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 (pAzF)) incorporated into yCD triple and the dibenzylcyclooctyne moiety of our PSMA targeting agent (DBCO-PEG4-AH2-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 proteins5 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,9 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 (pAzF) 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 (t1/2 at 50 °C = 117 h) over the wild-type enzyme (t1/2 at 50 °C = 4 h).20,21 In support of using this

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glycoprotein is upregulated in PCa,25,26 after androgen- (PSMA) is an ideal PCa target. This type II membrane for, the utilization of click chemistry to couple yCDtriple to a are then internalized via a PSMA-dependent process. There- coined inhibitor-directed enzyme prodrug therapy, or IDEPT. design of this platform is composed of a suicide enzyme and evoking cell killing through prodrug activation. As the therapeutic agents capable of targeting prostate tumor cells36,37 that have been demonstrated to deliver large molecular and biomolecular payloads to PSMA expressing PCa cells36,37 that plays an increased half-life that would allow for enzymatic activity after circulation in the bloodstream, and is relatively 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 PCa25,26 after androgen-deprivation therapy,77 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 pseudoirreversible manner, and consequently are potent inhibitors of PSMA enzymatic (carboxypeptidase) activity.35 In addition to their selective tumor uptake,33 these inhibitors 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 pseudoirreversible manner, and consequently are potent inhibitors of PSMA enzymatic (carboxypeptidase) activity.35 In addition to their selective tumor uptake,33 these inhibitors...
grew on CD selection plates in the presence of pAzF, but were not viable on CD selection plates when pAzF was omitted (lanes 2 and 3 in Figure 2). These results suggest that pAzF is incorporated via the pEVOL system, that pAzF must be present for functional yCDtriple to be produced from pET23α-yCDtripleF54X, and that yCDtriple-F54pAzF retains CD activity due to its ability to confer growth to E. coli GIA39(DE3) plated on CD selection medium.

The relationship between yCDtriple-F54pAzF protein expression and pAzF concentration in the culture medium was also evaluated to determine if full-length yCDtriple containing pAzF could be generated and purified. Protein yields not only depend on the presence of pAzF in the culture media, but also increase as the concentration of pAzF increases (Figure 3). In further support of pAzF incorporation into yCDtriple.

Figure 3. Yeast CDtriple-F54pAzF protein yields increase as pAzF 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 yCDtriple-F54pAzF (monomer) is 19.8 kDa. Molecular weight marker (M). (A) Yeast CDtriple-F54pAzF (upper band) and yCD triple -F54 pAzF (lower band) were analyzed by SDS-PAGE. Western blot analysis utilized polyclonal antiserum to yCD. yCD triple and yCD triple -F54 pAzF incorporated (lanes 1 and 2) and yCDtriple-F54pAzF (lanes 3 and 4) were probed with a rabbit polyclonal antibody. Yeast CDtriple (lanes 1 and 2) and yCDtriple-F54pAzF (lanes 3 and 4) were visualized with Coomassie Brilliant Blue. Molecular weight marker (M). (B) Coupling yCDtriple-F54pAzF with DBCO-PEG4-AH2-TG97 via click chemistry. Yeast CDtriple (lanes 1 and 2) and yCDtriple-F54pAzF (lanes 3 and 4) were probed with a rabbit polyclonal antibody. Yeast CDtriple (lanes 1 and 2) and yCDtriple-F54pAzF (lanes 3 and 4) were visualized with Coomassie Brilliant Blue. Molecular weight marker (M).

utilizing the pEVOL system, full-length yCDtriple could not be purified from expression cultures lacking pAzF, suggesting that His-tagged yCDtriple is not synthesized without the incorporation of pAzF (Figure 3). Roughly 9 mg of yCDtriple-F54pAzF 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 yCDtriple-F54pAzF 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-PEG4-AH2-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 PEG8 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 CDtriple-F54pAzF and DBCO-PEG4-AH2-TG97 were mixed at various molar ratios (1:5, 1:10, 1:25, 1:50, 1:100, 1:200, 1:500 (protein:DBCO-PEG4-AH2-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 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 yCDtriple-F54pAzF labeled with DBCO-PEG4-AH2-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, yCDtriple (no pAzF incorporated) reacted with DBCO-PEG4-AH2-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 yCDtriple-F54pAzF purified from rich media containing 1, 2, or 3 mM pAzF were observed. This suggests that the concentration of pAzF in the culture medium is the limiting factor for yCDtriple-F54pAzF yields, but does not influence the efficiency of click chemistry of the purified enzyme, further supporting the high fidelity of the pEVOL system for pAzF 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 pAzF incorporation and click chemistry conjugation. As noted, yCDtriple was engineered for pAzF incorporation at amino acid
position F54. After tryptic digest, amino acid F54 is part of the tryptic peptide FQK (residues 54–56). Therefore, a tryptic digest of yCDtriple was analyzed to identify the FQK tripeptide, and a tryptic digest of yCDtriple-F54pAzF was analyzed to identify peaks corresponding to the FQK and F′QK (F′ = pAzF) tripeptides. As predicted, the FQK tripeptide was seen at m/z 422.2294 (calculated mass: 422.22) for the tryptic digest of yCDtriple, the control protein that does not contain pAzF, and confirmed via MS/MS analysis. The tryptic peptide F′QK was not seen for yCDtriple-F54pAzF, and the absence of this peak may be due to the sensitivity of pAzF 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 yCDtriple, the control protein that does not contain pAzF, and confirmed via MS/MS analysis. The tryptic peptide F′QK was not seen for yCDtriple-F54pAzF, and the absence of this peak may be due to the sensitivity of pAzF to the laser pulse (355 nm) used in the MALDI procedure.

To confirm that click chemistry occurred at position F54, yCDtriple-F54pAzF modified by DBCO-PEG₄·AH₂·TG97 was subjected to a tryptic digest followed by mass spectrometry analysis. An ion corresponding to F′clickQK (F′click = pAzF modified by DBCO-PEG₄·AH₂·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 S4, a peak at m/z 1609.8473 was identified, corresponding to F′clickQK 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 (yCDtriple-F54pAzF modified by DBCO-PEG₄·AH₂·TG97), an inhibition (IC₅₀) study was performed with PSMA purified from LNCaP cells. The IC₅₀ values indicate the concentration of a molecule needed to inhibit PSMA enzymatic activity by 50%. The IDEPT agent (IC₅₀ = 41 nM) and parent compounds (DBCO-PEG₄·AH₂·TG97)
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$_{triple}$-F54AzF 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 exceed 100 mg/L. 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, 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 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 leuB6(AM) fhuA21 codA1 lacY1 tss-95 glv44-AS) λ<sup>−</sup> dadX3 pyrF101 his-108 argG6 ilvA634 thiE1 deoC1 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). The derived strain, GIA39(DE3), was used in genetic complementation studies and for protein purification. Photolysis of the aryl-azide of p-azido-l-phenylalanine (pAzF) 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$_{triple}$

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.
using NcoI and HinDIII restriction endonucleases, and subcloned into Ncol/HinDIII-digested pET23d vector DNA (Novagen (now EMD Millipore) Billerica, MA)).

Oligonucleotides MB713 (5′-GAATATTTGAGTGGATACGTGC-3′) and MB714 (5′-GGTCTGGCTGGTCTCTACATTCAATATCTTC-3′) were used to insert a single nucleotide into the stop codon of yCD̊ triple using QuikChange II Site-Directed Mutagenesis (Agilent Technologies, Santa Clara, CA). The subsequent frame-shift mutation at the yCD̊ triple stop codon allowed for fusion of the pET23d encoded 6× His tag to the C-terminus of yCD̊ triple. The resulting plasmid, pET23d:yCD̊ triple was confirmed by DNA sequencing.

To generate pET23d:yCD̊ triple-F54X, QuikChange II Site-Directed Mutagenesis (using oligonucleotides MB717 5′-GGTCACAACATGAGATAGCAAAAGGGATCCGC-3′ and MB718 5′-GCGGATCCCTTTTGTCTCATCTGTTTGA-CC-3′) was used to mutate the phenylalanine 54 codon of yCD̊ triple (in pET23d:yCD̊ 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.

The pET23d, pET23d:yCD̊ triple, pET23d:yCD̊ triple-F54X, and pEVOl-pAzF 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 and cytosine. Carbenicillin at 50 µg/mL (carb60), Research Products International Corp., Mt. Prospect, IL) was included to select for transformants harboring pET23d plasmids. Chloramphenicol at 100 µg/mL (cam50) was included to select for transformants harboring the pEVOl-pAzF plasmid. Where indicated, 1 mM pAzF (final) was added to cytisine containing minimal media.

**Protein Expression and Purification.** Yeast CD̊ triple-F54pAzF expression in the presence of 0.1, or 1 mM pAzF was performed as previously described with the following minor modifications.  

E. coli GIA39(DE3) cells expressing the pET23d:yCD̊ triple-F54X and pEVol-pAzF vectors were grown in 500 mL of 2× YT carb60 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 (OD600) of 0.6 was reached. After reaching an OD600 within this range, 75 mL of the culture was added to 3× His tag to the C-terminus of yCD̊ triple. The resulting plasmid, pET23d:yCD̊ triple was confirmed by DNA sequencing.

**Densitometry analysis was performed using NIH ImageJ (rsweb.nih.gov/jj/).** 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.

**IC50 Determination of the IDEPT Agent for PSMA.** PSMA inhibition studies were performed as previously published, with the following modifications. Working solutions of PSMA inhibitors and substrate (N-[4-(phenylazo)-benzoyl]-glutamyl-g-glutamic acid, PABGγG) were prepared in Tris-HCl buffer (50 mM Tris-HCl, pH 7.4). The concentration was used for all click chemistry calculations. Immunoblot analysis using rabbit polyclonal yCD antiseraum followed by goat anti-rabbit alkaline phosphatase-conjugated secondary antibody was performed as previously described.  

**Click Chemistry Reaction Conditions.** Yeast CD̊ triple-F54pAzF was modified via strain-promoted [3 + 2] azide–alkyne cycloaddition with DBCO-PEG4-AH2-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 yCD̊ triple monomer concentration was used for all click chemistry calculations. Immunoblot analysis using rabbit polyclonal yCD antiseraum followed by goat anti-rabbit alkaline phosphatase-conjugated secondary antibody was performed as previously described.

**DBC0-PEG4-AH2-TG97 Synthesis.** Detailed synthesis procedures of DBCO-PEG4-AH2-TG97 can be found in the experimental procedures section of the Supporting Information.
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 PABG/G (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% trisfluoroacetic acid in methanol (v/v) and vortexed. Each sample was then allowed to quench for 15 min on ice, and buffered by the addition of 25 μL K$_2$HPO$_4$ (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 on 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, EdgeBio, Gaithersburg, MD) according to the manufacturer’s protocol with the exception that an additional spin with 20 μL of ddH$_2$O 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-hydroxycinnamic 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:DBC0-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 cell 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 Heps, 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; pAzF, p-azidophenylalanine; PCD, prostate cancer; PSMA, prostate-specific membrane antigen; yCD, yeast cytosine deaminase

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therapy of prostate cancer using a PSMA inhibitor as a homing ligand. 


