

# Anthranilic acid analogs as diamagnetic CEST MRI contrast agents that feature an intramolecular-bond shifted hydrogen

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Diamagnetic chemical exchange saturation transfer (diaCEST) agents are a new class of imaging agents, which have unique magnetic resonance (MR) properties similar to agents used for optical imaging. Here we present a series of anthranilic acid analogs as examples of diaCEST agents that feature an exchangeable proton shifted downfield, namely, an intramolecular-bond shifted hydrogen (IM-SHY), which produces significant and tunable contrast at frequencies of 4.8–9.3 ppm from water. Five analogs of *N*-sulfonyl anthranilic acids are all highly soluble and produced similar CEST contrast at ~6–8 ppm. We also discovered that flufenamic acid, a commercial nonsteroidal anti-inflammatory drug, displayed CEST contrast at 4.8 ppm. For these N–H IM-SHY agents, the contrast produced was insensitive to pH, making them complementary to existing diaCEST probes. This initial IM-SHY library includes the largest reported shifts for N–H protons on small organic diaCEST agents, and should find use as multifrequency MR agents for *in vivo* applications. Copyright © 2014 John Wiley & Sons, Ltd.

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**Keywords:** chemical exchange saturation transfer; *N*-sulfonyl anthranilic acid derivatives; molecular imaging; *N*-aryl anthranilic acid derivatives

## 1. INTRODUCTION

Chemical exchange saturation transfer (CEST) contrast agents, first introduced in 2000 (1), are an alternative to traditional magnetic resonance (MR) contrast agents, which rely on direct enhancement of water relaxivity. The CEST mechanism involves saturation of labile protons on the agents via selective irradiation at their resonance frequencies. The signal loss is then transferred to surrounding bulk water through chemical exchange, leading to a reduction in water signal (2–4). This water signal loss (CEST contrast) results in an amplification of the signal from low-concentration protons through the multiple exchange events occurring during the saturation pulse. Because the CEST contrast is derived from irradiation at a specific proton frequency, it is easier to discriminate from other sources of signal change than  $T_1$  or  $T_2^*$  contrast. This frequency dependence of contrast also allows the simultaneous detection and discrimination of multiple agents within an image (5–7). Diamagnetic CEST (diaCEST) and paramagnetic CEST (paraCEST) agents have been the subjects of several recent reviews (8–11). DiaCEST agents, such as glucose (12–14), glycogen (15), *myo*-inositol (16), glutamate (17), creatine (18,19), *L*-arginine (20,21), glycosaminoglycans (22,23) and peptides (5,24–26), are attractive biocompatible materials, but compared with paraCEST agents (27), they suffer from reduced sensitivity owing to the relatively small chemical shift difference between their exchangeable protons and those of water (1–5.0 ppm). To address this issue, diaCEST agents with protons of increased chemical shift have been reported, including the thymidine analogs (5.5 ppm) (28) and iopamidol (4.2 and 5.5 ppm) (29,30). Most recently, we reported that the C2–OH in 2-hydroxybenzoic acid analogs

resonates between 8.7 and 10.8 ppm from water, with solute-to-water exchange rates ( $k_{sw}$ ) that are well suited to CEST imaging (31). Building upon that report, here we describe the anthranilic acid analogs: *N*-aryl derivatives, *N*-acyl derivatives and *N*-sulfonyl derivatives, as another class of IntraMolecular-Bond Shifted Hydrogens exchangeable proton (IM-SHY) diaCEST agents, based on the exchange of N–H protons instead of O–H (Scheme 1).

## 2. RESULTS AND DISCUSSION

Salicylic acid (1) displays CEST contrast at 9.3 ppm (31) (Fig. 1). This dramatic chemical shift derives from the low barrier

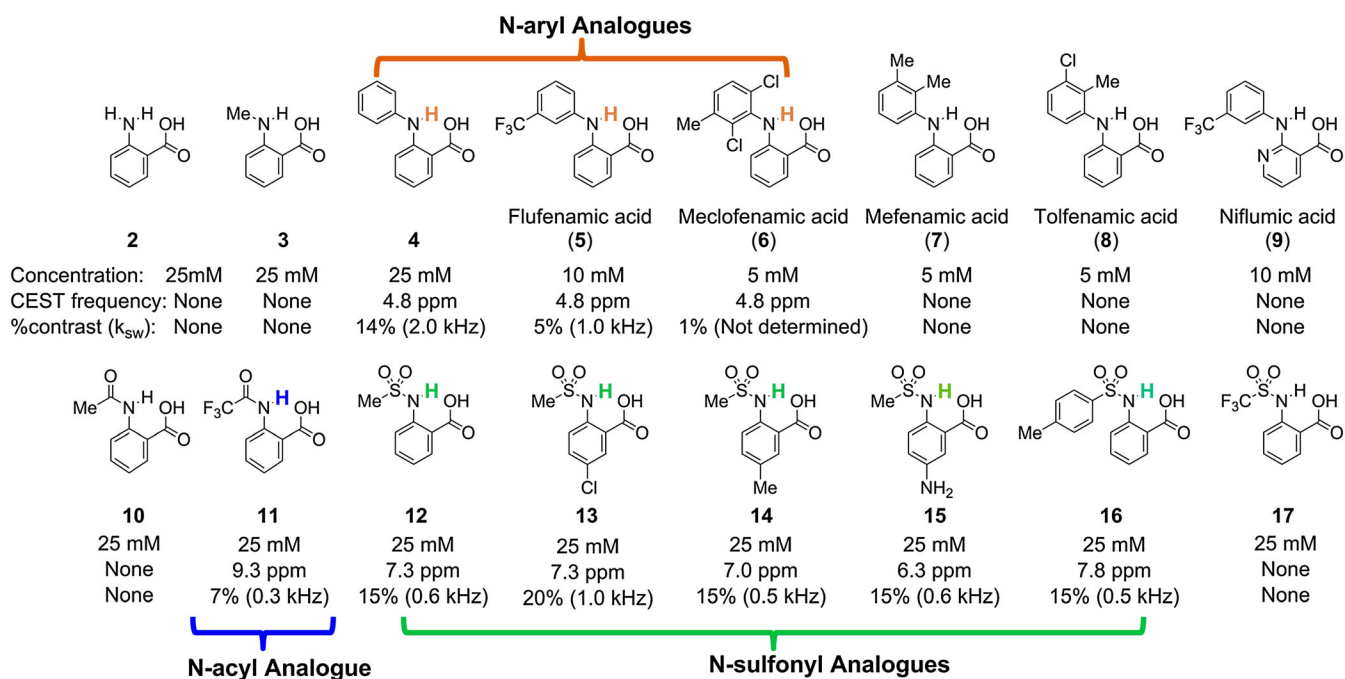
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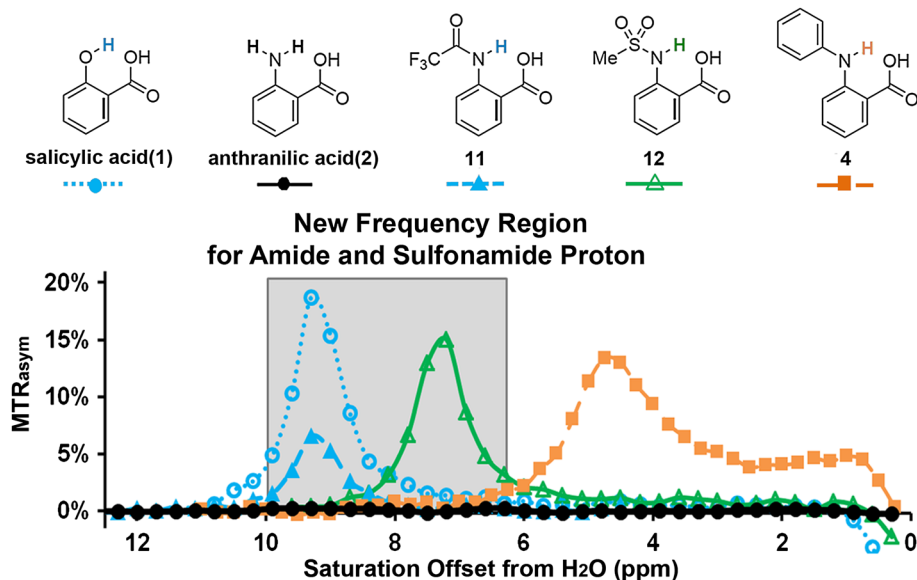
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**Scheme 1.** Chemical exchange saturation transfer (CEST) frequency (ppm), contrast (%) and  $k_{sw}$  (kHz) of anthranilic acid and its analogs. Experimental conditions: pH 7.1–7.5, using  $T_{sat} = 3$  s,  $B_1 = 3.6$   $\mu$ T. For Z-spectra, see Tables S1 and S2 in the Supporting Information. All the MR experiments were performed at 37 °C.



**Figure 1.** CEST contrast curves for representative salicylic acid (1) and anthranilic acid derivatives (2, 4, 11 and 12) at concentrations of 25 mM (pH 7.1–7.4) using  $B_1 = 3.6$   $\mu$ T,  $T_{sat} = 3$  s. The gray box indicates this group of agents includes a new frequency region for amide and sulfonamide protons.

hydrogen bond between the exchangeable phenolic proton and the carboxylate anion at neutral pH (32,33). We also determined that similar CEST signals could be observed in other compounds with the 2-hydroxybenzoic acid scaffold, representing a powerful new type of CEST agent, based on the principle of IM-SHY (31). We were interested in preparing similar agents with labile anthranilic rather than phenolic protons to explore further the capabilities of the benzoic acid core for generating CEST contrast. However, anthranilic acid (2), an N–H analog of salicylic acid, failed to produce contrast (Scheme 1, Fig. 1). To understand why, we measured the CEST contrast properties of a wide range of common anthranilic acid analogs, including those

with *N*-alkyl, *N*-aryl, *N*-acyl and *N*-sulfonyl substitutions (Scheme 1). Interestingly, significant contrast was observed in *N*-phenylanthranilic acid (4), although the labile protons resonate at 4.8 ppm, which is much lower than the 9.3 ppm observed in 1. At a relatively low saturation field strength ( $B_1 = 3.6$   $\mu$ T), 4 showed a broader peak in the CEST spectrum than 1 and 12 (Fig. 1b), indicating a faster exchange. Using the QUESP (QUantifying Exchange rates using Saturation Power dependence) experiment (34) we measured  $k_{sw} = 2.0$  kHz (Supporting Information, Fig. S1), which is slightly too fast to obtain optimal CEST contrast using the 3–5  $\mu$ T saturation pulses we are able to employ on our clinical scanners. Comparing the CEST signal between 4 and 2, the loss

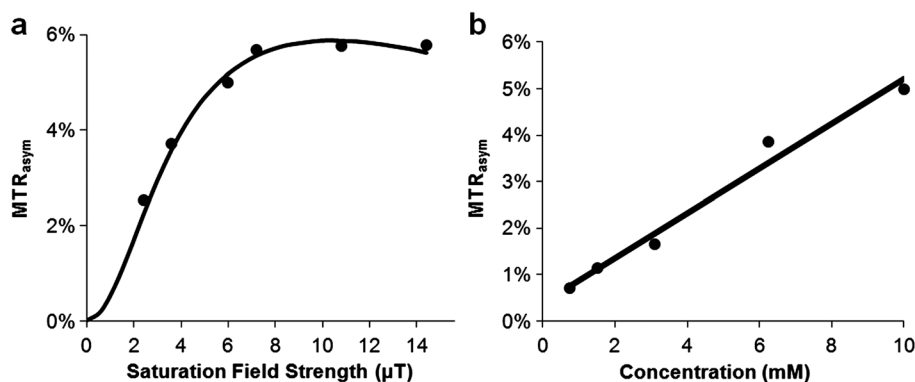
of CEST signal in **2** indicates that  $k_{sw}$  is too high. This is possibly due to the presence of the additional nonhydrogen-bonded C2 N-H proton, which might undergo a fast intramolecular exchange with the hydrogen-bonded proton. In addition, if we modify **2** through substitution of a methyl group for one of the amine protons (**3**), the CEST contrast is still absent, which implies that stereoelectronic influences are also important (Scheme 1). It is worth mentioning that *N*-phenylanthranilic acid analogs are commonly used as nonsteroidal anti-inflammatory drugs. The CEST properties were measured on five commercially available drugs: flufenamic acid (**5**), meclofenamic acid (**6**), mefenamic acid (**7**), tolfenamic acid (**8**) and niflumic acid (**9**). Their water solubility is generally low (~10 mM or lower). As shown in Scheme 1, flufenamic acid (**5**) showed similar CEST properties to **4**. The exchangeable proton resonates at 4.8 ppm, with  $k_{sw} = 1.0$  kHz. The CEST data of **6–8** indicated the importance of steric interaction on the proton exchange rate with water. Adding the chloro group ortho to the exchangeable N-H (**6**) reduced its water accessibility and the CEST contrast dropped to 1%. This is presumably because the exchange is too slow; however, it is difficult to quantify  $k_{sw}$  because of the small contrast. Increasing the steric hindrance through addition of methyl (**7** and **8**) eliminated the CEST signal. Niflumic acid (**9**), the pyridine analog of **5**, did not display any CEST contrast. One possible explanation is that the presence of the pyridine nitrogen tends to strongly hydrogen bond to water and alters the proton exchange of the IM-SHY -NH.

We next determined the detection limits of **5** with CEST, because it could potentially be translated into clinical applications (35). The solubility of **5** is quite poor at pH values below 7; however, 10 mM could be achieved in phosphate-buffered saline buffer at pH above 7.2. As shown by the QUESP data in Fig. 2 (a), the contrast is near maximal at  $B_1 > 6$   $\mu$ T, with a smaller  $k_{sw}$  (1.0 kHz) than that of **4**. The peaks in the Z-spectrum and the  $MTR_{asym}$  spectrum are also sharper than those of **4** (Table S1), which is also due to a slower  $k_{sw}$ . The contrast of **5** is nearly linearly dependent with concentration over a range from 0.75 to 10 mM (36) (pH 7.4), with 1.2% contrast observed at a concentration of 1.5 mM (Fig. 2b).

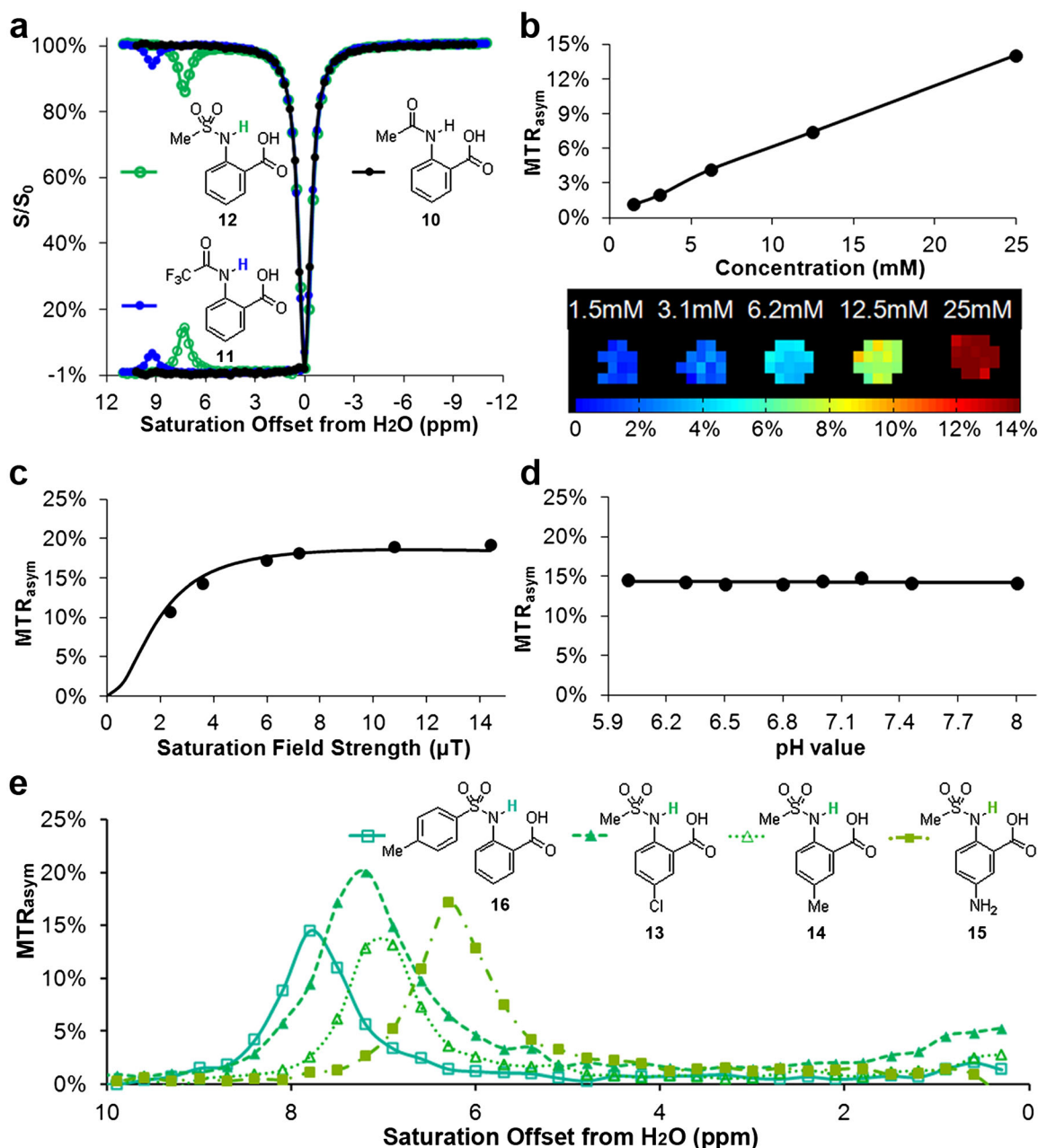
In an attempt to increase the chemical shift further to fit the slow to intermediate detection window of CEST ( $k_{sw} < \Delta\omega$ ) while still keeping  $k_{sw}$  slow enough to achieve efficient saturation using a  $B_1$  suitable for the MR hardware used in our *in vivo* scans, we investigated the C2 amide analogs of anthranilic acid. Amide N-H protons tend to be shifted further than amine protons,

although they also tend to exchange with water more slowly as well (**5**). As expected, **10** did not show any CEST contrast, presumably because the  $k_{sw}$  is too slow (Fig. 3a, Scheme 1). However, after modification of the structure to **11**, an example of a more acidic N-H proton, we observed CEST contrast with the labile proton resonating at 9.3 ppm, indicating a strong hydrogen bond interaction in water. The contrast produced by **11** is relatively low (6% at 25 mM,  $B_1 = 3.6$   $\mu$ T), because  $k_{sw}$  is relatively slow (0.3 kHz, see Supporting Information, Figs S2 and S3 for QUESP/pH details). Further increasing the acidity through 2-(methyl-sulfonamido) benzoic acid (**12**) resulted in more substantial contrast at 7.3 ppm (~15% at 25 mM,  $B_1 = 3.6$   $\mu$ T), based on adjusting the proton exchange of the IM-SHY-NH. According to our QUESP measurements, **12** displays a  $k_{sw} = 0.6$  kHz at pH = 7.1, which is quite similar to salicylic acid (**31**) and barbituric acid (Supporting Information, Fig. S5). Maximum contrast was achieved using  $B_1 = 6$   $\mu$ T or higher with ~90% of this contrast available at  $B_1 = 3.6$   $\mu$ T (Fig. 3c), which is near the maximum power we can apply using a parallel transmit body coil on our clinical scanners. More interestingly, the contrast and  $k_{sw}$  of **11** and **12** remained almost constant between the pH values 6 and 8 (Figs 3d and Figs S2–S4 in the Supporting Information). For comparison, salicylic acid (**1**), an alternative IM-SHY agent, possesses protons with  $k_{sw}$  that decrease dramatically over this range ( $k_{sw} = 2.4$  kHz at pH 6.5,  $k_{sw} = 0.4$  kHz at pH 7.8). This pH independence makes **11** and **12** ideal IM-SHY probes for *in vivo* quantification purposes. As expected, a nearly linear relationship between contrast and concentration was observed for **12** (Fig. 3b), with 1% CEST contrast produced at a concentration of 1.5 mM. Although the chemical shift is not as large as **1** or **11**, **12** represents the first diaCEST agent with labile N-H protons resonating at 7–8 ppm from water that produces significant contrast. This compound should be useful for multiple frequency detection and complementary to other existing diaCEST probes.

Encouraged by the result from **12**, we studied several commercially available analogs to check if the CEST contrast of this scaffold would tolerate chemical modification. As shown in Scheme 1 and Fig. 3(e), similar contrast was obtained upon chemical modification of the aniline ring (**13–15**), with the CEST frequency varying from 6 to 7.3 ppm. Placing a strong electron donating -NH<sub>2</sub> group (**15**) at the *para*-position to the C2-NH reduced the CEST frequency to 6.3 ppm, which is quite similar to the electronic effects we observed previously (**31**). Placing a -Cl at the *para*-position of the C2-NH (**13**) led to faster  $k_{sw}$



**Figure 2.** CEST properties of **5**. (a) QUESP data at 10 mM at pH = 7.4, with  $k_{sw} = 1.0$  kHz where the data are shown as points and the solid line represents the best fit after numerically solving the two-pool Bloch equations; (b) CEST contrast at 4.8 ppm as a function of concentration using  $B_1 = 3.6$   $\mu$ T (solid line: linear fitting).

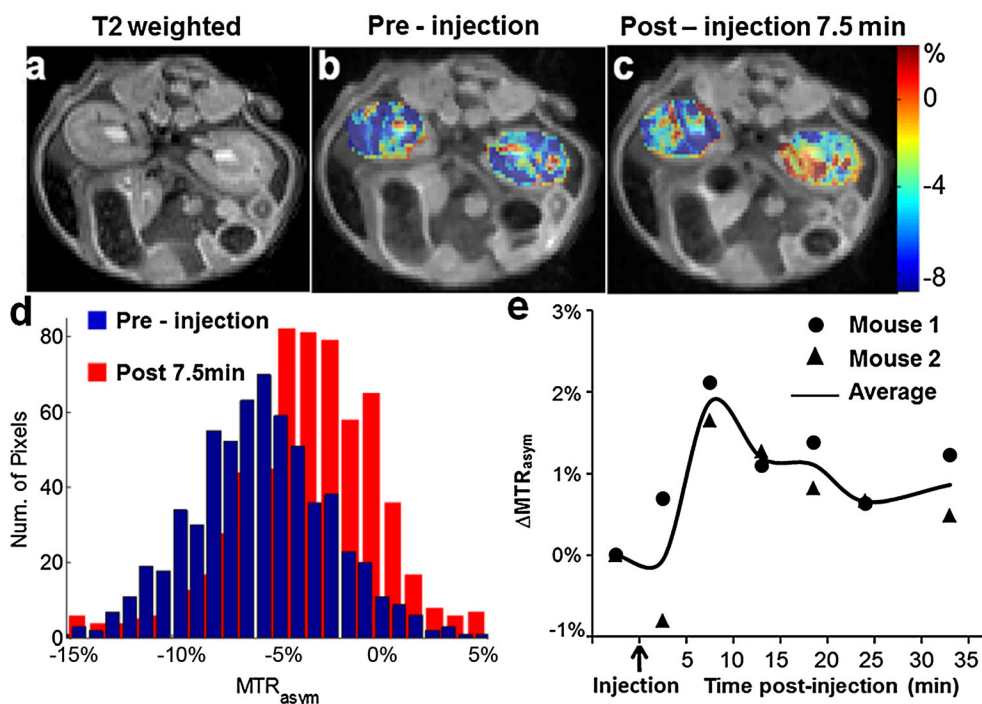


**Figure 3.** CEST properties of **10–16**. (a) Z-spectra and MTR<sub>asym</sub> for **10–12** at 25 mM, pH = 7.2,  $T_{\text{sat}} = 3$  s and  $B_1 = 3.6$   $\mu\text{T}$ ; (b) CEST contrast of **12** at 7.5 ppm as a function of concentration, using  $B_1 = 3.6$   $\mu\text{T}$ ; (c) QUESP data of **12** at 25 mM, pH = 7.1, with  $k_{\text{sw}} = 0.6$  kHz; (d) pH dependence of percentage contrast for **12**; and (e) analogs of **12** with different CEST peak frequencies from 6 to 8 ppm.

(1.0 kHz), and as a result a higher CEST contrast (~20%). Substitution of a phenyl for the methyl (**16**) resulted in deshielding with the chemical shift increased to 7.8 ppm. In comparison, replacing the methyl group in **12** with a  $-\text{CF}_3$  (**17**) resulted in loss of CEST contrast. As this group of agents, **12–16**, generated similar contrast to **1** in phantoms, we further chose to monitor *in vivo* the contrast in kidneys after administration into the tail vein of mice of the most sensitive, **13** (Fig. 4). The contrast was monitored over time, and compared with the pre-injection images (Fig. 4b); we observed a 2–3% increase in the CEST contrast 7.5 min after injection integrating from 7.0 to 7.6 ppm (Fig. 4b, c). The histogram in Fig. 4(d) indicates the pixelwise distribution of MTR<sub>asym</sub> values for mouse 1 pre- and post-injection. A negative MTR<sub>asym</sub> was

observed as baseline for the kidneys, which is presumably due to strong relayed NOE transfer of signal loss to water (37,38). As shown in Fig. 4(e), for both mice the contrast reached maximum at ~7.5 min post-injection

As shown above, anthranilic acid IM-SHY probes have larger shifts for their exchangeable protons than spherical lipoCEST agents (**10**), and similar shifts to those found for paraCEST probes such as Yb-DO3A-oAA (39). The shifts are not nearly as large as some of the Yb, Eu, Tm or Dy complexes described previously (40–43) or the cryptophane cages used for hyperCEST (43); however, because  $k_{\text{sw}}$  can be tuned to be as slow as 0.5–1 kHz through structure changes and is insensitive to pH in the physiologically relevant range, these IM-SHY probes are well suited for detection using saturation pulses attainable on clinical scanners.



**Figure 4.** *In vivo* contrast for **13**. (a)  $T_{2w}$  image; (b) overlay  $MTR_{asymp}$  map pre-injection for mouse 1; (c) overlay  $MTR_{asymp}$  map at 10 min post-injection for mouse 1; (d) histogram displaying the distribution of  $MTR_{asymp}$  for mouse 1 pre- and post-injection (c, d). (e) Dynamic time course of  $\Delta MTR_{asymp}$  based on regions of interest enclosing both left and right kidneys for the two mice using  $\omega_1 = 3.6 \mu\text{T}$  (circle: mouse 1; triangle: mouse 2; solid line, average value of mouse 1 and mouse 2).

A more detailed investigation of the steric and electronic factors for this scaffold is ongoing.

### 3. CONCLUSION

We have demonstrated that anthranilic acid provides a suitable scaffold for tunable IM-SHY diaCEST agents. Labile protons in *N*-aryl anthranilic acids (**4–6**) resonate at 4.8 ppm while for *N*-sulfonyl anthranilic acids (**12–16**) these resonate between 6 and 8 ppm and for **11** labile protons resonate at 9.3 ppm. Anthranilic acid analogs could be used for multicolor MR imaging, with one nonsteroidal anti-inflammatory drug, **5**, already administered to patients, having been identified among these analogs. The 2-sulfonamidobenzoic acid scaffold has been shown to allow chemical modification with labile protons that exchange in a non-pH-dependent manner, which could be advantageous for *in vivo* quantification. Additional studies are ongoing to improve our understanding of the relationship between CEST properties and molecular structure for these and other IM-SHY diaCEST agents.

## 4. EXPERIMENTAL SECTION

### 4.1. Phantom Preparation and Data Acquisition

Compounds **1–12** were purchased from Sigma Aldrich (St Louis, MO, USA). Compounds **13–17** were purchased from Enamine Ltd (Monmouth, NJ, USA). Samples were dissolved in 0.01 M phosphate-buffered saline at several concentrations from 1.5 to 25 mM depending on the solubility, 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. They were kept at 37°C during imaging. Phantom CEST experiments were performed

on a Bruker 11.7 T vertical bore MR scanner, using a 20 mm birdcage transmit/receive coil. CEST images were acquired using a Rapid Acquisition with Refocused Echoes (RARE) (RARE factor = 8) sequence with a continuous wave saturation pulse length of 3 s and saturation field strength ( $B_1$ ) from 1.2 to 14.4  $\mu\text{T}$ . The CEST Z-spectra were acquired by incrementing the saturation frequency every 0.3 ppm from  $-15$  to 15 ppm; repetition time ( $TR$ )/effective echo time ( $TE$ ) = 6 s/17 ms with linear phase-encoding, matrix size =  $64 \times 48$  and slice thickness = 1.2 mm. For determining  $k_{sw}$  using QUESP, Z-spectra were collected at  $B_1 = 1.2, 2.4, 3.6, 5.4, 7.2, 10.8$  and 11.4  $\mu\text{T}$ .

### 4.2. In Vivo Mouse Imaging

To evaluate whether the *N*-sulfonyl derivatives, **12–16**, could be detected after administration into live animals, we injected two mice with 60  $\mu\text{L}$  of a 0.25 M solution of compound **13** and collected CEST images. Images consisting of a single axial slice containing both kidneys were collected. To improve the temporal resolution and able to correct the  $B_0$  shift, we collected a partial Z-spectrum every 5 min by incrementing  $\Delta\omega$  over 10 frequencies ( $\pm 8.2, \pm 7.6, \pm 7.3, \pm 7$  and  $\pm 6.6$  ppm), and an average  $MTR_{asymp}$  (at  $\pm 7.6, \pm 7.3$  and  $\pm 7$  ppm). The imaging sequence employed is the same as for the phantoms, with the following parameters:  $B_1 = 3.6 \mu\text{T}$ , saturation duration ( $T_{sat}$ ) 3 s,  $TR$ /effective  $TE = 5$  s/16 ms with linear phase-encoding, matrix size  $96 \times 64$ .

### 4.3. Post-processing

CEST contrast was quantified using  $MTR_{asymp} = [S(-\Delta\omega) - S(+\Delta\omega)]/S_0$  for phantom and  $1 - S(+\Delta\omega)/S(-\Delta\omega)$  *in vivo* to increase the temporal resolution and reduce the motion where  $S(+\Delta\omega)$  represents water signal intensity with a saturation pulse applied at the frequency  $+\Delta\omega$  and  $S_0$  represents the water signal without a saturation pulse.

The Z-spectra were corrected pixel by pixel using a  $B_0$  map acquired using Water Saturation Shift Referencin (WASSR) as described in detail previously (9). To indicate the kinetics of CEST contrast upon injection of the agents, we subtracted the  $MTR_{\text{asym}}$  values at each time-point with a reference  $MTR_{\text{asym}}(0)$ , and plotted the averaged  $\Delta MTR_{\text{asym}}(t) = MTR_{\text{asym}}(t) - MTR_{\text{asym}}(0)$ , and plotted the averaged  $\Delta MTR_{\text{asym}}(t)$  of the whole kidney as a function of minutes post-injection. The solvent to water exchange rate ( $k_{\text{sw}}$ ) was calculated according to the QUEST and/or QUESP methods (34), which were considered as a simple and robust method for estimating  $k_{\text{sw}}$ , especially for the slow to intermediate exchange regime (44,45). In particular we numerically solved the two-pool model Bloch equations to fit the measured  $MTR_{\text{asym}}$  values as a function of different  $T_{\text{sat}}$  or  $B_1$  as described previously (34), with the following relaxation parameters for water and solute respectively, where  $R_{1w}$  is the longitudinal relaxation time for water and  $R_{2w}$  is the transverse relaxation time for water:  $R_{2w} = 0.9 \text{ s}^{-1}$ ,  $R_{1s} = 0.71 \text{ s}^{-1}$ ,  $R_{2s} = 39 \text{ s}^{-1}$ .  $R_{1w}$  was allowed to float between 0.33 and  $0.40 \text{ s}^{-1}$  to obtain the best fit. The QUESP/QUEST fittings are shown in the Supporting Information, Figs S1–S5.

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