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TITLE: Prostate-Specific Membrane Antigen (PSMA) Targeted Bio-orthogonal Therapy for Metastatic Prostate Cancer

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14. ABSTRACT During the first year of the project we have identified new monoclonal anti-PSMA antibody (mAb), which demonstrated high affinity to the PSMA receptor and excellent targeting of PSMA-expressing prostate cancer cells both <i>in vitro</i> and <i>in vivo</i> . We investigated details of the mAb and therapeutic complexes internalization in these cells and demonstrated rapid perinuclear localization of internalized agents. We have also synthesized and tested click-reactive components for <i>in vivo</i> therapy. First <i>in vivo</i> data for anti-PSMA click-based pretargeting have been obtained with NIR <i>in vivo</i> optical imaging. In addition, all regulatory reviews of the proposed animal procedures have been successfully completed.						
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1. Introduction

During the first year of the project we have identified new monoclonal anti-PSMA antibody (mAb), which demonstrated high affinity to the PSMA receptor and excellent targeting of PSMA-expressing prostate cancer cells both *in vitro* and *in vivo*. We investigated details of the mAb and therapeutic complexes internalization in these cells and demonstrated rapid perinuclear localization of internalized agents. We have also synthesized and tested click-reactive components for *in vivo* therapy. First *in vivo* data for anti-PSMA click-based pretargeting have been obtained with NIR *in vivo* optical imaging. To further understand the tumor delivery patterns contrast enhanced MRI was performed in several animals with PC3-Luc and PC3-PIP tumors. In addition, all regulatory reviews of the proposed animal procedures have been successfully completed.

During the second year we synthesized and characterized delivery components with highly cytotoxic antitubulin therapeutic agent, mertansine, and performed experiments with *in vitro* therapy in prostate cancer cells with PSMA-high and PSMA-low expressions. Efficient internalization and improved cell kill was detected in PSMA-positive PC-3-PIP cells which are specifically recognized by pretargeting 5D3 antibodies. Animal models of PC3-PIP (PSMA+) and PC3-flu (PSMA-) have been generated and tested with optical imaging using fluorescent labeled delivery components. A paper is in preparation for submission to *Theranostics* journal.

2. Keywords

Anti-PSMA monoclonal antibody, targeted therapy, *in vivo* imaging, antibody internalization, cytotoxic conjugates, two-component therapy, pretargeting

3. Accomplishments

What were the major goals of the project?

Specific Aims of the proposal are:

Aim 1. Synthesize and characterize anti-PSMA J591 mAb-based pretargeting and albumin based nanocarrier components. Optimize the therapeutic efficacy of the delivery strategy *in vitro* in PSMA(+) and PSMA(-) PCa cells.

Aim 2. To evaluate the therapeutic system in subcutaneous and intratibial metastatic mouse models using PSMA(+) C4-2 and PC3-PIP and PSMA(-) PC3 cell lines.

Specifically for the second year of the project the approved SOW includes the following activities:

Aim 1

Subtask 1: Dose response therapeutic study in PSMA-positive and negative prostate cancer cells, optimizing chemical linkers.

Subtask 2: Characterize cell kill mechanisms using cell viability, cell proliferation, and DNA-damage assays.

Aim 2

Subtask 1: generation of subcutaneous and intratibial models of human prostate cancer in mice.

What was accomplished under these goals?

Synthesis and characterization of the pretargeting components:

- (i) Functionalized 5D3 anti-PSMA monoclonal antibody with TCO functional group for click-chemistry conjugation and green AF488 fluorophore for microscopy.
- (ii) Functionalized 5D3 anti-PSMA monoclonal antibody with TCO functional group for click-chemistry conjugation and AF488 and pH-sensitive pRhodo (bright red at acidic pH) fluorophores.
- (iii) Functionalized F(ab')₂ domain of 5D3 mAb with TCO and AF488 fluorophore.

Synthesis and characterization of the cytotoxic carrier component: Human albumin conjugated with PEG-ylated tetrazine (Tt, reactive group for click chemistry), red fluorophores, rhodamine, and microtubule inhibitor mertansine (DM1) therapeutic agent using MCC hetero-bifunctional linker.

Therapeutic components internalization and cytotoxic effects have been measured in PSMA(+) PC3-PIP and PSMA(-) human prostate cancer cells in 2D cultures.

Main results:

- (i) Internalization of the pretargeting component is active receptor endocytosis and is minimally affected by known inhibitors of endocytosis (Fig. 1 A, B).
- (ii) Internalized mAb-receptor complexes are localized in low pH compartment next to the cell centrosome (Fig.1 C)

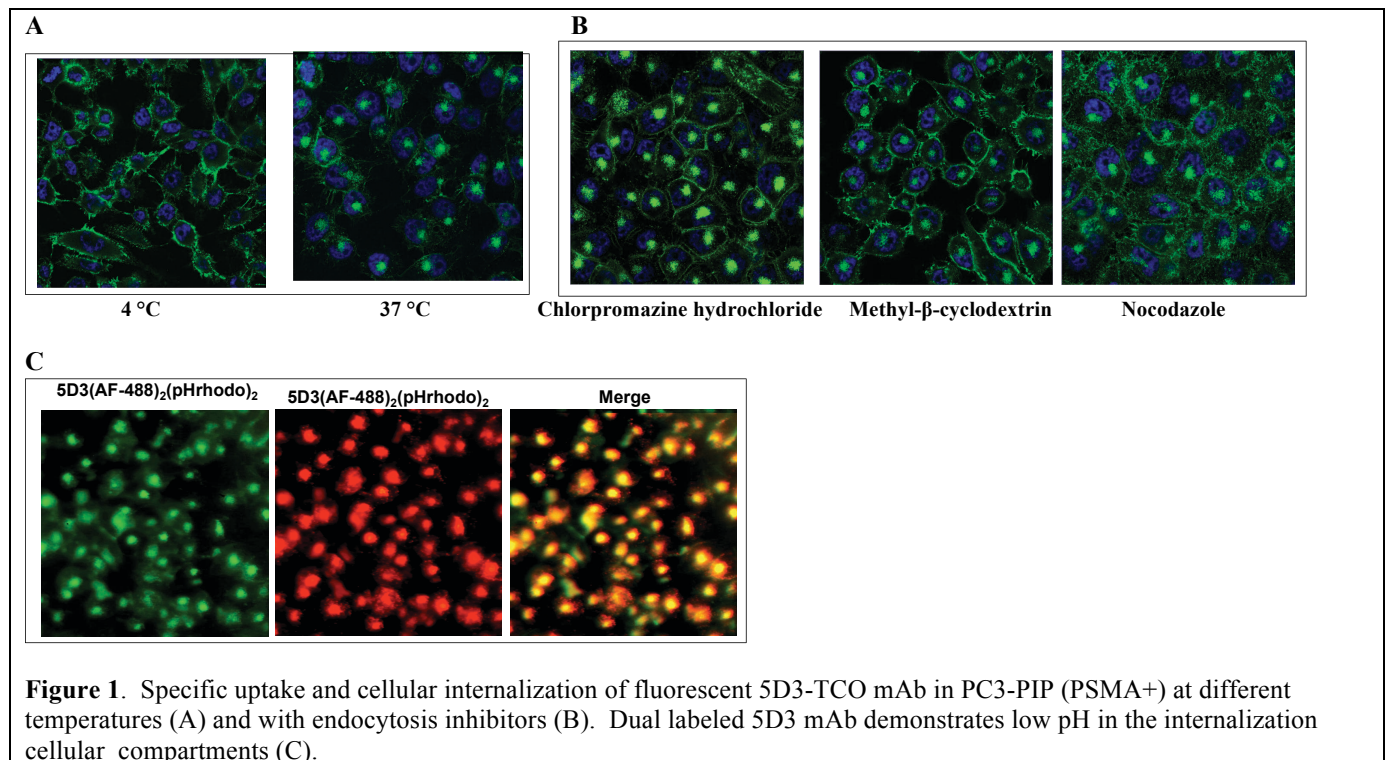
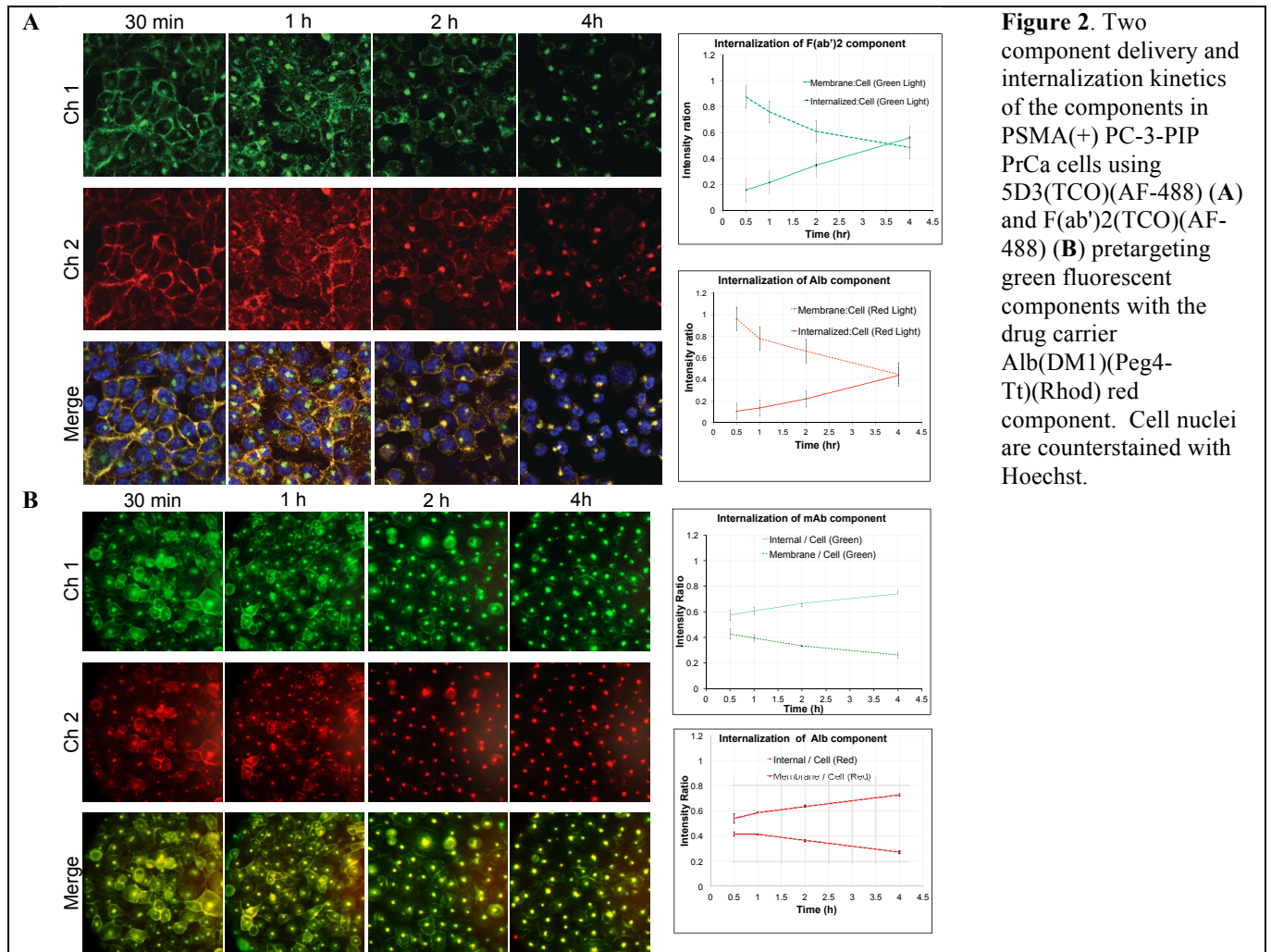
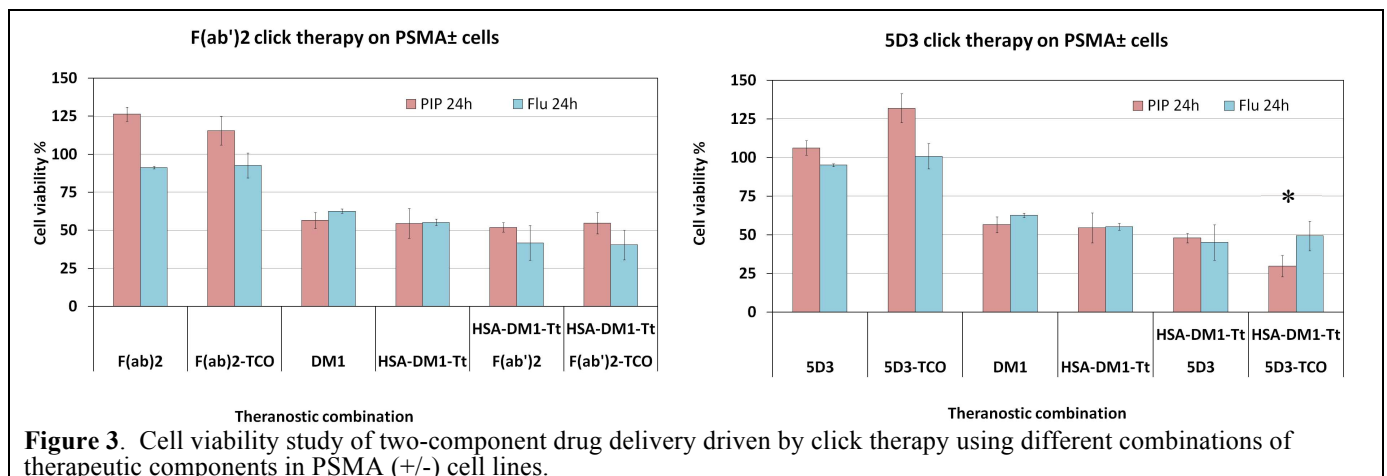


Figure 1. Specific uptake and cellular internalization of fluorescent 5D3-TCO mAb in PC3-PIP (PSMA+) at different temperatures (A) and with endocytosis inhibitors (B). Dual labeled 5D3 mAb demonstrates low pH in the internalization cellular compartments (C).

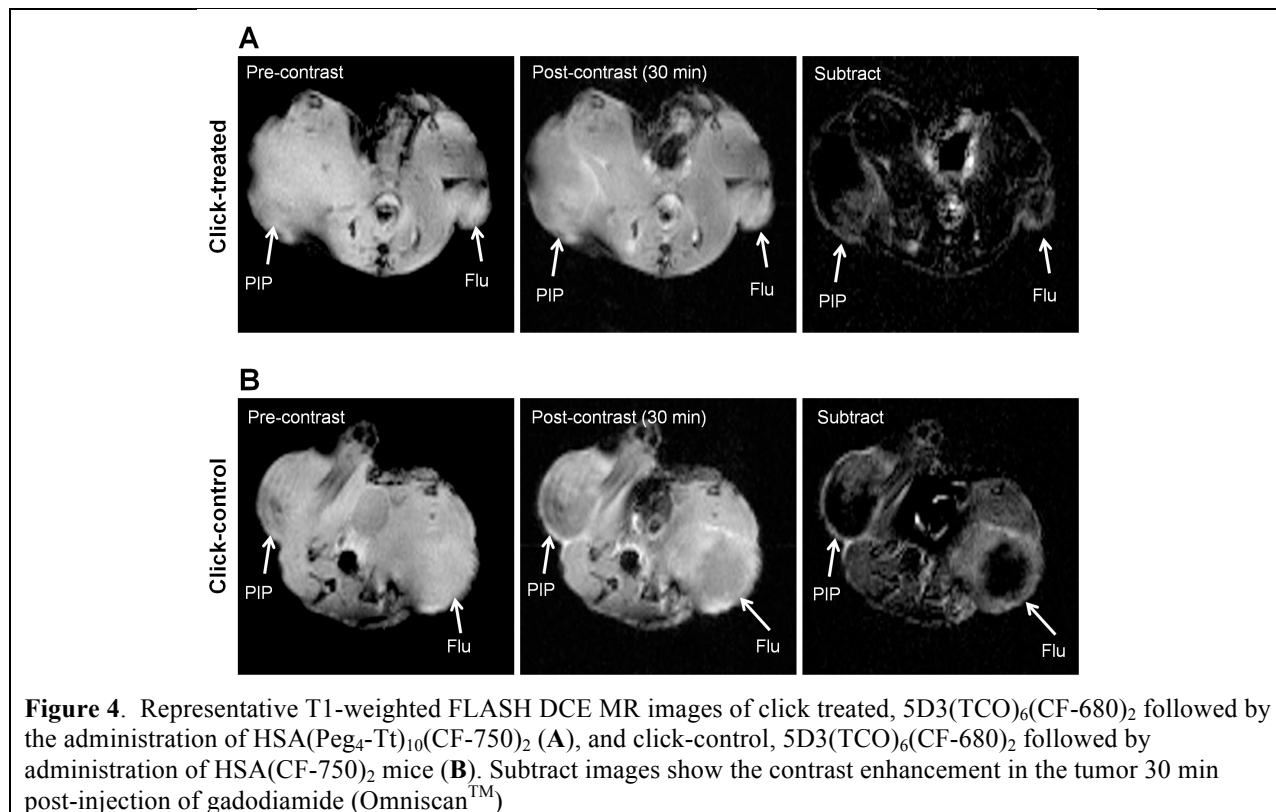
- (iii) Internalization of F(ab')₂ mAb domain is more efficient compared to the intact antibody (at 2 and 4 hours at 37 °C, Fig. 2 A&B).



(iv) Cell viability was assessed in vitro following PSMA-targeted two component pretargeting therapy in PSMA(+) and PSMA(-) prostate cancer cell lines. Highest treatment efficacy (lowest cell viability at 24h) was obtained in PSMA(+) PC-3-PIP cells treated with specific interacting components, 5D3-TCO and HAS-DM1-Tt. F(ab')₂ pretargeting did not substantially improved cytotoxicity in all cell samples (Fig. 3).



- (iv) We grow PC-3 xenografts in mouse models and have determined efficiency of delivery in xenografts of different size using dynamic MRI. Large xenografts have extensive poorly perfused core areas and should be excluded from *in vivo* treatment experiments, as shown in Fig. 4.



Deliverables:

- (i) 5D3(mAb)-TCO-A488/CF680 for fluorescent imaging guided pretargeting of PSMA
- (ii) 5D3(F(ab')₂)-TCO-A488/CF680 for fluorescent imaging guided pretargeting of PSMA
- (iii) 5D3(mAb)-TCO-pHrodo pH sensitive pretargeting component for measuring acidity in internalization compartments
- (iv) Albumin-PEG4-Tt- MCC-DM1-CF750/Rhodamine for image-guided drug delivery

How were the results disseminated to communities of interest?

Scientific presentations were given at laboratory seminars, JHU ICMIC seminar series, and the International Society for Magnetic Resonance in Medicine Annual Meeting.

What do you plan to do during the next reporting period to accomplish the goals?

During the next period of the project (year 3) we will apply the synthesized cytotoxic conjugates *in vivo* models of prostate cancer with different levels of expression of PSMA-receptors under optical imaging guidance. We will optimize the compounds for *in vivo* studies in orthotopic and metastatic mouse models of human prostate cancer. Specifically, timing of administration of the cytotoxic carrier (second component) will be optimized based on accumulation and retention of the first pretargeting component in the tumor. Biodistribution and pharmacokinetics of delivery components will be measured and tumor response will be determined by imaging and pathological studies. The efficacy of therapy and possible toxicity effects will also be assessed in treated animals.

4. Impact

What was the impact on the development of the principal discipline(s) of the project?

Novel two-component pretargeting system for specific therapy of PSMA-expressing prostate cancer can result in high efficacy and significantly reduced toxicity and side effects. Our synthesized therapeutic components (pretargeting and drug-carrier) are a first generation of such a system and are applicable to experimental therapy in animal models. Once optimized and validated future translation to clinic should be feasible.

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. Changes/Problems

- (i) There is a slight delay in developing of intratibial prostate cancer models due to the steep learning curve for intratibial injection of cancer cells.
- (ii) While internalization of F(ab')₂ domains of mAb is faster and more efficient than for the intact antibody, the therapeutic effect *in vitro* of mAb is significantly higher. We will explore this interesting results in *in vivo* system using intravital microscopy to validate intracellular delivery of therapeutic compounds.

6. Products

Publications:

Research materials (please see Accomplishments)

1. S Hapuarachchige, C Huang, J Flores, C Barinka, D Artemov. "MR and optical imaging study to evaluate effects on bioorthogonal click therapy in prostate cancer mouse models", ISMRM Annual Meeting Proceedings, 3593, Paris (2018).
2. D. Artemov "Treatment Monitoring Using Smart MRI Agents", Educational Course Theranostics & Novel Molecular Probes, ISMRM Annual Meeting, Paris (2018).

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name:	Dmitri Artemov, Ph.D.	Sean Lupold, Ph.D.	Sudath Hapuarachchige, Ph.D.	Colin Huang, B.S.	Martin Pomper, M.D./Ph.D.
Project Role:	PI	Co-investigator	Research Associate	Technician	Consultant
Research Identifier:					
Nearest person month worked:	3	0.6	6	6	
Contribution to the project:	<i>In vivo</i> imaging, data interpretation, supervising personal and insuring stable workflow	Providing prostate cancer cells, interpreting <i>in vitro</i> results	Chemical synthesis and characterization of therapeutic components, <i>in vivo</i> imaging	Maintaining cell cultures, performing routine tests, confocal microscopy and image processing	Consultancy in PSMA targeting and imaging
Funding support:	NIH/NCI	NIH/NCI	NIH/NCI	NIH/NCI	NIH/NCI

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Nothing to report

8. Special Reporting Requirements

Nothing to report

9. Appendices

ISMARM abstract

Title:

MR/Optical imaging study to evaluate effects on bioorthogonal click therapy in prostate cancer mouse models.

Authors:

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Synopsis (100 words):

Click therapy is a new therapeutic strategy for cancers minimizing the systemic toxicity and enhancing the therapeutic efficacy. This strategy is feasible in slow internalizing targeted cell surface receptors even after the binding of ligands giving sufficient time for blood clearance to unbound excess pretargeting component. Highly vascular and leaky late-stage tumors show high extravasation and EPR effect. In this study, we used PSMA-positive PC3-PIP and PC3-Flu (negative control) cells and mouse tumor models to evaluate internalization of and EPR factors. We observed that internalization of 5D3 antibody is too fast and has to reduce the internalization rate for click therapy. When the tumor is late-stage, 5D3 antibody can effectively extravasate and retain in the tumor microenvironment leading to the cluster formation with drug delivery component in tumor microenvironment.

Abstract: (750 words)**Introduction:**

Prostate cancer (PCa) is the second most frequently occurring cancer in men after lung cancer, and approximately 900,000 new cases of PCa are detected each year.¹ Initial treatment for localized disease is typically a combination of surgery and/or radiation therapy. Prostate-specific

membrane antigen (PSMA) is the clinically validated marker for prostate cancer; it is expressed on the surface of malignant PCa cells and currently is being explored as a target for specific imaging and therapy of PCa.² PSMA is an integral membrane glycoprotein that contains a short amino-terminal cytoplasmic domain, a single transmembrane domain, and an extracellular protease domain. PSMA has a molecular weight of approximately 100 kDa. PSMA is not shed into the circulation to any significant level. PSMA is extensively expressed by prostate adenocarcinomas at levels that greatly exceed those for normal prostate or other tissues, both in primary tumors and in bone and lymph metastases.³ PSMA is also expressed in the vasculature of multiple non-prostate tumors.⁴ Antibody-drug conjugates (ADC) are highly specific compounds that have a high affinity for the target cell expressing the receptor, and can deliver extremely cytotoxic drug molecules (that often cannot be used as a free drug due to severe systemic toxicity) directly to the target cell. Bioorthogonal click therapy is recently developed drug delivery strategy based on the sequential delivery of pretargeting target-specific antibody followed by the drug-loaded nano-carrier which will react each other by bioorthogonal click chemistry.^{5,6} However, the pharmacokinetics of the antibody cellular internalization and EPR effect play a vital role when applying click therapy prostate cancer models. In this study, we evaluated the internalization of antibody in PC3-PIP cells and extravasation of drug-loaded nanocarrier by EPR effects.

Methods:

The anti-PSMA 5D3 antibody was conjugated with *trans*-cyclooctene (TCO), labeled with a suitable fluorophore for *in vitro* and *in vivo* optical imaging and used as pretargeting component (Figure 1). As delivery component, albumin (HSA, Alb) was conjugated with pegylated tetrazine (Peg₄-Tt) and labeled with a suitable fluorophores for *in vitro* and *in vivo* imaging (Figure 1). In *in vitro* optical imaging, HER2(+) BT-474 and PSMA(+) PC3-PIP cells were treated with anti-trastuzumab-based Tz(TCO)₆(AF488)₂ and anti-PSMA-based 5D3(TCO)₆(AF-488)₂ (20µg/mL for 20 min at 37°C) respectively and fixed at 15, 30 min, 1, 6, 24 h for Zeiss LSM510-Meta confocal fluorescence imaging. In vivo optical imaging, athymic nude male mice with PC3-PIP and -Flu dual tumors were administered with 5D3(TCO)₆(CF-680)₂ (10mg/kg) and allowed for pretargeting the tumors and clearance of the excess in the blood for approximately 8 h. Then, delivery component, Alb(Peg₄-Tt)₁₀(CF-750)₂ or Alb(CF750)₂ (control) (100mg/kg) was

administered and continued the imaging for 48 h. The same set of mice were used for T1-weighted MR images of the tumors after the i.v. administration of gadodiamide (Omniscan™, 0.2 mmol/kg) using a horizontal bore, preclinical 9.4T Bruker Biospec MR spectrometer.

Results:

In vitro confocal images of Her2(+) BT-474 and PSMA(+) PC3-PIP cells treated with Tz(TCO)₆(AF488)₂ and 5D3(TCO)₆(AF488)₂ respectively were taken fixing at selected time points (figure 2). In vivo optical images of mice treated with 5D3(TCO)₆(CF-680)₂ followed by Alb(Peg₄-Tt)₁₀(CF-750)₂ were taken up to 48 h of post-injection (Figure 3). The graphs in figure 3 shows the uptake of pretargeting component 5D₃(TCO)₆(CF-680)₂ by tumors (B) and relative retention of delivery component, Alb(Peg₄-Tt)₁₀(CF750)₂ or Alb(CF750)₂ in the tumors (C). Figure 4 shows representative T1-weighted FLASH MR images of click treated, 5D3(TCO)₆(CF680)₂ followed by the administration of Alb(Peg₄-Tt)₁₀(CF750)₂ and click-control, 5D3(TCO)₆(CF680)₂ followed by administration of Alb(CF750)₂ mice. Subtract images show the enhancement of the contrast in the tumor microenvironment after the 30 min post-injection of gadodiamide (Omniscan™). In vivo MRI study revealed that late-stage tumors have rapid extravasation in the peripheral of the tumor. The Figure 3 also shows the subtracted images of tumors before and after administration of contrast agents.

Discussion:

Rapid internalization of pretargeting antibody diminishes the click reaction between two components on the cell surface. Unlike trastuzumab, anti-PSMA 5D3 antibody was internalized within 15-30 min in vitro conditions. In vivo optical images prove early-stage tumors has low extravasation tendency hence the pretargeting component can be populated on targeted cells. It can enhance the feasibility and therapeutic efficacy of click therapy on early-stage tumors. However, late-stage tumors have complex vasculature and high leakiness boosting the extravasation of nano-sized pretargeting and delivery component leading the click conjugation and cluster formation in the tumor microenvironment.

Conclusion:

References:

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5. Hapuarachchige S, Zhu W, Kato Y, Bioorthogonal, two-component delivery systems based on antibody and drug-loaded nanocarriers for enhanced internalization of nanotherapeutics. *Biomaterials* 2014;35(7):2346-2354.
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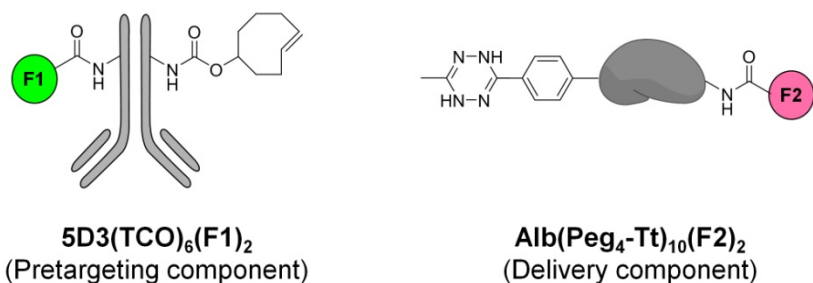


Figure 1. 5D3 antibody-based pretargeting component and HSA-based delivery component. (F1= AF-488 or CF-680 and F2= CF750).

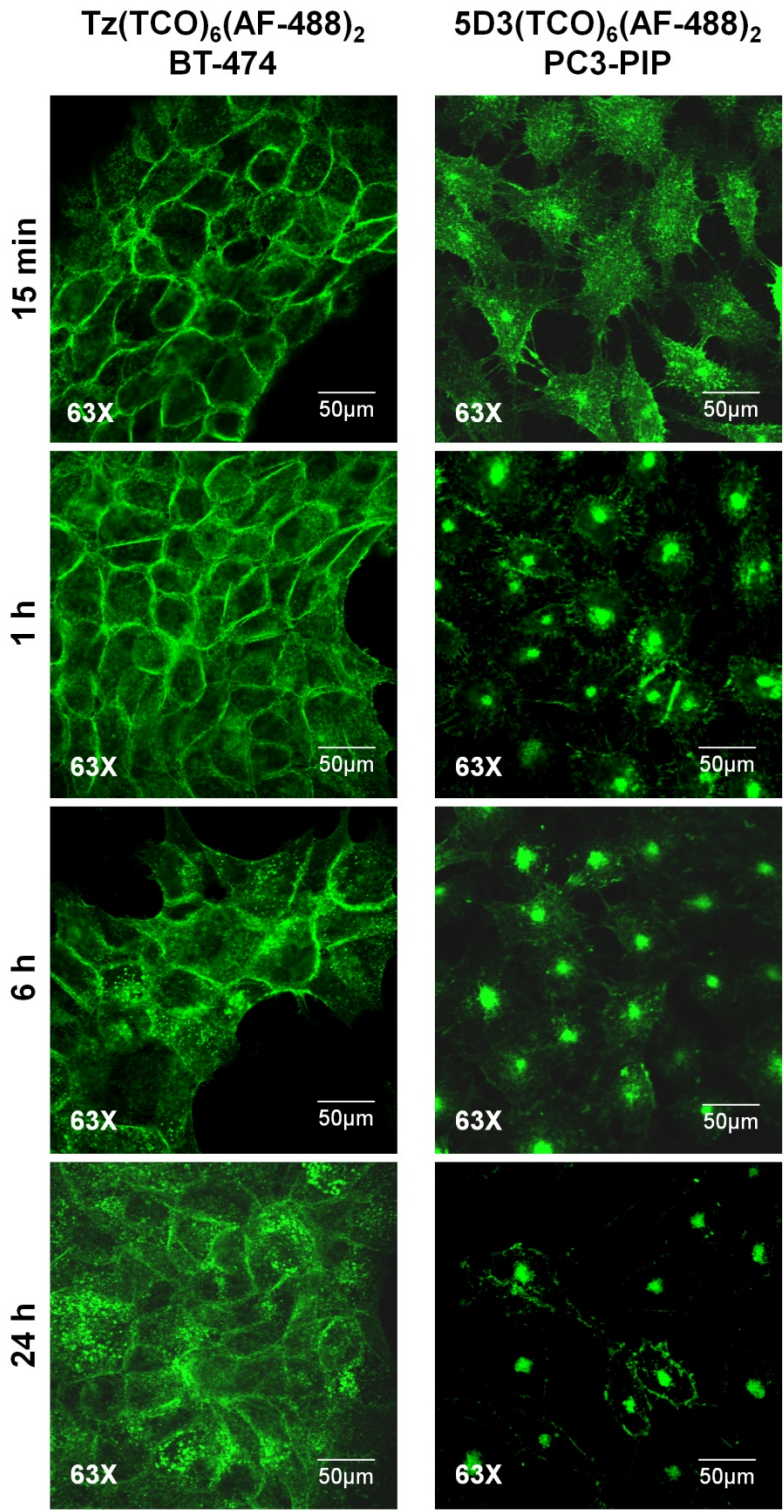


Figure 2. Comparison trastuzumab and 5D3 antibody-based pretargeting components in BT-474 and PC3-PIP cells.

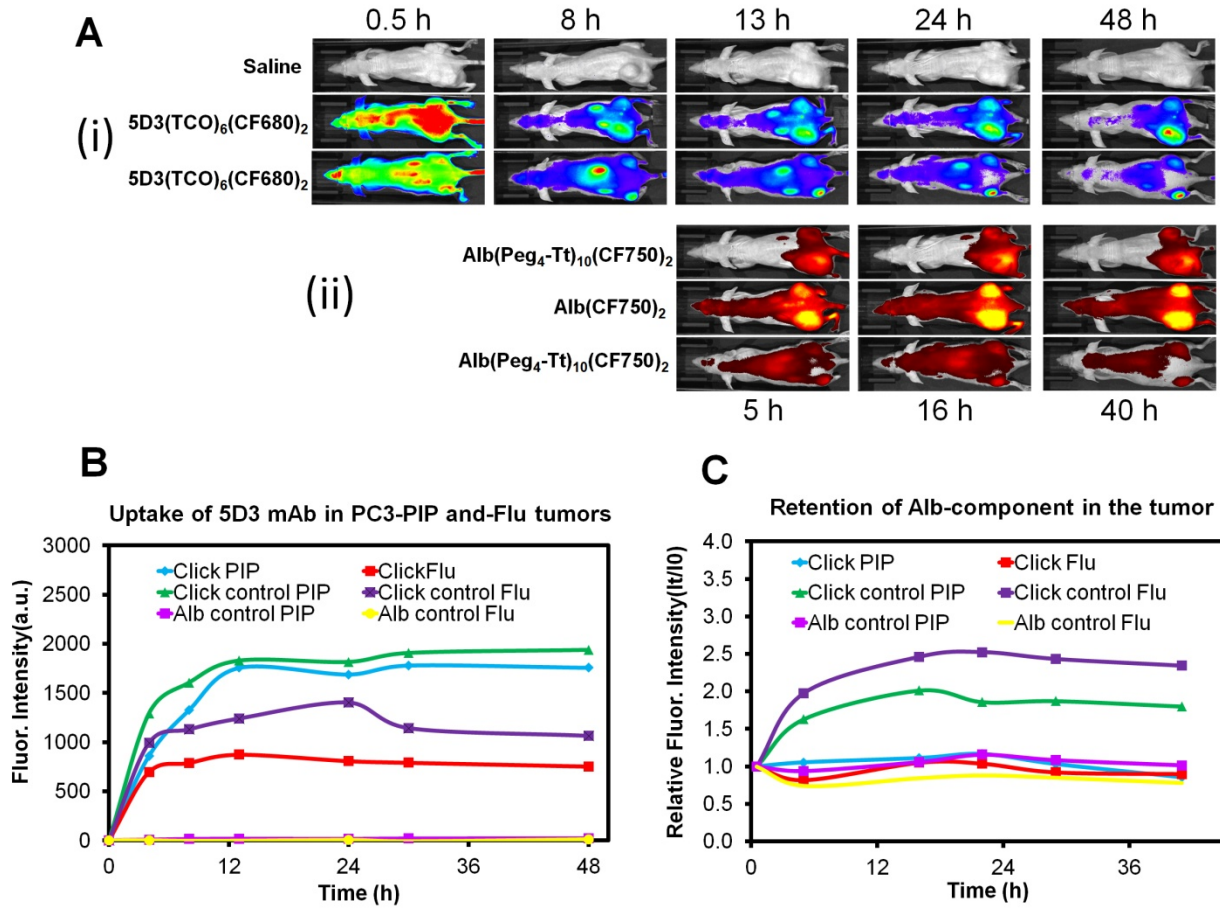


Figure 3. In vivo optical imaging study of two-component drug delivery in PC3-PIP and PC3-Flu dual tumor xenograft mouse models. (A) Tumor uptake of pretargeting 5D3(TCO)₆(CF680)₂ (panel-a) and retention of delivery component in the tumors (panel-b) at selected time points. (B) Pharmacokinetics of pretargeting component uptake by PC3-PIP and -Flu tumor models. (C) Relative retention of delivery component in tumors.

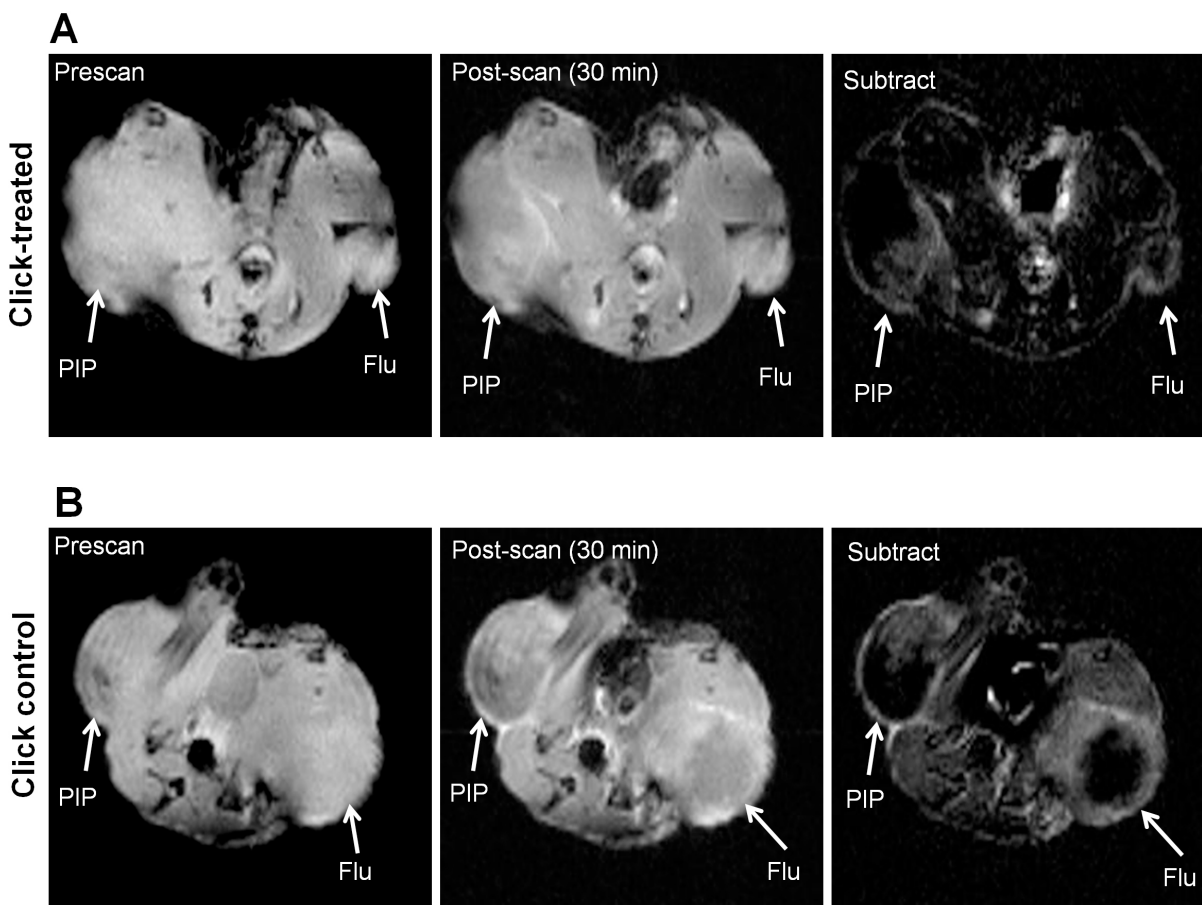


Figure 4. Representative T1-weighted FLASH MR images of click treated, $5D3(TCO)_6(CF680)_2$ followed by the administration of $Alb(Peg_4-Tt)_{10}(CF750)_2$ and click-control, $5D3(TCO)_6(CF680)_2$ followed by administration of $Alb(CF750)_2$ mice. Subtract images show the enhancement of the contrast in the tumor microenvironment after the 30 min post-injection of gadodiamide (OmniscanTM).