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A fluorescent, [^{18}F]-positron-emitting agent for imaging PMSA allows genetic reporting in adoptively-transferred, genetically-modified cells

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Abstract

Clinical trials that involve genome-edited-cells are growing in popularity, where CAR-T immunotherapy and CRISPR editing are more recognized strategies. Genetic reporters are needed to localize the molecular events that these cells cause in patients. Specifically, a non-immunogenic genetic reporter is urgently needed as current reporters are immunogenic due to derivation from non-human sources. Prostate-specific membrane antigen (PSMA) is potentially non-immunogenic due to its natural, low-level expression in select tissues (self-MHC display). PSMA overexpression on human prostate adenocarcinoma is also visible with certain contrast. We exploit these properties in a transduced, two-component, Human-Derived, Genetic, Positron-emitting and Fluorescent (HD-GPF) reporter system. Mechanistically analogous to the luciferase/luciferin

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Competing Interests

The authors have declared that no competing interest exists.

Supporting information

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This material contains additional experimental detail describing cell culture, vector construction, lentivirus production, imaging, data collection and additional supporting animal experiments.

reporter, PSMA is genetically encoded into non-PSMA expressing 8505C cells and tracked with ACUPA-Cy3-BF3, a single, systemically injected small-molecule that delivers both positron emitting fluoride (^{18}F) and a fluorophore (Cy3) to report on PSMA expression. PSMA-lentivirus transduced tissues become visible by Cy3 fluorescence, [^{18}F]-PET and γ -scintillated biodistribution. HD-GPF is visible at sub-cellular resolution, while a reduced PET background is achieved in *vivo*, due to rapid ACUPA-Cy3-BF3 renal excretion. Co-transduction with luciferase and GFP show specific advantages over popular genetic reporters in advanced murine models including: A 'mosaic' model of solid-tumor intratumoral heterogeneity, and a survival model for observing post-surgical recurrence. We report an advanced genetic reporter that is useful for tracking genetically modified cells from the deep tissue to the microscopic level by PET and fluorescence. This reporter system is potentially non-immunogenic and will therefore be useful in human studies. Due to derivation from prostate adenocarcinoma, translational ACUPA-Cy3-BF3 potential in radical prostatectomy is demonstrated.

Graphical Abstract

Introduction

With only a partial understanding of the consequences of human genome editing, we enter an era where federal approval is already granted to clinical trials that involve the genome-editing of patient cells. For examples, trials involving CAR-T immunotherapy or CRISPR cancer-gene-editing have grown in popularity^{1–3} despite unchecked expansion and tumorigenesis being a clear concern of therapeutic genome editing.^{4–5} Our contemporarily bioluminescent, PET, and fluorescent genetic reporter strategies^{6–9} are inadequate for tracking tumorigenesis in human genome editing trials, as they are derived from jellyfish, firefly, coral, bacterial, or viral sources that may trigger an immune response. New technology is needed to report on the adverse effects of genome editing in clinical trials (e.g. tumorigenesis and unchecked expansion).

One solution is a genetic reporter that is based human proteins like the prostate-specific membrane antigen (PSMA). PSMA is a type II transmembrane dimeric glycoprotein that is naturally present at low level in normal cranial, parotid, and renal tissue and therefore will

be potentially non-immunogenic. PSMA is overexpressed in 92% of prostate adenocarcinomas (Figure 1A),^{10–11} making it an attractive target for diagnosis and treatment of prostate cancer patients.^{10, 12–14} PSMA can be transduced via adenovirus to correctly express on the extracellular membrane of cells.¹⁵

We exploit PSMA using a PSMA-encoded lentivirus to transduce non-PSMA-expression cells (thyroid gland carcinoma 8505C). We detect our PSMA gene using ACUPA-Cy3-BF3, a novel small-molecule urea glutamate PSMA inhibitor that bears both a radioactive fluoride (¹⁸F) and a trimethine cyanine fluorophore (Cy3) for both PET and fluorescence imaging, respectively (Figure 1B).¹⁶ To simplify description, we name our two-component, single gene (PSMA) and single exogenous small molecule (ACUPA-Cy3-BF3) system the Human-Derived, Genetic, Positron-emitting and Fluorescent reporter (HD-GPF). We report the first ¹⁸F-PET/fluorescence genetic reporter that will be potentially non-immunogenic in patient clinical trials.

In the present study, we validate HD-GPF in PSMA-transduced-xenograft murine models to demonstrate HD-GPF utility for visualizing intertumoral heterogeneity (by PET, heterogeneity that exists between satellite lesions), intratumoral heterogeneity (by fluorescence, heterogeneity that exists within a primary lesion) and in real-time fluorescent-guided tumor surgery. HD-GPF is a superior-resolution-analogue of the luciferase/luciferin genetic reporter system, which is solely useful in bioluminescent imaging. We demonstrate that the substrate ACUPA-Cy3-BF3 is visible on PSMA transduced cancer cells at sub-cellular resolution (fluorescence), and can distinguish PSMA expressing tissue from normal tissue in full body PET scans. ACUPA-Cy3-BF3 can be retained in PSMA-positive tissues (> 48 h) and gives bright, intraoperative fluorescence signal at nanomolar-quantities. This allows delays to be introduced between contrast introduction, PET imaging and fluorescent exploration/xenograft removal. HD-GPF allows for sensitive, low-background, deep-tissue, and sub-cellular resolution genetic reporting of an exogenously expressed reporter gene (PSMA). As we derive this reporter system from prostate adenocarcinoma, our data additionally indirectly highlight ACUPA-Cy3-BF3 utility in radical prostatectomy.

Results

ACUPA-Cy3-BF3 fluorescent visualization of natural and transduced PSMA-expressing cell lines *in vitro*

ACUPA-Cy3-BF3 was synthesized and radiolabeled as described previously.¹⁶ PSMA is not detectable on the human thyroid carcinoma cell line (8505C). This makes 8505C ideal for HD-GPF imaging. A second-generation lentivirus plasmid was constructed by inserting the human PSMA gene after a elongation factor-1 α (*EF1 α*) promoter (Figure 1C). The resulting plasmid allows high efficiency expression in human cell lines. Following lentivirus transduction, ~100% PSMA⁺ 8505C cells were sorted and verified by flow cytometry to create a PSMA positive cell line (8505C+, Figure 1D). Non-transduced 8505C (8505C–), PC3 (PSMA[–]), and PC3-PIP (PSMA⁺) cell lines serve as respective PSMA[–] transduced, PSMA[–] non-transduced, and PSMA⁺ non-transduced controls for *in vitro* and *in vivo* experiments, respectively. Cells were transduced with firefly luciferase and enhanced GFP to allow HD-GPF to be compared to contemporarily used reporter genes in all experiments.

PSMA expression on all cell lines was verified by flow cytometry using the PSMA specific antibody, J591, bound to PSMA. PC3-PIP and 8505C+ cells expressed significant PSMA, while PC3 and 8505C- cells showed no PSMA expression (Figure 2A). ACUPA-Cy3-BF3 fluorescent binding to extramembrane PSMA was equivalent to J591 data. In fluorescent microscopy experiments, ACUPA-Cy3-BF3 (10–100 nM) showed similar affinity to both PC3-PIP and 8505C+ cells (Figure 2A, Figure S1). ACUPA-Cy3-BF3 bound to the cellular membrane and entered the cytosol of PSMA⁺ cells, suggesting PSMA internalization.^{19–20} ACUPA-Cy3-BF3 did not appear in the cell nucleus or exclusively accumulate in lysosomes as demonstrated by lack of Cy3 fluorescence correlation with DAPI or lysotracker fluorescence, respectively, in confocal microscopy (Figure S2–S3). ACUPA-Cy3-BF3 specific binding to PC3 and 8505- (PSMA⁻) cells was not observed (Figure 2A).

Competitive binding experiments confirm ACUPA-Cy3-BF3 specificity to PSMA⁺ cells (Figure 2B, G). Fluorescence intensity of PC3-PIP increase significantly when bound with ACUPA-Cy3-BF3 compared with no treatment (Figure 2B, E). When PSMA⁺ cells were pre-incubated with excess non-fluorescent PSMA inhibitor 30 min prior at a concentration ratio of 100:1, ACUPA-Cy3-BF3 fluorescence was significantly reduced (Figure 2B, F). In non-specific binding control experiments, PSMA⁺ cells were incubated with Cy3.18.OH, a trimethine cyanine dye that does not bear ACUPA.²¹ Cell specific binding to Cy3.18.OH was not observed (Figure 2B, G). The above data demonstrate that ACUPA-Cy3-BF3 has high binding affinity and specificity to cells expressing PSMA (PC3-PIP and 8505C+) and little background fluorescence to cells not expressing PSMA (PC3 and 8505C-) (Figure 2C).

PET and fluorescent visualization of PSMA expressing tumors *in vivo*

Having demonstrated PSMA specificity *in vitro*, HD-GPF utility was evaluated *in vivo*, in flank xenografts. A single, 100 μ Ci of [¹⁸F]-ACUPA-Cy3-BF3 was intravenously injected into mice bearing upper, bilateral PC3 and PC3-PIP flank tumors. Mice were scanned on an Inveon PET/CT at 2 h and 6 h post-contrast injection. PET imaging showed signal localization at PC3-PIP tumors as early as 1 week post-implantation, while PC3 tumors lack PET contrast despite PC3 tumors being larger due to faster growth (Figure S4).

[¹⁸F]-ACUPA-Cy3-BF3 PET resolved PSMA⁺ tissue but had a reduced presence in PSMA⁻ tissue (2–6 h post-contrast injection, Figure S4). ACUPA-Cy3-BF3 fluorescence was visible in delayed fluorescence imaging performed at least 24 h post ACUPA-Cy3-BF3 injection (Figure S5A–B). ACUPA-Cy3-BF3 fluorescent signal to noise improved when skin was removed (Figure S5C–D). PSMA⁻ tumors showed minimal uptake of ACUPA-Cy3-BF3 in PET and fluorescent modes 2–24 h post-injection.

In vivo HD-GPF PET and fluorescence data was corroborated with post-mortem, *ex vivo* gamma scintillation and fluorescence imaging. After 4 weeks post-implantation, mice were injected with [¹⁸F]-ACUPA-Cy3-BF3 and imaged by PET/CT (Figure 3A). [¹⁸F]-ACUPA-Cy3-BF3 uptake and exposure assays showed the greatest ACUPA-Cy3-BF3 accumulation in PC3-PIP tumors at 11.4 ± 3.4 %ID/g, which was 236-fold greater than PC3 tumors (2 h post-injection, Figure S6). [¹⁸F]-ACUPA-Cy3-BF3 uptake in heart, lung, liver, stomach, brain, spleen, and muscle were less than 0.04 %ID/g (6 h post-injection). Intestinal and renal

uptake was large at 2 h and 6 h post-injection (Figure S6, Figure 3B), indicating that ACUPA-Cy3-BF3 is excreted *via* gastrointestinal and renal organs. Organs were imaged for ACUPA-Cy3-BF3 affinity under the fluorescent microscope. Fluorescence imaging revealed Cy3 fluorescence in PC3-PIP tissue but not in PC3 tissue (Figure 3D, E first row).

HD-GPF enables PET and fluorescent visualization of intratumoral heterogeneity in 8505C+ flank tumors that are transduced to express PSMA

To demonstrate the sensitivity of HD-GPF, we mixed 8505C+ and 8505C– cells to generate a ‘mosaic’ model of 8505C intratumoral heterogeneity. 8505C cell mixtures containing 0%, 0.1%, 1%, 10% or 100% 8505C+ cells were subcutaneously xenografted in mice.

At 2 weeks and 4 weeks post-implantation, 8505C+ tumors (0.1%, 1%, 10%, and 100%) were visible by ACUPA-Cy3-BF3 PET imaging (Figure 4A–B, Figure S7). PET labeling was confirmed *ex vivo* with ACUPA-Cy3-BF3 fluorescence. ACUPA-Cy3-BF3 (Cy3) signal was visible in 0.1%, 1%, and 100% 8505C+ tumors that were resected at 24 h post-ACUPA-Cy3-BF3 injection, but not in 8505C– tumors (Figure 4C). In resected tumor tissue, PET and fluorescence intensity increase with higher percentage of 8505C+ cells. Intratumoral heterogeneity was observed in fluorescent histology (Figure 4D, third row, Figure S8) despite extensive premixing of 8505C+ and 8505C– ‘mosaic’ cell aliquots prior implantation. HD-GPF is useful in identifying significant dilutions (~1000 fold, 0.1% 8505C+) of PSMA-transduced cells in the PET (*in vivo*) and fluorescent (*ex vivo*) modes. Fluorescence imaging allows resolution of tumor margins after radioactive ^{18}F decay (i.e., > 24 h).

HD-GPF PET and fluorescent tumor visualization of metastases caused by transduced cells

8505C+ or 8505C– cells was systemically injected into NSG mice to evaluate HD-GPF in a metastatic xenograft model. *In vivo* bioluminescence imaging performed at 2–4 weeks post-8505C cell injection confirmed 8505C that expectedly embed in the lungs (Figure 5A, Figure S9B).^{18, 22} PET imaging with [^{18}F]-ACUPA-Cy3-BF3 (30 min post-injection, Figure 5A) confirmed bioluminescent signal in the lung, liver, and in a lower extremity joint of 8505C+ mice. [^{18}F]-ACUPA-Cy3-BF3 PET imaging was not visible in 8505C– tumors, and excess molecule was eliminated through in the kidneys, bladder, intestine (Figure 5A, Figure S9–S10).

Organs were resected and imaged *ex vivo* to confirm 8505C+ specific localization of ACUPA-Cy3-BF3. ACUPA-Cy3-BF3 fluorescence was present in 8505C+ lesions embedded in the lung, liver, and joint (Figure 5B) confirming *in vivo* PET imaging data. The distribution of visible lesions in the liver corroborate the punctate, ACUPA-Cy3-BF3 fluorescence signals. In 8505C– bearing mice, lesions in the liver were visible *ex vivo*, but PET and fluorescence signal were not. Gamma scintillation analysis comparing 8505C+ and 8505C– tissues show a statistically significant difference in [^{18}F]-ACUPA-Cy3-BF3 bound to the lung and liver. Activities from gamma scintillation were 23.0 ± 8.8 and 9.22 ± 2.08 %ID/g for the lung and liver of 8505C+ mice, respectively, and 0.63 ± 0.03 and 0.89 ± 0.35 %ID/g for lung and liver of 8505C– mice, respectively (Figure 5C). [^{18}F]-ACUPA-

Cy3-BF3 activity was present in the kidneys of both 8505C+ and 8505C– mice in similar quantity: 21.4 ± 7.4 %ID/g and 19.2 ± 2.9 %ID/g, respectively. This result suggests that unbound [^{18}F]-ACUPA-Cy3-BF3 is eliminated by renal excretion. Fluorescent histology of lung tissue reveal extensive, punctate infiltration of 8505C+ cells in the lung with GFP and Cy3 fluorescent colocalization (Figure 5D, Figure S11).

HD-GPF allows intraoperative post-mortem fluorescence-guided tumorectomy in mice

PET/CT or PET/MR scans do not accurately represent intraoperative patient anatomy, as tissue and organs shift depending on patient position and during dissection. Shifting tissues complicate the use of PET or MRI probes during real-time surgery. ACUPA-Cy3-BF3 labeled tissues remain fluorescent *in vivo*, even following radioactive ^{18}F decay (> 48 h), allowing surgeons to use fluorescence to track tissues found in PET scans, but shift during surgery in real time.

To assess the ability of ACUPA-Cy3-BF3 to assist in optical, image-guided tumor resection, an intravenous injection of ACUPA-Cy3-BF3 (7.5 nmols/mouse) was given to mice bearing both 8505C– (upper torso) and 8505C+ (pelvic area) tumors (4 weeks implantation). Surgery was performed 48 h post-ACUPA-Cy3-BF3-contrast injection to mimic a realistic delay that would occur between a nuclear medicine procedure and a surgery in a patient.

During tumorectomy, positive tumor margins were visible by both ACUPA-Cy3-BF3 and GFP imaging where fluorescence was used to guide the collection of margins to ensure negative 8505C+ margin at surgical conclusion. All mice bearing 8505C control tumors were imaged in the GFP channel to observe false positive/negative signal due to non-specific ACUPA-Cy3-BF3 binding or non-binding to 8505C+ tissue. Cy3 fluorescence accurately tracked with GFP fluorescence during surgery *in vivo* and *ex vivo* (Figure S12).

HD-GPF allows the generation of advanced survival models for quantitatively evaluating intraoperative fluorescent utility and post-surgical tumor regeneration due to positive margin

We developed a surgical survival model that allows HD-GPF for PET and fluorescent evaluation of surgical tumorectomy. Specifically, this model allows clear observation of unresected positive margin and clear, early observation of aggressive recurrence (i.e., amplification of positive margin due to recurrence).

8505C– or 8505C+ cells were xenografted in contralateral upper flanks of mice ($n = 5$, Figure 6A). We demonstrate that intravenously administrated [^{18}F]-ACUPA-Cy3-BF3 can visualize PSMA-positive tumor by PET imaging at high sensitivity. To mimic a theoretical delay that would take place between patient PET scanning and surgery, we introduced a delay to show that the contrast would continue to be visible in fluorescence-guided surgery. Mice were intravenously injected with ACUPA-Cy3-BF3 (7.5 nmols) 24–48 h prior to surgery. Tumor resection surgery was performed 24–48 h conventionally (visualization under white light) or with fluorescence-guidance technology. GFP optical filters were physically hidden from surgeons to ensure a blinded operation. As expected, pre- and intra-operative fluorescence imaging show Cy3 and GFP fluorescent colocalization in 8505C+ tissue. Using HD-GPF fluorescent guidance, 8505C+ tumors were completely resected.

8505C– flank tumors were only partially resected under white light and positive margins were only verified by GFP imaging. After that, the surgical site was sutured (Figure 6B, left) and mice were allowed to recover from anesthesia. Mice were monitored daily for 15 days where tumor regrowth was closely tracked. In 8505C– tumors, rapid regrowth was observed (Figure 6C, left). No regrowth of completely resected 8505C+ tumors was observed (Figure 6C, right).

In a variation of this experiment, mice were xenografted with two 8505C+ tumors (no 8505C– control). Surgery in right-flank 8505C+ tumors was revised until a negative margin was confirmed by ACUPA-Cy3-BF3 fluorescence. A deliberate positive margin (~10% confirmed by fluorescence imaging) was left in the left-flank tumor as a control (Figure 6B, right). Regrowth of the 8505C+ left-flank tumor (positive margin) was noted 15 days post-surgery, and regrowth tumor was visible with intravenous ACUPA-Cy3-BF3 (Figure 6C, right).

The resulting model is relevant to surgeries where minimal margins are important, i.e., in cases of perineural or vascular invasion, and in the generation of algorithms for robotic tumorectomy. The described model allows blinded comparison of white light surgery to fluorescence-guided surgery, which is especially important in developing surgical technique, minimizing resected healthy tissue (negative margin), and ensuring comprehensive removal of positive margin.

ACUPA-Cy3-BF3 safety studies

The safety profile of ACUPA-Cy3-BF3 was evaluated on different cell lines and in healthy mice. Cytotoxicity of ACUPA-Cy3-BF3 was not observed in 8505C–, 8505C+, A2780, and MDA-MB-231 at concentrations 50 μ M after 72h incubation (Figure S13). To evaluate systemic toxicity of ACUPA-Cy3-BF3, Balb/c mice ($n = 4$) were intravenously given 75 nmols (3.75 μ M/kg, 10-fold larger than the imaging dose), and mouse weight was monitored every other day. Weight loss was not observed over 14 days in ACUPA-Cy3-BF3 administrated mice (Figure S14A). After 14 days, mice were sacrificed and major organs were collected for pathological observation. Clinical pathology (blood analysis) showed no effect on hepatic and renal function (Supplementary Figure S14B–C, Table S1). Histopathology changes in brain, heart, lung, liver, kidney, spleen, and lymph node tissues were not observed (Figure S14D).

Discussion

There is no fluorescent protein or equivalent genetic reporter that allows for completely non-immunogenic, long-term tracking of genome-edited-cells in human trials (e.g. CAR-T or CRISPR). In this study, we show that a prostate-specific membrane antigen based reporter system, the Human-Derived, Genetic, Positron-emitting and Fluorescent reporter (HD-GPF) allows genetic reporting at high resolution, from the whole-animal, deep-tissue field of view of a PET scanner to the subcellular field of a confocal microscope. This enhanced resolution makes HD-GPF superior to contemporary, genetically encoded reporter systems. HD-GPF affords PET and fluorescence imaging in all stages of biological experimentation involving cells in cell culture, 3D cell cultures, and living organisms bearing xenografted tumors and

metastatic cancer. Transduced cells are visible using nanomolar quantities of a single injected small-molecule (ACUPA-Cy3-BF3).¹⁶ HD-GPF is additionally compatible with clinically available ⁶⁸Ga-HBEDCC for PET only imaging (Figure S15) or fluorescence-only contrast.²³ HD-GPF allows visualization of intratumoral heterogeneity and comprehensive sub-millimeter margin resection in real-time fluorescence-guided mouse xenograft surgery.

HD-GPF is a competitive alternative to other reporter systems

The single transduced gene and single exogenous chemical combination of HD-GPF makes it highly analogous to the luciferase (*hrl*), bioluminescence reporter system.²⁴ Competitive bioluminescent and fluorescent protein reporter systems are immunogenic and are limited in depth due to the limitations of visible to near-infrared light penetration, scattering, and absorbance by endogenous tissue. Stand-alone PET-only systems do not allow visualization at microscopic detail,^{25–26} while imaging combination systems (*hrl-mrfp-ttk*) require multiple exogenous chemicals with different pharmacokinetic properties (e.g., luciferin, [¹⁸F]-FHBG⁶).

With respect to PET-only reporter systems, ACUPA-Cy3-BF3 can serve as a competitive, multimodal substitute to 2-(3-{1-carboxy-5-[(6-18F-fluoropyridine-3-carbonyl)-amino]-pentyl}-ureido)-pentanedioic acid (18F-DCFPyL) and N-[N-[(S)-1,3-dicarboxypropyl]carbamoyl]-4-18F-fluorobenzyl-L-cysteine (18F-DCFBC). A PSMA reporter may potentially yield higher signal-to-noise ratio than the human sodium-iodide symporter (*NIS*) and type I thymidine kinase (*HSV-sr39tk*) reporter systems,¹⁵ and halotag transducible PET reporters.^{27–28}

Transduced PSMA is large in size relative to other reporter genes including GFP (27 kD), smURFP (32 kD, dimer of 16 kD),⁹ Firefly luciferase (65 kD), Renilla luciferase (36 kD),⁶ and Nanoluciferase (19 kD).²⁹ Lower molecular weight PSMA constructs that bind ACUPA-Cy3-BF3 may be advantageous; however, engineering attempts to reduce the molecular weight of the PSMA gene may induce immunogenicity (due to differences in major histocompatibility complex display) or would affect compatibility with corroborative antibody and immunotherapy reagents (e.g., J591 antibody).

HD-GPF is useful in prostate cancer

This is the first time we report ACUPA-Cy3-BF3 utility in mature human adenocarcinoma xenografts (Figure 3). PSMA is present in human prostate cancer; therefore, ACUPA-Cy3-BF3 has translational utility for distinguishing PSMA expressing prostate cancer from normal tissue in full body PET scans and on fluorescence imaging devices.

The ability to track prostate cancer before, during, and after surgery can lead to complete cancer removal and prolonged survival. PET is useful in identifying oligometastatic disease to avoid unnecessary surgery on patients with disseminated cancer and to ensure the completeness of tumor removal during surgery on new time-of-flight PET devices.³⁰ The fluorescent properties of ACUPA-Cy3-BF3 allow the identification of heterogeneous PSMA expression within a solid tumor.

ACUPA-Cy3-BF3 fluorescence at a lesion is present following radioactive decay for at least 48 h (Figure S12), allowing a delay to be introduced between contrast introduction, PET imaging and surgery. Pathologists can reevaluate ACUPA-Cy3-BF3 in PSMA-containing frozen tissue *ex vivo* after surgery. The real-time nature of fluorescence imaging allows quick response to physical manipulation during surgery and allows confirmation of tumor margin and local micrometastasis resection prior to the closure of a surgical site.³¹ A need for intraoperative, sub-millimeter margin determination is important in surgical cases where cancers are tightly wrapped around nervous or vascular tissue.³² ACUPA-Cy3-BF3 fluorescence would improve a pathologist's ability to observe margins *ex vivo* (intraoperative frozen section fluorescence histology).

HD-GPF is useful outside of prostate cancer

We report a transducible PSMA/ACUPA-Cy3-BF3 reporter gene system into cells that do not express PSMA, allowing PET/fluorescence imaging of tissue in clinical applications in both men and women that do not involve PSMA overexpression. In the case that there is a need for tracking living cell (e.g. CAR-T) in human patients, this reporter system can be utilized to provide the potential to noninvasively monitor the localization and expansion of PSMA-transduced cells. As PSMA is derived from human and will be potentially non-immunogenic, HD-GPF can assist in monitoring clinical immunotherapy.

[¹⁸F]-ACUPA-Cy3-BF3 vs current PET agents in PSMA prostate cancer

Gallium-68³³ and fluoride-18³⁴ PET contrast agents are becoming increasingly available because of their ideal pharmacokinetic properties and ability to precisely distinguish between disseminated and localized intraprostatic lesions in non-invasive deep tissue imaging. Unfortunately, PET imaging agents are less useful intraoperatively because PET requires complicated instrumentation, PET does not currently allow for the imaging of fine cancer margins that can shift during surgery, and lacks the sub-millimeter resolution that is useful to pathologists. For this reason, surgeons prefer to use fluorescent PSMA imaging agents to increase the accuracy of surgery.^{35–37} Fluorescent image-guided surgery (IGS) has been shown to reduce the incidence of positive surgical margins compared with white light surgery.³⁸ [¹⁸F]-ACUPA-Cy3-BF3 is advantageous over current PET equivalents because it is additionally fluorescent and could be used during radical prostatectomy, where a PET image generated upon ACUPA-Cy3-BF3 injection would be produced by a nuclear medicine department, discussed by a tumor board, and acted upon in the operating room > 48 h post-injection.

ACUPA-Cy3-BF3 vs current fluorescence agents in PSMA prostate cancer

Many fluorescent strategies employ nucleic acid aptamers,³⁹ small molecules,^{40–41} and engineered-antibodies^{38, 42} that bind to PSMA and are conjugated with fluorophores to assist in image-guided radical prostatectomy, lymph node resection, and tumor margin confirmation in intraoperative frozen section consults. In many preclinical trials, long wavelength, near-infrared Cy5 and Cy7 fluorophores are being explored.^{38, 42–44} These fluorophores have better penetration ability and low-background vs other fluorophores. However, the only current trial that is actively recruiting (NCT02048150), employs a short

wavelength Alexa fluor 488 PSMA-targeted monoclonal antibody with a large quantum yield.³¹

The ideal wavelength fluorophore for image guided surgery (IGS) is of debate. One must consider that in an ideal surgery, it is the opinion of two clinicians that determine decision-making during radical prostatectomy: the surgeon (urologist) and the intraoperative consult (pathologist). Fluorescent imaging equipment for IGS is expensive and not always available to the surgeon. While dissecting microscopes with fluorescent capabilities are commonly available. A pathologist would prefer short wavelength fluorophores like Cy3 in microscopy as it is resistant to photo-bleaching and has a large quantum yields (vs. Cy7).

Recent efforts combine PET and fluorescence agents in PSMA prostate cancer

Recent studies have combined radioisotopes (⁶⁸Ga, ⁶⁴Cu but not ¹⁸F) with fluorophores to form dual modality imaging probes for comprehensive non-invasive human imaging, for distinguishing localized tumors from oligometastatic disease, and for intraoperative pathologist-guided tumor margin confirmation.^{45–47} Placing PET and fluorescence modalities on the same small molecule could avoid complications associated with co-injected mixtures of separate contrast including receptor saturation, molecular pharmacology differences that give differences in blood clearance, non-specific tissue accumulation, and antigen affinity. [¹⁸F]-ACUPA-Cy3-BF3 is ideal because it is ¹⁸F bearing (i.e. not subject to gallium-68 generator depletion), its radiochemistry is simplified, and it contains an ideal fluorophore (Cy3) for margin determination by the intraoperative consult.

Conclusion

We develop a PET and fluorescence genetic reporter system (HD-GPF) based on PSMA for imaging genetically modified cells and tissues. HD-GPF is comprised of two parts: a single transduced gene and an ¹⁸F and trimethine cyanine bearing small molecule (ACUPA-Cy3-BF3) that allows for PET and fluorescence imaging of PSMA-transduced cells. PSMA-transduced cells are visible by PET and fluorescence at sub-cellular resolution, in full body PET scans, and in resected tissue at least 48 h post injection. ACUPA-Cy3-BF3 demonstrates rapid renal clearance from the blood, minimal hepatic accumulation, and reduced PET and fluorescent signal in tissues that do not express PSMA. ACUPA-Cy3-BF3 has ideal *in vivo* safety and sensitivity properties for additional use in PET/fluorescence-guided radical prostatectomy.

Materials and Methods

Materials and Methods are described in supporting information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Prostate-specific membrane antigen encoded, Human-Derived, Genetic, Positron-emitting and Fluorescent reporter (HD-GPF) allows for both PET and fluorescence imaging using a single gene and a single small molecule. (A) Crystal structure of the extramembrane domain of PSMA and residues with the substrate-binding cavity.¹⁷ Important residues of the active site of PSMA (red) are detailed in the inset.¹⁷ (B) Structure of the small-molecule inhibitor ACUPA-Cy3-BF3. The molecule contains radioactive fluoride (¹⁸F) for PET imaging (blue), a fluorescent molecule (Cy3, magenta), and a ureido pentanedioic acid for PSMA binding

(cyan). ACUPA-Cy3-BF3 allows for fluorescent and PET imaging at ~10 nM affinity¹⁶. Anaplastic thyroid gland carcinoma (8505C) cells do not express PSMA and serve as an ideal prototype to test the HD-GPF genetic reporter. (C) A schematic of two separate lentivirus vectors encoding PSMA or GFP/Luciferase. LTR, long terminal repeat; SD, splice donor; SA, splice acceptor; EF1 α , elongation factor 1 α promoter; and Ψ +, encapsulation signal.¹⁸ (D) 8505C were co-transduced with both PSMA and GFP/Luciferase (lentivirus) to create 8505C+, a cell line expressing all three reporter genes. As a control, 8505C were transduced with only GFP/Luciferase to give 8505C-, a PSMA-negative control cell line.

Figure 2.

Flow cytometry and microscopic analysis of PSMA expression and HD-GPF specificity. (A) Analytical flow cytometry with PSMA specific antibody (J591, detection with APC-secondary antibody, left panels) and ACUPA-Cy3-BF3 (100 nM) confocal fluorescent microscopy confirm appropriate PSMA expression levels in PC3-PIP, PC3, 8505C+, and 8505C- cells. Center panels show ACUPA-Cy3-BF3 fluorescence (red) in a single cell. Right panels show ACUPA-Cy3-BF3 (red)/Hoechst (blue) /GFP (green) /and lysotracker (white) merged fluorescence. Scale bar is 10 μ m. (B-C) Analytical mean fluorescent

intensity (MFI, Cy3) of untreated, 1 μ M ACUPA-Cy3-BF3, 1 μ M ACUPA-Cy3-BF3 in the presence of 100 μ M non-fluorescent competitor, and 1 μ M Cy3.18.OH (Cy3 without ACUPA) treated PC3-PIP (PSMA⁺, B) or PC3 (PSMA⁻, C). Error bars are $\pm$ SD, and ** is p < 0.01. (D-G) Representative epifluorescent images of PC3-PIP shown in B. Scale bar is 20 μ m.

Figure 3. PET and fluorescence imaging of ACUPA-Cy3-BF3 bound to PC3/PC3-PIP flank tumors. [18F]-ACUPA-Cy3-BF3 (2.5 nmols) was intravenously injected into the mice bearing PC3 and PC3-PIP tumors after 4 weeks post implantation. (A) Maximum intensity projections of a 20 min PET/CT scan at 6 h post contrast-injection. (B) Gamma scintillated biodistribution of organs that were harvested and weighed at 6 h post contrast-injection. All data points are detailed in the inset. Error bars are $\pm$ SEM and ** is $p < 0.01$. (C) Representative fluorescence imaging of an entire mouse showing fluorescence accumulation on PC3-PIP

tumor, but not PC3 tumor. (D) *Ex vivo* fluorescence imaging of ACUPA-Cy3-BF3 in select tissues. (E) PC3-PIP and PC3 tumors were sectioned and imaged in the fluorescent mode. Scale bar is 250 μm .

Figure 4.

HD-GPF allows imaging of dilute, heterogeneous PSMA-expression in transduced thyroid flank tumors. A mouse bore four tumors containing 0, 0.1, 1, and 100% 8505C+ cells in four flanks. (A) Both 8505C+ and 8505C- cells in 'mosaic' tissue (4 weeks post implantation) are visible by bioluminescent imaging. (B) ^{18}F -ACUPA-Cy3-BF3 maximum intensity projection PET images at 2 h post injection reveal 8505C+ content in mosaic tissue. (C) The mouse in B was sacrificed at 24 post-injection. Tumors were harvested and imaged in GFP and ACUPA-Cy3-BF3 fluorescent channels. (D) Tumors in C, were sectioned and imaged

using DAPI (Blue), GFP (Green), and ACUPA-Cy3-BF3 (Red) fluorescent filters on a microscope. Representative images are overlaid to show colocalization of ACUPA-Cy3-BF3 fluorescence and GFP expression in 8505C+ tissues. In heterogeneous 0.1% and 1% 8505C + tumors, a dashed, white or red line show the boundary of tissue expressing PSMA (red). Scale bar = 250 μ m.

Figure 5. HD-GPF detects small, tumorigenesis in multiple organs in a metastatic tumor murine model. 8505C+ or 8505C- cells were injected into NSG mice through the tail vein. (A) Tumorigenesis were visualized by luciferase bioluminescence (left panels) at 4 weeks post-8505C cell injection. [^{18}F]-ACUPA-Cy3-BF3 PET/CT (2 h post-injection) scans were performed to locate disseminated tumors and observe [^{18}F]-ACUPA-Cy3-BF3 elimination through kidneys, bladder, and intestine. (B) *Ex vivo* bright field and Cy3 fluorescent images of tumorigenesis in the lungs, liver, and joint. Arrows indicate visible reproducible

metastases in the liver (red, multinodular) and, most significantly the lower leg (cyan arrow). (C) Gamma scintillated biodistribution (2.5 h post-injection) of ACUPA-Cy3-BF3 in major organs. All data points are detailed in the inset. Error bars are $\pm$ SEM, and ** is $p < 0.01$. (D) Representative fluorescent histology show 8505C+ embedded in the lung by GFP and ACUPA-Cy3-BF3 fluorescence. Colocalization of GFP and Cy3 signals are observed.

Figure 6.

HD-GPF allows real-time guiding fluorescence guided tumorectomy and monitoring tumor regrowth. (A) Scheme illustrates workflow and xenograft implantation in hairless SCID mice. Two variations of the experiment are described. In the first variation (left), 8505C- and 8505C+ cells were xenografted in contralateral flanks of mice ($n = 5$), where the 8505C- tumor served as a control that cannot be observed with ACUPA-Cy3-BF3 fluorescence (left). A second variation (right) involved 8505C+ cells xenografted in contralateral flanks, where a right side-completely negative margin (due to aggressive surgery/large negative margin requirement) served as a control ($n = 5$). (B) Representative bright field images of mice before, immediately after surgery, and 15 days post-surgery. (C) Bright field and fluorescent imaging in a mouse that was intravenously injected with ACUPA-Cy3-BF3 (7.5 nmols) 24 h prior to the surgery and at the 15th day post-surgery. Only ACUPA-Cy3-BF3 fluorescence was used to guide primary tumor resection. GFP fluorescence was used to confirm surgery, but not to guide tumorectomy. Positive margins (left side) regrew aggressively post-surgery. No regrowth of resected (right side) 8505C+ tumors were observed.