Detection of carboxypeptidase G2 activity with chemical exchange saturation transfer magnetic resonance.

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Introduction. The bacterial enzyme carboxypeptidase G2 (CPG2) is a bacterial enzyme which mediates the release of the C-terminal glutamate residue from a wide range of N-acylating moieties including carboxyl, phenol or aniline mustard prodrugs (Fig. 1). CPG2 underpins a promising gene-directed enzyme prodrug therapy (GDEPT) strategy due to enter Phase I clinical evaluation (1). In GDEPT, the gene encoding the prodrug-activating enzyme is targeted selectively to the tumor prior to administration of the prodrug, resulting in the activation of the cytotoxic drug specifically in the tumor. The success of CPG2-based GDEPT relies on the careful optimization of the timing of injection of the prodrug following expression of the transgene and the generation of sufficient concentrations of CPG2 in the tumor. Non-invasive imaging strategies to monitor the prodrug-activating enzyme activity and its bio-distribution would thus be invaluable in guiding the successful translation of this promising therapeutic approach to the clinic. This study demonstrates how the differential exchange rates of amide (CPG2 substrates) and amine (glutamate) functional groups can be exploited to dynamically monitor CPG2 activity through the "activation" of the concentration-dependent chemical exchange saturation transfer MR signal induced by the CPG2-mediated release of glutamate.

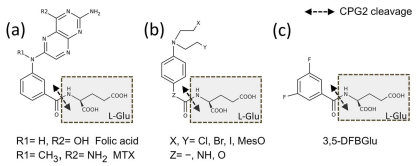

Figure 1. Carboxypeptidase G2 (CPG2)-mediated hydrolysis of the glutamate moiety of (a) folic acid and methotrexate (MTX), (b) nitrogen mustard prodrugs, and (c) the ^{19}F MRS reporter probe 3,5-difluorobenzoyl-L-glutamate.

Methods. All studies were performed on an 11.7T (Bruker) system equipped with a 5mm BBO probe. *CEST-NMR:* Z spectra of solutions of 10 mM glutamate, MTX, Folic acid and 3,5-DFBGlu were acquired at 37°C in PBS at pH 5-8. Z spectra were acquired using a series of spectra (NS=4, TR=17 s) with the saturation frequency at a different offset frequency from -5 to 5ppm from the water resonance (increment 0.2ppm). The frequency selective saturation was achieved using CW presaturation for 5sec and $\gamma B_1/2\pi \sim 280$ Hz. *In vitro detection of CPG2 activity in cells extracts*: 50 and 100 µl of extracts of stCPG2(Q)3 WiDr colon carcinoma cells, genetically-engineered to express CPG2, and control WiDr cell (LacZ WiDr), were added to a 12 mM solution of 3,5-DFBGlu and MTX, respectively, in 100mM phosphate buffer, 260µM ZnCl2, pH 7 at 37°C. Acquisition of a series of 5 spectra (same parameters described above) was started immediately after mixing the substrates, with saturation frequencies set to 9.6, 7.8, 7.6, 6.8 and 5.9 ppm and repeated over a period of 3 hours.

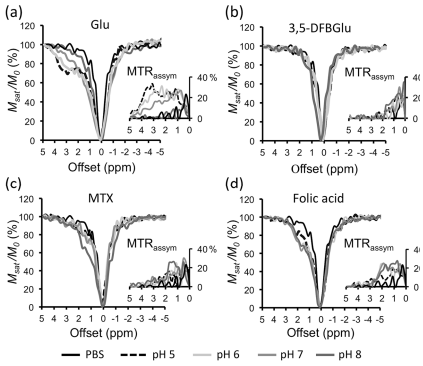

Figure 2. CEST z-spectra acquired at 11.7T of 10 mM (a) glutamate (Glu), (b) 3,5-DFBGlu, (c) methotrexate (MTX) and (d), folic acid in PBS at varying pH and at 37°C. ($MTR_{assym} = (M_{-\Delta\omega} - M_{+\Delta\omega})/M_0$, where $M_{-\Delta\omega}$ and $M_{+\Delta\omega}$ represent the bulk water signals acquired with selective RF saturation at $\pm\Delta\omega$ resonance offset from the water frequency)

Results. Glutamate demonstrates a sharp CEST peak in the z spectrum at +3 ppm offset from the water resonance (GluCEST), as previously demonstrated (2), whilst MTX, folic acid and 3,5-DFBGlu did not elicit any detectable CEST effect at 3.5ppm, the frequency of amide protons, at pH 5-8 (Fig 2.). The addition of MTX to the extracts of CPG2-expressing WiDr cells caused a time dependent increase of the CEST effect at +3ppm. CPG2 activity was confirmed through the increase in the concentration of 2,4-diamino-N^{10}-methylpteroic acid (DAMPA), measured by conventional ^{1}H-MRS (Fig 3). Importantly, DAMPA is not expected to elicit a CEST effect at +3 ppm, indicating that the increase in CEST signal correlates with the CPG2-induced increase in glutamate concentration. Figure 4 shows similar activation of the CEST effect following the addition of 3,5-DFBGlu to extracts of CPG2-expressing WiDr cells. CPG2 activity was also detected by the change in CEST signal at 2.1 ppm with improved sensitivity, which was confirmed by the comparison of the z spectra in the CPG2-expressing and control cell extracts acquired at the end of the experiment.

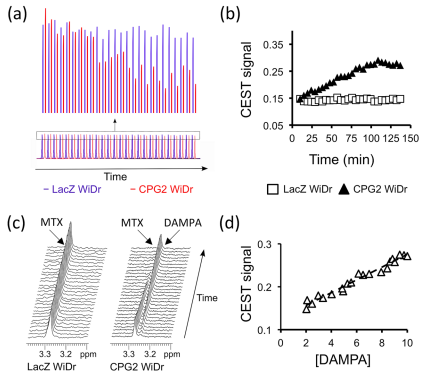

Figure 3. Detection of CPG2-mediated cleavage of MTX into 2,4-diamino-N10-methylpteroic acid (DAMPA) and glutamate. (a) Evolution of the bulk water signal acquired with RF saturation (+3ppm) over time, following addition of 10mM MTX to extracts of WiDr cells engineered to express CPG2 (CPG2 WiDr) or control LacZ WiDr), at pH 7 and 37 °C. (b) Evolution of the GluCEST signal over time, following the addition of 10mM MTX to the cell extracts. (c) Time series of ^{1}H MRS spectra acquired every 5 min showing the CPG2-mediated conversion of MTX into DAMPA in the same extract shown in (a). (d) CEST signal correlates with [DAMPA] in CPG2-expressing cell extracts after addition of 10mM MTX (r^2= 0.98, p<0.0001).

Discussion and Conclusion. This study demonstrates the potential of CEST-MRI to monitor CPG2 activity via its sensitivity to the CPG2-mediated release of glutamate, resulting in the "activation" of the GluCEST contrast. We have previously demonstrated the utility of ^{19}F MRS in combination with the imaging reporter 3,5-DFBGlu to monitor CPG2 activity *in vivo* (3). Although the ^{19}F MRS approach provides invaluable information on the level of CPG2 activity in the tumor, the inherent lack of sensitivity of conventional MRS precluded the assessment of the heterogeneous distribution of the enzyme activity within the tumor, an important prognostic factor for successful therapy. GluCEST MRI is an attractive method for metabolic imaging of glutamate (2), since it utilizes the bulk signal from water protons enabling the indirect detection of glutamate concentration at higher temporal and spatial resolution compared to its conventional direct detection by ^{1}H MRS. GluCEST MRI could thus provide a noninvasive and clinically available method to not just image the biodistribution of CPG2 mediated cleavage of the imaging reporter 3,5-DFBGlu, but also the entire portfolio of prodrugs utilized in GDEPT. This study thus encourages the translation of CEST-MRI to CPG2-based gene therapy *in vivo*.

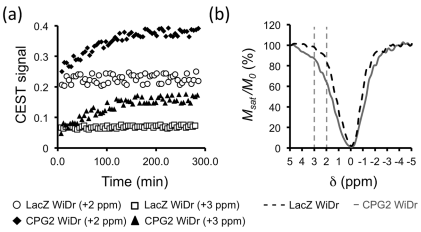

Figure 4. Detection of CPG2-mediated cleavage of 3,5-DFBGlu. (a) Evolution of the CEST signal over time, following the addition of 10mM 3,5-DFBGlu to extracts of WiDr cells engineered to express CPG2 (CPG2 WiDr) or control LacZ WiDr. (b) CEST z-spectra at 11.7T of CPG2 and LacZ WiDr cell extracts 6h following the addition of 10mM 3,5-DFBGlu, at pH 7 and 37°C.

References. (1) Hedley D. et *al.*, *Nat. Rev. Cancer* 2007.. (2) Cai K et *al.*, *Nat. Med.* 2012. (3) Jamin Y. et *al.*, *NMR Biomed.* 2011.

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