Monitoring the Accumulation of Natural Fruit-derived diaCEST Liposomes in B16 Melanoma

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Target Audience: Those working in the field of cancer, molecular imaging, nanomedicine and CEST/MT imaging.

Purpose: Nanosize containers containing imaging agents are becoming a powerful tool in oncology, allowing non-invasive assessment of tumor delivery, tumor retention, and tumor permeability. For instance, liposomes loaded with organic diamagnetic CEST (diaCEST) MR agents have been shown to detect enhanced tumor accumulation[1]. Recently a new family of CEST agents was reported, salicylic acid (SA) and analogues, which contain labile protons with chemical shifts from water larger than the usual 0-5ppm diaCEST range ($\Delta\omega$ = 8 -11 ppm), yet exhibit suitable exchange rates for improved detection at lower field strengths[2]. In this study, we developed SA-analogue loaded liposomes that are traceable using MRI. We first determined which agent was best retained by liposomes, which was found to be a natural fruit metabolite[3]. We then evaluated its use for *in vivo* monitoring of liposome accumulation in B16 melanoma graphs in mice. Studies were done with concurrent administration of tumor necrosis factor-alpha (TNF-α), a clinical agent known to increase tumor uptake of nanocarriers[4].

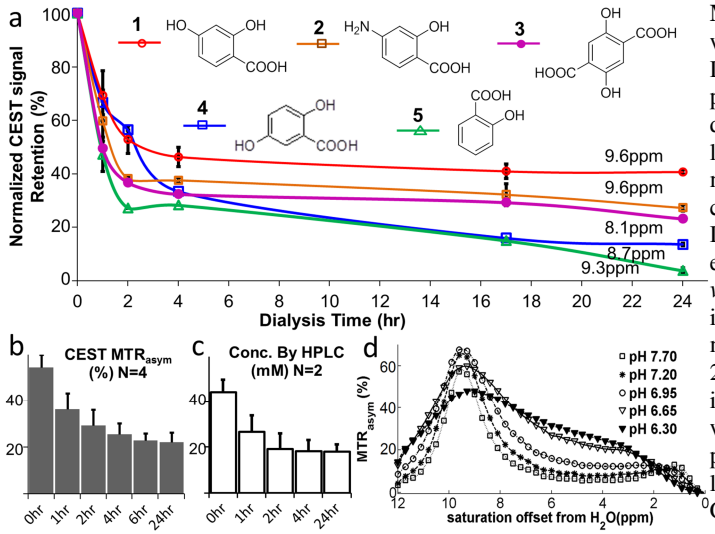

Fig. 1 a. Normalized retention of different SA analogues in liposomes (**1-5**) with N=4 for 2,4-DHB (**1**) and N=2 for others, with their peak $\Delta\omega$ at neutral pH labeled. For agent **1**, **b.c,d** are the CEST contrast changes, conc. changes measured by HPLC, and CEST contrast vs. pH, respectively.

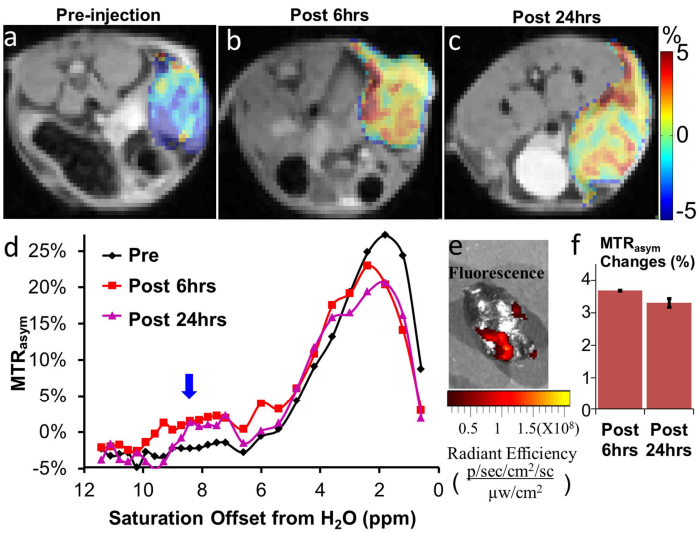

Fig.2 a.b.c: Overlaid CEST contrast map by averaging 8.4, 8.1 and 7.9 ppm at pre-, 6hrs and 24hrs post-injection. **d)** Averaged MTR_{asym} spectra of the tumor region at the 3 time points. **e)** Fluorescence validation. **f)** MTR_{asym} change with respect to pre-injection images (N=2).

Methods: *Liposomal retention studies:* Liposomes were prepared using the well-established thin film hydration method, a molar ratio of lipids of DSPC/CHOL/DSPE-PEG2000 = 1.62:1:3:0.08. Rhodamine B-labeling of phospholipids was included for validation by fluorescence imaging. The dried lipid layers then hydrated using solutions of several agents (100 mg lipid/ml in H_2O), including **1**) 2,4-Dihydroxybenzoic acid (2,4-DHB, a metabolite found in human plasma after cranberry juice or tart cherry consumption), **2**) 4-aminosalicylic acid (a NonSteroidal Anti-Inflammatory Drug, NSAID), **3**) 2,5-Dihydroxyterephthalic acid containing two exchangeable protons per molecule, **4)** 2,5-DHB and **5**) Salicylic Acid. *In vitro* retention of agents in liposomes was determined by both CEST imaging and high performance liquid chromatography (HPLC) measurements of samples collected at set time points post dialysis against 200 ml PBS at 37°C. *Mouse Model: 1x10[5]* B16 melanoma cells were inoculated subcutaneously in the lower flank of female C57BL/6 mice (6–8 weeks, n=3) and imaged 13-15 days post-inoculation. MR scans were performed both pre- and at 6 and 24 hrs post tail vein injection of 200 µl liposomes and 0.75 µg TNF-α. *Image acquisition and analysis:* *In vitro* CEST images were acquired on **a** Bruker 11.7T scanner using a RARE sequence with a CW saturation pulse. Z-spectra and MTR_{asym} spectra of a single slice were acquired by sweeping the frequency every 0.3 ppm from -12 to 12 ppm. Parameters were B_1=5.9 µT, T_{sat}=3 s, TR/effective TE=6 s/17-19 ms, and matrix size=96x64. For *in vivo* CEST imaging, 2-slice saturation-weighted images were acquired with an optimized frequency interval of every 0.3 ppm from 6.6 ppm to 11.4 ppm and every 0.6 ppm from 0 ppm to 6 ppm. Parameters: B_1 = 5.9 µT T_{sat} = 2 s, TR/effective TE = 5 s / 16 ms, matrix size = 64x48, and FOV = 20x18x1 mm. CEST was quantified as $MTR_{asym} = 1 - S_{+\Delta\omega} / S_{-\Delta\omega}$.

Results: Fig.1a shows the agent structures and retention. Among the agents, 2,4-DHB provided the best retention, with ~40% of the initial contrast remaining over 24 hrs. The normalized changes of CEST signal over time were consistent with the normalized molar concentration profile of agents within liposomes as determined by HPLC (e.g., see **Fig. 1 b,c for 2,4-DHB**). The MTR_{asym} spectra of 2,4-DHB liposomes revealed that acidic pH values broaden the CEST peak. The post-injection *in vivo* CEST spectra show a broad peak around 8.4 ppm (**Fig. 2 d**), presumably due to the low extracellular pH in the tumor. The averaged CEST contrast map over 8.4 ppm, 8.1 ppm and 7.8 ppm shows an enhanced region of liposomes at pre-, 6 hrs and 24 hrs post-injection. (**Fig. 2 a-c**). The liposomal uptake was validated by fluorescence imaging. A ~3-4% MTR_{asym} increase with respect to pre-injection images was observed in the tumor at both 6 and 24 hrs.

Discussion and Conclusion: Liposomes containing SA analogues exhibited similar initial CEST contrast, but only 2,4-DHB (**1**), a natural metabolite from fruits, displayed > 40% retention rates. Using 2,4-DHB, we successfully obtained 3-4% CEST contrast enhancement due to liposome accumulation in B16 melanoma *in vivo*.

References: [1]Chan, K., et al. WIREs Nanomed. Nanobiotechnol. 2013 doi: 10.1002/wnan.1246. [2]Yang, X. and Song, X. et al, Angew. Chem. Int. Ed., 2013, 52: 8116. [3] Prior, R, Am J Clin Nutr 2003,78(3) 570S. [4]Qiao,Y. , et al. Oncotarget,2011,2:59. Supported by NIH grants R01EB015031 and R01EB015032.