Direct detection of cytosine deaminase enzymatic activity using CEST MRI

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INTRODUCTION

Cytosine deaminase (CD) is an enzyme that converts the prodrug 5-Fluorocytosine (5-FC) into the active agent 5-fluorouracil (5-FU) and consequently can kill tumor cells. Thus, CD is a promising new approach for gene therapy of cancer.[1] However, due to variability in response and tumor heterogeneity between patients this approach can greatly benefit from non-invasive imaging in order to optimize treatment. Recently, the ability to assess the therapeutic effects of CD has been demonstrated with MRS/MRSI, [2,3] but spectroscopy is limited by low spatial resolution. Since CD removes exchangeable protons from its substrate, (**Fig. 1a**) we hypothesized that, by observing the changes in CEST signal between substrate and product, high special resolution CEST MRI[4] can be used to monitor CD activity. In this study we used cytosine as a surrogate substrate to reduce the impact on targeted cells, therefore allowing repetitive imaging of CD activity when applying to *in vivo*.

METHODS AND MATERIALS

Cloning: The gene encoding to the *E. Coli* cytosine deaminase was obtained from the American Type Culture Collection (ATCC Cat. 4099). The gene was sub-cloned into the pEXP5-CT expression vector (Invitrogen) and was expressed in-frame with a 6-histine tag under a T7 promoter. Both CD and the control protein, calmodulin (calm) were expressed in a cell-free expression system (Invitrogen). The translated proteins were used for the MRI experiments without further purification.

MRI: All *in vitro* MRI images were acquired on a 9.4T Bruker Avance system equipped with a 15 mm sawtooth RF coil. A modified RARE (TR=6.0 sec, effective TE = 43.2 ms. RARE factor =16, slice thickness=0.7 mm, FOV=14x14 mm, matrix size=128x128, resolution= 0.11x011mm^2, and NA=2) including a magnetization transfer (MT) module (one CW pulse, (ω_1) = 4.7 μT (200 Hz), 4sec) was used to acquire CEST weighted images from -6ppm to 6ppm (step=0.2-0.3ppm) around the water resonance (0ppm). The absolute water resonant frequency shift was measured using a modified WAter Saturation Shift Reference (WASSR) method[5], using the same parameters as in CEST imaging except TR=1.5 sec, t_{sat}= 500 msω_1 =0.5 μT (21.3 Hz) and sweeping from -2ppm to 2 ppm (step= 0.1ppm). Data processing was performed using custom-written scripts in Matlab. Z-spectra were calculated from the mean of ROI for each sample after B_0 correction. $MTR_{asym} = (S^{\Delta\omega} - S^{+\Delta\omega})/ S^{\Delta\omega}$ was computed at different offsets Δω (i.e. +2 ppm and +5.6 ppm).

RESULTS AND DISCUSSION

Differences in the CEST properties of cytosine and uracil were confirmed by their Z-spectra (**Fig. 1b**), indicating that the two compounds can be distinguished by the distinct CEST signals both at 2ppm and 5.6 ppm at physiological pH (i.e. pH 7.3) and temperature (i.e. 37^{o}C) (**Fig. 1b inset**). Quantitative study reveals that the CEST signal at 2ppm shows a higher sensitivity than at 5.6ppm, as shown in **Fig. 1c**, and will therefore be used for quantifying CD activity in the following studies.

In order to demonstrate that this new approach is able to quantitatively assess CD activity *in vivo*, CEST MRI was performed on the unpurified *in vitro* translation products of *E. coli* CD gene and calmodulin gene as control, which was verified by protein staining (**Fig. 2a**). The results indicate that the CEST signal at 2 ppm was effectively eliminated in the CD sample, but preserved in the control sample (**Fig. 2b**). As shown in **Fig. 2c**, a quantitative study was carried out on samples containing same substrate (cytosine) concentration, and different concentration of translated protein by diluting the translation product mixture with PBS for up to 1: 10. The dilution of enzyme solution (as quantified by relative concentration) was used to simulate the reduction of gene expression. Result showed that even in the presence of a very low relative concentration of translated CD, the reduced CEST signal was observable compared to the control sample. Increasing enzyme concentration results in a larger decrease of CEST (**Fig. 2d**) reflecting higher activity to convert the substrate (cytosine) to product (uracil). **Fig. 2d** also demonstrates a good linear range for quantifying the expression of CD gene before reaching high enzyme concentration, at which the reaction reaches a saturation point.

CONCLUSION

This is the first demonstration of direct detection and quantification of CD activity using CEST MRI *in vitro*. Since administration of the cytosine as a surrogate to prodrug 5-FC won't kill cells, the above findings suggests our approach has the potential to allow repetitive imaging for identifying the exact time and place of the CD expression in order to optimize the treatment regime.

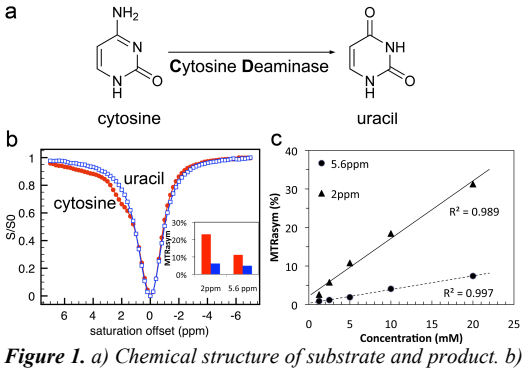

Figure 1. *a) Chemical structure of substrate and product. b) Difference in MTR_{asym} between the compounds. Cytosine shows high MTR_{asym} at 2ppm and 5.6 ppm, which is not observable for the uracil. c) Calibration curves of cytosine at 2 ppm and 5.6 ppm.*

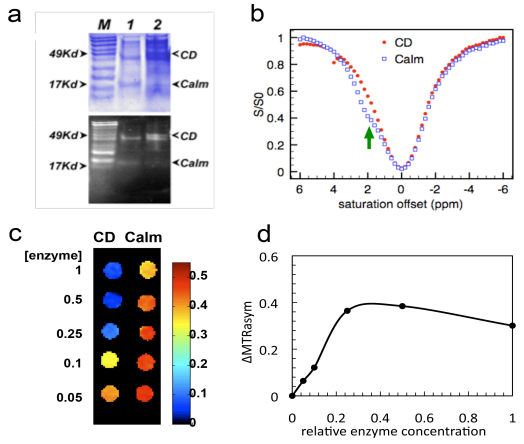

Figure 2. a) *In vitro expression of genes encoding CD (lane 1) and calmodulin (Calm; lane 2). Top: coomassie blue staining. Bottom: specific staining for integrated 6-HIS tag. M-molecular weight markers,* ***b)*** *z-spectra of the two samples from (a) after incubation with 25mM cytosine.* ***c)*** *MTRasym map at 2ppm of a MR phantom of samples containing different concentrations (relative) of enzyme with 25mM cytosine and* ***d)*** *the difference in CEST contrast of CD and Calm, ΔMTR_{asym}, was plotted versus enzyme concentration, indicating CEST MRI can be used for quantifying CD activity by the CEST signal at 2 ppm.*

Reference:

(1) Aghi, M.; Hochberg, F.; Breakefield, X. *The Journal of Gene Medicine* **2000**, *2*, 148-164.
(2) Aboagye, E.; Artemov, D.; Senter, P.; Bhujwalla, Z. *Cancer research* **1998**, *58*, 4075.
(3) Stegman, L. D.; et. al. *PNAS* **1999**, *96*, 9821-6.
(4) Ward, K. M.; Aletras, A. H.; Balaban, R. S. *J Magn Reson* **2000**, *143*, 79-87.
(5) Kim, M.; Gillen, J.; Landman, B. A.; Zhou, J.; van Zijl, P. C. M. .**2009**, *61*, 1441-1450.

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