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Development of Inhibitor-Directed Enzyme Prodrug Therapy (IDEPT) for Prostate Cancer

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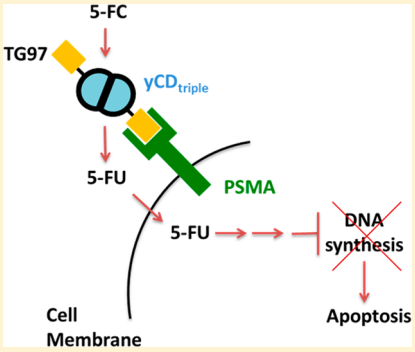
Supporting Information

ABSTRACT: Prostate cancer (PCa) is the second most common cause of cancer death among American men after lung cancer. Unfortunately, current therapies do not provide effective treatments for patients with advanced, metastatic, or hormone refractory disease. Therefore, we seek to generate therapeutic agents for a novel PCa treatment strategy by delivering a suicide enzyme (yCD_{triple}) to a cell membrane bound biomarker found on PCa cells (prostate-specific membrane antigen (PSMA)). This approach has resulted in a new PCa treatment strategy reported here as inhibitor-directed enzyme prodrug therapy (IDEPT). The therapeutic agents described were generated using a click chemistry reaction between the unnatural amino acid (*p*-azidophenylalanine (*p*AzF)) incorporated into yCD_{triple} and the dibenzylcyclooctyne moiety of our PSMA targeting agent (DBCO-PEG_4-AH_2-TG97). After characterization of the therapeutic agents, we demonstrate significant PCa cell killing of PSMA-positive cells. Importantly, we demonstrate that this click chemistry approach can be used to efficiently couple a therapeutic protein to a targeting agent and may be applicable to the ablation of other types of cancers and/or malignancies.



INTRODUCTION

Advances in unnatural amino acid incorporation into proteins have enabled the convenient functionalization of relevant proteins to a broad array of chemical and biochemical motifs utilizing azide−alkyne cycloaddition, or "click" chemistry.[1−4] This flexible strategy has been used for the *in vivo* fluorescent labeling of target proteins[5] and to generate therapeutically relevant agents such as bispecific antibodies.[6] In addition, click chemistry approaches have been applied to fluorescently label oligonucleotides,[7] to image modified cell-surface glycans,[8] and to target nanoparticles to cancer cells.[9] Work by Agard *et al.* demonstrating that strain-promoted [3 + 2] azide−alkyne cycloaddition (strain-promoted click chemistry) can be effectively used to couple biological molecules,[10] circumventing possible copper toxicity associated with copper(I)-catalyzed [3 + 2] cycloaddition between azides and alkynes, has expanded this already versatile click chemistry reaction. In order to improve cancer therapeutics and enlarge the arsenal against human diseases, we utilized strain-promoted click chemistry for protein−small molecule coupling. Our objective was to develop a targeted therapeutic enzyme platform specific for tumor cells (Figure 1). To this end, we report the site-specific incorporation of *p*-azidophenylalanine (*p*AzF) to outfit the suicide enzyme yeast cytosine deaminase (yCD) with a targeting molecule possessing selective and high affinity for the prostate tumor biomarker prostate-specific membrane antigen (PSMA).

The suicide enzyme yCD is responsible for the hydrolytic deamination of the nontoxic prodrug 5-fluorocytosine (5-FC) to the toxic chemotherapeutic agent 5-fluorouracil (5-FU).[11] Intracellularly, 5-FU is further converted into toxic antimetabolites that inhibit DNA synthesis and RNA function, resulting in apoptosis. In targeted therapeutic applications, cancer cells lacking the suicide enzyme also undergo apoptosis due to the phenomenon known as the bystander effect, whereby toxic antimetabolites produced by suicide enzyme containing cells diffuse to surrounding tumor cells. Since 5-FU is capable of nonfacilitated diffusion across cellular membranes, the CD/5-FC strategy generates a potent bystander effect.[12−14] This is particularly important for ablating tumors consisting of heterogeneous cancer cells that differentially express surface biomarkers. The targeting of yCD to cancer cells followed by prodrug administration thus results in a potent, localized cancer cell killing effect with minimal systemic toxicity. Yeast CD is a highly characterized enzyme that has been utilized in cancer therapy as part of larger fusion proteins,[15,16] DNA vectors,[17] and antibodies,[18] and was selected for use in phase I clinical trials for PCa.[19] In addition, our lab has generated a thermostable variant of yCD (yCD_{triple}) that displays an improved half-life ($t_{1/2}$ at 50 °C = 117 h) over the wild-type enzyme ($t_{1/2}$ at 50 °C = 4 h).[20,21] In support of using this

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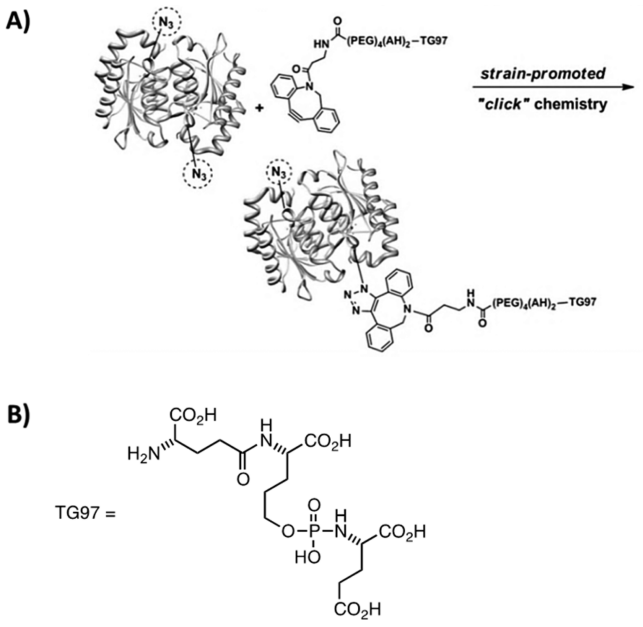

Figure 1. Click chemistry strategy. (A) Strain promoted [3 + 2] azide−alkyne cycloaddition between a therapeutic enzyme, yCD_{triple}, and a phosphoramidate inhibitor of PSMA, DBCO-PEG_4-AH_2-TG97 (Supporting Information Figure S1), to generate a stable triazole (IDEPT agent). Please note that both azide groups in yCD_{triple} are available for strain-promoted click chemistry. (B) TG97 (PSMA targeting agent) structure. Images were generated using the previously solved crystal structure of yCD_{triple} (Protein Data Bank accession code 1YSB).

enzyme for cancer therapeutics, a retroviral replicating vector encoding yCD_{triple}, in combination with 5-FC administration, is currently being utilized in a phase I/II clinical trial for the treatment of recurrent malignant glioma (registration number: NCT01156584, www.clinicaltrials.gov).[22] Since yCD_{triple} displays an increased half-life that would allow for enzymatic activity after circulation in the bloodstream, and is relatively small (35 kDa homodimer[23,24]) and flexible, it was selected as the therapeutic enzyme component of the PCa therapeutic.

The enzyme-biomarker prostate-specific membrane antigen (PSMA) is an ideal PCa target. This type II membrane glycoprotein is upregulated in PCa,[25,26] after androgen-deprivation therapy,[27] and is expressed in the neovasculature of most solid cancers.[28,29] Furthermore, PSMA is an active target for the delivery of PCa imaging and therapeutic agents.[30−34] Previously, we demonstrated that phosphoramidate-based inhibitors of PSMA bind to PSMA in a pseudoirreversibe manner, and consequently are potent inhibitors of PSMA enzymatic (carboxypeptidase) activity.[35] In addition to their selective tumor uptake,[33] these inhibitors have been demonstrated to deliver large molecular and biomolecular payloads to PSMA expressing PCa cells[36,37] that are then internalized via a PSMA-dependent process. Therefore, the utilization of click chemistry to couple yCD_{triple} to a small molecule PSMA inhibitor is expected to generate therapeutic agents capable of targeting prostate tumor cells and evoking cell killing through prodrug activation. As the design of this platform is composed of a suicide enzyme coupled to a potent biomarker inhibitor, this therapy has been coined inhibitor-directed enzyme prodrug therapy, or IDEPT.

RESULTS AND DISCUSSION

To engineer yCD_{triple} for click chemistry, the codon for amino acid position F54 was mutated to allow for *p*AzF incorporation into yCD_{triple}. Incorporation of *p*AzF was carried out using pEVOL technology, specifically pEVOL-*p*AzF, developed by the Schultz laboratory.[1,2] The pEVOL-*p*AzF vector encodes an orthogonal aminoacyl-tRNA synthetase (aaRS) and its cognate tRNA ($tRNA_{CUA}$).[2] The aaRS aminoacylates $tRNA_{CUA}$ with *p*AzF, which is then incorporated into a growing peptide at amber stop codons (UAG).[2] *p*AzF has been successfully incorporated into other proteins using this optimized pEVOL system and other unnatural amino acid incorporation systems similar to pEVOL.[2,38,39] Based on molecular modeling studies that identified surface aromatics previously deemed nonessential for enzyme function,[20] F54 of yCD_{triple} was selected for mutagenesis to an amber nonsense codon for *p*AzF incorporation. Since yCD_{triple} is a homodimer, two *p*AzF molecules are predicted to be present within the final dimeric enzyme, allowing for dual-valent targeting.

To determine if yCD_{triple} with *p*AzF incorporated at position F54 (F54*p*AzF) retains enzymatic activity, a genetic complementation assay was conducted utilizing *Escherichia coli* strain GIA39(DE3), a strain lacking *cod*A (CD) activity. *E. coli* GIA39(DE3) containing a functional CD will grow on cytosine supplemented minimal medium (cytosine as the only source of pyrimidines), since CD deaminates cytosine to uracil. Cells lacking a functional CD will only grow on nonselective minimal medium containing uracil.[40−42] *E. coli* GIA39(DE3) harboring the positive control pET23d:yCD_{triple} grew on cytosine supplemented minimal medium plates (CD selection), while the negative control, *E. coli* GIA39(DE3) containing both the pET23d and pEVOL-*p*AzF plasmids, only grew on nonselective minimal media (Figure 2). *E. coli* GIA39(DE3) harboring the pET23d:yCD_{triple}-F54X plasmid was viable on the nonselective minimal medium plates only, presumably due to protein truncation at position F54 in the absence of the pEVOL-*p*AzF vector and *p*AzF. Importantly, *E. coli* GIA39(DE3) cells harboring both pEVOL-*p*AzF and pET23d:yCD_{triple}-F54X

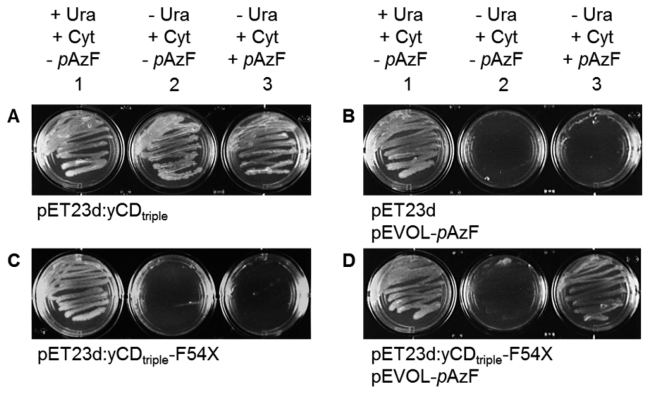

Figure 2. Genetic complementation of the CD deficient *E. coli* strain GIA39(DE3) by yCD_{triple} and yCD_{triple}-F54*p*AzF. Representative images of the various agar plates are shown. (A) Positive control, pET23d:yCD_{triple} (GIA39(DE3)). (B) Negative control, pET23d, pEVOL-*p*AzF (GIA39(DE3)). (C) pEVOL control, pET23d:yCD_{triple}-F54X (GIA39(DE3)). (D) pET23d:yCD_{triple}-F54X, pEVOL-*p*AzF (GIA39(DE3)). Lane 1, nonselective medium containing uracil. Lane 2, CD selection medium lacking uracil. Lane 3, CD selection medium supplemented with *p*AzF (1 mM). F54X, location of the TAG mutation. Cytosine (cyt), uracil (ura), *p*-azidophenylalanine (*p*AzF).

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grew on CD selection plates in the presence of *p*AzF, but were not viable on CD selection plates when *p*AzF was omitted (lanes 2 and 3 in Figure 2). These results suggest that *p*AzF is incorporated via the pEVOL system, that *p*AzF must be present for functional yCD_{triple} to be produced from pET23d:yCD_{triple}-F54X, and that yCD_{triple}-F54*p*AzF retains CD activity due to its ability to confer growth to *E. coli* GIA39(DE3) plated on CD selection medium.

The relationship between yCD_{triple}-F54*p*AzF protein expression and *p*AzF concentration in the expression culture medium was also evaluated to determine if full-length yCD_{triple} containing *p*AzF could be generated and purified. Protein yields not only depend on the presence of *p*AzF in the culture media, but also increase as the concentration of *p*AzF increases (Figure 3). In further support of *p*AzF incorporation into yCD_{triple}

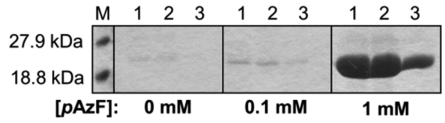

Figure 3. Yeast CD_{triple}-F54*p*AzF protein yields increase as *p*AzF concentration in the culture medium increases. SDS-PAGE analysis of the three protein elutions (lanes 1−3) performed during purification via nickel affinity chromatography. Predicted mass of yCD_{triple}-F54*p*AzF (monomer) is 19.8 kDa. Molecular weight marker (M).

utilizing the pEVOL system, full-length yCD_{triple} could not be purified from expression cultures lacking *p*AzF, suggesting that His-tagged yCD_{triple} is not synthesized without the incorporation of *p*AzF (Figure 3). Roughly 9 mg of yCD_{triple}-F54*p*AzF was obtained from a 500 mL culture using optimized expression conditions.

As discussed previously, click chemistry is a rapid, gentle methodology for coupling complementary molecules. In order to couple yCD_{triple}-F54*p*AzF to the PSMA targeting molecule, the PSMA inhibitor TG97 (Figure 1B) was outfitted with a strained alkyne capable of reacting specifically with azide containing proteins. The resulting molecule, DBCO-PEG_4-AH_2-TG97 (Figure 1A and Supporting Information Figure S1), is capable of targeting PSMA through the inhibitor core (TG97) and delivering biomolecular payloads coupled to the molecule through click chemistry strategies. Furthermore, this molecule meets the additional requirement of containing the equivalent of a PEG_8 spacer between the biomolecular payload and PSMA binding motif (TG97). A spacer of this length ensures that the biomolecular payload is sufficiently removed from the PSMA surface to allow the PSMA entrance lid to close over the inhibitor core after binding, while avoiding unproductive interactions between the payload and PSMA.[36,43] This class of phosphoramidate-based PSMA inhibitors has also demonstrated stability and specific tumor targeting *in vivo*, as exhibited by low nonspecific binding and a high tumor-to-blood ratio.[33]

An array of click chemistry conditions were investigated to optimize the coupling of the suicide enzyme and PSMA targeting agent. Yeast CD_{triple}-F54*p*AzF and DBCO-PEG_4-AH_2-TG97 were mixed at various molar ratios (1:5, 1:10, 1:25, 1:50, 1:100, 1:200, 1:500 (protein:DBCO-PEG_4-AH_2-TG97)), tested at a range of biologically relevant temperatures (rt, 37, 45, 50 °C), and analyzed at several reaction times (15, 30, 60, 90, 120 min) (Figure 4). The click chemistry reactions were analyzed via SDS-PAGE, since the clicked protein could easily be

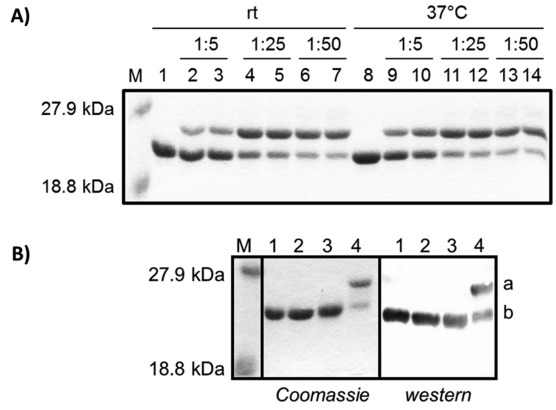

Figure 4. Click chemistry analysis. (A) Click chemistry optimization. Lanes 1 and 8, yCD_{triple}-F54*p*AzF (19.9 kDa); lanes 2−7 and 9−14, clicked yCD_{triple}-F54*p*AzF (upper band). Lanes 1, 3, 5, 7, 8, 10, 12, and 14, 90 min reaction; lanes 2, 4, 6, 9, 11, and 13, 30 min reaction. Molar ratios (1:5, 1:25, 1:50) and temperatures (rt, 37 °C) are indicated. Samples were resolved using SDS-PAGE and stained with Coomassie Brilliant Blue. Molecular weight marker (M). (B) Coupling yCD_{triple}-F54*p*AzF with DBCO-PEG_4-AH_2-TG97 via click chemistry. Yeast CD_{triple} (lanes 1 and 2) and yCD_{triple}-F54*p*AzF (lanes 3 and 4) were combined with DBCO-PEG_4-AH_2-TG97 (lanes 2 and 4) or PBS (control, lanes 1 and 3) using the 1:50 reaction discussed, and analyzed by SDS-PAGE. Western blot analysis utilized polyclonal antiserum to yCD. yCD_{triple} and yCD_{triple}-F54*p*AzF predicted monomer mass: 19.8 kDa. DBCO-PEG_4-AH_2-TG97 mass: 1276.32 Da. Noncoupled protein (b), DBCO-PEG_4-AH_2-TG97 coupled protein (a). Molecular weight marker (M).

identified by an observed shift in migration rate (band identities were confirmed with mass spectrometry analysis, Figure 5). Click chemistry efficiency was maximal at the 1:50 ratio, with a reaction time of 30−90 min. Elevated temperatures did not significantly influence click chemistry at molar ratios greater than 1:25. Densitometry analysis of the Coomassie Brilliant Blue stained SDS-PAGE gel indicates that the integrated optical density (IOD) value of yCD_{triple}-F54*p*AzF labeled with DBCO-PEG_4-AH_2-TG97 using a 1:50 molar ratio at rt for 90 min is 75% of the total IOD value for the lane, and this condition was selected for all future analyses (Figure 4). This is similar to the efficiency of click chemistry obtained with other proteins engineered to contain unnatural amino acids.[44,45] The control reaction, yCD_{triple} (no *p*AzF incorporated) reacted with DBCO-PEG_4-AH_2-TG97, did not demonstrate the shift in migration at the 1:50 ratio, supporting the hypothesis that the introduced azide is the site of modification (Figure 4). Notably, no differences in the click chemistry efficiencies using yCD_{triple}-F54*p*AzF purified from rich media containing 1, 2, or 3 mM *p*AzF were observed. This suggests that the concentration of *p*AzF in the culture medium is the limiting factor for yCD_{triple}-F54*p*AzF yields, but does not influence the efficiency of click chemistry of the purified enzyme, further supporting the high fidelity of the pEVOL system for *p*AzF incorporation. It should be noted that click chemistry optimization may need to be performed for new combinations of azide-containing proteins and DBCO-containing PSMA targeting agents, due to various charge and structural interactions that may influence the click chemistry reaction.

MALDI mass spectrometry (MS) analysis was conducted to verify the location of protein modification, with respect to both *p*AzF incorporation and click chemistry conjugation. As noted, yCD_{triple} was engineered for *p*AzF incorporation at amino acid

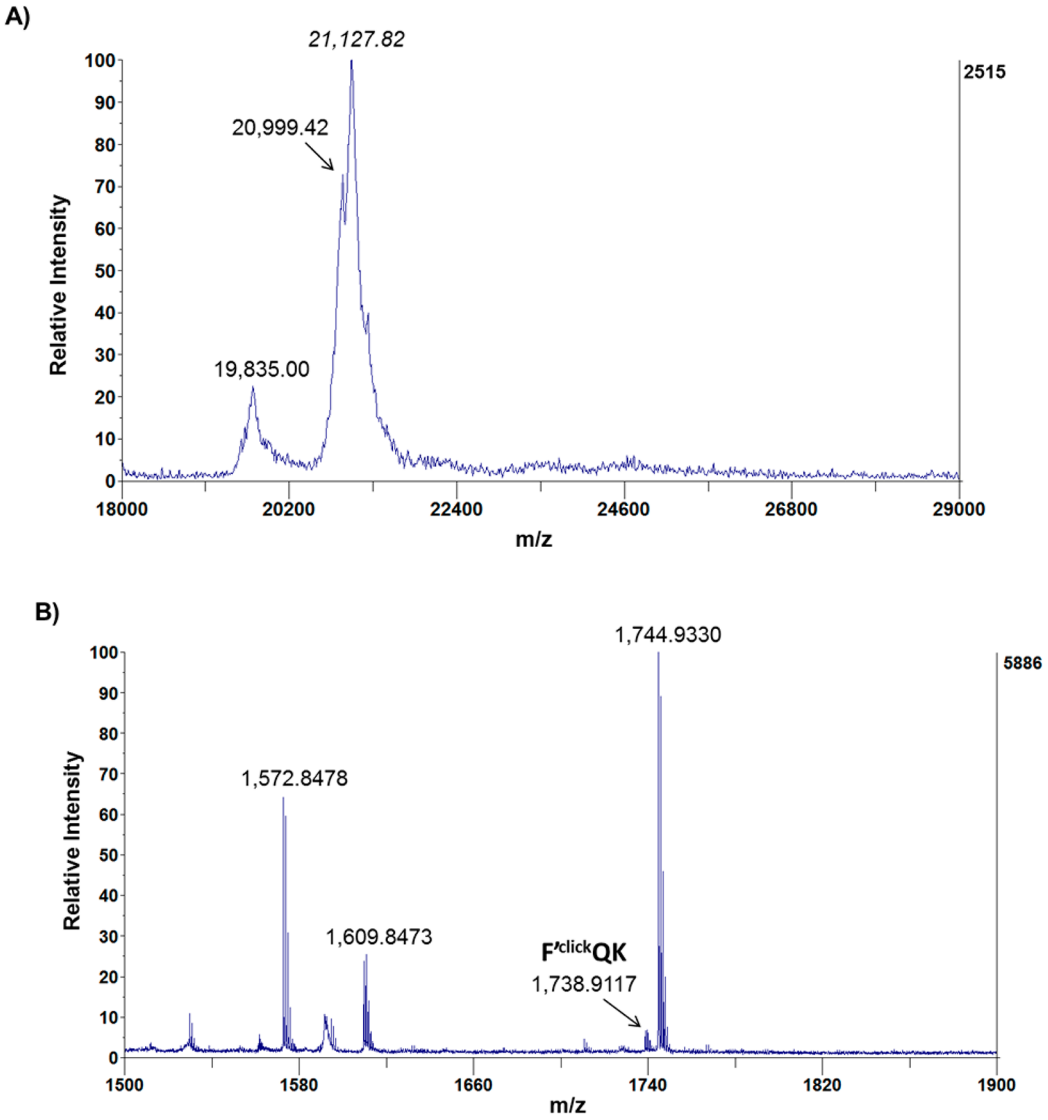

Figure 5. Mass spectrometry analyses. (A) Analysis of the 1:50 click chemistry reaction between yCD$_{triple}$-F54*p*AzF and DBCO-PEG$_4$-AH$_2$-TG97. A peak corresponding to yCD$_{triple}$-F54*p*AzF was seen at *m*/*z* 19 835.00 (expected average mass: 19 848 Da), and the DBCO-PEG$_4$-AH$_2$-TG97 modified yCD$_{triple}$-F54*p*AzF peak was seen at 21 127.82 (predicted mass: 21 124.32 Da), noted in italics. (B) Analysis of a tryptic digest of the click chemistry reaction between yCD$_{triple}$-F54*p*AzF and DBCO-PEG$_4$-AH$_2$-TG97.

position F54. After tryptic digest, amino acid F54 is part of the tryptic peptide FQK (residues 54−56). Therefore, a tryptic digest of yCD$_{triple}$ was analyzed to identify the FQK tripeptide, and a tryptic digest of yCD$_{triple}$-F54*p*AzF was analyzed to identify peaks corresponding to the FQK and F′QK (F′ = *p*AzF) tripeptides. As predicted, the FQK tripeptide was seen at *m*/*z* 422.2294 (calculated mass: 422.22) for the tryptic digest of yCD$_{triple}$, the control protein that does not contain *p*AzF, and confirmed via MS/MS analysis. The tryptic peptide F′QK was not seen for yCD$_{triple}$-F54*p*AzF, and the absence of this peak may be due to the sensitivity of *p*AzF to the laser pulse (355 nm) used in the MALDI procedure. Importantly, a peak corresponding to the wild-type FQK tripeptide (calculated mass: 422.22) could not be found for the tryptic digest of yCD$_{triple}$-F54*p*AzF. This suggests a high fidelity of *p*AzF incorporation into yCD$_{triple}$ when generating yCD$_{triple}$-F54*p*AzF, since the incorporation of phenylalanine at amino acid position 54 could not be identified with the mass spectrometry results.

To confirm that click chemistry occurred at position F54, yCD$_{triple}$-F54*p*AzF modified by DBCO-PEG$_4$-AH$_2$-TG97 was subjected to a tryptic digest followed by mass spectrometry analysis. An ion corresponding to F′^{click}QK (F′^{click} = *p*AzF modified by DBCO-PEG$_4$-AH$_2$-TG97) was found at *m*/*z* 1738.9117 (calculated mass: 1738.795 Da) (Figure 5). In further support of the click chemistry reaction occurring at amino acid 54, a peak at *m*/*z* 1609.8473 was identified, corresponding to F′^{click}QK after hydrolysis of the terminal glutamate of TG97 (Figure 5). TG97 glutamate hydrolysis was also seen during whole protein click chemistry analysis (peak at *m*/*z* 20 999.42, Figure 5).

To assess the PSMA binding capabilities of this first generation IDEPT agent (yCD$_{triple}$-F54*p*AzF modified by DBCO-PEG$_4$-AH$_2$-TG97), an inhibition (IC$_{50}$) study was performed with PSMA purified from LNCaP cells.[46] The IC$_{50}$ values indicate the concentration of a molecule needed to inhibit PSMA enzymatic activity by 50%. The IDEPT agent (IC$_{50}$ = 41 nM) and parent compounds (DBCO-PEG$_4$-AH$_2$-

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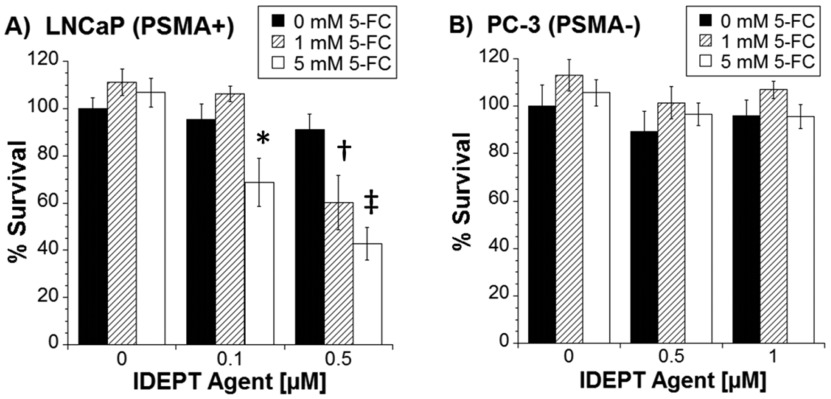

Figure 6. PCa survival after IDEPT. LNCaP (A), PC-3 (B) cells. Each bar (mean of experiments, $n = 3$, performed with 6 replicates) is expressed as a percentage of the value of the untreated PCa cells, which were defined as 100% viable. The percent SEM is indicated, and statistical significance was determined using Student's *t*-test with an α level of 0.05. (*), (†), and (‡) denote statistical significance when compared to the corresponding no drug control.

TG97, IC_{50} = 2.83 nM and TG97, IC_{50} = 27 nM) all exhibit low, nanomolar IC_{50} values indicative of PSMA inhibition. PSMA inhibition by noncoupled yCD_{triple}-F54*p*AzF could not be detected within 3 orders of magnitude of the IC_{50} of the IDEPT agent. These data suggest that the IDEPT agent demonstrates a high affinity for PSMA active site binding.

To demonstrate the functionality and therapeutic relevance of the IDEPT agent, *in vitro* cytotoxicity assays were performed. Human PCa cell lines, LNCaP (PSMA-positive) and PC-3 (PSMA-negative), were incubated with the IDEPT agent for 2.5 h, and then washed three times to remove any unbound IDEPT agent. The nontoxic prodrug 5-FC was added to the cells at 1 or 5 mM, and cell viability was assessed using Alamar Blue after incubation for 7 days (Figure 6). To avoid bone marrow depression and hepatotoxicity in patients receiving 5-FC, the prodrug plasma level must not exceed 100 mg/L.[47] To roughly correlate with this 5-FC plasma level, we used 1 mM 5-FC in our studies. Since it is difficult to mimic the variables associated with a tumor microenvironment in *in vitro* settings, such as changes in clearance, plasma levels, and tumor concentrations, and since multiple doses of 5-FC are utilized in the clinic,[19,47,48] the 5 mM 5-FC dose was selected to represent the accumulation of 5-FC in the tumor microenvironment that may occur after multiple 5-FC doses.

In the PSMA-positive LNCaP cells, an enhanced cell killing effect was observed as the concentration of IDEPT agent and 5-FC increased. In contrast, this correlation was not observed in PC-3 cells (note the higher concentration of IDEPT agent used), suggesting that the IDEPT agent was unable to target the PSMA-negative cell line. Furthermore, the dose of TG97 used is nontoxic, and TG97 in combination with 5-FC does not produce a cell killing effect (Supporting Information Figure S2). Notably, 0.5 μM IDEPT agent combined with 5 mM 5-FC resulted in 57% cell death. This cell killing mechanism is predicted to result from surface targeted IDEPT agents producing 5-FU after 5-FC administration via yCD_{triple} catalysis.

As an initial benchmark, we sought to achieve a comparable level of prodrug-mediated cell killing with a protein targeting strategy (IDEPT), to that achieved with cells stably transfected with yCD_{triple}. Previous experiments from our lab that evaluated yCD_{triple}-5FC mediated cell killing in stably transfected rat C6 glioma cells[21] achieved 45% cell killing in the presence of 5 mM 5-FC. Fifty-seven percent cell death was observed when 0.5 μM IDEPT agent was administered to LNCaP cells followed by a 5 mM 5-FC treatment. We believe that the IDEPT/5-FC treatment outcome in these initial experiments is similar, if not slightly better, than the 5-FC-mediated cell killing in stably transfected C6 glioma cells, and thus serves as a comparison to other treatment modalities. Further improvements to the therapeutic efficacy may involve multiple IDEPT agent and 5-FC administrations, as well as utilizing the IDEPT strategy to augment other PCa treatment strategies.

CONCLUSION

This proof-of-concept study represents a novel contribution to the development of targeted enzyme therapeutics for the treatment of PCa. Our novel IDEPT agent displays cancer cell targeting capabilities and enzymatic activity, suggesting that click chemistry reactions can be employed not only to generate individualized, combinatorial, curative treatment strategies for advanced PCa, but also for the development of enhanced therapeutics that may improve the current standard of care used to treat other cancers and diseases.

EXPERIMENTAL PROCEDURES

General Materials and Methods. Oligonucleotides used for yCD_{triple} mutagenesis experiments were obtained from Integrated DNA Technologies (Coralville, IA). Restriction endonucleases were purchased from New England Biolabs (Ipswich, MA). The cytosine deaminase (CD) and orotidine 5′-phosphate decarboxylase deficient *Escherichia coli* strain GIA39 (*thr*-1 *leu*B6(AM) *fhu*A21 *cod*A1 *lac*Y1 *tsx*-95 *gln*V44-(AS) λ^- *dad*X3 *pyr*F101 *his*-108 *arg*G6 *ilv*A634 *thi*E1 *deo*C1 *glt*-15) was obtained from the *E. coli* Genetic Stock Center (CGSC #5594) and was lysogenized with λDE3 according to the manufacturer's directions (Novagen (now EMD Millipore) Billerica, MA). The derived strain, GIA39(DE3), was used in genetic complementation studies and for protein purification.[41] Photolysis of the aryl-azide of *p*-azido-L-phenylalanine (*p*AzF) was limited by conducting relevant preparations and experiments under yellow light. LNCaP and PC-3 human prostate cancer cell lines were obtained from the American Type Culture Collection. All reagents were purchased from Sigma-Aldrich (St. Louis, MO) or JT Baker (Avantor Performance Materials, Center Valley, PA) unless otherwise indicated.

Construction of yCD_{triple} Vectors. The thermostable yCD, "yCD_{triple}", previously designed and generated via computational design, was excised from pETHT:yCD_{triple}

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using *Nco*I and *Hin*DIII restriction endonucleases, and subcloned into *Nco*I/*Hin*DIII-digested pET23d vector DNA (Novagen (now EMD Millipore) Billerica, MA)).[20,21] Oligonucleotides MB713 (5′-GAAGATATTGGTGAGGTAGAGC-ACGCAGCAAGC-3′) and MB714 (5′-GCTTGCTGCGTG-CTCTACCTCACCAATATCTTC-3′) were used to insert a single nucleotide within the stop codon of yCD_{triple} using QuikChange II Site-Directed Mutagenesis (Agilent Technologies, Santa Clara, CA). The subsequent frame-shift mutation at the yCD_{triple} stop codon allowed for fusion of the pET23d encoded 6× His tag to the C-terminus of yCD_{triple}. The resulting plasmid, pET23d:yCD_{triple}, was confirmed by DNA sequencing.

To generate pET23d:yCD_{triple}-F54X, QuikChange II Site-Directed Mutagenesis (using oligonucleotides MB717 5′-GGTCACAACATGAGATAGCAAAAGGGATCCGC-3′ and MB718 5′-GCGGATCCCTTTTGCTATCTCATGTTGTGA-CC-3′) was used to mutate the phenylalanine 54 codon of yCD_{triple} (in pET23d:yCD_{triple}) to an amber stop codon, "F54X". The resulting plasmid was confirmed by DNA sequencing.

Genetic Complementation in *E. coli* GIA39(DE3). Genetic complementation studies were performed as previously described for the selection of functional CD mutants, with the modifications outlined below.[21,41] The pET23d, pET23-d:yCD_{triple}, pET23d:yCD_{triple}-F54X, and pEVOL-*p*AzF vectors were used to transform *E. coli* GIA39(DE3). Isolated GIA39(DE3) transformants were streaked from minimal medium containing uracil onto minimal medium containing uracil or cytosine. Carbenicillin at 50 μg/mL ($carb^{50}$, Research Products International Corp., Mt. Prospect, IL) was included to select for transformants harboring pET23d plasmids. Chloramphenicol at 100 μg/mL (cam^{100}) was included to select for transformants harboring the pEVOL-*p*AzF plasmid. Where indicated, 1 mM *p*AzF (final) was added to cytosine containing minimal media.

Protein Expression and Purification. Yeast CD_{triple}-F54*p*AzF expression in the presence of 0, 0.1, or 1 mM *p*AzF was performed as previously described with the following minor modifications.[2] *E. coli* GIA39(DE3) cells harboring the pET23d:yCD_{triple}-F54X and pEVOL-*p*AzF plasmids were grown in 500 mL of 2 × YT $carb^{50}$ cam^{100} medium (2 × YT (1 L) contained 16 g tryptone, 10 g yeast extract, and 5 g NaCl) at 37 °C with constant shaking at 225 rpm until an optical density at 600 nm (OD_{600}) between 0.85 and 0.9 was reached. After reaching an OD_{600} within this range, 75 mL of the culture was added to 3 flasks. To each flask, 25 mL of *p*AzF (dissolved in 2 × YT $carb^{50}$ cam^{100}) or 2 × YT $carb^{50}$ cam^{100} was added, such that the final *p*AzF concentrations were 0, 0.1, or 1 mM. Protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) and L-arabinose to final concentrations of 1 mM and 0.02%, respectively, for 14 h at 30 °C with constant shaking at 225 rpm. The 100 mL expression cultures were harvested by centrifugation at 4000 × *g* for 30 min and the cell pellets were frozen at −80 °C. Protein purification was completed using the nickel-nitrilotriacetic acid (Ni-NTA) Spin Kit (Qiagen, Valencia, CA) with the following modifications: Cells were resuspended in 2 mL of lysis buffer supplemented with lysozyme (1 mg/mL) and Halt Protease Inhibitor Cocktail (1×, EDTA-free, Thermo Scientific, Rockford, IL), four washes (wash buffer contained 30 mM imidazole) were performed, and the protein was eluted five times with 150 μL of elution buffer. Protein purification was analyzed using 15% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) gels under reducing conditions followed by Coomassie Brilliant Blue staining. Yeast CD_{triple}-*p*AzF was purified to near (>95%) homogeneity.

Large scale purification of yCD_{triple}-F54*p*AzF was performed as described above, using 500 mL of 2 × YT $carb^{50}$ cam^{100} medium containing 3 mM *p*AzF. Cell lysates were prepared under native conditions as described in the QIA*expressionist* handbook (Qiagen, Valencia, CA). Briefly, cells were resuspended in lysis buffer (2 mL lysis buffer/g cell pellet) supplemented with 1 mg/mL lysozyme and 1× Halt Protease Inhibitor Cocktail. Following sonication, the lysate was centrifuged at 10 000 × *g* for 45 min at 4 °C. This centrifugation step was repeated with the resulting cleared lysate. Yeast CD_{triple}-F54*p*AzF was purified using Ni-NTA metal-affinity chromatography according to the manufacturer's protocol for batch purification under native conditions, with the exceptions that four washes (wash buffer contained 40 mM imidazole) were performed and the protein was eluted with five aliquots of elution buffer. Protein purification was analyzed using a 15% SDS-PAGE gel as described above. Yeast CD_{triple}-F54*p*AzF was purified to near homogeneity (>95%). Eluted fractions were pooled and dialyzed against 50 mM NaCl, 50 mM Tris-HCl, pH 7.5 or phosphate buffered saline (PBS, 10 g/L NaCl, 0.25 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4) at 4 °C. Protein concentration was determined by the Bio-Rad Protein Assay (Bradford method) (Bio-Rad, Hercules, CA) using a bovine serum albumin standard.

For expression and purification of yCD_{triple}, *E. coli* GIA39-(DE3) cells expressing pET23d:yCD_{triple} were grown in 2 × YT $carb^{50}$ at 37 °C at 225 rpm until an OD_{600} of 0.6 was reached. Protein expression was induced for 4 h by addition of 1 mM IPTG (final). Cells were harvested by centrifugation at 4000 × *g* for 30 min, and the cell pellets were frozen at −80 °C. The generation of cell lysates and protein purification were performed as described for the preparative purification of yCD_{triple}-F54*p*AzF.

DBCO-PEG_4-AH_2-TG97 Synthesis. Detailed synthesis procedures of DBCO-PEG_4-AH_2-TG97 can be found in the experimental procedures section of the Supporting Information.

Click Chemistry Reaction Conditions. Yeast CD_{triple}-F54*p*AzF was modified via strain-promoted [3 + 2] azide−alkyne cycloaddition with DBCO-PEG_4-AH_2-TG97 using the following method. Reactions were performed in PBS at the molar ratios, temperatures, and time periods described in the text and resolved on a 15% SDS-PAGE gel as described above. The yCD_{triple} monomer concentration was used for all click chemistry calculations. Immunoblot analysis using rabbit polyclonal yCD antiserum followed by goat anti-rabbit alkaline phosphatase-conjugated secondary antibody was performed as previously described.[21] Densitometry analysis was performed using NIH ImageJ software (rsbweb.nih.gov/ij/). The integrated optical density (IOD) of each band on the Coomassie Brilliant Blue stained gel was determined, and compared to the corresponding sum of IOD values for all bands in each lane.

IC_{50} Determination of the IDEPT Agent for PSMA. PSMA inhibition studies were performed as previously published,[49,50] with the following modifications. Working solutions of PSMA inhibitors and substrate (*N*-[4-(phenylazo)-benzoyl]-glutamyl-γ-glutamic acid, PABGγG) were prepared in Tris-HCl buffer (50 mM Tris-HCl, pH 7.4). A working solution of purified PSMA was prepared on ice using

Tris-HCl buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100) such that a 15−20% conversion of substrate to product was achieved in the absence of inhibitor. Each reaction mixture was prepared by adding 25 μL of an inhibitor solution (serially diluted concentrations providing 10−90% PSMA inhibition), or Tris-HCl buffer (50 mM Tris-HCl, pH 7.4) for controls, and 25 μL of PABGγG (1 μM final) to 175 μL Tris-HCl buffer. The PSMA enzymatic reaction was initiated by the addition of 25 μL of the PSMA working solution. Samples were incubated in a 37 °C water bath with constant shaking for 15 min, and the reaction was terminated by the addition of 25 μL of 2.5% trifluoroacetic acid in methanol (v/v) and vortexed. Each sample was allowed to quench for 15 min on ice, and buffered by the addition of 25 μL K_2HPO_4 (0.1 M). Samples were then centrifuged for 10 min at 7000 × *g*. An 85 μL aliquot of the supernatant was analyzed for product formation via RP-HPLC, and the IC_{50} values were determined using KaleidaGraph 3.6 (Synergy Software).

Mass Spectrometry Characterization of IDEPT Agents. MALDI mass spectrometry was performed with a Sciex 4800 MALDI TOF/TOF Analyzer. Whole protein spectra were collected using Linear High Mass mode with sinapic acid matrix. For whole protein MALDI, the samples were desalted and concentrated using a C8 hydrophobic tip (NuTip C-08, Glygen Corp., Columbia, MD), followed by a 2-fold dilution with matrix. When applicable, excess DBCO-PEG_4-AH_2-TG97 compound was removed using Performa DTR Gel Filtration Cartridges (EdgeBio, Gaithersburg, MD) according to the manufacturer's protocol with the exception that an additional spin with 20 μL of ddH_2O was used to elute the protein. Default calibration was used unless otherwise stated, and internal calibration was performed using myoglobin and the +2 charged bovine serum albumin peak.

Trypsin digestion of yCD_{triple} and DBCO-PEG_4-AH_2-TG97 modified yCD_{triple} was performed in solution by the addition of trypsin (1.5 μg/mL final, Promega, Madison, WI) to the protein sample suspended in 100 mM bicarbonate buffer, pH 7.5. Digested samples were then diluted with water to lower the bicarbonate concentration, and mixed 1:1 with matrix. Mass spectrometry of digested peptides was collected using positive reflector mode using α-cyano-4-hydroxcinnamic acid as matrix. Internal calibration was obtained by using peptide peaks from the yCD_{triple} enzyme that were not expected to be modified by a click chemistry reaction. This allowed accurate masses of peptides modified by the click chemistry reaction to be obtained. MS/MS spectra were obtained in both positive and negative mode using 1 kV MS/MS mode, and default calibrations were used for the MS/MS spectra.

***In Vitro* Cytotoxicity Assays.** For cell culture experiments, the IDEPT agent was prepared as described in the text and above, with additional purification steps. The 1:50 click chemistry reaction of protein:DBCO-PEG_4-AH_2-TG97 was performed, and analyzed via denaturing SDS-PAGE and mass spectrometry. The IDEPT agent was then purified to remove excess DBCO-PEG_4-AH_2-TG97 using Ni-NTA chromatography as described above. After mass spectrometry analysis of the eluted fractions, IDEPT agent containing fraction(s) were pooled. Buffer exchange (into 50 mM Tris-HCl, 50 mM NaCl, pH 7.5) was performed using a 7 kDa molecular weight cutoff Zeba Spin Desalting Column (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. The recovered IDEPT agent was subjected to an additional buffer exchange step using an additional Zeba Spin Desalting Column. The recovered protein was then sterilized using a 0.22 μm syringe filter, and protein concentration was determined by the Bio-Rad Protein Assay (Bradford method) (Bio-Rad, Hercules, CA) using a bovine serum albumin standard. IDEPT stock solutions were prepared in phosphate- and L-glutamine-free RPMI 1640 medium, supplemented with 1× Penicillin−Streptomycin−Glutamine (Gibco, Life Technologies Corp., Grand Island, NY).

Human prostate cancer cells lines, LNCaP (PSMA-positive) and PC-3 (PSMA-negative) cells, were routinely grown in a humidified incubator at 37 °C containing 5% CO_2. Unless otherwise indicated, complete HyClone classical medium was used and cells were passaged using 0.25% trypsin-0.53 mM EDTA. Complete HyClone classical medium consisted of HyClone RPMI 1640 (Thermo Scientific, Rockford, IL) containing 2.05 mM L-glutamine and 25 mM Hepes, supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 1× Penicillin−Streptomycin−Glutamine (Gibco, Life Technologies Corp., Grand Island, NY, 1 U/mL penicillin, 1 μg/mL streptomycin, 292 μg/mL L-glutamine final), and 1× sodium pyruvate (Gibco, Life Technologies Corp., Grand Island, NY, 1 mM final). For cell survival assays, cells were plated in 96-well format on BD BioCoat Poly-D-Lysine coated plates (BD Biosciences, San Jose, CA) at a density of 9000 cells/well. After adherence for 48 h (roughly 70% confluency), media was removed (via inversion of plates), and cells were incubated with the IDEPT agent (0, 0.1, or 0.5 μM for LNCaP cells; 0, 0.5, or 1 μM for PC-3 cells) for 2.5 h, with mild agitation every 30 min. Following this incubation, the IDEPT agent was removed by inversion of the 96-well plate. Cells were washed three times by the addition of complete HyClone classical medium to each well, followed by inversion of the 96-well plate to remove the media. Following the final wash, HyClone classical medium containing 0, 1, or 5 mM 5-FC was added. After 5-FC incubation for 7 days, cell viability was analyzed via the redox-indicator dye Alamar Blue (AbD Serotec, Oxford, UK) according to the manufacturer's instructions (530 nm excitation/590 nm emission). All experiments were performed in triplicate. The data were plotted with a standard error of the mean bar, and analyzed for statistical significance using Student's *t*-test with an α-level of 0.05.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

DBCO-PEG_4-AH_2-TG97 structure and detailed synthesis methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil; aaRS, aminoacyl-tRNA synthetase; CD, cytosine deaminase; IDEPT, inhibitor-directed enzyme prodrug therapy; *p*AzF, *p*-azidophenylalanine; PCa, prostate cancer; PSMA, prostate-specific membrane antigen; yCD, yeast cytosine deaminase

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