus hyperfine coupling constants (a_N and a_P respectively) are changed, as depicted on the EPR spectrum (fig 1) allowing a distinct substrate (acetate form, A) or product (hyrolyzed form, B) visualization using the OMRI approach with frequency-selective irradiation of one EPR line. Nitroxides were evaluated *in vitro* and substrate hydrolysis was also evaluated *in vivo* in mice.

Methods: Nitroxide molecules - These enzymatic probes have their hyperfine coupling constants modified upon hydrolysis. The acetylated form, A (a_P = 38.7G; a_N = 15.6G; electronic saturation at 5426 MHz) has its peaks shifted by ~8 MHz from the hydrolyzed form, B (a_P = 43.1G; a_N = 15.0G; electronic saturation at 5418 MHz).

OMRI setup - A 0.2T C-shaped resistive MRI system (Magnetom Open Viva Siemens) together with a Transverse-Electric TE011-mode resonant cavity (Bruker) were used. The ^{1}H-frequency was 8.24 MHz and the electron frequency was around 5.4 GHz.

Animal Preparation - 200µl of freshly prepared nitroxide molecule at 24mM was orally administered in mice (n=3, CB57/CRL). They were then anesthetized and placed on a thermostatic bed for image acquisition.

Imaging Protocol - 1) A first set of 18-second images were acquired at 5426 MHz using a fully balanced steady-state sequence - TrueFISP3D (resolution = 0.5×1×1 mm^3) with/out electron spin saturation. 2) A similar data set was then acquired at 5418 MHz. 3) A 5-minute TrueFISP3D image of resolution 0.5×0.5×0.5 mm^3 without electron spin excitation was also generated at each frequency for keyhole reconstruction. 4) A 3-minute gradient echo anatomical image – FLASH3D (resolution = 0.5×0.59×1.0 mm^3) was also acquired.

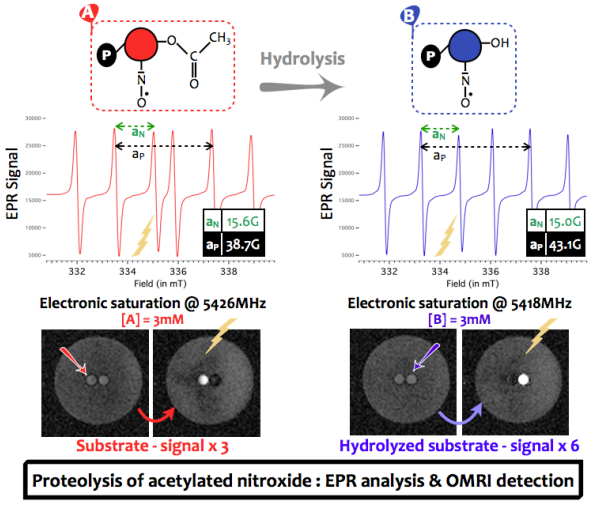

Figure 1 represents a rough sketch of the nitroxide molecule with the acetylated form (A) and the hydrolyzed form (B). The 6-EPR peaks of the nitroxide is also displayed with their different a_N and a_P values. The OMRI *in vitro* images of the selective Overhauser enhancements are also depicted in this figure.

Image Processing - 3D keyhole reconstruction resulted in OMRI images with electron spin saturation (OMRI-**on**) or without (OMRI-**off**). Overhauser enhancements were then calculated as a ratio of signal intensity of OMRI-**on** images to that of OMRI-**off**.

Results: *In vitro*, no spontaneous hydrolysis from A to B was observed. Preliminary *in vivo* results (n=3) showed a clear Overhauser enhancement of the acetylated form (A @ 5426 MHz) immediately after gavage and later on the hydrolyzed form (B @ 5418 MHz). At 5426 MHz, an average signal enhancement of 5.0 ± 0.8 was either observed in the stomach or in the small intestine; showing the presence of A. Simultaneously, at 5418 MHz, an average signal enhancement of 4.8 ± 3.3 revealing the hydrolyzed form was obtained in the stomach. For the mouse illustrated in fig.2, a maximum Overhauser enhancement of 5.8 was observed at 15mins post-gavage at 5426 MHz and of 8.5 at 36mins at 5418 MHz. Both enhancements were seen inside the stomach, more precisely at its base as validated in the superimposed image (fig 2).

Discussion: The feasibility in the use of this probe was clearly demonstrated *in vivo* through highly resolved OMRI. Even if this substrate had a low specificity as well as a low affinity with a certain class of enzymes (data not shown), high signal amplifications undoubtedly showed an enzymatic process occurring in the stomach of the mice. This appealing molecular imaging strategy monitors and localizes both the substrate and its associated enzymatic product at two distinct frequencies. In conclusion, this line-shifting OMRI probe-design would be a good candidate to target abnormal proteolysis using specific peptide sequences.

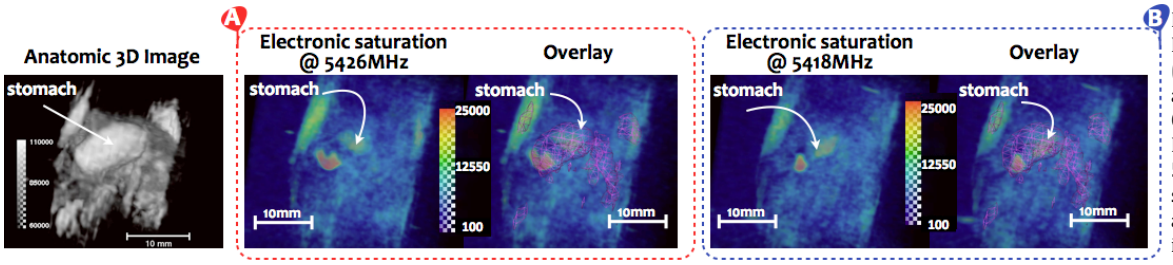

Figure 2: *In vivo* images. A Maximal-Intensity-Projection (MIP) of the T_1-weighted anatomic image (left). Box A – OMRI-on image at 5426MHz; Box B – OMRI-**on** image at 5418MHz. "Overlay" – superimposed image of the anatomic image and OMRI-**on** image to localize the presence of the probe.

References: 1. Mellet.P et al. New concepts in molecular imaging: non-invasive MRI spotting of proteolysis using an Overhauser effect switch. PLoS One 2009;4(4):e5244. 2. Koonjoo.N et al. *In vivo* Overhauser-enhanced MRI of Proteolytic Activity. Contrast Media Mol Imaging 2014 Sep-Oct;9(5):363-371.