Published in final edited form as:

RSC Adv. 2014 ; 4(18): 9003–9011. doi:10.1039/c3ra47161f.

A self-internalizing mitochondrial TSPO targeting imaging probe for fluorescence, MRI and EM†

Lynn E. Samuelson^a, Bernard M Anderson^a, Mingfeng Bai^a, Madeline J Dukes^a, Colette R. Hunt^a, Jonathon D. Casey^a, Zeqiu Han^b, Vassilios Papadopoulos^c, Darryl J. Bornhop^a

^aDepartment of Chemistry, The Vanderbilt Institute for Chemical Biology and Vanderbilt-Ingram Cancer Center, Vanderbilt University, VU Station B 351822 Nashville, Tennessee 37235-1822, USA

^bDepartment of Biochemistry & Molecular and Cellular Biology, Georgetown University Medical Center, BSB Room 315, 3900 Reservoir Road NW, Washington, DC 20057, USA

^cThe Research Institute of the McGill University Health Centre, Departments of Medicine, Biochemistry, and Pharmacology and Therapeutics, McGill University, Montreal, QC, Canada

Abstract

Advances in probes for cellular imaging have driven discoveries in biology and medicine. Primarily, antibodies and small molecules have been made for contrast enhancement of specific proteins. The development of new dendrimer-based tools offers opportunities to tune cellular internalization and targeting, image multiple modalities in the same molecule and explore therapeutics. The translocator protein (TSPO) offers an ideal target to develop dendrimer tools because it is well characterized and implicated in a number of disease states. The TSPO-targeted dendrimers reported here, primarily CIPhIQ-PAMAM-Gd-Liss, are cell membrane permeable nanoparticles that enable labeling of TSPO and provide contrast in fluorescence, electron microscopy and magnetic resonance imaging. The molecular binding affinity for TSPO was found to be 0.51 μM , 3 times greater than the monomeric agents previously demonstrated in our laboratory. The relaxivity per Gd^{3+} of the CIPhIQ₂₃-PAMAM-Gd₁₈ dendrimer was 7.7 and 8.0 $\text{mM}^{-1} \text{s}^{-1}$ for r_1 and r_2 respectively, approximately double that of the clinically used monomeric Gd^{3+} chelates. *In vitro* studies confirmed molecular selectivity for labeling TSPO in the mitochondria of C6 rat glioma and MDA-MB-231 cell lines. Fluorescence co-registration with Mitotracker Green® and increased contrast of osmium-staining in electron microscopy confirmed mitochondrial labeling of these TSPO-targeted agents. Taken collectively these experiments demonstrate the versatility of conjugation of our PAMAM dendrimeric chemistry to allow multi-modality agents to be prepared. These agents target organelles and use complementary imaging modalities *in vitro*, potentially allowing disease mechanism studies with high sensitivity and high resolution techniques.

darryl.bornhop@vanderbilt.edu.

†Electronic supplementary information (ESI) available. See DOI: 10.1039/c3ra47161f

Introduction

Chemical tools capable of multiple, diverse functions offer new methodologies for exploring basic biology, imaging and drug delivery with and without targeting moieties. Organic nanoparticles are used to make multifunctional agents that are then applied to complex biological problems *in vitro* and *in vivo*.^{1,2} These multi-functional agents provide the power to assess multiple perspectives and gain valuable information not possible or practical by using several single agents. For example, one multifunctional agent can be synthesized for detection with multiple imaging modalities and provide more robust information compared to a mono-functional agent. Multifunctional agents for electron microscopy (EM) and fluorescence microscopy make organelles visually distinguishable by EM while providing live *in vitro* or *in vivo* assessment *via* fluorescence microscopy. Fluorescence can be utilized *in vitro* and in small animal *in vivo* studies while another modality, such as MRI, would be required for human imaging. However, fluorescence can also be used *in vivo* for image-guided surgery.³ Nanomaterials are commonly used as multipurpose scaffolds to provide a combination of receptor targeting, imaging, drug delivery and tuning pharmacokinetics.⁴ Continued development of new tools is needed to further advance the fields of imaging, drug delivery and to intercalate biological systems both *in vitro* and *in vivo*.

Nanomaterials that target well characterized biological receptors expedite the validation of the probe as well as provide new tools to further understand the system. The translocator protein (TSPO) has been widely characterized, has several small molecule ligands and has been the target for molecular imaging agents of numerous diseases including cancer, multiple sclerosis and neurodegenerative processes using small molecule radiotracers or dye-conjugated ligands.⁵⁻⁹ TSPO has been shown to be associated with a number of biological processes that are signatures for tissues' well-being, including cell proliferation, apoptosis, steroidogenesis, and immunomodulation.⁵⁻⁷ While it has been used as a target for nuclear imaging in humans,^{10,11} TSPO's importance to steroid production and cholesterol transport continue to make it an important biomarker for targeted imaging that has not met its full potential.¹²⁻¹⁴ Recognizing the importance of TSPO, our laboratory has reported the synthesis and biological evaluation of several small molecule optical TSPO imaging agents as well as one dendrimeric agent.¹⁵⁻²⁴ We have demonstrated *in vivo* detection using fluorescent TSPO-targeted molecular imaging in preclinical models of breast cancer²⁵ and breast cancer metastases.²⁴ With the dendrimeric agent, we have demonstrated improved binding affinity as well as the ability to image with fluorescence and EM.

Adapting small, targeted imaging agents to multimodal systems requires advances in chemistry and characterization of molecules. Poly(amido amine) (PAMAM) dendrimers and other organic nanomaterials have been used as backbones in a variety of biomedical applications, with or without targeting, including cellular labeling and tracking as well as drug delivery.²⁶ PAMAMs and other dendrimers are ideal agents for multifunctional targeted imaging due to their controlled structure, multiple attachment sites and ease of synthesis. To accomplish this, often multiple moieties are attached to a single dendrimer, making characterization of each moiety attachment complicated. While EM and dynamic light scattering offer important information on size and shape of nanomaterials, they do not provide information regarding the distribution of attachment efficacy of multiple small

molecules attached to a single dendrimer. To quantitate ligands, MALDI-TOF MS and NMR are important tools.

Recognizing the need for improved multi-modal imaging, we capitalize on our previous observations with the fluorescent dendrimer,²² and incorporate Gd^{3+} (ref. 27) into the imaging agent to make CIPhIQ₂₃-PAMAM-Gd₁₈-Liss, a TSPO-targeted agent with a magnetic resonance signature. The scope of the TSPO-targeted dendrimer agents was further expanded to include EM imaging of mitochondria *via* live cell labelling with the probe and post-fixation staining with osmium. The approach presented here is to use PAMAM dendrimers to build a molecule that enters a cell under normal physiological conditions and selectively labels the protein of interest without the need for genetic manipulation, which can be time consuming, disrupt native cell function and is not practical for future translation into the clinic.

Materials and methods

General methods

G(4)-PAMAMTM dendrimer in a 10% w/w solution of methanol was purchased from Fischer Scientific and pipetted into a preweighed vial immediately before use. The methanol was evaporated under a stream of $Ar_{(g)}$ and the resulting viscous oil was dissolved in water, frozen and lyophilized to give a fluffy white powder. 1-(2-Chlorophenyl)isoquinoline-3-carboxylic acid CIPhIQ acid was synthesized in our laboratory as previously reported.^{28,29} All 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and DOTA derivatives were purchased from Macrocyclics and used as arrived. All other chemicals were purchased from Fischer Scientific and used as is unless otherwise indicated. Cells lines were purchased from ATTC and grown according to the provided guidelines. MALDI-TOF MS were obtained on a PerSeptive Biosystems Voyager-DE STR mass spectrometer. Freshly recrystallized *trans*-indole acrylic acid (IAA) was used as the matrix in a 10 mg mL⁻¹ solution of DMSO. The plate was spotted with 1 μ L of a 10 : 1 solution of matrix to analyte. NMR spectra were obtained on a 400 MHz Bruker AV-400 instrument with a 5 mm *Z*-gradient broadband inverse probe. NMR spectra were obtained in d₆-dimethyl sulphoxide. UV-vis spectra were obtained on a Shimadzu 1700 UV-vis spectrophotometer. Fluorescence spectra were obtained using a ISS PCI spectrofluorometer at room temperature. MR relaxivities were obtained using a Maran 0.5T NMR scanner.

CIPhIQ₂₃-G(4)-PAMAMTM-DO3A^tBu₁₈ (1)

CIPhIQ₂₃-G(4)-PAMAMTM (202 mg, 9 μ moles) and DOTA^tBu (179 mg, 97 μ moles) were dissolved in 2 and 1 mL of DMSO respectively. The 1,4,7,10-tetraazacyclododecane-1,4,7-tris-(*t*-butyl acetate)-10-acetic acid mono(*N*-hydroxysuccinimide ester) (DOTA^tBu-NHS) solution was added dropwise to a stirring solution of CIPhIQ₂₃-G(4)-PAMAMTM over a 10 minute period. The reaction was stirred overnight and purified with the Amicon Centrifugation filters (5 kDa MWCO) following the procedure used for CIPhIQ₂₃-G(4)-PAMAMTM purification and lyophilized to give 163.2 mg (100% yield) of CIPhIQ₂₃-G(4)-PAMAMTM-DOTA^tBu₁₈. The molecular weight was determined using MALDI-TOF MS to

be 31 000 a.m.u. ^1H NMR (300 MHz, CDCl_3) δ 8.8 (1H, s), 8.6 (1H, s), 8.2–8.5 (20H, m), 7.5–8.0 (9H, multi), 2.9 (3H, bs) and 3.1–3.5 (208H, m), 1.4 (108, s) ppm.

CIPhIQ₂₃-G(4)-PAMAMTM-DO3A₁₈

CIPhIQ₂₃-G(4)-PAMAMTM-DOTA⁴Bu₁₈ (256.0 mg, 9 μ moles) was dissolved in 3 mL of neat trifluoroacetic acid (TFA) and stirred overnight. The solution was diluted 10 fold with water and purified with Amicon Centrifugation filters (5 kDa MWCO) following the procedure used for CIPhIQ₂₃-G(4)-PAMAMTM purification and lyophilized to give 120 mg (100% yield). The molecular weight was determined using MALDI-TOF MS to be 27 000. ^1H NMR (300 MHz, CDCl_3) δ 8.8 (1H, s), 8.6 (1H, s), 8.2–8.5 (5H, m), 7.5–8.0 (20H, multi), 2.9 (3H, bs) and 3.1–3.5 (73H, m) ppm.

CIPhIQ₂₃-G(4)-PAMAMTM-DO3A₁₈-Lissamine

CIPhIQ₂₃-G(4)-PAMAMTM-DO3A₁₈ (4.8 mg, 148 nmoles) was dissolved in 1 mL of dimethylformamide (DMF). Next, 12.8 μ L of a 17.3 mM solution of Lissamine rhodamine B sulfonyl chlorideTM (221 nmoles) was added to the stirred dendrimer solution. The reaction was stirred overnight followed by purification in Amicon Centrifugation filters (5 kDa MWCO) as described above and lyophilized to give 2.8 mg (57% yield) of a pink fluffy solid. The presence of the fluorophor was confirmed with UV-vis (max absorbance at 551 nm) and fluorescence (ex. 551 nm, em. 586 nm) spectroscopies.

CIPhIQ₂₃-G(4)-PAMAMTM-DO3AGd₁₈³⁺ (2)

CIPhIQ₂₃-G(4)-PAMAMTM-DO3A₁₈ (22 mg, 679 nmoles) was dissolved in water. Next, 1 mL of a 9.1 mg mL⁻¹ solution of GdCl₃ (9.1 mg, 25 μ moles) was added to the dendrimer solution. The reaction was stirred at room temperature for 24 hours and purified with Amicon Centrifugation filters (5 kDa MWCO) following the procedure used for CIPhIQ₂₃-G(4)-PAMAMTM purification then lyophilized to give 24.7 mg (100% yield). The molecular weight was determined using MALDI-TOF MS to be 30 500 Da.

CIPhIQ₂₃-G(4)-PAMAMTM-DO3A-Gd₁₈³⁺-Lissamine (3)

CIPhIQ₂₃-G(4)-PAMAMTM-DOTAGd₁₈³⁺ (5.3 mg, 145 nmoles) was dissolved in 1 mL of dimethylformamide (DMF). Next, 12.7 μ L of a 17.3 mM solution of Lissamine rhodamine B sulfonyl chlorideTM (220 nmoles) was added to the stirred dendrimer solution. The reaction was stirred overnight followed by purification in Amicon Centrifugation filters (5 kDa MWCO) as described above and lyophilized to give 3.7 mg (69% yield) of a pink fluffy solid. The presence of the fluorophor was confirmed with UV-vis (max absorbance at 551 nm) and fluorescence (ex 551 nm, em. 586 nm) spectroscopies.

Cell internalization experiments

G(4)-PAMAMTM-Lissamine derivatives: either 20 000 C6 rat glioma or 40 000 MDA-MB-231 breast cancer cells were plated into collagen coated glassbottom microscopy (MatTeckTM) dishes and allowed to grow for two days in the ATCC recommended cell media. After two days, the cells had good morphology and were attached, however were not confluent. G(4)-PAMAMTM-Lissamine compounds (with or without other moieties) were

diluted to 1 μM with media from a 10 mg mL^{-1} stock solution in DMSO. The media over the cells was replaced with the media containing the fluorophor and incubated for 6–12 hours. The media was then poured off and the cells were carefully rinsed 3–4 times with Dulbecco's Phosphate Buffered Saline (DPBS). The cells were imaged live. Both white light and fluorescence pictures were obtained. For fixed cells, the rinsed cells were incubated at room temperature for 30 minutes with a 4% paraformaldehyde solution. The cells were carefully washed 4 times with DPBS and stored under DPBS at 4 $^{\circ}\text{C}$ until imaged (not longer than 1 week, typically overnight). Immediately before imaging, the fixed cells were incubated with a 25 nM solution of mitotracker green (MTG) for 10 minutes. Excess MTG was removed by 4 washings with DPBS and the cells were imaged. Fluorescence bleeding was tested with cell plates that were dosed with either MTG or ClPhIQ₂₃-PAMAM-Liss-Gd₁₈ (**3**) and imaged using both filter sets. No signal bleed-through was seen for either molecule when imaged with the improper filter set.

Radioligand binding assay

C6 rat glioma cells (cultured in Dulbecco's modified Eagle medium (DMEM)-F12 medium (Gibco/Invitrogen) supplemented with 0.5% FBS and 2.5% horse serum (HS) at 3.7% CO_2) were scraped from 150 mm culture dishes into 5 mL of phosphate buffered saline (PBS), dispersed by trituration, and centrifuged at $500 \times g$ for 15 min. Cell pellets were resuspended in PBS and assayed to determine protein concentration. The binding studies with [³H]PK11195 on 30 μg of protein from cell suspensions were performed as previously described.³⁰ The data was analyzed using PRISM software (vs. 4.0, GraphPad, Inc., San Diego, CA). In PRISM, the one binding site competition assay wizard was used, which incorporates the equation: $Y = \text{bottom} + (\text{top} - \text{bottom}) / (1 + 10^{(X - \log \text{EC}_{50})})$. For the ClPhIQ₂₃-PAMAM-Gd₁₈ dendrimer, the IC_{50} was found to be 510 nM on a per ClPhIQ ligand basis with a goodness to fit error of 0.9394.

Preparation of TEM samples

C6 cells were plated in a 4 cm culture dish, allowed to propagate to near 75% confluency and treated with a 32 μM ClPhIQ₂₃-PAMAM-Gd₁₈ solution in Dulbecco's Modified Eagle Medium. After incubation at 37 $^{\circ}\text{C}$ for 10 hours, the C6 cells were then washed three times with a 0.1 M solution of sodium cacodylate buffer to remove any extracellular ClPhIQ₂₃-PAMAM-Gd₁₈. The cells were subsequently fixed for one hour using a 4% paraformaldehyde solution. The fixed cells were post-fixed using a 1% osmium tetroxide solution, dehydrated using ethanol, pelletized and sliced into 80 nm thick sections. The sections were placed on a 300 mesh copper grid and imaged on a Phillips CM-12 Electron Microscope.

Relaxivity of Gd Dendrimers

Samples were prepared at 5 concentrations (in Gd) from 1 μM to 1 mM, depending on the availability of sample. Pre-written pulse sequences were used to obtain the relaxation at each concentration for both T_1 and T_2 which varied the time intervals between the pulse and detecting the population that had relaxed. The data was graphed and fitted to a three parameter exponential rise to max curve to give the relaxation at each concentration. Each

sample was run 3 times with 15 minute intervals between trials. The relaxivity was determined by graphing the relaxation *versus* concentration and fit to a line. The slope of the line is the relaxivity.

Results and discussion

Synthesis

Adaptation of TSPO-targeted agents to multi-modal agents based on PAMAM dendrimers expands the current utility of these agents to include multiple imaging modalities on one molecule. The imaging agent, CIPhIQ₂₃-PAMAM-Gd₁₈-Liss dendrimer, was synthesized as shown in Scheme 1 starting with CIPhIQ₂₃-PAMAM and reacting tri-*t*-butyl-DO3A-NHS in dimethyl sulphoxide (DMSO) to give CIPhIQ₂₃-PAMAM-^tBu-DO3A₁₈ (**1**). Next, in two steps, the *t*-butyl groups were removed by treatment with tri-fluoroacetic acid (TFA) and the resulting carboxylates on the DO3A were chelated with gadolinium to give compound **2**. Typically, reaction of 30 equivalents of tri-*t*-butyl-DO3A resulted in an average attachment of 23 metal chelates per CIPhIQ₂₃-PAMAM (Fig. S1, see ESI,†). Characterization with NMR (pre-chelation) and MALDI-TOF MS (pre and post chelation) verified the desired reactions were completed (see ESI Fig. S1,†). In the last step, Lissamine rhodamine B sulfonyl chlorideTM (Liss), a red fluorescent dye, was reacted with CIPhIQ₂₃-PAMAM-Gd₁₈ (**2**) in dimethylformamide (DMF) to produce a TSPO targeted molecule with optical, MR and EM visualization capabilities.

Purification of small molecule imaging agents is challenging, time consuming and requires column chromatography or prep-HPLC under conditions compatible with dyes and/or inorganic chelates. An advantage of conjugating small molecules to dendrimers is the ease of purification. For purification of these dendrimers, low molecular weight by-products were removed by diafiltration with a 5 kiloDalton (kDa) molecular weight cut off (MWCO). Nuclear magnetic resonance (NMR) and matrix assisted laser desorption ionization mass spectrometry (MALDI-TOF MS) were used to measure the distributions of CIPhIQ, tri-*t*-butyl-DO3A and Liss molecules along with Gd³⁺ ions and calculate the average number of ligands attached to the dendrimer. Both the presence and absence of chemical shifts in the NMR as well as the relative integrations of key peaks were used to estimate the number of ligands attached. The aromatic proton integrations of CIPhIQ ligands attached to the PAMAM were compared to the amide peaks associated with the PAMAM before modification as previously reported.²² Integration of the *t*-butyl methyl resonance at 1.4 ppm in the proton NMR spectra of CIPhIQ₂₃-PAMAM-^tBuDO3A₁₈ was compared to the aromatic protons from the CIPhIQ ligands attached to the dendrimer and the amide protons to determine the ratio of TSPO ligands to the metal chelate and thus the number of DO3A molecules attached.

MALDI-TOF MS was essential in characterizing the average number of ligands attached to the PAMAM. This was accomplished using a mean molecular weight value of PAMAM and the subsequent products from each reaction. As shown in Table 1, the average molecular weight fluctuated as expected with the addition of ligands or removal of protecting groups. The fluorophore attachment was observed through characterization with MALDI-TOF MS (31 000 Da), UV-vis (max absorbance at 551 nm) and fluorescence (ex. 551 nm, em. 586

nm) spectroscopy. Reaction with 1.5 equivalents of Liss resulted in an average of 1 Liss per dendrimer. No significant changes in the spectra of Liss were observed between the conjugated and unconjugated Liss dyes. Following synthesis, these TSPO targeted PAMAMs were characterized for their potential in biomedical research applications.

TSPO specificity

To ensure that the dendrimer conjugation did not disrupt binding to TSPO, a competition study between CIPhIQ₂₃-PAMAM-Gd₁₈ (**2**) and tritiated [³H]PK11195 (TSPO ligand with nM binding affinity)^{12–14} was completed. Displacement studies using increasing concentrations of **2** were performed in the presence of 15 nM [³H]PK11195 to obtain the IC₅₀ curve (Fig. 1). Radioligand binding assays were also performed on an acetate capped aminoethyl CIPhIQ (Ac-aminoethyl CIPhIQ) compound (structure in Fig. 1). The [³H]PK11195 ligand was used due to the similarities in structure between CIPhIQ and PK11195, making PK11195 the best previously characterized ligand to compare the CIPhIQ binding with. The CIPhIQ₂₃-PAMAM-Gd₁₈ was found to have a *K*_i of 510 nM per CIPhIQ ligand while the Acaminoethyl CIPhIQ has a binding affinity of 1.5 μM (Fig. 1). The radioligand binding assay indicates that the PAMAM-conjugated CIPhIQ ligand does not significantly disrupt binding to TSPO, but rather enhances it 3 fold on a per CIPhIQ ligand basis.

MR relaxivity

Incorporation of the chelated Gd³⁺ ions was intended for MRI contrast enhancement with a targeted agent. The TSPO targeted dendrimer showed increased relaxation of water molecules compared to Magnevist™ on a per Gd³⁺ ion basis. The *T*₁ and *T*₂ relaxation rates of five different concentrations in Gd³⁺, diluted in saline were measured for CIPhIQ₂₃-PAMAM-Gd₁₈, CIPhIQ₂₃-PAMAM-Gd₁₈-Liss, PAMAM-Gd₁₈ and Magnevist were all measured (Tables S1 and S2, ESI,†). The *T*₁ relaxivity of PAMAM-Gd₁₈ and CIPhIQ₂₃-PAMAM-Gd₁₈ is greater than that of Magnevist™ by about 1.6 on a per molecule basis or 29 on a per Gd³⁺ ion basis (Table S1, ESI,†). A similar trend was observed for the *T*₂ measurements of PAMAM-Gd₁₈ and CIPhIQ₂₃-PAMAM-Gd₁₈ (Table S1, ESI,†). *T*₂ relaxivity was not increased as significantly as *T*₁ but is still 1.4 and 33 times better than Magnevist™ on per molecule and per Gd³⁺ ion basis respectively (Table S1, ESI,†).

The relaxivities of dye containing, CIPhIQ₂₃-PAMAM-Gd₁₈-Liss were measured to be 4.0 and 3.8 mM⁻¹ s⁻¹, slightly lower than Magnevist™'s 4.8 and 5.6 mM⁻¹ s⁻¹ for *T*₁ and *T*₂ respectively (Table S1, ESI,†). A similar effect was observed when Cy5.5 was attached to the Gd₁₈-PAMAM rather than Liss (Table S2, ESI,†). The Cy5.5-PAMAM-Gd relaxivities were slightly higher at 5.56 and 5.95 mM⁻¹ s⁻¹ for *T*₁ and *T*₂ respectively. Interestingly, the order in which the Liss dye was added effected the relaxivity measurement. Synthesis in which Liss was reacted with PAMAM first resulted in values higher than Magnevist™, albeit not as high as Gd-PAMAMs without dye attached. Treating PAMAM-Gd₁₈ with the dye conjugation reaction conditions resulted in no differences in the relaxivity measurements. The cause of the decreased values is unclear but likely relates to a change in the solution properties that occurs when dye is added to PAMAM-Gd compounds.

With the exception of CIPhIQ₂₃-PAMAM-Gd₁₈-Liss, the relaxivity values are consistent with recently published PAMAM-Gd molecules when the amount of Gd³⁺ and generation of the dendrimer are taken into account.³¹ For MRI contrast enhancement, either relaxivity needs to be increased significantly or millimolar concentrations need to be delivered. Although the increase in relaxivity with PAMAMs is encouraging, optimization of the chemistry to increase relaxivity is required for viable MR contrast agents.²⁶

Fluorescent cell labeling

The importance of cellular fluorescence imaging in biology has been well established,^{32,33} chemical tools to target intercellular proteins without perturbation of the cell offer the ability to incorporate other functionalities such as a second imaging modality and/or drug could further advance *in vitro* capabilities of these systems. With that in mind, we used MDA-MB-231 human breast cancer and C6 rat glioma cells to label mitochondria in live cells with CIPhIQ₂₃-PAMAM-Gd₁₈-Liss (Fig. 2). No perturbation of the cellular membrane was performed, indicating that the imaging agent is labeling the mitochondria *via* normal biological processes. Examining the overlay of the fluorescence and DIC images indicates that CIPhIQ₂₃-PAMAM-Gd₁₈-Liss is perinuclear (Fig. 2, Panels A & B); as expected since TSPO is primarily located on the mitochondria. The control compound, PAMAM-Liss, did not label the cells even at higher concentrations of molecule, nor was it visible at increased fluorescence integration times (Fig. 2, Panels C & D). As with the CIPhIQ₂₃-PAMAM-Liss, the indication is that the CIPhIQ moiety is necessary for the labeling and/or internalization of the functionalized dendrimer into the cell.

Further cellular fluorescence studies were completed in C6 rat glioma cells, using the cellular labeling procedure described for the MDA-MB-231 cells, yet after incubation, the cells were fixed with 4% paraformaldehyde and treated with 25 nM Mitotracker GreenTM (MTG) immediately before imaging. MTG is a commercially available small molecule for labeling mitochondria and has fluorescence properties (ex. 490 nm & em. 516 nm) complementary for co-imaging with CIPhIQ₂₃-PAMAM-Gd₁₈-Liss (**3**).

Individual fluorescent images of MTG and compound **3** are shown in Fig. 3A and B respectively illustrating that there is substantial similarity of the labelling site. Panel 3C depicts the fluorescence overlay for both the imaging agents, where the red of CIPhIQ₂₃-PAMAM-Gd₁₈-Liss (**3**) combines with the green signal from MTG to give a mostly yellow image. Note that very little green or red is seen, with the yellow color dominating the image. The co-incubation evidence suggests that the CIPhIQ₂₃-PAMAM-Gd₁₈-Liss (**3**) and MTG are located in the same intracellular location as seen with the CIPhIQ₂₃-PAMAM-Liss (**6**), suggesting that the mitochondria is labelled by our new agent. The cellular images presented here, suggest that CIPhIQ₂₃-PAMAM-Gd₁₈-Liss (**3**) has been internalized into the cell and effectively transported to the mitochondrial membrane.

Electron microscopy imaging

Electron microscopy (EM) is extremely powerful in its ability to image biological structures from 20 down to 1 nm; however, it has been difficult to use it for ultrahigh resolution expression mapping and specific protein studies.^{34–36} Molecules that could function as both

fluorescent and EM contrast agents provide tools for correlating fluorescence observations with the high resolution of EM and minimal changes to the experiment. To further examine the utility of the different CIPhIQ-PAMAM molecules as multi-modal imaging agents we performed a few preliminary EM studies. Capitalizing on the TSPO specificity exhibited by the gadolinium dendrimers as shown *via* fluorescence imaging, we hypothesized that the electron dense Gd^{3+} molecules would create EM contrast enhancement of TSPO in C6 cells.

To look at EM enhancement by these dendrimers, C6 rat glioma cells were dosed with CIPhIQ₂₃-PAMAM-Gd₁₈ (**2**) using the same procedure completed with the fluorescent experiments and prepared for EM in a lead and uranium free environment; however, osmium tetroxide was included in the prep as a secondary fixative.³⁷ A set of cells not dosed with CIPhIQ₂₃-PAMAM-Gd₁₈ served as the control. The differences between the labeled and control cells at 2650× magnification are apparent over numerous mitochondria in the CIPhIQ₂₃-PAMAM-Gd₁₈ treated cells (Fig. 4A) as exhibited by enhanced contrast on the mitochondrial membrane, where TSPO is located. There is visually decreased contrast on the mitochondria of unlabeled samples (Fig. 4D). Magnification of the mitochondrial regions by 7650× and 25 000× for both samples further illustrates these differences (Fig. 4B, C, E & F). The mitochondria for cells treated with CIPhIQ₂₃-PAMAM-Gd₁₈ (**2**) are at least 2-times darker than control.

The enhanced contrast of CIPhIQ₂₃-PAMAM-Gd₁₈ (**2**) treated cells was initially attributed to the accumulation of the high-Z, electron dense Gd^{3+} ions chelated to the dendrimer. After energy evaluation using Energy Dispersive X-ray Analysis (EDX) was completed to examine the elemental composition of these electron dense regions, the enhanced contrast on the mitochondria was found to be caused by osmium instead of the complexed gadolinium (Fig. S2, ESI,†). Since, osmium tetroxide is known to coordinate amines,^{38,39} it is possible that the increased contrast was created anywhere there is a build-up of TSPO-targeted dendrimer, which contains a high concentration of amines, on the mitochondrial membrane of treated cells. Cells not treated with CIPhIQ₂₃-PAMAM-Gd₁₈ were processed the same, yet no contrast enhancement was observed. Additional experiments to confirm osmium tetroxide binds to PAMAM were completed by reacting osmium tetroxide and PAMAM. With every PAMAM tested, the addition of osmium tetroxide yielded the formation of a dark precipitate. Increased EM contrast of osmium tetroxide treated PAMAMs was observed compared to non-osmium reacted PAMAM. Both observations suggest that reaction with osmium tetroxide enhances EM contrast post-fixation. While further investigations are required, this increased EM contrast offers the potential for using PAMAMs to study protein structure *in vitro* simultaneously with both EM and fluorescence. Since any number of biological agents can be targeted with PAMAMs, it should be possible to use this class of chemistries to interrogate a wide array of proteins. Furthermore, with the ability to conjugate numerous fluorophores (or other small molecule imaging agents), live cell multi-modal (color) imaging should be possible prior to fixation and EM imaging, allowing several levels of resolution to aid in mechanism of action determinations.

Conclusion

A new TSPO-targeted multi-modal chemical tool for cellular imaging with fluorescence and EM was synthesized and validated. Binding studies confirmed the affinity (K_d) for CIPhIQ₂₃-PAMAM-Gd₁₈ and Ac-aminohexyl CIPhIQ to be 510 and 1500 nM respectively and for TSPO and that when used to label live and fixed cells, the specificity was maintained. Studies with fluorescent cellular imaging show the potential of this agent to target high expressing TSPO cells and that the TSPO targeted dendrimers enter cells under physiological conditions and label mitochondria. New chemical tools with the ability to deliver a dendrimer to intracellular structures in live, diseased cells has potential to impact preclinical studies on cellular processes, detection, progression and treatment of diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by grants from the National Science Foundation (Bes-0323281), Department of Defense (W81XWH-04-1-0432) and the National Institute of Health (5P20 GM072048-4).

References

1. Culver J, Akers W and Achilefu S, Multimodality molecular imaging with combined optical and SPECT/PET modalities, *J. Nucl. Med.*, 2008, 49, 169–172. [PubMed: 18199608]
2. Saovapakhiran A, D'Emanuele A, Attwood D and Penny J, Surface Modification of PAMAM Dendrimers Modulates the Mechanism of Cellular Internalization, *Bioconjugate Chem.*, 2009, 20, 693–701.
3. Nguyen QT and Tsien RY, Fluorescence-guided surgery with live molecular navigation – a new cutting edge, *Nat. Rev. Cancer*, 2013, 13, 653–662. [PubMed: 23924645]
4. Parveen S, Misra R and Sahoo SK, Nanoparticles: a boon to drug delivery, therapeutics, diagnostics and imaging, *Nanomedicine*, 2012, 8, 147–166. [PubMed: 21703993]
5. Hardwick M, Fertikh D, Culty M, Li H, Vidic B, et al., Peripheral-type benzodiazepine receptor (PBR) in human breast cancer: correlation of breast cancer cell aggressive phenotype with PBR expression, nuclear localization, and PBR-mediated cell proliferation and nuclear transport of cholesterol, *Cancer Res.*, 1999, 59, 831–842. [PubMed: 10029072]
6. Lacapere JJ and Papadopoulos V, Peripheral-type benzodiazepine receptor: structure and function of a cholesterol-binding protein in steroid and bile acid biosynthesis, *Steroids*, 2003, 68, 569–585. [PubMed: 12957662]
7. Papadopoulos V, Lecanu L, Brown RC, Han Z and Yao ZX, Peripheral-type benzodiazepine receptor in neurosteroid biosynthesis, neuropathology and neurological disorders, *Neuroscience*, 2006, 138, 749–756. [PubMed: 16338086]
8. Takano A, Piehl F, Hillert J, Varrone A, Nag S, et al., *In vivo* TSPO imaging in patients with multiple sclerosis: a brain PET study with [18F]FEDAA1106, *EJNMMI Res.*, 2013, 3, 30. [PubMed: 23618062]
9. Ching AS, Kuhnast B, Damont A, Roeda D, Tavittian B, et al., Current paradigm of the 18 kDa translocator protein (TSPO) as a molecular target for PET imaging in neuroinflammation and neurodegenerative diseases, *Insights Imaging*, 2012, 3, 111–119. [PubMed: 22696004]
10. Garnier M, Boujrad N, Ogwuegbu SO, Hudson JR Jr, Papadopoulos V, The polypeptide diazepam-binding inhibitor and a higher affinity mitochondrial peripheral-type benzodiazepine receptor sustain constitutive steroidogenesis in the R2C Leydig tumor cell line, *J. Biol. Chem.*, 1994, 269, 22105–22112. [PubMed: 8071335]

11. Roivainen A, Nagren K, Hirvonen J, Oikonen V, Virsu P, et al., Whole-body distribution and metabolism of [*N*-methyl-¹¹C](*R*)-1-(2-chlorophenyl)-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide in humans; an imaging agent for *in vivo* assessment of peripheral benzodiazepine receptor activity with positron emission tomography, *Eur. J. Nucl. Med. Mol. Imaging*, 2009, 36, 671–682. [PubMed: 19050880]
12. Casellas P, Galiegue S and Basile AS, Peripheral benzodiazepine receptors and mitochondrial function, *Neurochem. Int.*, 2002, 40, 475–486. [PubMed: 11850104]
13. Decaudin D, Castedo M, Nemati F, Beurdeley-Thomas A, De Pinieux G, et al., Peripheral benzodiazepine receptor ligands reverse apoptosis resistance of cancer cells *in vitro* and *in vivo*, *Cancer Res.*, 2002, 62, 1388–1393. [PubMed: 11888910]
14. Papadopoulos V, Baraldi M, Guilarte TR, Knudsen TB, Lacapere JJ, et al., Translocator protein (18 kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function, *Trends Pharmacol. Sci.*, 2006, 27, 402–409. [PubMed: 16822554]
15. Bai M, Rone MB, Papadopoulos V and Bornhop DJ, A novel functional translocator protein ligand for cancer imaging, *Bioconjugate Chem.*, 2007, 18, 2018–2023
16. Bai M, Sexton M, Stella N and Bornhop DJ, MBC94, a conjugable ligand for cannabinoid CB 2 receptor imaging, *Bioconjugate Chem.*, 2008, 19, 988–992.
17. Bai M, Wyatt SK, Han Z, Papadopoulos V and Bornhop DJ, A novel conjugable translocator protein ligand labeled with a fluorescence dye for *in vitro* imaging, *Bioconjugate Chem.*, 2007, 18, 1118–1122.
18. Manning HC, Goebel T, Marx JN and Bornhop DJ, Facile, efficient conjugation of a trifunctional lanthanide chelate to a peripheral benzodiazepine receptor ligand, *Org. Lett.*, 2002, 4, 1075–1078. [PubMed: 11922786]
19. Manning HC, Goebel T, Thompson RC, Price RR, Lee H, et al., Targeted molecular imaging agents for cellular-scale bimodal imaging, *Bioconjugate Chem.*, 2004, 15, 1488–1495.
20. Manning HC, Merchant NB, Foutch AC, Virostko JM, Wyatt SK, et al., Molecular imaging of therapeutic response to epidermal growth factor receptor blockade in colorectal cancer, *Clin. Cancer Res.*, 2008, 14, 7413–7422. [PubMed: 19010858]
21. Manning HC, Smith SM, Sexton M, Haviland S, Bai M, et al., A peripheral benzodiazepine receptor targeted agent for *in vitro* imaging and screening, *Bioconjugate Chem.*, 2006, 17, 735–740.
22. Samuelson LE, Dukes MJ, Hunt CR, Casey JD and Bornhop DJ, TSPO targeted dendrimer imaging agent: synthesis, characterization, and cellular internalization, *Bioconjugate Chem.*, 2009, 20, 2082–2089.
23. Wyatt SK, Manning HC, Bai M, Bailey SN, Gallant P, et al., Molecular imaging of the translocator protein (TSPO) in a pre-clinical model of breast cancer, *Mol. Imag. Biol.*, 2010, 12, 349–358.
24. Wyatt SK, Manning HC, Bai M, Ehtesham M, Mapara KY, et al., Preclinical molecular imaging of the translocator protein (TSPO) in a metastases model based on breast cancer xenografts propagated in the murine brain, *Curr. Mol. Med.*, 2012, 12, 458–466. [PubMed: 22348613]
25. Wyatt SK, Manning HC, Bai MF, Bailey SN, Gallant P, et al., Molecular Imaging of the Translocator Protein (TSPO) in a Pre-Clinical Model of Breast Cancer, *Mol. Imag. Biol.*, 2010, 12, 349–358.
26. Svenson S and Tomalia DA, Dendrimers in biomedical applications-reflections on the field, *Adv. Drug Delivery Rev.*, 2005, 57, 2106–2129.
27. Nwe K, Milenic D, Bryant LH, Regino CA and Brechbiel MW, Preparation, characterization and *in vivo* assessment of Gd-albumin and Gd-dendrimer conjugates as intravascular contrast-enhancing agents for MRI, *J. Inorg. Biochem.*, 2011, 105, 722–727. [PubMed: 21463567]
28. Newman AH, Lueddens HWM, Skolnick P and Rice KC, Novel Irreversible Ligands Specific for Peripheral Type Benzodiazepine Receptors – (±), (+), and (–)-1-(2-Chlorophenyl)-*N*-(1-Methylpropyl)-*N*-(2-Isothiocyanatoethyl)-3-Isoquinolinecarboxamide and 1-(2-Isothiocyanatoethyl)-7-Chloro-1,3-Dihydro-5-(4-Chlorophenyl)-*H*-2-1,4-Benzodiazepin-2-One, *J. Med. Chem.*, 1987, 30, 1901–1905. [PubMed: 2821259]

29. Manning HC, Goebel T, Marx JN and Bornhop DJ, Facile, efficient conjugation of a trifunctional lanthanide chelate to a peripheral benzodiazepine receptor ligand, *Org. Lett*, 2002, 4, 1075–1078. [PubMed: 11922786]
30. Garnier M, Dimchev AB, Boujrad N, Price JM, Musto NA, et al., *In Vitro* Reconstitution of a Functional Peripheral-Type Benzodiazepine Receptor from Mouse Leydig Tumor-Cells, *Mol. Pharmacol*, 1994, 45, 201–211. [PubMed: 8114671]
31. Bryant LH, Brechbiel MW, Wu CC, Bulte JWM, Herynek V, et al., Synthesis and relaxometry of high-generation (G = 5, 7, 9, and 10) PAMAM dendrimer-DOTA-gadolinium chelates, *J. Magn. Reson. Imaging*, 1999, 9, 348–352. [PubMed: 10077036]
32. Culver J, Akers W and Achilefu S, Multimodality molecular imaging with combined optical and SPECT/PET modalities, *J. Nucl. Med*, 2008, 49, 169–172. [PubMed: 18199608]
33. Saovapakhiran A, D'Emanuele A, Attwood D and Penny J, Surface modification of PAMAM dendrimers modulates the mechanism of cellular internalization, *Bioconjugate Chem.*, 2009, 20, 693–701.
34. de Jonge N, Peckys DB, Kremers GJ and Piston DW, Electron microscopy of whole cells in liquid with nanometer resolution, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, 106, 2159–2164. [PubMed: 19164524]
35. Giepmans BNG, Bridging fluorescence microscopy and electron microscopy, *Histochem. Cell Biol*, 2008, 130, 211–217. [PubMed: 18575880]
36. Koster AJ and Klumperman J, Electron microscopy in cell biology: integrating structure and function, *Nat. Cell Biol*, 2003, S6–S10.
37. Bozzola J and Russel LD, Specimen Staining and Contrast Methods for Transmission Electron Microscopy, in *Microscopy: Principles and Techniques for Biologists*, Jones and Bartlett Publishers, Boston, 2nd edn, 1999, pp. 121–147.
38. Chatt J, Leigh GJ and Paske RJ, Reaction of complexes of Ruthenium and Osmium Halides with Ammonia, Amines and Hydrazine, *J. Chem. Soc*, 1969, 854–859
39. Li G, Graham A, Chen Y, Dobhal MP, Morgan J, et al., Synthesis, comparative photosensitizing efficacy, human serum albumin (site II) binding ability, and intracellular localization characteristics of novel benzobacteriochlorins derived from vic-dihydroxybacteriochlorins, *J. Med. Chem*, 2003, 46, 5349–5359. [PubMed: 14640543]

Fig. 1. Binding of CIPhIQ to TSPO both on and off the PAMAM. The IC₅₀ curve of the competitive binding assay CIPhIQ₂₃-PAMAM-Gd₁₈ (left) and Ac-aminohexyl CIPhIQ (right) with radiolabeled [³H]PK11195 illustrate the three fold better binding affinity (by molecule) or 88 fold lower binding affinity per CIPhIQ molecule.

Fig. 2.

Live cell imaging of TSPO in human breast cancer cells. Cellular images of MDA-MB-231 cells dosed with CIPhIQ₂₃-PAMAM-Gd₁₈-Liss (3) and PAMAM-Liss indicated that the CIPhIQ ligand is required for internalization and labelling the cytoplasmic space. Panel A: red fluorescence image of CIPhIQ₂₃-PAMAM-Gd₁₈-Liss (3); Panel B: DIC and red fluorescence overlay of CIPhIQ₂₃-PAMAM-Gd₁₈-Liss (3); Panel C: red fluorescence image of PAMAM-Liss; Panel D: DIC image of PAMAM-Liss.

Fig. 3.

CIPhIQ₂₃-PAMAM-Gd₁₈-Liss labels the mitochondria. C6 Rat Glioma cells were co-incubation with CIPhIQ₂₃-PAMAM-Gd₁₈-Liss and the commercial mitochondrial fluorescent label, Mitotraker Green (MTG). The representative image indicates that CIPhIQ₂₃-PAMAM-Gd₁₈-Liss and MTG occupy the same areas of the cell. Panel A: fluorescence of MTG. Panel B: fluorescence of CIPhIQ₂₃-PAMAM-Gd₁₈-Liss (3). Panel C: fluorescence overlay of MTG and CIPhIQ₂₃-PAMAM-Gd₁₈-Liss (3). Yellow indicates co-registration. Panel D: the fluorescence overlay of MTG and CIPhIQ₂₃-PAMAM-Gd₁₈-Liss (3) onto the DIC image.

Fig. 4.

CIPhIQ₂₃-PAMAM-Gd₁₈ label mitochondria and can be imaged with high contrast in the EM. TEM images of CIPhIQ₂₃-PAMAM-Gd₁₈ dosed (A–C) and undosed cells (D–F). (A): Representative image of CIPhIQ₂₃-PAMAM-Gd₁₈ dosed cells at 2650× magnification. (B): Representative image of CIPhIQ₂₃-PAMAM-Gd₁₈ dosed cells at 7100× magnification. (C): Representative image of CIPhIQ₂₃-PAMAM-Gd₁₈ dosed cells at 25 000× magnification. (D): Representative image of undosed cells at 2650× magnification. (E): Representative image of undosed cells at 7100× magnification. (F): Representative image of undosed cells at 25 000× magnification.

Scheme 1.
Synthesis of TSPO targeted MR/optical/EM.

Table 1MALDI-TOF MS determined average MW and the corresponding number of chelators or chelated Gd³⁺

Compound	Ave MW	Ave # DO3A or Gd (per PAMAM)
G(4)-PAMAM	13 738	n/a
ClPhIQ ₂₀ -PAMAM	19 500	n/a
ClPhIQ ₂₀ -PAMAM-DO3A/Bu ₂₃	31 000	23.0
ClPhIQ ₂₀ -PAMAM-DO3A ₂₃	27 000	24.7
ClPhIQ ₂₀ -PAMAM-Gd ₂₃	30 500	22.3 (Gd ³⁺)