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A Human GRPr-Transfected Ace-1 Canine Prostate Cancer Model in Mice

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Abstract

BACKGROUND—A versatile drug screening system was developed to simplify early targeted drug discovery in mice and then translate readily from mice to a dog prostate cancer model that more fully replicates the features of human prostate cancer.

METHODS—We stably transfected human cDNA of the GRPr bombesin (BBN) receptor subtype to canine Ace-1 prostate cancer cells (Ace-1^{huGRPr}). Expression was examined by ¹²⁵I-Tyr⁴-BBN competition, calcium stimulation assay, and fluorescent microscopy. A dual tumor nude mouse xenograft model was developed from Ace-1^{CMV} (vector transfected Ace-1) and Ace-1^{huGRPr} cells. The model was used to explore the in vivo behavior of two new IRDye800-labeled GRPr binding optical imaging agents: 800-G-Abz4-t-BBN, from a GRPr agonist peptide, and 800-G-Abz4-STAT, from a GRPr antagonist peptide, by imaging the tumor mice and dissected organs.

RESULTS—Both agents bound Ace-1^{huGRPr} and PC-3, a known GRPr-expressing human prostate cancer cell line, with 4–13nM IC₅₀ against ¹²⁵I-Tyr⁴-BBN, but did not bind Ace-1^{CMV} cells (vector transfected). Binding was blocked by bombesin. Ca²⁺ activation assays demonstrated that Ace-1^{huGRPr} expressed biologically active GRPr. Both Ace-1 cell lines grew in the flanks of 100% of the nude mice and formed tumors of ~0.5 cm diameter in 1 week. In vivo imaging of the mice at 800nm emission showed GRPr+: GRPr– tumor signal brighter by a factor of two at 24 h post IV administration of 10 nmol of the imaging agents. Blood retention (4–8% ID at 1 h) was greater by a factor >10 and cumulative urine accumulation (28–30% at 4 h) was less by a factor 2 compared to a radioactive analog of the t-BBN containing agent, ¹⁷⁷LuAMBA, probably due to binding to blood albumin, which we confirmed in a mouse serum assay.

CONCLUSIONS—The dual tumor Ace-1^{CMV}/Ace-1^{huGRPr} model system provides a rapid test of specific to nonspecific binding of new GRPr avid agents in a model that will extend logically to the known Ace-1 orthotopic canine prostate cancer model.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Keywords

NIRF imaging; GRP; bombesin; canine prostate cancer

INTRODUCTION

Several aspects of prostate cancer diagnosis and therapy could be advanced with more cancer specific imaging and therapeutic agents. Cancer specific nuclear, MRI, and ultrasound imaging agents are therefore being explored for tumor location and therapeutic monitoring [1–4]. Active Surveillance (AS) is now a clinically proven procedure for sparing the prostate gland, but it is limited to a small subset of patients with the lowest risk disease and short life expectancy [5]. The subset of patients offered AS might be increased if focal therapies could be more accurately delivered as adjuvants to AS. But both AS and focal therapies have been limited by the lack of imaging agents that are specific for the prostate cancer [6–8]. Our goal is to create such prostate cancer specific agents.

Gastrin Releasing Peptide Receptor (GRPR) is a neuroendocrine receptor family, two members of which are specifically over-expressed on human breast, prostate, and other cancers [9–11]. Of particular interest is the expression in 100% of fresh frozen primary human prostate cancer tissues and precancerous PIN cells, and lack of expression in benign prostatic hyperplasia (BPH) and normal prostate tissue [12]. Radiolabeled peptide GRPR binders have been tested in humans as imaging and radio-therapeutic agents, and near infrared fluorescent (NIRF) derivatives have been tested in animals as potential real time intraoperative Optical Surgical Navigation agents [13–16]. While the clinical data on some GRPR targeted analogs have been positive, significant improvements are needed that require further drug discovery efforts. For example, a GRPR agonist, $^{177}\text{LuAMBA}$ (AMBA: $\text{DO3A-CH}_2\text{CO-G-4-aminobenzoyl-Q-W-A-V-G-H-L-M-NH}_2$), caused dose-limited vomiting in humans at 100 μg doses, presumably due to stimulation of excess gastrin release [17], as GRP (bombesin, BBN) is known to stimulate this effect [18]. Radiolabeled antagonists for nuclear imaging and radiotherapy are therefore desirable and are in preclinical studies [19–22].

Effective drug discovery and development research in the area of focal and local gland sparing therapies requires efficient and reproducible cell and mouse models for screening new candidate molecules in vitro and in vivo, and the models need to be scalable to large animal species that recapitulate the human disease. The latter need is driven by the fact that surgeons and interventionists will be the end users. Human cancer cells, such as PC-3 prostate cancer cells, express two GRPR subtypes, GRPr (BB2), and NMBR (BB1), complicating early research, and PC-3 cells do not grow consistently (~60% in this laboratory) in nude mice. There is also a dearth of useful large animal models of human prostate cancer. We have created a new cell line and developed a mouse model for screening of GRPr binding peptide ligands to resolve these problems. The Ace-1 canine prostate cancer cell line [23–25] is known to grow consistently within the prostate gland of immune compromised beagles [26]. In the present work, the Ace-1 cell line was permanently transfected with human GRPr and validated in vitro and in vivo using nude mice bearing

both Ace-1^{huGRPr} expressing and Ace-1^{CMV} (vector transfected, not GRPr expressing) xenograft tumors. We used the model to explore the behavior of a Near Infrared Fluorescent (NIRF) labeled GRPr agonist peptide we recently synthesized [13] and a newly created NIRF labeled GRPr antagonist peptide.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were conducted according to NIH guidelines, and according to protocols approved by the Institutional Animal Care and Use Committee of The Ohio State University.

Chemical Syntheses and Analyses

800-G-Abz4-t-BBN, 800-G-Abz4-STAT, and bombesin were synthesized, purified, and characterized by our published method [13], using preparative HPLC for final purification. Further details of the syntheses are in the Supporting Information. STAT is a GRPR antagonist created by Llinares [27–29]. Purity and identity of both agents were analyzed by HPLC and mass spectra. HPLC purity was >95% in a single peak on analytical HPLC and mass spectral identity was confirmed by single parent ion peaks consistent with the actual masses (see Supporting Table S1 for data). Both peptide conjugates were soluble enough in water for in vitro cell studies; however, for mice injections at >10 nmol in 100 μ l (>10 mM), agents were first dissolved in DMSO and then diluted tenfold with PBS. We measured fluorescence signals for quantitative assays in a Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek, Highland Park, VT). The excitation/emission settings were 764nm excitation (ex)/809nm emission (em). Fluorescence emission was roughly doubled and more stable in 30–60 μ M bovine serum albumin (BSA), hence ~30 μ M BSA was added for all fluorescent measurements in vitro. Fluorescent molecules are known to display unpredictable nonlinearity as concentrations increase in solution, generally attributable to self-association [30]. Linearity of fluorescence signal detection with agent concentration was therefore demonstrated between 1–100nM in 30 μ M BSA with LOD <1 nM (Fig. S1). Measured liquid samples were then diluted into this range for quantitation. The 800-G-Abz4-STAT had ~30% lower fluorescence intensity than 800-G-Abz4-t-BBN at 794 ex/809 em. AMBA was generously supplied by Bracco SpA (Milan, Italy) [31].

Cell Culture, Plasmids, Stable Transfection, and Receptor Activity

Cell lines—Human prostate adenocarcinoma cell line, PC-3, was obtained from American Type Culture Collection (ATCC) CAT # CRL-1435. Canine prostate cancer cell line, Ace-1 was isolated and characterized by co-author TR [26]. All cell lines were maintained at 37°C with 5% CO₂ in DMEM (PC-3) and DMEM-F12 (Ace-1) supplemented with 10% fetal bovine serum and 100U penicillin-streptomycin. Cells were passaged twice per week.

Establishment of GRPr stable expression cell line—Ace-1 cells expressing human GRPr (Ace-1^{huGRPr}) were generated by transfecting Ace-1 cells with a plasmid containing human GRPr cDNA (OriGene Technologies, Inc., Rockville, MD). Control cell clones (Ace-1^{CMV}) were generated by transfecting Ace-1 cells with empty plasmid (OriGene

Technologies). Transfection was performed using Lipofectamine 2000 Transfection Reagent (ThermoFisher Scientific, Carlsbad, CA) according to the manufacturers' instructions. Stable expression cell clones were selected by incubating cells in culture medium containing 600 µg/mL G418 by a published method [32].

Real-time PCR (qRT-PCR)—Total RNA was extracted from Ace-1 and Ace-1^{huGRPr} cells using the Absolutely RNA Miniprep Kit (Agilent Technologies, Santa Clara, CA). Total RNA (0.5µg) was reverse transcribed using SuperScript II Reverse Transcriptase and oligo (dT)^{12–18} primer (Invitrogen, Carlsbad, CA). qRT-PCR was performed for human GRPr (BB2) and the canine housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), using the QuantiTect SYBR® Green PCR Kit (Qiagen, Inc., Qiagen St. 1, Hilden, Germany) and the following primers (NCBI uses “GRPR”: to refer to BB2, a.k.a. GRPr): hGRPR-2S (Forward: TGGCTTTGGGAGACCTGCTCCT), hGRPR-2AS (Reverse: GGCCGGACAATGGCTTTGTATCTGT), K9GAPDH-3S (Forward: CCCACTCTTCCACCTTCGAC), K9GAPDH-2AS (Reverse: AGCCAAATTTCATTGTCATACCAGG). The canine GAPDH primers were reported previously [33]. The human primers were chosen from two different primer pairs designed for this gene. These primers were compared side-by-side and the primer pair that had the best amplification and qRT-PCR product melting characteristics was chosen. The primer pairs were designed using Primer-BLAST software (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>). In order to confirm primer specificity, qRT-PCR products were verified by electrophoresis on a 2% agarose gel and stained with ethidium bromide to confirm a single amplification product of the expected size. All of the products within a PCR reaction were then purified using the QIAquick PCR Purification Kit (Qiagen, cat. No. 28106) and sequenced by the Plant-Microbe Genomics Facility at The Ohio State University using a 3730 DNA Analyzer (Applied Biosystems, Grand Island, NY) and BigDye Terminator Cycle Sequencing chemistry (Applied Biosystems). Sequences were verified by a BLAST search using the NCBI website.

In Vitro Radioligand Binding Assays

PC-3 and Ace-1 cells were seeded at 30,000/well in a 96-well plate in triplicate for each reaction. Cell culture medium was replaced 24 h later with 100µl of binding buffer (RPMI 1640, 20mM HEPES, 0.1% BSA w/v, 0.5mM PMSF, and 0.1mg/mL bacitracin) at room temperature and the plate was then kept at 4 °C for 30 min to allow the medium to cool slowly. The plates were then processed for the individual assays as described below. Finally, the last step in all ligand binding assays was to incubate ligand bound cells on ice for 1 h, then wash five times with ice-cold wash buffer (25mM HEPES, 150mM NaCl, pH 7.4), and lyse the cells in 100 µL of 1N NaOH. Solutions were then transferred to scintillation tubes and radioactivity was measured using an automated gamma counter (PerkinElmer Wizard II, Model 2480). The data were analyzed using GraphPad Prism 5 and reported as mean±SE.

Competition binding assay—The buffer was replaced with 60 µL of binding buffer containing a constant concentration of ¹²⁵I-Tyr⁴-BBN (0.22 µCi/mL) (Perkin Elmer) and a range of concentrations (0.1nM—1µM) of test peptides (Tyr⁴-BBN, 800-G-Abz4-t-BBN, and 800-G-Abz4-STAT) [13]. The Tyr⁴-BBN binding data were always included as a

positive quantitative control because cell based IC₅₀ results on the same molecule can vary significantly by laboratory and are best compared as a ratio to a well-known standard.

Saturation binding assay—The buffer in the top half plate was replaced with binding buffer containing 1.5 μ M Tyr⁴-BBN, and the lower half plate with binding buffer, followed by 1 h incubation on ice. The buffers were then removed and substituted with 60 μ L of binding buffer containing a gradient concentration (0.0313–16 nM) of a mixture of Tyr⁴-BBN: ¹²⁵I-Tyr⁴-BBN at 24:1. The top half plate data represented the nonspecific binding.

Screening of stably transfected clones—The buffer over plated cells was replaced with 60 μ L of binding buffer containing a mixture of 14.89nM Tyr⁴-BBN plus 0.2367 μ Ci-mL ¹²⁵I-Tyr⁴-BBN (a concentration close to saturating of the GRPr on the cell surface), and 1.86nM Tyr⁴-BBN plus 0.0295 μ Ci-mL ¹²⁵I-Tyr⁴-BBN (a concentration close to the IC₅₀). After this assay, further studies used Ace-1^{huGRPr}-8 and Ace-1^{CMV}-1.

Calcium activation assay—Metabolic activity of the transfected huGRPr was measured using a commercial kit (Invitrogen, Grand Island, NY) following the directions included in the kit. Briefly, each cell line was seeded at 30,000/well in a 96-well plate and allowed to attach overnight. Cells were then washed with assay buffer containing 2.5mM probenecid followed by incubation in 100 μ L assay buffer containing Fluo-4 dye and probenecid for 30 min at 37°C and 5% CO₂ and then for a further 30 min at RT. Probenecid was added to reduce the baseline signal by inhibiting extrusion of Fluo-4 indicator out of cells according to manufacturer's instruction. Twenty μ L 1 $\times$ Hank's balanced salt solution with or without 100nM AMBA (final concentration) was added. Intracellular calcium mobilization was recorded at room temperature for 60 s in the Synergy4 plate reader for fluorescence 485nm ex/520nm em.

Direct Binding Assays in Cells

The fluorescence microscopy assay has been described previously [13]. Briefly, PC-3, Ace-1^{huGRPr}, and Ace-1^{CMV} cells were seeded in duplicate on eight-well chamber slides and allowed to attach overnight. Cell culture medium was then replaced with 200 μ L binding buffer containing 1–2 μ M 800-G-Abz4-t-BBN or 800-G-Abz4-STAT. Cells were incubated at 37°C for 2 h followed by washing four times with 300 μ L of binding buffer and once with 300 μ L of wash buffer (25mM HEPES 150mM NaCl at pH 7.4) containing 1 μ g/mL DAPI. For the blocking studies 1 h incubation was used. The chamber slide scaffold was then removed. Each chamber was then covered by a drop of aqua-poly mount and a coverslip, and sealed with clear nail polish. Cells were observed and imaged with an Olympus IX81 microscope using an 800nm emission filter set. Cell sizes were compared by measuring their average area using light microscopy and the imaging software with the Olympus IX81 microscope. Fifty cells were measured for averaging.

Pharmacokinetics and Metabolism Studies

Mouse serum protein binding assay—800-G-Abz4-t-BBN or 800-G-Abz4-STAT was incubated in 500 μ L of fresh Balb/c mouse serum at a concentration of 6.4 μ M at room temperature for 10 min. The concentration was chosen based on expected intravenous

imaging doses of 10 nmol in a 20 g mouse with a blood factor of 0.078 [34]. The solution (400 μ L) was then loaded into an Amicon unit (0.5 mL, 10 K) and centrifuged at 12,000 g for 15 min, leaving ~10% of the liquid remaining above of the filter. Samples (1 μ L) from filtrate, residual, and original solution were loaded in triplicate into 96-well plates containing 99 μ L PBS with 0.2% BSA. Fluorescence intensity was measured as described as above. Fluorescence units for each fraction were calculated by fraction volume $\times$ fluorescent unit/ μ L. The amount of test peptide stuck to the membrane was calculated as the fluorescence signal of (original solution)–(filtrate solution)–(residual solution). The two test peptides naturally bound to the membranes of size exclusion filtering devices when assays were run in PBS, but only to ~10% in the serum assays. Filtrate fluorescence was near zero, while ~90% of total fluorescence remained above the 10,000 MW cutoff filter in the remaining serum solution. The ~10% remaining on the membrane could be either protein bound or free peptide. Hence the data indicate that the peptides are >90% serum protein bound at the 6.4 μ M concentrations to be tested in vivo.

Blood clearance—Normal female Balb/c mice, 6- to 8-week old, were used. Blood samples (5 μ L) were collected from the saphenous vein at 2, 30 min, 1, 2, 4, 8, and 24 h post-injection (p.i.), and loaded into a 96-well plate with each well containing 95 μ L of PBS with 0.15% EDTA (pH 8.5) and 0.2% BSA. Mice urine was collected until 3 h post administration. For analysis, 1 μ L of urine was loaded into the 96-well plate in triplicate. Blood and urine samples from an uninjected control mouse were used as a negative control. Fluorescence intensity was measured using the BioTek Synergy H4 plate reader. The total blood fluorescence (% ID/blood) (ID is injected dose) for each mouse was calculated as (blood volume $\times$ fluorescent unit per μ L blood)/(fluorescent unit per μ L ID $\times$ 100), and 3 h urine excretion for each mouse was calculated as (% ID/urine) equals (urine volume $\times$ fluorescent unit/ μ L urine)/(fluorescent unit per μ L ID $\times$ 100). Blood volume was calculated based upon mouse weight [34]. Fluorescence change in blood was plotted versus time using Microsoft Excel software. All data are presented as mean $\pm$ SD. A Student's *t*-test (Microsoft Excel Software) was employed to analyze the difference between two points. A *P*-value of 0.05 was considered to be statistically significant.

Metabolism in mouse serum—The 800-G-ABz4-t- BBN agonist was added to fresh mouse serum to make the same starting concentration that was achieved in the pharmacokinetics in vivo study. The serum samples were sealed under an atmosphere of CO₂ and were allowed to stand at 37°C. They were analyzed by HPLC at 1 min, 1 h, and 3 h as previously reported [35]. The serum proteins were separated from the analytes by 2% of SDS and then precipitated with 50% ice-cold ethanol plus 50% of ACN (the peptides are stable in ethanol as verified by HPLC). The samples were centrifuged at 12,000g for 20 min at 4°C. The liquid phase was analyzed by HPLC. Separate control samples were run with buffer and peptides, plus buffer with IRdye800-CW as a control sample. The HPLC of collected fractions were monitored via 800nm emission fluorescence using a Shimadzu RF-10AXL fluorescence HPLC detector. The agonist was successfully separated from serum proteins using the SDS, and subsequently demonstrated a mouse serum half-life of approximately 90 min (Fig. S2). The 800-G-ABz4-STAT antagonist, however, could not be

separated from mouse serum proteins or from bovine albumin at any SDS concentration achievable, thus preventing analysis of its stability.

In Vivo Mouse Imaging

Dual tumor mouse model—Male nude mice (Tac:Cr: [NCr]-Foxn1nu homozygous), 4- to 6-week old, were purchased from Taconic Farms Inc (Rensselaer, NY). Ace-1^{huGRPr} and Ace-1^{CMV} cells (6×10^6) in 100 μ L PBS were inoculated subcutaneously in the right and left flanks, respectively. Tumor size was measured every 24–48 h and the volume was calculated using the formula: length $\times$ width² $\times$ 0.52. The diet for the mice was changed from regular to fluorescence reduced (CAT# TD.97184, Harlan, WI) chow 1 week before imaging.

Imaging—When the tumors grew approximately to 150mm³, the mice were injected via tail vein with 10nmol 800CW-G-Abz4-t-BBN or 800CW-G-Abz4-t-STAT in 100 μ L. The whole animals were imaged using two imagers: a CRi Maestro white light excitation imager (CRi Inc., Woburn, MA) exciting with a xenon arc lamp filtered for 684–729 nm and a 745 nm long pass emission filter, and a laser excitation Fluobeam^{TM} 800 NIR imaging system (Fluoptics, Grenoble, France) which uses a 780nm laser (7mW/cm²) for excitation and a >820nm emission filter. Imaging was performed immediately after euthanization of the mice on whole intact mice and then again after removing with the skin from the torso. Mice were euthanized and imaged at 3, 6, 15, 24, and 36 h, p.i. Ace-1^{huGRPr} and Ace-1^{CMV} tumors. Similar sizes of skeletal muscle were then excised and imaged using the Fluobeam. The intensity of fluorescence of dissected tumors and muscle were measured using Image J software and relative ratios were calculated (n=2–4).

RESULTS AND DISCUSSION

Figure 1 shows the structure of the two peptide conjugates. The Abz4 Linker was used successfully to connect the truncated BBN (7–14) agonist to a DO3A chelator to create the clinical radiotheranostic agent, ¹⁷⁷LuAMBA [31], and to create 800-G-Abz4-t-BBN [13] and now the 800-G-Abz4-STAT conjugate. The peptides used are both well characterized: t-BBN is primarily an internalizing agonist and STAT is primarily a surface bound antagonist [27,29,36–40]. Lantry found an internalized to surface bound ratio of ~2 at 40 min incubation of ¹⁷⁷LuAMBA in GRPR expressing human prostate cancer PC-3 cells, and the same ratio for ¹²⁵I-BBN. Mansi duplicated Lantry's result, using ¹¹¹In-AMBA and extended it to the antagonist peptide, finding that the internalized versus cell surface bound radioactivity increased at 2 h incubation to five for the agonist, and remained at two for the antagonist.

Competition binding of the two NIRF agents with ¹²⁵I-Tyr⁴-BBN in PC-3 cells demonstrated that labeling with the IRdye-800 through the Abz4 spacer maintained GRPr specificity and nM binding IC₅₀. Figure 2A displays the binding curves. ¹²⁵I-Tyr⁴-BBN bound in the range published for that ligand [41–43]. 800-G-Abz4-STAT and 800-G-Abz4-BBN IC₅₀ values of 13 versus 4 nM were comparable to ¹¹¹InDO3AAbz4 analog values of 14 and 1 nM, respectively, and to the ¹⁷⁷LuAMBA analog value of 2.5 nM. The Abz4 Linker was created for the clinical ¹⁷⁷LuAMBA agent to favor binding to GRPr and NMBR over BB3. Interestingly, the minus four overall charge on IRdye800 did not weaken the specific

binding to GRPR compared to the charge neutral metal chelates when the Abz4 Linker group is the structural intermediary. The Abz4 Linking group appears to be a very reliable insulator for the truncated bombesin peptides [31,44,45].

The serum binding (Fig. 2B) was strong enough at 6 μM peptide that a binding constant $>10 \mu\text{M}$ is suggested, or multiple binding sites at lesser K_d values. In any event, albumin binding of this type of molecule is likely of a far lower avidity than the nM GRPr receptor binding (vide infra), but can encourage hepatobiliary excretion in vivo as is the case for the prototype dicyanine, the FDA approved Indocyanine green [46]. The current data suggest that these molecules will exist primarily bound to albumin in blood and interstitial spaces, because the doses dictated for NIRF detection lead to blood concentrations in the range where serum albumin concentration ($\sim 600 \mu\text{M}$) will control the agent's bound to free ratio (i.e., $K_{eq} [\text{SA}] = [\text{agent}][\text{SA}]/[\text{agent}]$). Our cell binding buffer contains 30 μM BSA and the cell binding and imaging data rule out a significant competition between albumin and the receptor.

Characterization of GRPr Expression in Ace-1 Cells

Ace-1 cells did not bind the ^{125}I -Tyr⁴-BBN, nor either of the NIRF agents. Canine bombesin peptides show high sequence homology with human and terminate in the same sequence [47], suggesting that the canine Ace-1 cell line expresses few GRPr. In addition, quantitative RT-PCR showed that the Ace-1 cells had very low levels of canine GRPr mRNA in contrast to the primary carcinoma tissues from which the Ace-1 were derived, which had abundant GRPr mRNA [48]. Stable transfection generated 10 canine Ace-1 clones with the plasmid containing human GRPr (Ace-1^{huGRPr}), and eight clones with the empty vector (Ace-1^{CMV}). A rapid radioligand-based screen showed that GRPr presence was strongly detectable in several Ace-1^{huGRPr} clones but weakly or at background levels in Ace-1 parent cells and Ace-1^{CMV} clones (Fig. 3A). To further confirm specific expression, GRPr mRNA levels of six clones were determined using real-time RT-PCR. Figure 3B shows that GRPr mRNA in Ace-1^{huGRPr} clones 2, 8, and 9 was 20-, 35-, and 28-fold greater than that in the human PC-3 cells that naturally express GRPr, but RNA expression of GRPr was not detectable in Ace-1^{CMV} clones 1 and 6. GRPr numbers in the Ace-1^{huGRPr} clones 8 and 9 were further defined using a saturation assay, since RNA levels do not guarantee cell surface expression that is necessary for efficient imaging.

Saturation radioligand binding curves in Figure 3C show that Ace-1^{huGRPr}-8 and Ace-1^{huGRPr}-9 clones expressed high levels of human GRPr on their surface with a total receptor per cell of 693,269 and 537,935, respectively versus 133,905 (GRPr plus NMBR) in the PC-3 cell line. GRP receptor expression was not detectable in the Ace-1 parent cells or in the Ace-1^{CMV}-1 clone. Compared with PC-3 cells, the Ace-1^{huGRPr} cells also had a somewhat smaller radius and hence approximately smaller surface area as measured in two dimensions: PC-3 cells = $394 \pm 190 \mu\text{m}^2$; Ace-1^{huGRPr} = $203 \pm 64 \mu\text{m}^2$. This suggests 8- to 10-fold higher density of huGRPr expression on the Ace-1^{huGRPr} cells we used in binding and animal experiments. The curve shape in Figure 3C demonstrated saturation of the receptors and hence, specific receptor binding of the ligand.

The activity of the huGRPr was determined in Ace-1^{huGRPr}-8, Ace-1^{CMV}-1, and positive control PC-3 cells using a Ca^{2+} mobilization assay. The assay requires that the ligand bind

and stimulate activation of the receptor. For this experiment we used the thoroughly characterized clinical candidate, AMBA, a known internalizing agonist to GRPR [31]. The results shown in Figure 3D demonstrate that at least some of the human GRPr expressed on the canine prostate cancer cells is metabolically active and internalizing.

The binding ability of the two peptides to the GRPr expressing and non-expressing cells was observed directly by incubating cells with 800-G-Abz4-t-BBN and 800-G-Abz4-STAT. The former compound is known to bind avidly and specifically to GRPR on PC-3 cells [13]. As shown in Figure 4A, binding of both compounds was observed in both Ace-1^{huGRPr}-8 and PC-3 cells, but not in Ace-1^{CMV}-1 cells. Specific binding to the Ace-1^{huGRPr}-8 cells was also demonstrated by blocking the binding of the two peptides with BBN (Fig. 4B).

We conclude from the binding and activation data that the human GRPr receptor has been successfully transfected into Ace-1 canine prostate cancer cells. Ace-1^{huGRPr}-8 and Ace-1^{CMV}-1 cells were therefore used in the in vivo experiments on xenograft tumors in nude mice. Despite the GRPr presence, the Ace-1^{huGRPr}-8 and Ace-1^{CMV}-1 cells grew at roughly same rate in media and as xenografts in nude mice.

Mouse Serum Binding and Stability

The stability in mouse serum was measured by fluorescence detection HPLC of standing solutions (Fig. S2). The rate of loss of the parent peak in the HPLC showed ~1.5 h half-time for the 800-G-Abz4-t-BBN, but could not be measured for 800-G-Abz4-STAT due to strong protein binding. One and half hours is consistent with the known stability ranges of STAT [49–51] and the truncated BBN (7–14) [28,52,53], with STAT being somewhat more stable. However, Linder showed that metabolism of BBN (7–14) based ¹⁷⁷Lu-AMBA molecule in human plasma was greater than 10-fold slower than in mouse plasma. Mouse serum and plasma stability studies are useful for interpreting mouse biological studies, but do not predict analogous human stability that will in turn affect bioavailability in humans.

Pharmacokinetics

The blood clearance pattern observed in normal mice (Fig. 5A) was a very rapid initial blood clearance, 70–80% within 2 min, followed by a slower blood clearance, especially of the 800-G-Abz4-STAT, with both agents culminating in nearly complete clearance by 24 h. While overall the two compounds showed a similar pattern, the blood fluorescence intensity values (%ID/blood) of 800-G-Abz4-STAT were significantly higher than those of 800-G-Abz4-BBN at 2 min (32.8% vs. 16.9%, $P<0.05$), 30 min (9.3% vs. 5.4%, $P<0.05$), 1 h (8.2% vs. 3.6%, $P<0.05$), and 2 h (7.8% vs. 2.4%, $P<0.05$) and remained different at 4 h (5.5% vs. 1.1%, $P<0.05$) and 8 h (4.4% vs. 0.5%, $P<0.01$). Cumulative urinary excretion within 4 h was the same for the two agents with 28.2% and 29.9%/ID, respectively, indicating 60–70% unaccounted for in blood and urine. Somewhat higher fluorescence intensities were observed in skin, liver, lungs, liver, and GI in mice injected with 800-G-Abz4- STAT compared to 800-G-Abz4-BBN, even at 36 h post administration (Fig. 5B).

The total extracellular space in mice is about threefold greater than blood, hence the 60% unaccounted for in urine and blood (Fig. 5A) could be in interstitial space and does not necessarily imply significant hepatic or other specific organ uptake. We noticed highly

fluorescent fecal pellets in mice housed for a few hours after the urine was collected, suggesting at least some hepatic excretion, but coprophagy of residual fluorescent urine coated pellets complicates the observation. In dissected animals sacrificed at 36 h post administration, skin, kidney, and GI were the brightest under the fluorescent imager, with liver intermediate. The antagonist left slightly more signal in all organs. These data, while not quantitative, are consistent with the quantitative blood clearance data, and all of these data are consistent with stronger protein binding for the 800-G-Abz4-STAT.

¹⁷⁷LuAMBA is a chelated analog of t-BBN used in 800-G-Abz4-t-BBN. ¹⁷⁷LuAMBA initial clearance half-time in mice ($T_{1/2}=3.1\pm0.1$ h) was slower than either of the 800-conjugates tested, and urine accumulation was greater (~60 % in 1 h). Only <35% of the injected dose of the 800 conjugates was accounted for between blood and the urine accumulated. Strong albumin binding probably reduced initial renal clearance rate compared to the more hydrophilic ¹⁷⁷LuAMBA. Protein binding and hepatobiliary excretion is characteristic of indocyanine green, the prototype dicyanine structure from which the IRdye800 Label is derived [46,54].

Dual Tumor Nude Mouse Model

The Ace-1^{CMV} and Ace-1^{huGRPr} lines were easily cultured and demonstrated very rapid growth, more rapid than PC-3 by a factor of two, and had more reliable growth when implanted into nude mice. The tumors in nude mice took <1 week to grow to 0.5cm diameter, were not necrotic, and there was 100% growth in >50 cell xenografts. Despite the GRPr presence, the Ace-1^{huGRPr}-8 and Ace-1^{CMV}-1 cells grew at same rate in media and as xenografts in nude mice, allowing for rapid reliable screening of new agents with paired statistical data in dual tumor mice. A further advantage of the Ace-1^{huGRPr} cells is that they contain only the most commonly expressed of the two GRPR subtypes (GRPr and NMBR) known in human tumors, making a more specific screening system.

The imaging studies were conducted in dual tumor nude mice (Ace-1^{CMV}/Ace-1^{huGRPr}). In a previous study, 10 nmol of 800-G-Abz4-BBN was used for imaging in PC-3 tumors in mice [13]. Pilot studies (Figs. S3 and S4) were conducted in the new dual tumor mice testing time and dosage. We then created groups of dual tumor Ace-1^{CMV}/Ace-1^{huGRPr} nude mice, administered 10 nmol of test agent per mouse and euthanized mice groups (N=4) at 3–36 h. Mice were imaged with the Fluobeam imager (Fig. 5C). Tumor signal gradually increased out to 24 h p.i. and then diminished with no significant difference between the two agents. For both agents, the ratios of Ace-1^{huGRPr} tumor to muscle and Ace-1^{huGRPr} to Ace-1^{CMV} increased from 3 to 15 h post administration and then decreased between 24 and 36 h (Fig. 5D). The two tumors were significantly different ($P<0.05$) between 6 and 24 h. Ace-1^{CMV} tumor to muscle ratios were not significantly different.

Fluorescent imaging data are not reliably quantitative due to high attenuation of the optical signal by tissue, but the data in Figure 5C and D qualitatively and semi-quantitatively demonstrated differences between the two tumors in each animal but not a difference between the agonist and the antagonist test molecules, despite different blood clearance curves and brightness that was somewhat greater for the 800-G-Abz4-t-BBN agent (Fig. S1). It is noteworthy that the smallest ratio in Figure 5D is for the GRPr positive to GRPr

negative tumors, suggesting that the dual tumor model is a more stringent test of a new molecule's ability to image the GRPr receptor than an in vivo test using a single GRP receptor positive tumor ratio against muscle.

The fact that the Ace-1 canine cell lines grows readily in immune-compromised dogs will allow us to test lead candidates in a large animal model whose prostate gland is approximately the same size as in men. We recently reported preliminary success with this Ace-1^{huGRPr} canine model [55]. The ability to transfect the Ace-1 cell line with human receptors makes it a promising candidate cell line for research screening on further variants containing the other GRPR subtypes, such as the NMBR expressed in human prostate cancer, and the BB3 subtype found in human pancreas [56], as well as for screening other receptors potentially useful in prostate cancer therapy.

CONCLUSIONS

The Ace-1 canine prostate cancer cell line has been successfully transfected with a human prostate cancer GRPr receptor that is expressed on the cell surface and remains functional. The transfected Ace-1^{huGRPr} cell can be grown reproducibly and efficiently in culture and in nude mice, and imaged with an NIRF agonist and antagonist that bind specifically to the human GRPr receptor. The dual tumor Ace-1^{CMV}/Ace-1^{huGRPr} model system provides a rapid semi-quantitative estimate of specific to nonspecific binding of new GRPr specific agents in a mouse model that can be scaled up to the Ace-1 orthotopic canine prostate cancer model to enable surgical and focal therapies to be tested on a humansized gland.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Chemical structures of GRPr binding NIRF agents: 800-G-Abz4-t-BBN (top) and 800-G-Abz4-STAT (bottom). t-BBN (Q-W-A-VG- H-L-M-NH₂) and STAT (F*Q-W-A-V-G-H- L-NH₂ where F* is D-Phenylalanine and is 3S,4S-amino-6 methyl heptanoic acid) refer to the agonist and antagonist peptide, respectively. The linker between the peptide and 800 is G-Abz4 (4-aminobenzoyl) in both molecules.

Fig. 2.

In vitro characterization of GRPR binding NIRF agents. **A.** Competition binding (IC₅₀) with ¹²⁵I-Tyr⁴-BBN in PC-3 cells: BBN (square), 800-G-Abz4-t-BBN (closed circle), 800-G-Abz4-STAT (open circle). **B.** Mouse serum protein binding of 800-G-Abz4-t-BBN (agonist) and 800-G-Abz4-STAT (antagonist).

Fig. 3.

In vitro characterization of stably transfected Ace-1 cells. **A.** GRPr expression level from competition binding of ^{125}I -Tyr⁴-BBN to the cells, normalized to PC-3 cells. CMV is the empty vector. Well counts per minute (CPM) were normalized by cell number and then normalized to CPM of PC-3. **B.** GRPr RNA expression plotted relative to PC-3. **C.** Saturation binding assay in of GRPr expressed in cell lines using ^{125}I -Tyr⁴-BBN: Ace-1^{hu}GRPr-8 (Ace-1-GRPr8, open circle), Ace-1^{hu}GRPr-9 (Ace-1-GRPr9, closed circle), Ace-1^{CMV}-1 (Ace-1-CMV1, open square), Ace-1 (open triangle), and PC-3 (closed square). **D.** Induction of mobilization of intracellular calcium by AMBA (100 nM) in PC-3 positive control (left graph) and Ace-1^{hu}GRPr-8 and Ace-1^{CMV}-1 cell lines (right graph). Results are the average of triplicate runs corrected by subtracting buffer control runs.

Fig. 4.

A. Direct binding of 800-G-Abz4-t-BBN (top row) and 800-G-Abz4-STAT (bottom row) in Ace-1^{huGRPr}, Ace-1^{CMV}-1, and PC-3 cell lines detected by fluorescence microscopy. Blue stain is DAPI; red to pink is detected >800 nm fluorescence. **B.** Ace-1^{huGRPr} cells binding 800-G-Abz4-t-BBN and 800-G-Abz4-STAT in the presence or absence of bombesin (BBN, 366 μ M) for 1 h. BBN blocked the binding of 800-G-Abz4-t-BBN completely and 800-G-Abz4-STAT largely at 1 and 1.5 μ M agent (image of 800-G-Abz4-t-BBN at 1 μ M, not shown). Magnification is 200 $\times$, bar is 50 μ m.

Fig. 5.

In vivo characterization of the NIRF agents. **A.** Fluorescence intensity changes in blood of Balb/c mice over time after injection of 5 nmol 800-G-Abz4-STAT and 800-G-Abz4-t-BBN; urine accumulations were 29.6% and 28.2% ID, respectively. **B.** Fluobeam NIRF images of dissected tissues of Balb/c mice dissected 36 h after 10 nmol administrations of agents. **C.** Fluobeam NIRF images of Ace-1^{huGRPr} (right flank) and Ace-1^{CMV} (left flank) tumor mice over time after 10 nmol administration of agents (top row, 800-G-Abz4-t-BBN; bottom row, 800-G-Abz4-STAT). **D.** The graphs show quantitative Fluobeam data plotted for the tumors and similar sizes of skeletal muscle mass, as ratios of Ace-1^{huGRPr} tumor/Ace-1^{CMV} tumor (GRPr/CMV), Ace-1^{huGRPr}/muscle (msl), and Ace-1^{CMV}/muscle.