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Reporter Protein-Targeted Probes for Magnetic Resonance Imaging

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Abstract

Contrast agents for magnetic resonance imaging are frequently employed as experimental and clinical probes. Drawbacks include low signal sensitivity, fast clearance and non-specificity that limit efficacy in experimental imaging. In order to create a bio-responsive MR contrast agent, a series of four Gd(III) complexes targeted to the HaloTag reporter were designed and synthesized. HaloTag is unique among reporter proteins for its specificity, versatility, and the covalent interaction between substrate and protein. In similar systems, these properties produce prolonged in vivo lifetimes and extended imaging opportunities for contrast agents, longer rotational correlation times, and increases in relaxivity (r_1) upon binding to the HaloTag protein. In this work we report a new MR contrast probe, **2CHTGd,** which forms a covalent bond with a target protein and results in a dramatic increase in sensitivity. A 6-fold increase in r_1, from 3.8 $mM^{-1}s^{-1}$ to 22 $mM^{-1}s^{-1}$, is observed upon **2CHTGd** binding to the target protein. This probe was designed for use with the HaloTag protein system which allows for a variety of substrates (specific for MRI, florescence, or protein purification applications) to be used with the same reporter.

Magnetic resonance imaging (MRI) is a diagnostic tool in both research and clinical applications due to its capacity to render images of high spatial and temporal resolution without the need for ionizing radiation.[1] Contrast agents, such as chelated Gd(III), can be utilized in clinical MR imaging to improve sensitivity, resolution, and allow more rapid scanning times.[2] While valuable information can be attained using generic contrast agents, their effectiveness is limited due to their non-specificity, rapid clearance, and low relaxivity.[2]

In order to create new generations of MR agents that overcome these shortcomings, a focus of our research has been the development of bio-responsive contrast agents that bind to a target protein, and report on enzyme activity or cation binding.[3] Here, we describe a system to be used for tracking the expression of a reporter protein using a MR contrast agent.

Contrast agent relaxivity is determined by the interaction between a Gd(III) ion and nearby water protons.[2] This interaction is described by several parameters including the number of coordinating water molecules (q), the exchange rate of the inner-sphere water (k_{ex} or $1/\tau_M$), and the rotational correlation time of the complex (τ_R).[2] The fast molecular motion of small molecule complexes is the factor most limiting the observed relaxivity. A common strategy to increase relaxivity is to employ the receptor-induced magnetization enhancement (RIME)

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Supporting information: Complete reference 6, Supplemental Chart 1, Supplemental Table 1, Supplemental Table 2, Supplemental Figure 1. Materials and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

effect in which the motion of a contrast agent is coupled to that of a target protein, thereby increasing the τ_R and increasing relaxivity.[4] One example of such an agent is MS-325 (Ablavar®), a small molecule contrast agent targeted to human serum albumin (HSA). Upon binding to HSA there is an increase in τ_R from 115 ps to 10 ns, and a concurrent increase in relaxivity from to 5.5 $mM^{-1}s^{-1}$ to 25 $mM^{-1}s^{-1}$ (at 60 MHz, 37 °C).[4] As a result of the improved relaxivity and blood pool lifetime, MS-325 has recently been approved for clinical MR angiography.[5]

Here, we present a series of MR contrast agents developed to utilize a RIME strategy. These complexes target and covalently bind a commercially available modified haloalkane dehalogenase reporter (HaloTag) developed by Promega.[6] Wildtype haloalkane dehalogenase proteins are a class of bacterial enzymes that use a catalytic triad active site to catalyze the hydrolysis of the haloalkane substrate to a primary alcohol.[7] The HaloTag active site is located at the bottom of a 15 Å binding pocket where the wildtype active site histidine has been mutated to a phenylalanine, leaving the enzyme unable to hydrolyze the covalent ester intermediate.[6] One haloalkane substrate covalently binds to HaloTag under physiological conditions and acts as a suicide inhibitor of the modified protein.[6]

A number of HaloTag-targeted substrates have been generated by conjugating various functionalities onto the distal end of the haloalkane targeting group, including fluorophores, biotin, quantum dots, and purification resins.[6,8] These ligands have been utilized in a variety of reporter experiments, including pulse-chase, time-course, and fate-mapping.[9] All of these substrates utilize the same 14 atom, chlorine-terminated haloalkane reactive moiety designed to optimally bind the HaloTag protein.

In this report we describe the synthesis of a series of four MR probes containing a Gd(III) chelate and a haloalkane substrate connected by linkers of varying lengths (Figure 1). These derivatives were designed to systematically shorten the distance between the Gd(III) chelate and the surface of the protein in order to exploit the RIME effect and to optimize binding to the protein. We hypothesized that restricting local motion of the Gd(III) chelate near the flexible linker would result in longer rotational correlation times and therefore, increased relaxivity. The complexes are referred to by the number of carbons in the linker excluding the carbonyl carbon, the HaloTag targeting group, and the Gd(III) ion (**1CHTGd, 2CHTGd, 3CHTGd** and **4CHTGd**).

The synthesis of the amine-terminated HaloTag targeting moiety is described in Supplemental Scheme 1. 1-amino-2-ethoxyethanol was carbobenzyloxy (CBZ) protected using benzyl chloroformate and TEA to produce **S1**. 1-bromo-6-chlorohexane was coupled in 40% KOH in water using *t*-butyl ammonium hydroxide as a phase transfer catalyst to produce **S2**. The protecting group was removed via hydrogenation with a palladium on carbon catalyst to produce the amine-terminated haloalkane species (**S3**) for peptide coupling to the Gd(III) chelate-linker molecule.

The protected 3-carbon (**1c**) and 4-carbon linker (**1d**) arms (benzyl 4-bromobutyrate and benzyl 5-bromovalerate respectively) were synthesized by benzyl protecting 4-bromobutyric acid and 5-bromovaleric acid using benzyl alcohol and esterification agents DIC and DPTS (Scheme 1). These protected linkers were conjugated to the *t*-butyl protected DO3A macrocycle via a S_N2 reaction using K_2CO_3 and reflux conditions to produce **2a, 2c**, and **2d**.

Benzyl protection of 3-bromopropionic acid (**1b**) was achieved in high yield using a previously described method (Scheme 1).[10] 3-bromopropionic acid was heated to reflux with benzyl alcohol in toluene using *p*-toluene sulfonic acid monohydrate as a catalyst. A new procedure for synthesis of a monopropionate derivative of DOTA is described in Scheme 2b.[11,12] The propionate linker (**1b**) was coupled to the *t*-butyl DO3A macrocycle

using an anion exchange resin (Amberlite IRA-410 resin) at room temperature to produce **2b**.

The final products were prepared using the approach shown in Scheme 2. The benzyl protecting group on the linker arm was removed via hydrogenation using a palladium on carbon catalyst to produce the free carboxylic acid. The haloalkane moiety was peptide coupled to the free acid of the *t*-butyl DO3A-linker using DIC, DPTS, and TEA. The *t*-butyl protecting groups were removed with triflouroacetic acid, and the final complexes were produced by metalation with $GdCl_3$ maintaining the pH below 6. The metalated complexes were purified by reverse-phase HPLC and characterized by MS. No aggregation was observed in relaxometeric measurements.

The HaloTag protein was expressed recombinantly after subcloning the target protein into the vector pMCSG7 to include an N-terminal His_6-Tag followed by a TEV protease cleavage site. The protein was expressed in BL21(DE3) cells and purified by Nickel affinity chromatography. The affinity tag was proteolytically removed with TEV protease, and HaloTag protein was further purified by ion exchange and gel-filtration chromatography (the resulting protein was >99% pure by SDS-PAGE). The HaloTag protein was labeled with five-fold molar excess of Gd(III) complex at 4 °C overnight. The excess was removed via desalting column followed by dialysis overnight into 10 mM MOPS pH 7.4 resulting in a 1:1 Gd(III) complex-protein species. The labeled proteins were concentrated to a starting concentration of ~600 μM for all relaxivity analyses.

Longitudinal relaxivities of the unbound agents were determined in water and the protein-bound contrast agents in 10 mM MOPS at 1.5 T, 37 °C (Table 1)[13]. The agents that possess shorter linkers (**1CHTGd** and **2CHTGd**) had relaxivities similar to those of reported agents coordinating one water molecule.[2] It is possible that an unfavorable geometry prevents the carbonyls in **3CHTGd** and **4CHTGd** from coordinating the Gd(III) providing an additional coordination site for water, resulting in their slightly larger observed relaxivities.

The relaxivities of the protein-bound contrast agents do not follow a trend with linker length and vary significantly, from 7.6 $mM^{-1}s^{-1}$ to 22.0 $mM^{-1}s^{-1}$ (Table 1). The bound **2CHTGd** demonstrated the largest difference in relaxivity with a six-fold increase from 3.8 $mM^{-1}s^{-1}$ to 22.0 $mM^{-1}s^{-1}$. This increase in relaxivity is similar to that of MS-325, which increases from 5.5 $mM^{-1}s^{-1}$ to 25 $mM^{-1}s^{-1}$ (1.5 T, 37 °C) when bound to HSA.[4b] This result reflects an effective linker length that couples the rotational correlation time of the Gd(III) chelator to the HaloTag protein. Complexes **1-, 3-**, and **4CHTGd**, showed less increase in relaxivity upon binding to the protein; **3CHTGd** and **4CHTGd** resulted in the smallest change in r_1, and **1CHTGd** had an intermediate increase in r_1 when protein-bound.

This range in signal enhancement was visualized by acquiring MR images at 1.5 T (Figure 2). The phantoms (100 μM solutions of **1CHTGd, 2CHTGd, 3CHTGd, 4CHTGd, 1CHTGd- Pro, 2CHTGd-Pro, 3CHTGd-Pro, 4CHTGd-Pro**) confirmed the longitudinal relaxivity results. A difference of a single carbon had a significant effect on the relaxation properties of the protein bound agent.

The relatively low relaxivities of the bound **1-, 3-**, and **4CHTGd** complexes, compared to **2CHTGd**, can be attributed to one or a combination of unoptimized relaxation parameters, τ_R, τ_M, or *q*. As discussed previously, relaxivity increases achieved from binding a protein can be impacted by rapid local motion of the Gd(III) chelator. For example MP-2269, a serum albumin-targeted contrast agent that is similar to MS-325, achieves only half the protein-bound relaxivity of MS-325 due to its τ_R being an order of magnitude shorter.[14] The short τ_R of the protein- bound MP-2269 is attributed to the internal flexibility which allows free rotation of the Gd(III) complex.[14] In addition, several amino acid residues have the

potential to coordinate Gd(III) (such as the hydroxyl groups in serine and threonine, amines in lysine, and carboxylic acid in glutamic and aspartatic acids). It is possible for nearby residues to displace the inner-sphere water of the bound-contrast agent causing coordinative saturation and a decrease in observed relaxivity.[4a,15]

2CHTGd will facilitate magnetic resonance molecular imaging of cellular events by binding to the HaloTag reporter protein. Recently, an extracellular HaloTag-receptor protein was generated[9b] that precludes the need to develop a cell permeable contrast agent and will allow systemic delivery of **2CHTGd**. The reduced molecular motion of a cell-membrane anchored HaloTag is expected to improve bound-relaxivity through an increase in the τ_R of the complex. Increased signal-to-noise will be achieved by the large increase in relaxivity of the protein-bound **2CHTGd** complex and rapid clearance of low-relaxivity, free contrast agent. The covalent bond formation between the contrast agent and the protein will result in prolonged in vivo lifetimes of the probe which in turn will extend imaging opportunities.

A potential limitation of a 1:1 contrast agent : target protein model is that the low Gd(III) payload may limit our ability to visualize the target by MRI. However, it has been shown that monomeric contrast agents with high affinity and specificity for their target protein have a lower detection limit if the target protein is clustered in an area of high local concentration (microdomain).[16] Gd(III) concentration at the target site can be further increased through several approaches, included increasing the number of HaloTag receptors through genetic means or utilizing several Gd(III) complexes per haloalkane targeting groups.[17] Together, **2CHTGd** and concurrent advancements in HaloTag technology should allow for sensitive MR imaging of cellular processes at a level that, to date, has been difficult to achieve.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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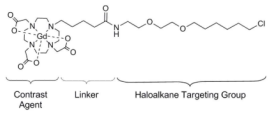


Figure 1.
HaloTag-targeted contrast agents. A macrocyclic Gd(III) chelator is connected to a haloalkane group via a flexible linker to generate a HaloTag-targeted contrast agent. Four complexes, with linkers of 1 to 4 carbons (shown), are described in this study.

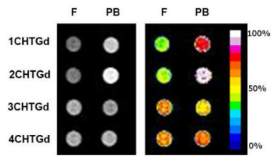
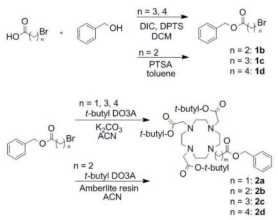
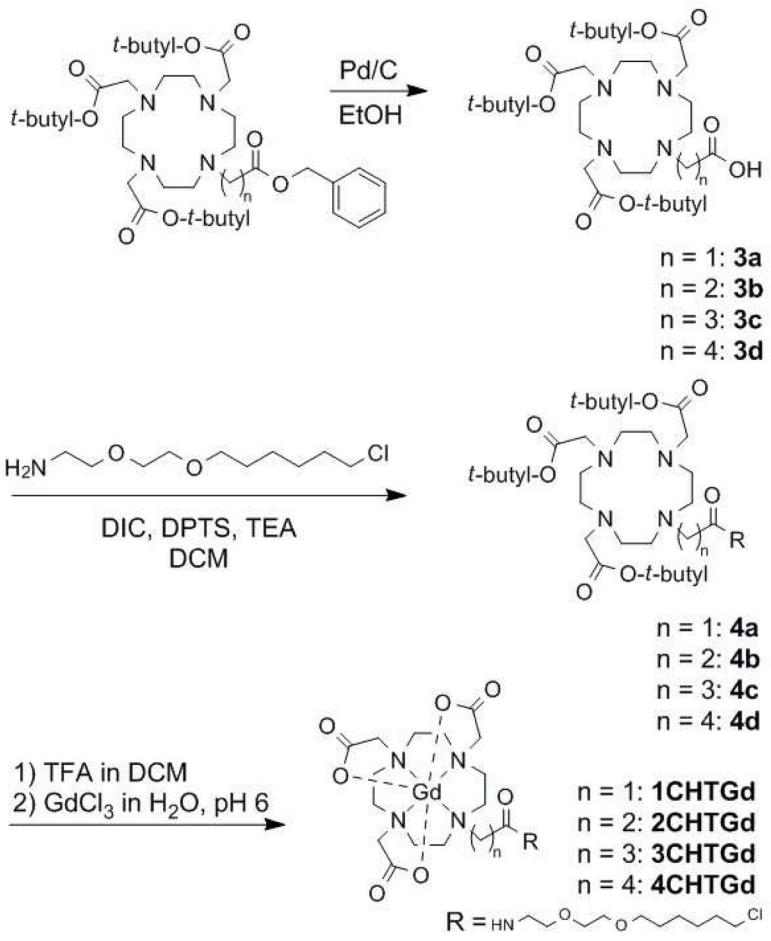


Figure 2.
MR images of HaloTag-targeted contrast agents. Grey-scale (left panel) and intensity-scale (right panel) T_1-weighted images of the free (F) and protein-bound (PB) contrast agents (100 μM Gd(III)) obtained at 1.5 T (TR 150 ms, TE 3.6 ms).



Scheme 1.

Two synthetic routes coupling Gd(III) chelators and variable length linkers towards the formation of a HaloTag-targeted contrast agent series.



Scheme 2.

Synthesis of macrocyclic Gd(III) contrast agents connected to haloalkane moieties by linkers of varying length.

Table 1

Longitudinal relaxivities of free (F) and protein-bound (PB) HaloTag targeted contrast agents at 60 MHz, 37 °C.

Sample	r_1 – F ($mM^{-1}s^{-1}$)	r_1 – PB ($mM^{-1}s^{-1}$)	Fold Change
1CHTGd	3.6 ± 0.2	13.4 ± 1.3	3.7
2CHTGd	3.8 ± 0.1	22.0 ± 2.2	5.8
3CHTGd	5.1 ± 0.1	7.6 ± 0.7	1.5
4CHTGd	6.4 ± 0.5	8.6 ± 1.1	1.3