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A Biomarker-Responsive T_{2ex} MRI Contrast Agent

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Abstract

Purpose—This study investigated a fundamentally new type of responsive MRI contrast agent for molecular imaging that alters T_2 exchange (T_{2ex}) properties after interacting with a molecular biomarker.

Methods—The contrast agent Tm-DO3A-oAA was treated with nitric oxide (NO) and O_2 . The R_1 and R_2 relaxation rates of the reactant and product were measured with respect to concentration, temperature and pH. CEST spectra of the reactant and product were acquired using a 7 T MRI scanner and analyzed to estimate chemical exchange rates and r_{2ex} relaxivities.

Results—The reaction of Tm-DO3A-oAA with NO and O_2 caused a 6.4-fold increase in the r_2 relaxivity of the agent, while r_1 relaxivity remained unchanged, which demonstrated that Tm-DO3A-oAA is a responsive T_{2ex} agent. The effects of pH and temperature on the r_2 relaxivities of the reactant and product supported the conclusion that the product's benzimidazole ligand caused the agent to have a fast chemical exchange rate relative to the slow exchange rate of the reactant's ortho-aminoanilide ligand.

Conclusion— T_{2ex} MRI contrast agents are a new type of responsive agent that have good detection sensitivity and specificity for detecting a biomarker, which can serve as a new tool for molecular imaging.

Keywords

responsive agent; T_{2ex} MRI; chemical exchange; molecular imaging; nitric oxide

INTRODUCTION

T_2 -exchange (T_{2ex}) MRI contrast agents are fundamentally different from the common contrast agents used for MRI (1-5). T_{2ex} contrast agents possess a labile proton or bound water molecule with a chemical exchange rate of $10^5 - 10^8$ Hz (6). The stochastic chemical exchange of protons between the T_{2ex} agent and bulk water causes the net precession of the bulk water protons to become broadly distributed, especially when the labile proton has a large chemical shift. This change in the net precession of the water protons causes a faster

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R_2 ($1/T_2$) relaxation rate for the water. Importantly, this chemical exchange process does not affect the R_1 ($1/T_1$) relaxation rate of the bulk water.

We investigated whether a T_{2ex} agent can detect a molecular biomarker (7). We hypothesized that a biomarker-responsive change in R_2 relaxation rate can be compared to a biomarker-unresponsive “control” R_1 relaxation rate to detect the biomarker in a manner that is independent of the agent’s concentration. Although T_{2ex} has been investigated for more than 40 years (8-10), its utility for detecting molecular biomarkers has not been explored.

To develop a responsive T_{2ex} MRI contrast agent, we first considered Yb-1,4,7,10-tetraazacyclododecane-(*N,N,N'*-triacetic acid)-(N'''-orthoaminoanilide) (Yb-DO3A-oAA) that changes its chemical exchange rate after reaction with NO and O₂ (11). The chemical shifts of the labile protons of Yb-DO3A-oAA are -11 and +8 ppm. We hypothesized that replacing Yb(III) with Tm(III) may cause larger chemical shifts for the labile protons of this agent, based on comparisons of similar lanthanide chelates with Yb(III) and Tm(III) (12), which would lead to a stronger T_{2ex} relaxation effect (Eq. [S3]) (3). This report describes the R_1 and R_2 relaxation rates and the CEST MR properties of Tm-DO3A-oAA before and after reaction with NO and O₂.

METHODS

Chemical reactions and preparation of samples

The synthesis of the contrast agent Tm-1,4,7,10-tetraazacyclododecane-(*N,N,N'*-triacetic acid)-(N'''-orthoaminoanilide) (Tm-DO3A-oAA; Figure S1), reactions with NO (Figure S2), and details regarding sample preparations are provided in the Supplementary Information.

R_1 and R_2 relaxation rate measurements

A spin echo MRI protocol was used to measure R_1 relaxation rates with a 12.7 ms echo time; 10 repetition times between 18.339 and 10000 msec; 1 mm slice thickness; 782×782 μm in-plane resolution; 5×2.5 cm field of view; and 64×32 matrix using a 7 T Bruker Biospec MRI scanner. The same protocol was used to measure R_2 relaxation rates with a 15-960 ms echo time in increments of 15 ms; 40 sec repetition time; 1.07 mm slice thickness; 400×400 μm in-plane resolution; 5.12×2.56 cm field of view; and 128×64 matrix size. All MRI experiments were performed at 37.3°C, except for temperature-dependent studies that were performed from 27°C to 48°C (Figure S4). To calculate the R_1 and R_2 relaxation times, the signal amplitudes were fitted with a monoexponential function with a constant to account for a possible DC offset using ParaVision® PV5.1 (Bruker Biospin, Inc., Billerica, MA).

CEST MRI studies

CEST spectra were obtained at 1-30 mM concentrations of the reactant and 30 mM of the product treated with NO and O₂ gas, at 37.3°C. A HW-Conc fitting method was used to correlate CEST signals with concentrations (13). MRI studies were performed with a CEST-FISP MRI protocol using a 7 T Bruker Biospec MRI scanner (14). The FISP acquisition

used a 1.624 ms TE; 3.248 ms TR; 422.41 ms scan repetition time; 30° excitation angle; linear encoding; unbalanced “FID” mode; 1 mm slice thickness; 391 × 391 μm in-plane resolution; 5 × 5 cm field of view; and 128x128 matrix size. CEST saturation consisted of a series of Gaussian shaped pulses with 10 μs interpulse delays. Saturation was applied at a peak power of 20 μT and a bandwidth of 650 Hz for 2.995 sec. Images were reconstructed using ParaVision® pv5.1. Signal amplitudes were measured using ImageJ to create a CEST spectrum (15).

The baseline was not flat in some CEST spectra due to sample heating and/or power drift from the amplifier that caused a change in the tip angle of the FISP imaging sequence. To correct for this sloped baseline, the baseline region with no CEST peaks was fit with a straight line, and this line was subtracted from the CEST spectrum. CEST spectra were smoothed with a cubic spline and a sum of four Lorentzian line shapes was fit to each smoothed CEST spectrum using Matlab R2012B (Mathworks, Inc., Natick, MA) (16,17). A Levenberg–Marquardt least squares fitting routine optimized the center, width, and amplitude of each Lorentzian line to fit the lines to the experimental data. Because the chemical shift of the direct saturation of water was fitted, this method automatically compensated for B₀ inhomogeneity during the CEST MRI studies (18). The amplitude of the Lorentzian line shape represented the % CEST value.

Chemical Exchange Rate Estimations

Experimental CEST spectra of nine samples of Tm-DO3A-oAA at 1 to 30 mM concentration were simultaneously fit with the Bloch-McConnell equations using a four-pool model (19,20). The estimate of the chemical exchange rate is largely dependent on fitting the line width of the CEST peak while the estimate of the concentration is largely dependent on fitting the CEST peak amplitude (21). Thus, the chemical exchange rate can be accurately estimated without also requiring the fitting to provide an accurate estimate of the concentration and/or the number of equivalent protons on the agent. The T₁ and T₂ time constants of water, the B₀ shift, and a scale factor that accounts for transceiver coil characteristics were fit for each sample, because each sample had a different concentration and location in the magnet bore. The B₁ power, chemical exchange rates, and chemical shift offsets of the three labile pools were simultaneously fit for all phantoms, because these characteristics are the same for each sample regardless of concentration or location in the magnetic field. The fitting was performed by using a 3 second, 9 μT continuous wave pulse instead of a series of Gaussian shaped pulses. To confirm the validity of this approximation, we simulated CEST spectra with a Gaussian pulse train or a continuous wave pulse using the other parameters determined from the fitting process, and observed that the simulated CEST spectra had a difference that was much less than the residual of our fitting to the experimental data. Because linear encoding was used for image acquisition, our simulated CEST spectra included 0.211 s of evolution without RF irradiation, corresponding to the time required to reach the center of k-space acquisition.

Swift-Connick Plot

Swift-Connick plots were generated to show the relationships between chemical exchange rates and r_{2ex} relaxivities for the reactant, based on CEST signals at -49, -29, and +19 ppm

at 7 T magnetic field strength (details are provided in Supplementary Information) (3). To gain insights about the product, three-dimensional Swift-Connick plots were generated to show the relationships between chemical exchange rates, chemical shifts, and r_{2ex} relaxivities at 7 T magnetic field strength, for one or two exchangeable protons.

RESULTS

Synthesis and T_{2ex} characterization

Tm-DO3A-oAA was synthesized in five steps (Figure S1). The reaction of Tm-DO3A-oAA with NO and O₂ gas was confirmed with HPLC and its product was characterized by high-resolution mass spectroscopy (Figure 1a). The formation of the product was consistent with previous reports (22,23).

The R_1 and R_2 relaxation rates of serially-diluted samples of the reactant and product treated with NO and O₂ gas were used to determine r_1 and r_2 relaxivities (Figure 1b). The r_2 relaxivity of Tm-DO3A-oAA increased 6.4-fold after the reaction, from $0.21 \pm 0.020 \text{ mM}^{-1}\text{s}^{-1}$ to $1.32 \pm 0.20 \text{ mM}^{-1}\text{s}^{-1}$. The r_1 relaxivities of the reactant and product did not change. The r_2 relaxivity values showed that the reactant had a good detection sensitivity that is within an order of magnitude of the sensitivity of T_1 contrast agents, and the product had good detection sensitivity that was comparable to the sensitivity of T_1 contrast agents (which typically have r_1 relaxivities of approximately $1\text{--}4 \text{ mM}^{-1}\text{s}^{-1}$). Notably, this detection sensitivity also depends on the endogenous T_2 relaxation time of tissues, which may reduce the detection sensitivity during *in vivo* studies. These results also demonstrated good detection specificity because T_2 -weighted MR contrast could be compared to T_1 -weighted MR contrast to detect the biomarker in a concentration-independent manner (Figure 1c).

The R_2 relaxation rates of the reactant and the product of reaction with NONOate were dependent on pH (Figure 2). Both the reactant and the product showed an increase in R_2 when the pH was increased from 3.5 to 5.8, which was attributed to faster base-catalyzed chemical exchange involving the multiple acetate ligands. For pH values higher than 5.8, the R_2 value of the reactant initially decreased to reach a minimum at pH 7.1 and then this value increased at higher pH. The product showed a 1.5 fold higher R_2 for the product versus the reactant at pH 7.1. This different dependence on pH was attributed to the presence of the product's benzimidazole arm, because benzimidazoles typically have a pK_a of ~ 7 . The R_1 rate of both the reactant and the product did not change between pH 3.5 to 8, which provided additional evidence that the pH-dependent T_2 relaxation was due to a T_{2ex} effect.

CEST MR studies and analyses of chemical exchange rates

We acquired CEST spectra of the reactant and product of reaction with NO and O₂ gas. The two labile groups of protons in the reactant produced three CEST signals at -49 ppm , -29 ppm , and $+19 \text{ ppm}$ (Figure 3a). The generation of three CEST signals may be due to multiple conformations of the lanthanide chelate, which has been reported for other CEST agents (24,25), and/or may be arise from a water molecule that is bound to the chelate in addition to the labile protons on the aminoanilide ligand. All CEST signals of the reactant showed an enhancement when the concentration of the agent was increased to 10 mM, but

these CEST signals decreased at higher concentrations (Figure 3b). This loss of CEST at higher concentrations was attributed to a decrease in saturation caused by faster R_2 relaxation at higher concentrations. For comparison, the product from treatment with NO and O_2 gas did not show a CEST signal (Figure 3c), which differed from the observation of CEST at +500 ppm for similar Tm(III) complexes (26). This lack of a CEST signal indicated that the product had a faster chemical exchange rate than the reactant.

We simultaneously fit a series of CEST spectra of the reactant at different concentrations using Bloch-McConnell equations, which determined that the chemical exchange rates of these CEST signals at -49 ppm, -29 ppm, and +19 ppm were 8,400 Hz, 14,500 Hz, and 4,700 Hz, respectively. The 95% confidence intervals of our fittings were $\pm 15.7\%$, $\pm 17.9\%$, and $\pm 17.1\%$ for CEST signals at -49 ppm, -29 ppm, and +19 ppm, respectively, which indicated good fitting despite the weak CEST signals generated by this agent. Based on a Swift-Connick plot, these chemical exchange rates from CEST MRI studies were estimated to generate r_{2ex} relaxivities of 0.045, 0.090, and 0.019 $mM^{-1}s^{-1}$, respectively (Figure 4a) (3,8). The sum of these r_{2ex} relaxivities is 0.154 $mM^{-1}s^{-1}$, while the measured r_2 relaxivity was 0.210 $mM^{-1}s^{-1}$ (Figure 1b). The difference between these values, 0.056 $mM^{-1}s^{-1}$, was attributed to the r_2 relaxivity mechanisms of the Tm(III) chelate that are attributed to properties other than chemical exchange. Assuming that the product also has the same 0.056 $mM^{-1}s^{-1}$ dipolar r_2 relaxivity attributed to the Tm(III) chelate, then the r_{2ex} relaxivity of the product is 1.26 $mM^{-1}s^{-1}$.

An increase in temperature caused an increase in r_2 relaxivity for the reactant (Figures 4b, S3), which agreed with a previous report (3). An increase in temperature should cause an increase in chemical exchange rate, which enhances the r_{2ex} relaxivity of the reactant that has chemical exchange rates that are slower than the exchange rate at the peak of the Swift-Connick plot (Figure 4a). In contrast, an increase in temperature caused a decrease in the r_{2ex} relaxivity of the product treated with NO and O_2 gas, indicating that the product has a chemical exchange rate that is faster than the exchange rate at the peak of a Swift-Connick plot. The r_1 relaxivities of the reactant and the product remained unchanged with temperature, further supporting that the product is a T_{2ex} contrast agent.

To estimate the chemical exchange rate of the product, we generated a Swift-Connick plot as a function of chemical shift and exchange rate. The scale of this plot is different for one exchangeable benzimidazole proton or two protons on a bound water molecule. Based on our temperature-dependent study (Figure 4b), the chemical exchange rate must be greater than the exchange rate at the peak of the Swift-Connick plot, and therefore must lie in the colored region of these plots. To achieve the estimated r_{2ex} value of 1.26 $mM^{-1}s^{-1}$ in this colored region of these plots, the chemical exchange rate of the product must range from 1.8×10^6 to 3.1×10^8 Hz if one proton is responsible for the chemical exchange, and from 7.4×10^5 to 9.5×10^8 Hz if two protons are responsible for the chemical exchange.

Validation studies

The r_1 and r_2 relaxivities were negligible for both the reactant and product without Tm(III) when treated with NO and O_2 gas, confirming that agent needs a large chemical shift caused by Tm(III) to generate T_{2ex} relaxation (Figure 5a). Samples of the metal $TmCl_3 \cdot H_2O$

without chelator showed a 3-fold decrease in r_2 relaxivity and a 2-fold decrease in r_1 relaxivity when reacted with NO and O₂ (Figure 5b). The decrease in relaxation was attributed to complexation between Tm(III) and NO or oxidative byproducts of NO and O₂ that reduced water access to the metal ion. These validation studies showed that potential demetallation of the contrast agent after reaction with NO could not cause the observed increase in R_2 relaxation and the invariant R_1 relaxation (Figure 2b).

DISCUSSION and CONCLUSIONS

These studies demonstrated that Tm-DO3A-oAA is a responsive T_{2ex} MRI contrast agent. The change in r_2 relaxivity after reaction with NO and O₂, and without a corresponding change in r_1 relaxivity, is a hallmark of the T_{2ex} process. The effects of pH on r_2 relaxivities indicated that the change in r_{2ex} was due to the formation of the benzimidazole ligand of the product. Notably, the faster chemical exchange rate of the product may be due to chemical exchange of the benzimidazole protons with bulk water, and/or chemical exchange of bulk water with a bound water molecule coordinated to the chelate that interact with the benzimidazole ligand. Only one bound water molecule is coordinated to the reactant and product, because the r_1 relaxivity of the agent did not change. Regardless of the exact source of the product's chemical exchange rate, this faster rate caused a change in r_{2ex} relaxivity without a change in r_1 relaxivity that was used to detect nitric oxide in a concentration-independent manner.

Future studies to assess chemical reaction rates with NO and *in vivo* studies that evaluate the effect of pH and temperature on biomarker detection are warranted to further demonstrate the utility of responsive T_{2ex} contrast agents. *In vivo* studies are also warranted to assess the detection sensitivity of T_{2ex} agents relative to potentially short T_2 relaxation times in tissues. Furthermore, other chemical designs should be explored to detect other types of biomarkers, including other metabolites, enzymes, ions, proteins or nucleic acids, and to measure environmental conditions such as pH, hypoxia, redox state, or temperature (7,27). This report provides a guideline for establishing these other agents as responsive T_{2ex} contrast agents.

Responsive T_{2ex} MRI contrast agents have an outstanding combination of advantages for molecular imaging relative to other MRI contrast agents. T_1 and T_2 MRI contrast agents have chemical exchange rates of $10^7 - 10^9$ Hz that generate excellent detection sensitivity as low as ~ 10 and ~ 1 μ M, respectively (28, 29). However, the concentration of the agent can also change the R_1 and R_2 relaxation rate of the system, which compromises the specificity for detecting a biomarker with these agents (30). CEST MRI contrast agents can possess multiple biomarker-responsive and unresponsive “control” CEST signals that can be compared to improve the specificity for detecting the intended biomarker (12, 31, 32). However, the slow $10^2 - 10^4$ Hz exchange rate of CEST agents creates low detection sensitivity, requiring >1 mM concentration of a contrast agent for detection (33). T_{2ex} contrast agents have an intermediate chemical exchange rate of $10^4 - 10^7$ Hz, which provides the ‘best of both worlds’ by retaining good detection sensitivity and specificity for detecting the intended biomarker in a concentration-independent manner. Therefore, responsive T_{2ex} agents represent an intriguing new paradigm for molecular imaging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A responsive T_{2ex} contrast agent. **(a)** The reaction of Tm-DO3A-oAA in the presence of nitric oxide and oxygen. **(b)** The R_1 and R_2 relaxation rates before and after reaction with NO and O₂ gas were measured at 37.3°C. The reactant was measured at pH 6.77 ± 0.06 , and the product was measured at pH 6.90 ± 0.12 . Error bars represent the standard deviation of measurements, and some error bars are smaller than the data symbol. **(c)** The ratio of the relaxation rates for the reactant or product are concentration-independent. This ratio is higher for the product than the reactant, which can be used to indicate the detection of nitric oxide.

Figure 2.

The pH dependence of R_1 and R_2 relaxation rates of the reactant and product after treatment with with NO and O_2 gas. Samples were measured at 5 mM concentration and at 37.3°C . Error bars represent the standard deviation of measurements, and some error bars are smaller than the data symbol.

Figure 3.

The CEST spectrum of Tm-DO3A-oAA before and after reaction with NO and O₂ gas. (a) The CEST spectrum of 30 mM of the reactant showed three CEST peaks. (b) The concentration dependence of the CEST signal of Tm-DO3A-oAA before reacting with NO gas and O₂ is shown with lines that represent the results of a HW-conc fitting method that does not account for R₂ relaxation (30). (c) The CEST spectrum of the product at 30 mM concentration did not show CEST peaks. All samples were tested at 37.3°C and pH 7.2.

Figure 4.

The estimation of the chemical exchange rate of the product. (a) A Swift-Connick plot of the reactant relates the expected r_{2ex} relaxivity with the chemical exchange rate of CEST signals at -49 ppm (red), -29 ppm (green) and +19 ppm (blue) at 7 T magnetic field strength. The chemical exchange rates determined from CEST MRI are shown as a dot on each Swift-Connick line plot. (b) The temperature dependence of r_1 and r_2 relaxivities of Tm-DO3A-oAA before and after reacting with NO and O₂ gas shows that the r_2 relaxivity of the reactant increases with increasing temperature, and the product has a r_2 relaxivity that decreases with increasing temperature. The reactant was measured at $\text{pH } 6.77 \pm 0.06$, and the product was measured at $\text{pH } 6.90 \pm 0.12$. Error bars represent the standard deviation of measurements, and some error bars are smaller than the data symbol. (c) A Swift-Connick plot shows the expected relationship between chemical shift, chemical exchange rate, and r_{2ex} relaxivity of the product at 7 T magnetic field strength. Scale bars are shown for one benzimidazole proton and two protons on a bound water molecule. As shown by Figure 4b, the chemical exchange rate must lie in the colored region of this plot. The estimated r_{2ex} relaxivity of $1.26 \text{ mM}^{-1} \text{ s}^{-1}$ for the product is shown as a black line for one proton and a white line for two protons in the plot.

Figure 5.

Relaxation rates of the chelator and ion. The R₁ and R₂ relaxation rates of (a) DO3A-oAA and (b) Tm(III) before and after reacting with NO gas and O₂ are shown as a function of concentration. Relaxation rates were measured at 37.3°C. The pH of the solution of DO3A-oAA was adjusted to 6.76 ± 0.06 before reaction and 6.88 ± 0.1 after reaction. The pH of the solution of TmCl₃ was adjusted to 6.68 ± 0.08 before reaction and 6.61 ± 0.05 after reaction with NO, respectively. Error bars represent the standard deviation of measurements, and some error bars are smaller than the data symbol.