MRI Contrast Agents

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Salicylic Acid and Analogues as diaCEST MRI Contrast Agents with Highly Shifted Exchangeable Proton Frequencies**

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Magnetic resonance imaging (MRI) has been widely used as a diagnostic method to detect changes in soft tissue due to its exquisite spatial resolution. One of the standard methods to detect pathologies involves injection of magnetic resonance (MR) contrast agent, such as the gadolinium(III) complexes routinely used for angiography.[1] Chemical exchange saturation transfer (CEST) contrast agents are a new alternative, which have become popular owing to the unique features of these agents.[2] One of the features of CEST probes is that MR contrast can be produced by a variety of organic diamagnetic compounds having exchangeable protons,[3] such as glucose,[4] glycogen,[5] myo-inositol,[6] glutamate,[7] creatine,[8] L-arginine,[9] glycosaminoglycans,[10] nucleic acids,[11] and peptides.[12] The CEST contrast mechanism involves selective irradiation of labile protons on the diamagnetic CEST (diaCEST) agent to perturb their signal, with this signal change then transferred to water by exchange between these labile protons and bulk water.[5] Because a number of common metabolites possess labile protons, there can be challenges in discriminating the signal loss associated with the CEST agent of interest and background,[2c] especially between 1 to 3.6 ppm from water. Recently, iopamidol, a computed tomography (CT) agents approved for clinical use, was reported to produce strong CEST contrast at 4.2 and 5.5 ppm.[13] Herein we show that salicylic acid (**1**), one of the main metabolites of aspirin, possesses a suitable exchangeable proton that resonates 9.3 ppm from water, a frequency far removed from all other organic diaCEST agents reported to date. Furthermore, the intramolecular hydrogen bonding found in salicylic acid analogues[14,15] results in strong CEST contrast properties. Seven salicylic acid analogues (**4**–**10**) produce similar contrast to **1**, with labile protons up to 10.8 ppm from water. We were able to measure the proton exchange rate for **1**, determine optimum saturation conditions, and detect this agent in the kidneys of mice after intravenous (IV) administration.

Balaban and co-workers have tabulated a number of organic compounds that display CEST contrast from 1 to 6 ppm, including barbituric acid (**2**) and D-glucose (**3**).[3] As shown in Figure 1, the MTR_{asym} spectra of **1**, **2**, and **3** were compared at the same scale. The phenol proton in **1** displays contrast at a much larger chemical shift using a saturation field strength (ω_1) = 7.2 μT. The maximum CEST contrast occurred at 9.3 ppm from water. Presumably, at neutral pH, the deprotonated carboxylic anion forms a strong hydrogen bond to the phenol proton and results in this dramatic shift.[15] The signal of **1** dropped greatly at either lower pH (< 6) when a significant amount of the carboxylate becomes protonated, or at higher pH (> 11) when the phenol proton becomes deprotonated (see the Supporting Information for details).

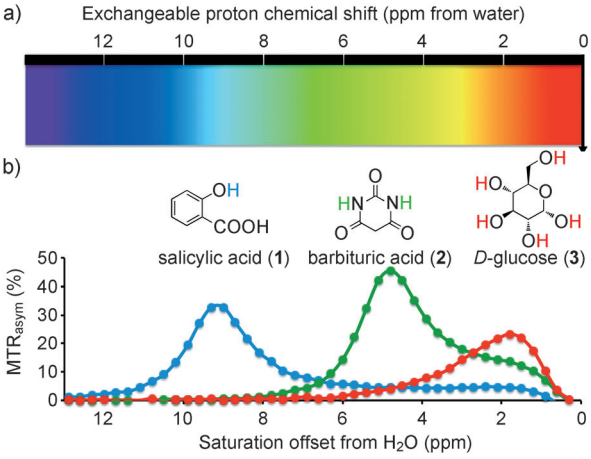

Figure 1. Depiction of the color spectrum for diaCEST agents. a) Range of exchangeable proton shifts observed presently for diaCEST agents; b) CEST contrast curves for three representative agents: salicylic acid (**1**), barbituric acid (**2**), and D-glucose (**3**) at concentrations of 25 mM, pH 7.0, 37 °C using $\omega_1 = 7.2$ μT, $t_{sat} = 3$ s for saturation.

We next measured the CEST properties of compound **1** in vitro. Figure 2a shows a Z-spectrum and MTR_{asym} spectrum for the compound. The proton exchange rate (k_{sw}) with water for **1** was measured as a function of pH using the QUESP experiment[16] (Figure 2b,c). Compound **1** has a k_{sw} = 1.2 ks^{-1} at 25 mM, pH 7.0, and k_{sw} is strongly dependent on the

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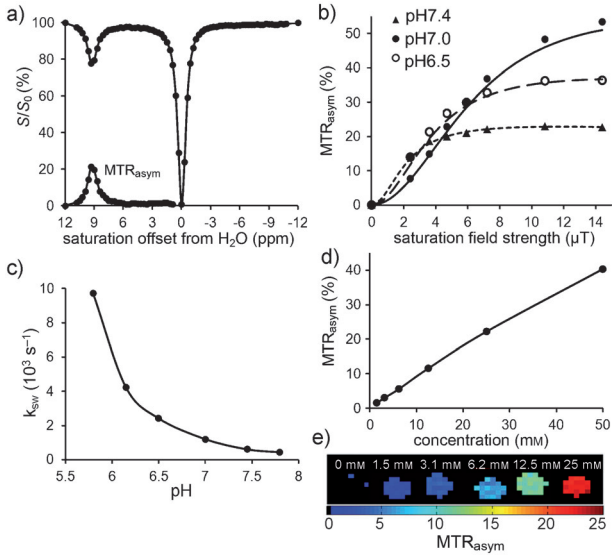
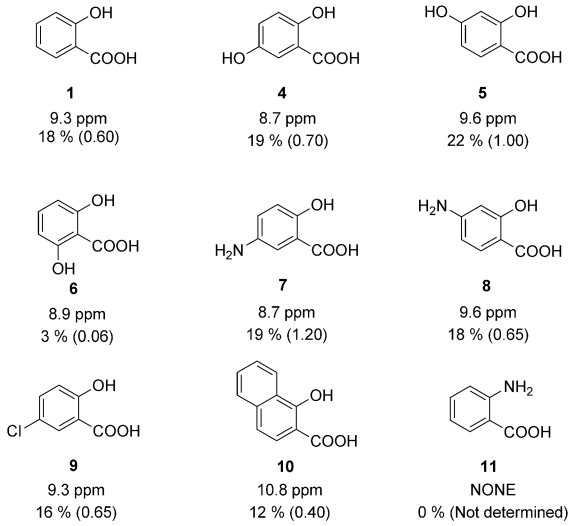


Figure 2. CEST properties of **1** at 37 °C. a) Z-spectrum and MTR_{asym} for 25 mM at pH 7.0 using $\omega_1 = 3.6$ μT; b) QUESP data for 25 mM, at pH values 6.5, 7.0, 7.4; c) pH dependence of k_{sw} based on QUESP data; d) CEST contrast at 9.3 ppm as a function of concentration, using $\omega_1 = 7.2$ μT, pH 7.0; e) CEST contrast map on phantom with assorted concentrations using $\omega_1 = 7.2$ μT, pH 7.0.



Scheme 1. CEST signals [ppm] for C2-OH and contrast [%] (exchange rate [ks^{-1}]) of salicylic acid and its analogues. Experimental conditions: CEST agent concentration = 25 mM, pH 7.1–7.4, using $t_{sat} = 3$ s, $\omega_1 = 3.6$ μT. For Z-spectra, see the Supporting Information.

pH (Figure 2b). Above pH 6.0, k_{sw} is below the chemical shift difference at 11.7 T ($\Delta\omega = 4{,}650$ Hz), placing the rates in the slow exchange NMR regime and making this agent well-suited for CEST imaging. As can also be seen, for pH 7.0, maximum contrast was produced by $\omega_1 = 10.8$ μT, although using 7.2 μT or higher would in practice produce similar results. The use of these saturation conditions resulted in CEST contrast = 4 % at 1.5 mM concentration (Figure 2d,e). This is a respectable sensitivity, especially considering in vivo tissue background (see the Supporting Information).

Based on these results, eight analogues of **1** were also tested to determine how electronic effects related to the phenol ring would modify this contrast (Scheme 1). Placing an OH or NH_2 group (**4** or **7**) at the *para* position to the phenol C2-OH reduced the chemical shift to 8.7 ppm, which could be slightly increased to 9.6 ppm by attaching an OH or NH_2 group (**5** or **8**) at the C4 position of the salicylic acid. In the case of 2,6-dihydroxybenzoic acid (**6**), instead of an increase, the contrast dropped dramatically owing to a drop in k_{sw} to 60 s^{-1}, although the chemical shift remained similar. A more interesting result was obtained for 1-hydroxy-2-naphthoic acid (**10**). The naphthalene ring with more electron delocalization helped to shift the CEST peak signal further to 10.8 ppm. Anthranilic acid (**11**), with the substitution of an NH_2 for the OH adjacent to the carboxylic acid, did not show any contrast.

As seen in Figures 1 and 2 and Scheme 1, these analogues possess exchangeable protons that resonate at the furthest downfield from water of all organic CEST agents, and further than the 6 ppm protons of thymidine analogues we reported recently.[11] While these protons are not nearly as shifted as the 45 to 52 ppm found for bound water in paramagnetic Eu^{3+} complexes[17] or the −600 ppm found in paramagnetic Tb^{3+} complexes,[18] the exchange rates are slow enough relative to the chemical shift difference with water to not produce significant exchange-based T_2 relaxation, which can darken pixels containing CEST agents.[19] The saturation field strengths shown in Figure 2b can be generated in many imaging coils, making these analogues suitable diaCEST agents. The shifts are approaching, but not quite as large, as those seen for the carbamate protons that generate CEST contrast on Yb^{3+} agents (ca. −16 ppm),[20] osmotic stressed lipoCEST agents based on Tm^{3+} complexes (ca. 18 ppm), or Dy^{3+} complexes (ca. 45 ppm),[21] and larger than the spherical lipoCEST preparations.[22] Furthermore, the salicylic acid scaffold can tolerate chemical modification (Scheme 1). This can allow the conjugation of this type of probe to polymers,[23] nanoparticles,[24] or hydrogels,[9a] which are suitable for a variety of drug- or cell-based therapies.

To evaluate whether **1** could be detected after administration into live animals, we injected two mice with 60 μL of a 0.25 M solution of compound **1** and collected CEST images. Images consisting of a single axial slice containing both kidneys were collected. Because the B_0 inhomogeneity was less than 200 Hz for these animals, we decided to use a two-point collection method to limit the scan time, and a 7.2 μT saturation field strength to reduce the sensitivity to B_0 inhomogeneity. We observed a pronounced increase in the CEST contrast at 9.3 ppm which peaked in the kidneys 7 min after injection (Figure 3b,c), indicative of probe uptake. The average CEST contrast was 6.0 ± 0.8 % over the whole kidney (Figure 3b,c,e), with the contrast persisting for 8 min as displayed in Figure 3e. The advantage of the large shift is evident from Figure 3d, where the peak of **1** is far removed from the large MTR_{asym} seen in the right kidney between 1–4.5 ppm, which is the chemical shift of many exchangeable protons seen on common metabolites such as glucose, creatine, L-glutamine, L-glutamate, glutathione, and others.

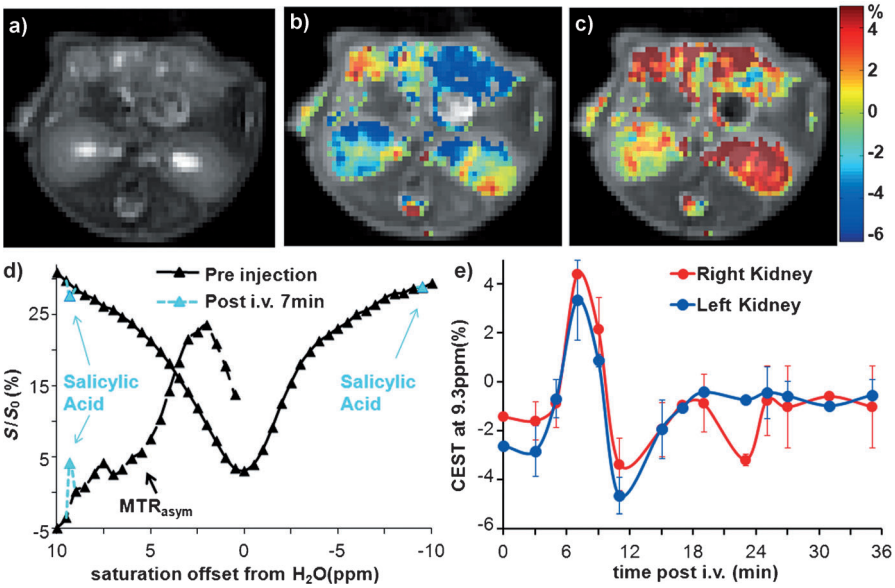


Figure 3. In vivo contrast for **1**. a) T2w image; b) overlay MTR_{asym} (9.3 ppm) map pre-injection; c) overlay MTR_{asym} (9.3 ppm) map at 7 min post-injection; d) Z-spectra and MTR_{asym} for a region of interest (ROI) enclosing the entire right kidney with pre-injection data (black) and 7 min post-injection (light blue); e) dynamic time course of the MTR_{asym} (9.3 ppm) for ROIs enclosing the whole left kidney and right kidney. $\omega_1 = 7.2\ \mu T$ ($n = 2$).

Furthermore, the intensity of the total water signal is 25% larger at 9.3 ppm compared to at 5 ppm owing to less direct saturation.

The magnitude of the contrast detected is similar to that shown previously by Longo and co-workers[13a] for iopamidol, with a difference in the optimal time to observe the contrast (7 min for 15 μM **1** versus 45 min for 48.5 μM iopamidol). Furthermore, simple continuous-wave irradiation was chosen to detect **1** because of the robustness of this method; however, more advanced saturation methods, such as SAFARI,[25] OPARACHEE,[26] FLEX,[27] CERT,[28] two-frequency irradiation,[29] or LOVARS,[30] may improve the detection of this contrast and will be evaluated in future studies.

One of the attractive features of **1** and its analogues is the wealth of literature on the pharmacokinetics, formulation, and toxicity. Compound **1**, a well-known nonsteroidal anti-inflammatory drug (NSAID), is a component of human diets[31] and is found at elevated levels in the serum of vegetarians.[32] Salts and esters of salicylic acid have been administered to patients so that low millimolar concentrations are achieved in plasma for the treatment of rheumatoid arthritis.[33] Aspirin is a prodrug of salicylic acid and has beneficial effects in a variety of conditions, including inflammation and cancer.[34] We expect these probes to be translatable on the basis of the widespread testing that has been performed on these and similar compounds in patients over many decades.

In conclusion, we have demonstrated that salicylic acid (**1**) and its analogues (**4**–**10**) are a promising new set of diaCEST probes. As a quick in vivo evaluation, **1** was injected into mice and 6% contrast was obtained in kidneys. This type of low-toxicity probe, especially **1**, could improve the sensitivity of existing CEST methods. More MRI studies on the pharmacokinetics and tumor uptake of formulated salicylic acid and its analogues are now under investigation in our laboratories.

Experimental Section

Phantom preparation and data acquisition: All of the compounds were purchased from Sigma Aldrich (St. Louis, MO). Samples were dissolved in 0.01M phosphate-buffered saline (PBS) at concentrations from 1.5 mM to 100 mM, and titrated using high-concentration HCl/NaOH to various pH values ranging from 6 to 8. The solutions were placed into 1 mm glass capillaries and assembled in a holder for CEST MR imaging. The samples were kept at 37 °C during imaging. Phantom CEST experiments were taken on a Bruker 11.7 T vertical bore MR scanner, using a 20 mm birdcage transmit/receive coil. CEST images were acquired using a RARE (RARE = 8) sequence with CW saturation pulse length of 3 s and saturation field strength (B_1) from 1.2 μT to 14.4 μT. The CEST Z-spectra were acquired by incrementing saturation frequency every 0.3 ppm from −15 to 15 ppm for phantoms; TR = 6 s, effective TE = 17 ms, matrix size = 64 × 48 and slice thickness = 1.2 mm.

Animal preparation and data acquisition: In vivo images were acquired on a Bruker Biospec 11.7 T horizontal bore MR scanner, with one axial slice of 1.5 mm thickness obtained through the medulla of both kidneys. CEST images with saturation frequencies of ±9.3 ppm were acquired repeatedly every 100 s. both pre- and post-injection. Image parameters were similar to those for the phantom except for TR/TE = 5 s/15 ms, with optimized $B_1 = 7.2\ \mu T$. For MRI, the BALB/c mice ($n = 2$) weighing 20–25 g (Charles River Laboratories, Wilmington, MA) were anesthetized by using 0.5–2% isoflurane and placed in a 23 mm transmit/receive mouse coil. Breath rate was monitored throughout the in vivo MRI experiments using a respiratory probe. A 60 μL volume of a 0.25 M solution of **1** in PBS (pH 7) was slowly injected by a catheter into the tail vein. CEST contrast was quantified by $MTR_{asym} = (S^{-\Delta\omega} - S^{+\Delta\omega})/S_0$ for phantom and $MTR_{asym} = (S^{-\Delta\omega} - S^{+\Delta\omega})/S^{-\Delta\omega}$ in vivo to normalize the conventional magnetization transfer of tissue, where $S^{-\Delta\omega}$ and $S^{+\Delta\omega}$ refer to the water signal intensity with a saturation pulse applied at the frequencies $-\Delta\omega$ and $+\Delta\omega$, respectively. For details of the data processing, see the Supporting Information.

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