Published in final edited form as:

J Biol Inorg Chem. 2014 February ; 19(2): 271–279. doi:10.1007/s00775-013-1058-5.

GdDO3NI, a nitroimidazole-based T₁ MR contrast agent for imaging tumor hypoxia *in vivo*

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Abstract

Tumor hypoxia is known to affect sensitivity to radiotherapy and promote development of metastases. Therefore the ability to image tumor hypoxia *in vivo* could provide useful prognostic information and help tailor therapy. We previously demonstrated *in vitro* evidence for selective accumulation of GdDO3NI (GdDOTA monoamide conjugate of 2-nitroimidazole), a magnetic resonance imaging (MRI) T₁ shortening agent, in hypoxic cells grown in tissue culture. We now report *in vivo* evidence for accumulation of GdDO3NI, in hypoxic tumor tissue *in vivo*. Our data show that GdDO3NI accumulated significantly ($p < 0.05$) in the central, poorly perfused regions of rat prostate adenocarcinoma AT1 tumors (3-fold higher concentration than control agent) and showed better clearance from well-perfused regions and completely clearance from the surrounding muscle tissue. Inductively coupled plasma mass spectroscopy (ICP-MS) confirmed that more GdDO3NI was retained in the central region compared to either the periphery or the control agent. These results show the utility of GdDO3NI to image tumor hypoxia and highlight the potential of GdDO3NI for application to image guided interventions for radiation or hypoxia activated chemo therapy.

Keywords

Magnetic resonance imaging; hypoxia; nitroimidazole; prostate tumors

Introduction

Oxygen is essential for tissue health and survival. It is also an important variable in the treatment of many medical conditions including tumors, peripheral vascular disease and

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stroke. Hypoxia is known to affect the radiation sensitivity of tumors and promote development of metastases [1]. Further, the extent of hypoxia can vary greatly in radiographically similar tumors in many cases [2]. Therefore the ability to image hypoxia *in vivo* on an individual basis could not only provide useful prognostic information but could also be useful for developing hypoxia-targeted therapeutic approaches [1, 3]. Given the advent of novel, hypoxia-targeted therapies in various stages of development [4-6], the ability to stratify patients based on the extent of tumor hypoxia could be crucial.

Tissue oxygenation status can be assessed in *ex vivo* tissue samples or *in vivo* using a variety of approaches (both invasive and noninvasive). *Ex vivo* methods of assessing hypoxia in tissues include immunohistochemical staining for intrinsic markers of hypoxia (e.g., carbonic anhydrase IX (CA-IX) and hypoxia inducible factor-1 (HIF-1)) [7] as well as for adducts of exogenously administered nitroimidazoles [8]. Invasive techniques include polarographic needle electrodes [9] or fiber-optic fluorescence-based probes to obtain quantitative pO₂ measurements but these are limited to tumors that are readily accessible [10, 11]. As reviewed recently [11, 12], noninvasive hypoxia imaging approaches can be broadly classified into non-MR and MR based techniques; some are in preclinical development stage while others are in use clinically. Non-MR-based methods include: fluorescence imaging [13], phosphorescence quenching [14], near-infrared spectroscopy [15], SPECT [16] and PET [17] imaging. MR-based methods include: blood oxygen level dependent (BOLD) imaging [18], electron paramagnetic resonance (EPR) [19], fluorocarbon relaxometry using echo planar imaging for dynamic oxygen mapping (FREDO) [20] and proton imaging of siloxanes for mapping tissue oxygenation levels (PISTOL) [21].

An important strategy for imaging hypoxia exploits the principle of selective enzyme mediated reduction of the nitro group in 2-nitroimidazole containing compounds under hypoxic conditions [22]. Though initial studies concentrated on using 2-nitroimidazole analogs to sensitize hypoxic tumors to ionizing radiation [23], soon the prospect of these compounds as potential noninvasive hypoxia-homing probes was realized and several radiolabeled nitroimidazole derivatives have since been developed to image hypoxic tissues *in vivo* [10, 11]. Currently, [¹⁸F]fluoromisonidazole (F-MISO) [10], [¹⁸F]fluoroazomycin arabinoside (FAZA) [24], [¹⁸F]-EF5 [25], [¹⁸F] fluoroerythronitroimidazole (FETNIM) [26] and [⁶⁴Cu]-diacetyl-bis(N(4)-methylthiosemicarbazone (Cu-ATSM) [27] are being evaluated as hypoxia sensitive PET imaging probes while [¹⁹F] Tri-Fluoromisonidazole (TF-MISO) [28] has been used as a fluorine MRI probe to qualitatively image hypoxia. Recently, we developed a GdDOTA monoamide conjugate of 2-nitroimidazole (GdDO3NI) as a ¹H MR based hypoxia targeting agent and showed preferential accumulation of the agent in 9L glioma cells incubated with the agent under hypoxic conditions (8-fold compared to normoxia) [29].

Since MR-based noninvasive imaging is particularly suitable for high resolution longitudinal measurements, in this study, we investigated the potential of GdDO3NI for use as a novel hypoxia targeting MRI T₁ contrast agent. A similar structure lacking the nitroimidazole moiety, GdDOTA-(n-butyl)monoamide (GdDO3ABA), was used as control [29]. Here, we compare the kinetics of GdDO3NI and GdDO3ABA (Fig.1a and 1b, respectively) uptake

and washout from tumors and the tissue biodistribution of these agents after a single, bolus intravenous injection into rats bearing large, hypoxic prostate tumors.

Materials and Methods

The contrast agents, GdDO3NI and GdDO3ABA (molecular weights 0.839 kD and 0.615 kD) respectively, were synthesized as published previously [29]. All MR studies were performed on an INOVA 4.7T (Agilent Technologies, Santa Clara, CA) horizontal-bore MR system (200 MHz for ^1H). Due to the low molecular weights, both agents can be considered as freely diffusible small molecular agents.

Spin- lattice relaxivity (r_1) measurements

In vitro measurements of agent T_1 relaxivity (r_1) was performed at 37°C using two serial dilution phantoms (0- 4 mM), one in phosphate buffered saline (PBS) and another in 1% agarose to simulate tissue (Fig. S.1). For the R_1 measurements, a spin-echo sequence was used to acquire images at several TR values (0.1-6 s) and a fixed TE of 12 ms. The r_1 relaxivities were determined from the slopes of plots of relaxation rate (R_1) vs. concentration.

Kinetics of GdDO3NI vs. GdDO3ABA

All the animal experiments were approved by the Institutional Animal Care and Use Committee at UT Southwestern Medical Center, Dallas. For *in vivo* studies comparing GdDO3NI with GdDO3ABA, 10 Copenhagen rats were implanted subcutaneously with syngeneic Dunning prostate R3327-AT1 tumors [30]. Imaging studies were performed when the tumor sizes reached ~3 cc. The anesthetized animals (1.5% isoflurane in 1 L/min inhaled gas) were passively restrained and the body temperature was maintained at 37°C by a warm circulating water pad. The subcutaneous tumors growing on the leg of each rat were placed in a 3.5 cm diameter home-built volume coil and multi-slice T_1 -weighted images (TR/TE = 200/10 ms, FOV= 5 cm × 5 cm, matrix=128 × 128, slice thickness = 1 mm) were obtained pre and post injection of 0.1 mmol/kg body wt of either GdDO3NI or GdDO3ABA (n= 5 each). Images were acquired every 30 seconds during baseline and up to 3 minutes post injection followed by acquisition every minute up to 15 minutes and then once every 10 minutes up to 145 min post injection. The Gd concentration was estimated from the image data using the relationship, $[\text{Gd}] = (R_{1,\text{post}} - R_{1,\text{pre}})/r_1$, where $R_{1,\text{pre}}$ and $R_{1,\text{post}}$ are the relaxation rates and r_1 is the relaxivity of the contrast agent in a 1% agar phantom.

Data Analysis

All data analyses were performed using MATLAB (Mathworks Inc, Natick, MA) routines developed in-house. First a region-of-interest (ROI) was drawn on the T_2 -weighted scout image to delineate the tumor. The percentage signal enhancement map at 90 s post injection relative to pre injection (baseline) signal intensity was used to segment the total tumor region into well-perfused region (% enhancement at 90 s ≥ 50%) and the remaining as poorly-perfused region (% enhancement at 90 s < 50%). Of the voxels in the poorly perfused regions, those that showed <10% enhancement at 90 minutes post injection were also excluded, since they were more likely to represent necrotic regions. The well and poorly

perfused regions were designated as “periphery” and “center”, respectively, as these correspond well to the empirically observed locations of the majority of voxels in the well and poorly perfused regions. The statistical significance of differences in the normalized mean intensities and concentrations at each time point were estimated by using a 2-tailed unpaired student t-test.

Histology and Immunohistochemistry

To confirm the presence of hypoxic tissue, tumors samples were collected for histological and immunohistochemical evaluation after staining using the hypoxia marker, pimonidazole hydrochloride (Hypoxyprobe-1; NPI, Inc., Belmont, MA). Pimonidazole was dissolved in saline and injected via a tail vein (60 mg/kg) and ninety minutes later, the rats were euthanized. Tumors were excised and sections were cut through the center of the tumor. These tumor sections were then fixed in 10% formalin and embedded in paraffin. Before H&E staining and immunohistochemistry, 5 μ m thick cut sections were deparaffinized in xylene and ethanol and rehydrated in water. Hypoxic regions were detected using mouse monoclonal antibody Mab1 (1:50; NPI, Inc.) and FITC-conjugated goat antimouse secondary antibody (1:100 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA). After mounting with Vectashield medium (Vector Laboratories, Burlingame, CA), the slides were imaged using a fluorescence microscope (DeltaVison RT, Applied Precision, Issaquah, WA) equipped with a CoolSNAP^{HQ} cooled charge-coupled device digital camera (Photometrics, Tucson, AZ) with a computer-controlled motorized stepping stage. The images were reconstructed using the SoftWoRx 3.7 imaging software package (Applied Precision, Issaquah, WA).

Inductively Coupled Plasma - Mass Spectroscopy (ICP-MS)

To determine the amount of retained gadolinium, ICP-MS analysis of select regions of the tissue was also performed on a subset of the animals used in imaging studies. 5 mm thick tumor sections (n=3 rats) were cut and further segmented into periphery and center regions using a scalpel. These samples were then frozen immediately in liquid nitrogen and sent for ICP-MS analysis (Galbraith Laboratories, Inc., Knoxville, TN). The results were compared using 1-way ANOVA followed by Tukey's multiple comparison test.

Results

MRI reveals higher retention of GdDO3NI in Dunning prostate R3327-AT1 tumors

The r_1 relaxivity of GdDO3NI and GdDO3ABA were determined to be $5.21 \pm 0.04 \text{ mM}^{-1} \text{ s}^{-1}$ and $4.74 \pm 0.03 \text{ mM}^{-1} \text{ s}^{-1}$, respectively in PBS, from imaging data collected at 4.7T while those in a tissue simulating agarose phantom (1% w/v) were $5.45 \pm 0.29 \text{ mM}^{-1} \text{ s}^{-1}$ and $4.76 \pm 0.27 \text{ mM}^{-1} \text{ s}^{-1}$, respectively (supplementary data, Fig. S.1). The values determined in agarose were used to convert intensities measured *in vivo* into concentrations of the respective contrast agents. The kinetics of the two contrast agents were compared following intravenous injections of GdDO3NI or GdDO3ABA in Dunning prostate R3327-AT1 rat tumor-bearing animals (n=5). The data were segmented into well perfused and non-necrotic poorly perfused regions as described in the supplementary data (Fig S.2). These are henceforth referred to as “periphery” and “center” regions, respectively. Qualitatively, the

normalized time course signal intensities showed a clear difference in the enhancement patterns for the two agents (GdDO3NI and GdDO3ABA) and between peripheral and central regions (Fig 2 a-f). In the case of GdDO3NI, hyperintense regions were observed in the center of the tumor at 145 min post injection of the contrast agent when compared to pre-injection images (Fig 2c). While some hyperintensity was also observed in the center of the tumor in the case of GdDO3ABA, the intensity values were generally much lower than with GdDO3NI (~19% over baseline intensities, on average, for GdDO3NI vs. ~5% for GdDO3ABA). Comparing ratios of tumor to muscle intensities, a statistically significant difference ($p < 0.05$) in contrast enhancement was observed between peripheral (well-perfused and consequently well-oxygenated) regions and central (poorly perfused and potentially hypoxic) regions at late time points (80-145 min post injection) in animals given GdDO3NI (Fig. 3a) whereas for GdDO3ABA, no statistically significant difference between these regions was observed at late time points (Fig. 3b). Both agents showed a statistically significant difference in the two regions at early time point as well (up to 8 min post injection for GdDO3ABA and up to 15 minutes post injection for GdDO3NI).

Quantitative comparison of the time course of Gd concentration obtained by MRI showed a statistically significant difference in the Gd concentration at late time points (100 -145 min) in the central regions of tumors between animals ($n=5$) injected with GdDO3NI and GdDO3ABA (Fig. 4a). No statistically significant differences in the clearance patterns were observed in tumor periphery (Fig. 4b) or thigh regions (Fig. 4c). At 145 min post-injection, the Gd concentrations in the center regions of the tumor were $16.6 \pm 4.8 \mu\text{M}$ for GdDO3NI as compared to $5.3 \pm 5.8 \mu\text{M}$ GdDO3ABA. At the same time, the periphery showed a higher concentration of GdDO3ABA than GdDO3NI (Table 1). The time to reach peak concentration was faster for GdDO3ABA than for GdDO3NI for all 3 regions analyzed (thigh muscle, tumor periphery and tumor center). Gd concentration maps created at 145 minutes post injection of contrast agents clearly reveal higher uptake of GdDO3NI at foci in central regions of the tumor (Supplementary, Fig S3) when compared to GdDO3ABA (Fig S4).

Ex vivo tumor sample analysis correlates GdDO3NI retention with presence of hypoxia in Dunning prostate R3327-AT1 tumors

Histology (Fig 5a) and pimonidazole staining and imaging by immunofluorescence (Fig 5b) confirmed the existence of three zones in the tumor: a) necrotic center surrounded by a region composed of sparse vasculature and hypoxic regions and a viable well perfused periphery. Inductively coupled plasma mass spectroscopy (ICP-MS) analysis of tumor sections (*ex vivo*) was performed to quantify the Gd concentration. The tumor sections were cut to delineate the periphery and core to mimic MR segmentation procedure used here following which the samples were sent for ICP-MS analysis. The concentration of gadolinium (Gd) (Fig 6) was significantly higher in the central tumor region of GdDO3NI injected animals ($n=3$) when compared to its periphery as well as tumor regions of GdDO3ABA injected animals ($n=3$). On average ~ 50 fold higher retention of Gd was observed by ICP-MS in central regions of tumors that were injected with GdDO3NI when compared to GdDO3ABA.

Discussion

The vital role of hypoxia in the development and malignant progression in various cancers has been well studied. Hypoxia is also an important factor in determining the efficacy of radiotherapy, photodynamic therapy and some chemotherapies, and information related to hypoxic regions could be clinically important [3]. Our study tries to address this issue by evaluating a novel hypoxia targeting MR contrast agent GdDO3NI with respect to the following attributes, a) being an effective reporter of hypoxia, suitable for noninvasive *in vivo* studies and b) enabling high-resolution ^1H MR imaging to show the heterogeneity of hypoxia distribution in solid tumors.

Macrocyclic compounds and their derivatives are widely used as chelating agents in imaging probe development as they can form highly stable chelates with lanthanide metals [31]. In addition, the coordination chemistry of these compounds has been well studied [32, 33], allowing for the design of targeted probes for *in vivo* applications. Previously, radiolabeled lanthanide 2-nitroimidazole conjugates with DOTA derivatives were synthesized for imaging of hypoxia [34-37]. To our knowledge, our work is the first report on a 2-nitroimidazole derivative as a ^1H magnetic resonance imaging (MRI) probe to detect hypoxia. GdDO3ABA, was used as the 'control agent' as it has a similar structure to GdDO3NI without the hypoxia targeting nitroimidazole moiety. Having previously demonstrated the hypoxia-targeting ability, *in vitro*, using cells incubated under hypoxic conditions[29], we have now successfully validated the novel Gd-based nitroimidazole as a T_1 contrast agent (GdDO3NI) for MR imaging of hypoxia.

Since our main objective was to evaluate the hypoxia targeting capability of GdDO3NI, the choice of *in vivo* (tumor) models was of paramount importance. Another focus of this study was to determine the *in vivo* contrast agent kinetics as well as the ability to detect the spatial distribution of hypoxia in tumor models. Therefore, we chose large (~3 cc) Dunning prostate R3327-AT1 tumors as they have been shown using ^{19}F MR oximetry to be extensively hypoxic (mean $p\text{O}_2$ values of 3.5 ± 1.5 torr) [30]. In future studies, spontaneous or orthotopic tumor models might be appropriate, as well, for testing therapeutic interventions based on imaging hypoxia (e.g. tumor stratification for use of hypoxia-targeting chemotherapeutics).

The observed signal intensities and derived concentrations of the agents increased fastest in the well-perfused regions of tumors because of rapid contrast agent influx (Fig 4b). This was followed by fast wash-out, irrespective of the contrast agent (GdDO3NI or GdDO3ABA). In contrast, central regions, typically characterized by deficits in vascular supply, showed a delay in signal build-up corresponding to a delayed uptake for both contrast agents (Fig 4a), as also reported for this tumor type previously for Gd-DTPA[38]. More GdDO3NI was retained in the central regions at late time points than GdDO3ABA and we attribute this retention to the presence of hypoxia activated binding of GdDO3NI. The wash-in kinetics were different for both contrast agents (Table 1) with increased time to peak intensities in all regions for GdDO3NI compared to GdDO3ABA. This is consistent with expected kinetics for a targeted agent versus an untargeted one [39]. GdDO3NI and GdDO3ABA were also observed in the peripheral regions as well. In general the tumor vasculature, even in the well

perfused region, is chaotic and poorly functional [40] and regions of hypoxia can co-exist with vasculature within the volume of an MRI voxel ($390\ \mu\text{m} \times 390\ \mu\text{m} \times 1000\ \mu\text{m}$ in this case). Similarly, agents that are non-specific for hypoxia also accumulate in hypoxic or necrotic regions by passive diffusion and demonstrate enhanced retention [41]. Indeed, GdDTPA (non-hypoxia specific clinical contrast agent) based pharmacokinetic parameters have been shown to correlate with hypoxia [42]. Fitting a first order decaying exponential function of the form $C=C_0 + Ae^{-t/t_1}$ to the late time concentration time-course (85-145 min) yields a residual concentration C_0 of $13 \pm 2\ \mu\text{M}$ in the central region for GdDO3NI compared to $-2 \pm 4\ \mu\text{M}$, *i.e.* complete clearance, for GdDO3ABA. Thus the combination of differential kinetics and retention at late time points adds confidence to hypoxia-specific retention in the central regions of the tumor. Pimonidazole staining of the excised AT1 tumor tissues confirmed the presence of extensive hypoxia throughout the central regions of the tumor (Fig 5b). Also, ICP-MS analysis of the AT1 tumor samples revalidated the presence of excess amounts of Gd in the central regions of GdDO3NI injected animals (Fig 6) as observed from the concentration maps (Fig. 4a and Table 1). Interestingly, the amount of GdDO3NI found by ICP-MS in the central tumor region ($549\ \text{ppm} = 549\ \mu\text{g Gd/g tissue}$) was significantly higher than that estimated by MRI using a presumed intracellular relaxivity equal to that measured in 1% agarose. If one assumes that 1 g of tissue contains $\sim 0.6\ \text{mL}$ of water, then the concentration of Gd in this region could be as high as $5.84\ \text{mM}$. This is ~ 365 -fold higher than the concentration estimated by MRI ($16\ \mu\text{M}$). If anything, one would expect that the r_1 of the agent would be higher when bound to a larger macromolecular structure inside the cell so the MRI estimate of $16\ \mu\text{M}$ would be an upper limit if the agent has full access to water. This surprising result suggests that much of the GdDO3NI trapped in hypoxic cells does not have full access to water, *i.e.*, has a much lower relaxivity than that measured for the complex in aqueous media, such that the MRI underestimates the true amount of GdDO3NI in cells. This is entirely consistent with a previous study by Terreno, et al. [43], who showed that the relaxivity of Gd-HPDO3A when loaded into cells by electroporation *versus* pinocytosis differs considerably. These differences in relaxivity were ascribed to differences in the intracellular location of the agent (cytoplasm using electroporation versus endosomal vesicles using pinocytosis). This same group later showed by using photochemical release of endosomal GdHPDO3A molecules entrapped by pinocytosis that the relaxivity of the agent increased considerably as the agent moved into the cytoplasm [44]. Further modeling of this data suggested that the difference in proton relaxation rates between the cytoplasmic and the extracellular compartment is higher than the exchange rate of water molecules across the cellular membrane. Given the current uncertainty in the intracellular location and chemical environment of GdDO3NI when trapped in hypoxic cells, it is not unreasonable to assume that the agent is confined to a space within cells that has either limited water access or that water exchange between that environment and extracellular water is slow. Based upon the tissue concentration of GdDO3NI estimated by ICP-MS, one can estimate that the effective r_1 relaxivity of the trapped agent is only $0.015\ \text{mM}^{-1}\text{s}^{-1}$. In any case, the injected dose used in this study ($0.1\ \text{mmol/kg body wt}$) corresponds to a typical dose of Gd-DTPA used in the clinic and was sufficient to clearly highlight regions of hypoxia (Fig 2).

An important parameter for imaging is the acquisition resolution. Irrespective of the imaging technique or modality, every value that is obtained is an ensemble average of the microscopic variations present in the measured voxel. The current clinical hypoxia imaging resolution reported using PET imaging of ^{18}F -MISO is $\sim 5 \text{ mm} \times 5 \text{ mm}$ whereas the new pre-clinical PET scanners can achieve $1 \text{ mm} \times 1 \text{ mm}$ resolution [45]. Our data, using contrast agent GdDO3NI, were acquired at anatomical ^1H MR resolution of $390 \mu\text{m} \times 390 \mu\text{m}$, which is achievable on clinical MR scanners. We were limited by temporal resolution (30 s at early time points) in our selection of acquisition resolution; even higher resolution would have been possible for imaging a single time point.

In this study, GdDO3NI was shown to reveal hypoxia at anatomical MRI resolution with standard dosing. Since MRI is non-invasive, this agent also has the potential for imaging modulation of tumor hypoxia by hyperoxic or hypoxic interventions. It could also help us better understand treatment-induced changes in tumor oxygenation, particularly hypoxia targeting therapy. This capability could have profound implications on cancer treatment as it is practiced today and lead to more personalized therapeutic decisions. Further evaluation of the targeting efficiency of GdDO3NI at different levels of hypoxia as well as subcellular localization, *in vitro*, will help in better understanding the mechanism and quantification of data, potentially leading to quantitative oximetry. Given the preliminary results reported here, a further one-to-one comparison study using PET in the preclinical setting would be beneficial in providing further support of the clinical translation potential of MRI hypoxia reporters like GdDO3NI. The development of an appropriate theoretical model for contrast kinetics of an MRI-based hypoxia targeting agent, that takes into account the arterial input function, would be critical to for quantitative analysis of the data. To the best of our knowledge, such a model has not been published to date and will be a focus of our efforts in the near future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported in part by grants from the Norman Hackerman ARP grant (010019-0056-2007), the National Institutes of Health (R21CA132096 and RO1CA115531), the Southwestern Small Animal Imaging Research Program (U24 CA126608) and the Robert A. Welch Foundation (AT-584). MRI experiments were performed at the Advanced Imaging Research Center with support from a NIH National Institute of Biomedical Imaging and Bioengineering resource grant (P41EB015908). We would like to thank Dr. Li Liu and Abhijit Bugde for helpful advice.

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ABBREVIATIONS

BOLD	Blood oxygen level-dependent (contrast)
CAIX	Carbonic anhydrase IX
⁶⁴Cu-ATSM	⁶⁴ Cu-diacetyl-bis(<i>N</i> ⁴ -methylthiosemicarbazone)
DOTA	
EPR	Electron paramagnetic resonance
¹⁸F-MISO	Fluoromisonidazole
¹⁸F-FAZA	¹⁸ F-fluoroazomycin-arabinofuranoside
¹⁸F-FETNIM	¹⁸ F-fluoroerythronitroimidazole
¹⁸F-FETA	¹⁸ F-fluoroetanidazole
FREDOM	Fluorocarbon relaxometry using echo planar imaging for dynamic oxygen mapping
Gd	Gadolinium
GdDO3ABA	Gd chelated DOTA monoamide conjugate of 2-nitroimidazole

GdDO3NI	Gd chelated DOTA-(n-butyl)monoamide
HIF-1	hypoxia inducible factor-1
HMDSO	Hexamethyldisiloxane
MR	Magnetic resonance
MRI	Magnetic resonance imaging
PET	Positron emission tomography
PISTOL	Proton Imaging of Siloxanes to map Tissue Oxygenation Levels
pO₂	partial pressure of oxygen in tissue
SPECT	Single photon emission computed tomography
T1-wt	Spin-lattice relaxation time weighted
T2-wt	Spin-spin relaxation time weighted
TF-MISO	Tri-fluoromisonidazole

Figure 2.

Grayscale magnetic resonance T_2 weighted (a,d) and color T_1 -wt (b,c,e,f) images of Copenhagen rat thighs bearing syngeneic Dunning prostate R3327-AT1 tumors following injection of 0.1 mmol/kg body wt GdDO3NI (a-c) and GdDO3ABA (d-f) pre injection (b,e) and 145 min post injection (c,f). The tumor and thigh muscle are labeled “T” and “M”, respectively.

(a)

(b)

Figure 3.

Comparing time course of normalized mean intensities ($n = 5$) for well perfused (periphery) and poorly perfused regions (center) of Dunning prostate R3327-AT1 tumors in animals injected with 0.1 mmol/kg body wt of (a) GdDO3NI or (b) GdDO3ABA. Normalization was performed with respect to mean intensity in thigh muscle tissue for each rat before averaging. Regions of graph shaded in gray represent time interval showing a statistically significant difference ($p < 0.05$, 2-tailed unpaired t-test) between the two agents at each measurement time-point in the interval.

(a)

(b)

(c)

Figure 4.

Kinetics of mean Gd concentration ($n=5$) for (a) poorly perfused (central) regions of tumors, (b) well perfused (peripheral) region of tumors and (c) thigh muscle tissue in animals injected with 0.1 mmol/kg body wt GdDO3NI or GdDO3ABA. Regions of graph shaded in gray represent time intervals showing a statistically significant difference ($p < 0.05$, 2-tailed unpaired t-test) between the two agents at each measurement time-point in the interval.

Figure 5.

Shows the a) cellularity by H&E staining and (b) hypoxia distribution by pimonidazole based immunohistochemical staining in a Dunning prostate R3327-AT1 tumor section. The pimonidazole staining clearly showed extensive hypoxia in regions of the tumor. The black and white scale bars represent 0.5 cm.

**

Figure 6.

Inductively coupled plasma mass spectroscopy (ICP-MS) analysis of tumor sections *ex vivo*. The central or core regions of Dunning prostate R3327-AT1 tumors of animals injected with 0.1 mmol/kg body wt GdDO3NI agent showed significantly enhanced retention of Gd³⁺ clearly validating GdDO3NI as hypoxia targeting agent. ** represents $p < 0.05$ following 1-way ANOVA.

Table 1

Contrast agent kinetic parameters by magnetic resonance imaging in Copenhagen rat thighs bearing syngeneic Dunning prostate R3327-AT1 tumor following injection of 0.1 mmol/kg body wt GdDO3NI or GdDO3ABA. Time to peak refers to the time post contrast agent injection at which the listed peak concentrations were observed. Final concentrations were measured at 145 min post injection.

	GdDO3NI			GdDO3ABA		
	time to peak (min)	peak conc. (uM)	final conc. (uM)	time to peak (min)	peak conc. (uM)	final conc. (uM)
Thigh muscle	1.5	48 ± 7	1 ± 10	0.5	38 ± 11	-2 ± 7
Tumor periphery	2.5	119 ± 25	12 ± 6	1.5	119 ± 9	16 ± 6
Tumor center	22.5	39 ± 18	17 ± 5	14.5	29 ± 16	5 ± 6