Thiol Complexes of Gadolinium for Imaging Therapy-Induced Oxidative Stress in Pre-Clinical Tumors

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Introduction: Oxygen is required as the terminal electron acceptor in mitochondrial respiration, and under conditions of hypoxia there can be paradoxically increased levels of reactive oxygen species (ROS). In turn, high levels of ROS can lead to compensatory production of antioxidants via pathways mediated by the Nrf2 transcription factor and the Antioxidant Response Element (*ARE*). *ARE*-driven responses can include the increased production of reduced thioredoxin, reduced glutathione, and transfer of reduced cysteine equivalents between intracellular and extracellular pools [1]. Hyperglycemia and exposure to 2-deoxygluxose (2DG) can also lead to an oxidative stress response [2]. Increased thiol/disulfide ratio in the extracellular milieu is associated with increased tumor resistance to radiotherapy and platinum-based drugs which act by inducing oxidative stress. We present the development of DOTA- and DO3A-based thiol complexes of gadolinium that are responsive to the extracellular thiol/disulfide ratio. These complexes covalently bind human serum albumin (HSA) at the conserved Cys^{34} position under normal oxidizing conditions, but are designed to be competed off by endogenous thiols in highly reducing microenvironments. We propose a method to exploit the differential r_1 relaxivities of the bound *vs.* unbound forms to detect therapy-mediated changes in tumor redox status in a pre-clinical model.

Methods: Two previously-described DOTA-based, and 3 novel DO3A-based, thiol complexes of gadolinium (fig. 1) have been synthesized and characterized in these studies. Relaxivity measurements were made at 37°C and pH 7.4 in buffered saline in the absence ($r_{1,free}$, table 1) or presence of 0.66 mM HSA. The r1 relaxivity of the bound fraction of each molecule ($r_{1,bound}$, table 1) was calculated from a knowledge of the corresponding $r_{1,free}$ and the apparent equilibrium association constant of each molecule with HSA (K_A, table 2). Equilibrium binding was measured by means of rapid filtration through 30 kDa MWCO centrifugal filters [3]. While covalent binding to HSA at Cys^{34} is expected, the binding to commercial HSA in solution exhibited equilibrium characteristics that were consistent with the commercial HSA being partially bound at the Cys^{34} position to endogenous small molecule thiols such as cysteine or homocysteine. The K_A values reported in table 2 are internally consistent for a given lot of HSA (Sigma-Aldrich). Sensitivity to solution thiol content was demonstrated by measuring K_A in the presence of varying amounts of either homocysteine or the corresponding thiol complex of Eu (table 3). Quantitative dynamic contrast-enhanced MRI (DCE-MRI) requires knowledge of the arterial input function [4]. Additionally, quantitation with "responsive" Gd agents such as Gd-3c-SH and Gd-4b-SH may require dual boluses – of a non-responsive Gd agent followed by the responsive agent [5]. Since these Gd thiol molecules spontaneously bind circulating plasma albumin, we have sought to identify a "saturating" dose of each Gd thiol such that at relatively long times post-injection, the change in tissue T1 (ΔT1) is only a weak function of the injected Gd dose. DCE-MRI experiments were performed on MIA-PaCa-2 tumor xenograft bearing SCID mice using a GRE sequence (TR=72ms, TE=2.5ms, α=75°, matrix=128x128). Using the identified Gd dose, DCE-MRI T1 measurements were made on untreated mice and mice treated 24 h previously with 2DG (500 mg/Kg, i.p.) (fig. 2).

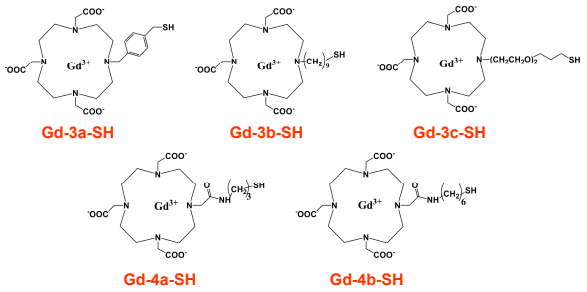

Fig. 1: DO3A- and DOTA-based thiol complexes of gadolinium.

Results: The relaxivities of the 5 complexes depicted in fig. 1 are reported in table 1. The $r_{1,bound}$ of Gd-3a-SH was lower than its $r_{1,free}$ – a desirable quality for highlighting regions in an MRI image with unbound Gd agent. However, Gd-3a-SH and Gd-3b-SH both appear to bind HSA at multiple sites and not just at the desired Cys^{34} site (table 2). Gd-3c-SH, Gd-4a-SH and Gd-4b-SH all experience an increase in r1 upon binding to HSA, and appear to bind to a single site on HSA with good affinity (table 2). Binding of Gd-3c-SH, Gd-4a-SH and Gd-4b-SH to HSA could be competitively inhibited by homocysteine (table 3). Binding of Gd-3c-SH to HSA was even more effectively inhibited by the competing presence of Eu-3c-SH (table 3). Based on high K_A and appreciable differences in $r_{1,free}$ *vs.* $r_{1,bound}$, Gd-3c-SH and Gd-4b-SH were advanced to in vivo studies as the most promising candidate molecules. The change in tumor T1 following injection of a Gd-based agent will be dependent on both the injected dose (AIF) as well as the relaxivity of the agent. MIA-PaCa-2 tumor bearing SCID mice were imaged by DCE-MRI following injection of either Gd-3c-SH or Gd-4b-SH, each at 3 different doses – 0.03, 0.05 and 0.1 mmole/Kg. Change in tumor T1 (ΔT1) was characterized as a function of both injected dose and time post-injection of Gd for both agents. Reduction in tumor T1 at 60 min post-injection at the 0.05 mmole/Kg dose was greater than at the 0.03 mmole/Kg dose but statistically similar to the 0.1 mmole/Kg dose for both agents. In addition, tumor ΔT1 at 60 min was greater for Gd-4b-SH than for Gd-3c-SH at the 0.05 mmole/kg dose (= "saturating" dose). Tumor ΔT1 at 60 min post-Gd-4b-SH was smaller in MIA-PaCa-2 tumors in mice that were treated 24 h earlier with 2DG, compared to tumors in control mice (fig. 2). This is consistent with the reported increase in *ARE*-driven events in MIA-PaCa-2 tumor cells following 2DG challenge [2].

Complex	$r_{1,free}$ (1/mM-s)	$r_{1,bound}$ (1/mM-s)
Gd-3a-SH	2.37 ± 0.18	1.54 ± 0.15
Gd-3b-SH	2.27 ± 0.37	3.28 ± 0.17
Gd-3c-SH	2.18 ± 0.58	3.15 ± 0.51
Gd-4a-SH	3.86 ± 0.44	4.54 ± 0.14
Gd-4b-SH	3.23 ± 0.33	4.74 ± 0.32

Table 1: r_1 relaxivities at 37ºC & 7 Tesla.

Complex	Interaction Energy at Cys^{34} (kcal/mole)	K_A (mM^{-1})
Gd-3a-SH	-410	Multiple sites
Gd-3b-SH	-471	Multiple sites
Gd-3c-SH	-453	22 ± 1.4
Gd-4a-SH	-290	5.0 ± 1.5
Gd-4b-SH	-510	64 ± 16

Table 2: Apparent equilibrium binding to Human Serum Albumin (HSA) at 37ºC.

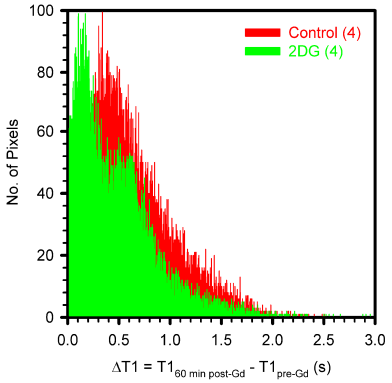

Fig. 2: MIA-PaCa-2 tumor ΔT1 at 60 min following 0.05 mmole/Kg Gd-4b-SH is decreased at 24 h post-therapy with 2DG (green) relative to control tumors (red).

Inhibitor, mM	Gd-3c-SH	Gd-4a-SH	Gd-4b-SH
None	22 ± 1.4	5.0 ± 1.5	64 ± 16
Homocys., 0.5	10.6 ± 3.4	2.0 ± 0.12	17 ± 2.2
Homocys., 1.0	6.6 ± 2.7	1.5 ± 0.2	6.1 ± 0.54
Homocys., 2.0	3.4 ± 0.84	0.87 ± 0.05	3.6 ± 0.23
Eu-X-SH, 0.5	6.2 ± 1.2	-	-
Eu-X-SH, 1.0	3.0 ± 0.91	-	-
Eu-X-SH, 2.0	~ zero	-	-

Table 3: Lowering of K_A by two competing thiols.

Conclusion: Tumor ΔT1 at 60 min following i.v. administration of Gd-4b-SH is a function of the HSA-bound/unbound fractions of the Gd when a "saturating" dose of the agent is utilized; an increase in the unbound fraction would lead to a reduction in tumor ΔT1. The observed decrease in tumor ΔT1 in treated tumors relative to control tumors would be consistent with an *ARE*-driven reduction of the tumor microenvironment following the 2DG challenge. Experiments are underway to validate these MRI findings against histopathological measures of treatment-related changes in tumor redox status.

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References: [1] Hayes JD, McMahon M, *TIBS* **34:**176-188, 2008; [2] Coleman et al., *Free Rad. Biol. Med.* **44:**322-331, 2008; [3] Caravan et al., *Inorg. Chem.* **40:**6580-6587, 2001; [4] Tofts PS, *JMRI* **7:**91-101, 1997; [5] Beauregard et al., *Proc. ISMRM* 1998, 53.