A High-Affinity, High-Stability Photoacoustic Agent for Imaging Gastrin-Releasing Peptide Receptor in Prostate Cancer

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Abstract

Purpose: To evaluate the utility of targeted photoacoustic imaging (PAI) in providing molecular information to complement intrinsic functional and anatomical details of the vasculature within prostate lesion.

Experimental Design: We developed a PAI agent, AA3G-740, that targets gastrin-releasing peptide receptor (GRPR), found to be highly overexpressed in prostate cancer. The binding specificity of the agent was evaluated in human prostate cancer cell lines, PC3 and LNCaP, and antagonist properties determined by cell internalization and intracellular calcium mobilization studies. The imaging sensitivity was assessed for the agent itself and for the PC3 cells labeled with agent. The in vivo stability of the agent was determined in human plasma and in the blood of living mice. The in vivo binding of the agent was evaluated in PC3 prostate tumor models in mice, and was validated ex vivo by optical imaging.

Results: AA3G-740 demonstrated strong and specific binding to GRPR. The sensitivity of detection in vitro indicated suitability of the agent to image very small lesions. In mice, the agent was able to bind to GRPR even in poorly vascularized tumors leading to nearly 2-fold difference in photoacoustic signal relative to the control agent.

Conclusions: The ability to image both vasculature and molecular profile outside the blood vessels gives molecular PAI a unique advantage over currently used imaging techniques. The imaging method presented here can find application both in diagnosis and in image-guided biopsy. Clin Cancer Res; 20(14); 3721–9. ©2014 AACR.

Introduction

Clinical diagnosis of prostate cancer is made upon histologic examination of the tissue obtained by biopsy, indicated for patients with abnormal PSA levels or rectal examination (1). The biopsy is not lesion directed as the imaging technique currently used to guide the procedure, transrectal ultrasound (TRUS), lacks the specificity to identify the malignancies and is used only to provide anatomical references within prostate. Although imaging plays an important role in all aspects of prostate cancer patient management, from diagnosis to treatment, currently no modality serves as a diagnostic tool. As articulated by the experts in the field, to allow more accurate characterization of the disease, an imaging modality should ideally be able to provide anatomical, functional, and molecular information (2, 3). Photoacoustic imaging (PAI), a technique closely related to ultrasound, may be able to impart just such fusion of information by imaging vasculature as well as molecular signatures of prostate cancer (4). As a potential clinical tool in management of prostate cancer, PAI has been investigated in small animals as a modality that would enable better localization and detection based on the increased vascularization (5–7) and hypoxia (8) in tumors. A few targeted nanoparticle-based molecular imaging agents have been reported but they have not been tested in vivo (9, 10). In this study, we develop a PAI agent that binds to gastrin-releasing peptide receptor (GRPR) with high affinity and specificity and as such supplies molecular information that complements intrinsic functional and anatomical details provided by endogenous absorbers, hemoglobin, and deoxyhemoglobin. The ability to image both vasculature and molecular profile outside the blood vessels gives PAI a unique advantage over currently one of the most promising imaging techniques, TRUS that utilizes microbubble contrast agents (11). Although targeted microbubbles do offer molecular information, because of their large size, they are capable of imaging targets found only on or in the blood
vessels. Another technique that is showing great promise for improved prostate cancer diagnosis is the molecular imaging technique currently used to guide biopsy, indicated for patients with abnormal PSA levels or rectal examination. The biopsy is not lesion directed as the imaging technique currently used to guide biopsy, lacks the specificity to identify the malignancies and is used only to provide anatomical references within prostate. Current clinical diagnosis of prostate cancer can be greatly improved by utilization of an imaging modality that allows better characterization of the disease through evaluation of functional and molecular status in a detected lesion. Molecular photoacoustic imaging (PAI), with good depth penetration in combination with high contrast and resolution, has a great potential to be clinically utilized for noninvasive, nonionizing visualization of prostate lesions. The ability to image both vasculature and molecular profile outside the blood vessels gives PAI a unique advantage over currently used imaging techniques. The imaging method presented here can find clinical application both in diagnosis and in image-guided biopsy.

Translational Relevance
Prostate cancer diagnosis is made upon histologic examination of the tissue obtained by biopsy, indicated for patients with abnormal PSA levels or rectal examination. The biopsy is not lesion directed as the imaging technique currently used to guide the procedure, transrectal ultrasound (TRUS), lacks the specificity to identify the malignancies and is used only to provide anatomical references within prostate. Current clinical diagnosis of prostate cancer can be greatly improved by utilization of an imaging modality that allows better characterization of the disease through evaluation of functional and molecular status in a detected lesion. Molecular photoacoustic imaging (PAI), with good depth penetration in combination with high contrast and resolution, has a great potential to be clinically utilized for noninvasive, noninvasive visualization of prostate lesions. The ability to image both vasculature and molecular profile outside the blood vessels gives PAI a unique advantage over currently used imaging techniques. The imaging method presented here can find clinical application both in diagnosis and in image-guided biopsy.

Materials and Methods

General
All Fmoc amino acids and Rink Amide resin were purchased from EMD Millipore. Peptide syntheses were carried out following the standard solid phase Fmoc synthesis. Analysis and purification of the peptides were performed using the Dionex Summit high-performance liquid chromatography (HPLC) system (Dionex Corporation) and reverse phase HPLC column Higins Analytical (Higgins Analytical; C18, 4.6 mm × 250 mm). The mobile phase was 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in 90% acetonitrile (CH$_3$CN) in water (solvent B). Matrix-assisted laser desorption/ionization mass spectrometry was performed by the Canary Center proteomics facility on AB Sciex 5800 TOF/TOF System. The absorbance measurements were performed using Cary50 (Varian), fluorescence measurements using FluoroMax4 (Horiba).

Dye selection
The dyes, IRDyeQC1 (Li COR), Hilyte750 (Anaspec), Alexa750 (Life technologies), ATTO740 (ATTO-Tec), RD800 and RD831 (BioVentures Inc.), indocyanine green (ICG; Spectrum chemicals), and methylene blue (emp Biotech GmbH) were dissolved in a minimal amount of dimethylformamide (DMF) and diluted with PBS to a final concentration of 10 μmol/L. Capillary tubes were filled with dye solutions, sealed and embedded in agar phantom. PA signal was determined at maximum absorption wavelength for each dye using the PA instrument described previously (29). For the photobleaching study, the dyes were dissolved in a minimal amount of DMF and diluted with PBS to a final concentration of 10 μg/mL concentration, placed in an eppendorf tube and irradiated with laser light using maximum absorption wavelength for 30 minutes. Photobleaching was determined by the change in absorbance after irradiation.

Imaging agent synthesis
Peptides, GGGdFQWAVGHStaL-NH$_2$ and GGGdFQWAVGHStaAQLV-NH$_2$, were dissolved in PBS to 10 μmol/L. Capillary tubes were filled with dye solutions, sealed and embedded in agar phantom. PA signal was determined by incubating PC3 cells with 3 pmol/L of AA3G-740 or CAA3G-740 in PBS for 15 minutes at 4°C. The reaction mixture was injected directly onto the HPLC column, and the separation of the product mixture followed using absorbance at 740 nm. The imaging agents had a retention time of 22.9 minutes and m/z of 1732.4717.

Cell binding studies
Human prostate cancer cell lines, PC3 and LNCaP, were obtained from American Type Culture Collection and were grown according to the supplier’s instructions. Cells (3 × 10$^5$) were incubated with 3 pmol/L AA3G-740 or CAA3G-740 in PBS for 15 minutes at 4°C. Specificity of the binding was determined by incubating PC3 cells with 3 pmol/L of AA3G-740 and varying amount of bombesin (1.5 × 10$^{-11}$, 1.5 × 10$^{-10}$, 1.5 × 10$^{-9}$, 1.5 × 10$^{-8}$ mol/L). All samples were done in triplicates.
Cell internalization studies
PC3 cells (0.5 million) were incubated with 1.5 nmol/L AA3G-740 in PBSA (PBS containing 1% BSA) for 30 and 60 minutes at 37°C. The same cellular uptake study was performed after 30-minute preincubation with 0.5% sodium azide. The cells were washed three times with cold PBSA and the cell fluorescence analyzed using the Becton Dickinson FACS Aria III instrument. All samples were done in triplicates.

Intracellular calcium mobilization
Intracellular calcium was detected using Fluo-4 NW Calcium Assay Kit (Life Technologies) as per manufacturer’s instructions. PC3 cells (35 × 10⁶) were seeded in a 96-well plate one day ahead of the experiment. Each well was loaded with 100 µL Fluo-4 dye in assay buffer and incubated for 30 minutes at 37°C, followed by 30 minutes incubation at room temperature. The cell fluorescence (494 excitation/516-nm emission) was determined before and 30 minutes after the addition of AA3G-740 (1, 2.5, 10, and 25 nmol/L) of AA3G-740 agent dissolved in PBS for 30 minutes at 37°C. After washing three times with cold PBS, the cells were suspended in 50 µL PBS and placed directly in the dimple of the PA instrument for determination of photoacoustic signal. All samples were done in triplicates.

Sensitivity study
Solutions containing different amounts (50, 100, 250, and 500 pmol/L) of AA3G-740 agent dissolved in PBS were placed directly in the imaging dimple of the Nexus 128 photoacoustic instrument (Endra Lifesciences) and PA signal detected using 750-nm wavelength. Different number of PC3 cells (0.1, 0.2, 0.5, 1, and 3.5 million) was incubated with 2.5 nmol/L AA3G-740 in PBS for 30 minutes at 37°C. After washing three times with cold PBS, the cells were suspended in 50 µL PBS and placed directly in the dimple of the PA instrument for determination of photoacoustic signal. All samples were done in triplicates.

Imaging agents’ stability and blood metabolism
Of note, 3 nmol/L solutions of AA3G-740 andCAA3G-740 in PBS were incubated in 500 µL pooled male human plasma (Innovative research) for 30 and 60 minutes. After addition of 1 mL acetonitrile, plasma proteins were removed by centrifugation and supernatant injected directly onto the HPLC column. The HPLC conditions were the same as those described above for the purification of the imaging agents described above. Similarly, metabolism of AA3G-740 in blood was tested 30 and 60 minutes after injection of the agent using the same HPLC method. All samples were done in triplicates.

Photoacoustic imaging of GRPR in PC3 tumors in mice
All animal studies were performed in accordance with the Guidelines for the Care and Use of Research Animals established by the Stanford University (Stanford, CA). Eight-week-old nude male mice (Charles River) were injected with 3 million PC3 cells into the rear leg flank. Mice bearing 0.5 to 0.7 cm PC3 tumors (n = 6 for AA3G-740, n = 4 for CAA3G-740) were photoacoustically imaged using a commercial photoacoustic computed tomography system ( refs. 30, 31; Nexus 128, Endra Inc.). The system uses a tunable nanosecond pulsed laser (7 ns pulses, 20 Hz pulse repetition frequency, about 7 mJ/pulse on the animal surface, wavelength range 680 nm to 950 nm) and 128 unfocused ultrasound transducers (with 5 MHz center frequency and 3 mm diameter) arranged in a hemi-spherical bowl filled with water. We used 750-nm light with 100 views and 100 pulses/view. For each animal, we first obtained pre-injection data at 750 nm. The imaging agent (10 nmol/L) was dissolved in 200 µL PBS (pH 7.4) and administered to mice via tail-vein injection. The data were acquired 30 and 60 minutes after the injection of the imaging agent. The agent was dissolved in PBS. Volume rendered three-dimensional (3D) photoacoustic images are reconstructed off-line using data acquired from all 128 transducers from all views using a filtered back-projection algorithm. The algorithm corrects for pulse to pulse variations in the laser intensity and small changes in the temperature that affect acoustic velocity in the water. The reconstructed 3D raw data are then analyzed using AMIDE software (32).

Ex vivo studies
Mice were sacrificed 30 and 60 minutes postinjection of 10 nmol/L AA3G-740 (n = 3 for each time point). Optical imaging of the excised organs was carried out using an IVIS200 system (Caliper Life Sciences) and ICG filter set. The exposure time for images was 10 seconds. Quantitative analysis was performed using the Living Image 4.0 software.

Results
To select a signaling molecule to be conjugated to the GRPR-binding peptide that will allow high sensitivity detection, we compared the intensity of the photoacoustic signal of the dyes absorbing in the near infrared region, focusing on the longer wavelengths cca. 750 nm, where the background signal is minimal and the intensity of the laser light is at its maximum. As a comparison, we also evaluated two FDA-approved dyes, methylene blue and ICG that are frequently used in photoacoustic studies. Four dyes,IRDyeQD-1, ATTO740, RD-831, and ICG, showed high photoacoustic signal (Fig.1A) but because ATTO740 demonstrated superior photostability (Fig. 1B and Fig. 1C), it was selected as the most suitable signaling moiety for our imaging agent. The dye was conjugated to the targeting peptide through a triple glycine linker that was shown to allow retention of the peptide’s binding affinity (33, 34). The targeting peptide was a D-Phe6-bombesin[6–14] with modifications at positions 13 and 14 that impart antagonist properties, high binding and stability toward peptidases (Fig. 1D). The control agent, CAA3G-740, contained the same linker and dye but a scrambled peptide, HDfGWSAaqv-NH2. Besides photoacoustic, ATTO 740 shows a fluorescent signal (Fig. 1D) as well that enables bimodal imaging and efficient binding characterization in cell culture by FACS. Spectral characterization of the
conjugate indicated 750 nm to be the wavelength with maximum photoacoustic signal (Fig. 1E).

The binding of the agents was tested in two prostate cancer cell lines with relatively low and high level of GRPR expression, LNCaP and PC3 (19, 35). The GRPR specificity of AA3G-740 was demonstrated by the intense labeling of PC3 cells that was efficiently blocked in the presence of bombesin in a dose-dependent manner (Fig. 2A and Supplementary Information). Conversely, LNCaP cells that have lower levels of GRPR showed minimal binding of AA3G-740 (Fig. 2B). Control agent, CAA3G-740, did not show appreciable binding to PC3 cells (Fig. 2C).

As agonists and antagonists demonstrate different behavior after binding to the receptor, we investigated functional properties of AA3G-740 by evaluating the extent of its internalization and the effect that the binding to GRPR has on intracellular calcium mobilization (Fig. 3). Although agonists show fast and massive internalization of the ligand-receptor complex and cause calcium mobilization, antagonists are largely cell surface bound and do not affect calcium signaling. Both studies indicated that AA3G-740 has antagonistic properties. The amount of the agent that was bound to the cell surface exceeded the internalized portion at both time points tested. Similarly to demobesin 1, a well-investigated GRPR antagonist, the surface bound AA3G-740, reached a plateau at 30 minutes, while the internalized portion continued to rise over time (21). Calcium mobilization assay further supported the antagonistic function of AA3G-740 as there was no increase in intracellular calcium even at high doses of the agent. The slight decrease in fluorescence after the addition of the agent at high doses could be explained by the intracellular, intramolecular quenching between the dye used for calcium sensing and ATTO 740.

The sensitivity of detection was determined for the agent itself and for the cells that have been labeled with the agent (Fig. 4). We could detect as low as 100 pmol/L AA3G-740 with linear dependence of the photoacoustic signal on the concentration of the agent. The sensitivity of 100 pmol/L represents only 1% of the commonly used 10 nmol/L.
imaging dose, suggesting aptness for in vivo imaging. The smallest number of AA3G-740–labeled PC3 cells that demonstrated photoacoustic signal significantly higher than the background was found to be 0.5 million. Considering that there are around 10^8 cells in 1 cm^3 tumor (36), the determined cell sensitivity indicates that the agent has the potential to detect very small lesions.

The stability of the agent was determined in human male plasma as well as in blood of living mice (Fig. 5). The agent was highly stable in human serum, as only parent compound was observed by HPLC, without detectable decomposition occurring during the 1 hour of incubation. In living mice as well, the major peak observed in blood at 30 minutes postinjection was of the parent AA3G-740 agent. The indication of a possible adduct formation was apparent in the existence of small peaks with longer retention times (Fig. 5B). At one hour postinjection, the intact agent was still the major component but its low level indicated a short blood lifetime (Supplementary Information).

The efficacy of the agent to bind to and detect GRPR in vivo was evaluated in prostate cancer xenografts implanted in nude mice (Fig. 6). Mice bearing PC3 tumors were injected with 10 nmol/L AA3G-740 or CAA3G-740 and imaged at 30 and 60 minutes postinjection. High photoacoustic signal was detected at both time points in mice injected with AA3G-740, whereas CAA3G-740 administration resulted in a signal barely exceeding the intensity of the preinjection background. Different accumulation of the agents was confirmed ex vivo by optical imaging (Fig. 6B).

Accumulation of the agent in various organs was determined ex vivo by optical imaging (Fig. 7). The highest signal was observed in gallbladder and liver, followed by intestine kidneys and spleen.

**Discussion**

Prostate cancer diagnosis and detection can be greatly improved by utilization of an imaging modality that allows better characterization of the disease through describing both functional and molecular status in the detected lesion. In this study, we develop a molecular agent for PAI that can complement intrinsically present signal of blood vessels.
The agent, AA3G-740, was designed to target GRPRs found to be overexpressed in prostate cancer. The antagonist GRPR peptide, dFQWAVGHStaL-NH₂, was labeled via a triple glycine linker to the ATTO-740 dye, that showed high photoacoustic signal and good photostability. The specificity of AA3G-740 for GRPR was demonstrated in GRPR highly expressing PC3 cells by the ability of bombesin to prevent binding of the agent to the cells. Low-expressing LNCaP cells showed minimal binding of the agent.

The conjugation of the dye was not found to change the functional properties of the antagonist peptide as the binding of the conjugate did not lead to fast internalization, expected from an agonist. In addition, binding of the agent did not seem to have any effect on the calcium signaling in cells. It was first shown for somatostatin and later for GRPR receptors that antagonists, although internalized at a much lower extent than agonists, demonstrate preferable in vivo tumor targeting properties, possibly due to strong, high efficiency labeling. Besides for these important in vivo characteristics, it was essential to confirm the antagonist function of our imaging agent because of the dose of the agent needed for photoacoustic and optical imaging. Although agonists have mitogenic properties, antagonists have been proposed as anticancer agents that have shown no toxicity in clinical trials at high doses (37) and are as such suitable as agents requiring more than tracer level for imaging.

Figure 4. Sensitivity of detection. A, the photoacoustic signal was linearly dependent on the amount of the agent. The lowest amount that could reliably be detected above the background was found to be 100 pmol/L. B, the lowest detectable number of PC3 cells labeled with AA3G-740 was determined to be 0.5 million.

Figure 5. Agent stability. The stability of the agent was determined in human male plasma (A) and in mouse blood (B). There was no evidence of decomposition within one hour of incubation in human plasma (gray line, 30-minute incubation; dotted line, 60-minute incubation). In mouse blood, at 30 minutes postinjection, the major peak corresponded to the intact agent with minute amount of possible peptide adducts also visible (arrow). The three spectra in B correspond to data from three mice. There was no indication of proteolysis occurring in the blood.
The sensitivity of AA3G-740 *in vitro* was found to be sufficient to detect as low as 1% of the commonly used 10 nmol/L imaging dose and to allow imaging of as little as 0.5 million cells. Such excellent sensitivity is particularly important in view of the size of the signaling moiety used in the agent. Most of the agents developed for PAI are...
nanoparticles that have masses several orders of magnitude higher than ATTO-740. Found high sensitivity suggests that on a mass level, ATTO-740 provides a more intense photoacoustic signal than most nanoparticles, and as such is more likely to have a better safety profile for *in vivo* applications.

A recent study described rapid metabolism of $^{68}$Ga labeled bombesin antagonist in blood of healthy volunteers that resulted in more than 50% agent decomposition at only 20 minutes postinjection (28). Although AA3G-740 contains the same antagonist peptide, we did not observe any decomposition during 1-hour incubation in human male plasma. The deficiency of the $^{68}$Ga and ATTO740-labeled GRPR antagonist can be explained by differences in size, charge, or hydrophobicity that leads to differential susceptibility to proteolysis. In blood of mice, at 30 minutes postinjection, the major peak was of the parent AA3G-740 with no indication of proteolysis. The other peaks that were observed in HPLC chromatogram had longer retention times indicating possible adducts with molecules from the blood. Short blood lifetime, expected from an agent of this size, was evident from a low level of AA3G-740 present in blood at 60 minutes postinjection.

Considering the blood lifetime, we evaluated *in vivo* targeting of AA3G-740 and CAA3G-740 at 30 and 60 minutes postinjection in tumor-bearing mice (Fig. 6). As a blocking study would require potentially a lethal dose of bombesin, the specificity of the binding was established through the use of the control agent designed not to bind to GRPR. At both time points, there was a clear distinction between the two agents. Although binding of AA3G-740 led to an intense photoacoustic signal, close to 2-fold higher than the background, the injection of the control agent increased minimally over time. In addition, the signal in tumors of mice injected with the control agent decreased over time, suggesting that the signal observed at 30 minutes postinjection was most likely due to the agent present in the vasculature (Fig. 6C).

The images in Fig. 6 clearly illustrate the importance of having molecular information in addition to the blood vessel network status. The preinjection photoacoustic signals that correspond to tumor vasculature were noticeably different in two PC3 tumors presented in the Figure, suggestive of their low and high vasularization state. Nonetheless, the active agent was able to reveal the GRPR status of the tumor despite its poor vascularization, while the inability of CAA3G-740 to bind to the receptor led to minimal photoacoustic signal in a richly vascular lesion. Besides indicating the specificity of AA3G-740, these results suggest that molecular information unrelated to vascularization can be a differentiating point for benign and malignant prostate lesions with similar vascular status.

Optical *ex vivo* imaging of the major organs at 1 hour postinjection indicated hepatobiliary excretion as gallbladder, liver, and intestine displayed the highest photoacoustic signal. Similar clearance route was observed for some of the other GRPR antagonist agents (18, 26, 38). There are several issues that need to be addressed in the future studies. The AA3G-740 was evaluated in mice at only two time points due to its short blood lifetime. Although we found 30 minutes postinjection to be a sufficient time to allow specific binding of the agent, for a potential clinical translation it would be beneficial to extend the blood lifetime and redetermine the optimal imaging time. As the lifetime greatly depends on the size of the agent, we will explore pegylation as a means to increase the size and improve pharmacokinetics of the agent. In addition, as linkers seem to play an important role for tumor targeting, we will investigate amino acids with different size and charge to optimize the targeting efficiency of the agent (18). Finally, it will be important to determine the targeting ability of the agent in other prostate tumor models such as orthotopic, androgen dependent, and independent.

This is the first report of molecular PAI of prostate cancer in small animals. As exemplified by the presented data, PAI using GRPR-specific agent afforded visualization of the molecular profile of the tumors with relatively high resolution and specificity. The imaging method presented here can find application in diagnosis as well as in image-guided biopsy. One of the most pressing issues in prostate cancer is overdiagnosis and overtreatment, caused by our current inability to differentiate between an aggressive and an indolent disease. The differentiation between the low- and high-risk tumors could be enabled by the use of agents, similar to the one presented here, that target the molecular differences between the lesions. Prostate imaging using PAI has a great potential for clinical translation and this study warrants further investigations in that direction.

**Disclosure of Potential Conflicts of Interest**

S.S. Gambhir is a consultant/advisory board member for Endra and VisualSonics. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

Conception and design: J. Levi, S.S. Gambhir
Development of methodology: J. Levi, S.S. Gambhir
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Levi
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Levi, S.S. Gambhir
Writing, review, and/or revision of the manuscript: J. Levi, S.S. Gambhir
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Levi, A. Sathirachinda, S.S. Gambhir
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