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A Switchable Site-Specific Antibody Conjugate

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Abstract

Genetic incorporation of unnatural amino acids (UAAs) provides a unique approach to the synthesis of site-specific antibody conjugates that are homogeneous and better defined constructs than random conjugates. Yet, the yield varies for every antibody, and the process is costly and time-consuming. We have developed a switchable α GCN4-Fab conjugate that incorporates UAA p-acetylphenylalanine. The GCN4 peptide is used as a switch, and antibodies fused by GCN4 can direct the α GCN4-Fab conjugate to target different cancer cells for diagnosis, imaging, or therapeutic treatment. More importantly, this switchable conjugate demonstrated an impressive potential for pretargeted imaging *in vivo*. This approach illustrates the utility of an orthogonal switch as a general strategy to endow versatility to a single antibody conjugate, which should facilitate the application of UAA-based site-specific conjugates for a host of biomedical uses in the future.

Graphical Abstract

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Notes

The authors declare no competing financial interest.

Supporting Information

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Supporting Figures S1–S13, Table S1, and detailed descriptions of the experimental materials and methods applied in this study (PDF)

RESULTS AND DISCUSSION

Rationale

Recently, a 14-mer peptide sequence from the yeast transcription factor GCN4 was fused to antibodies, thereby directing the targeting of GCN4-selective CAR-T cells to CD19- or CD20-positive tumor cells.¹⁹ The fact that this “GCN4 tag” has a low probability of immunogenicity and satisfactory *in vivo* stability and is orthogonal to human proteins¹⁹ suggests that it may be usable as a switch to mediate the targeting of GCN4-selective antibody conjugates (Scheme 1). Primary IgGs could be readily fused with the GCN4 peptide in order to direct α GCN4 antibody conjugates toward their cognate antigens on tumor surfaces. For proof of concept, we picked trastuzumab to target HER2-positive breast cancer² and FMC63 to target α CD19-positive B-cell lymphoma,²⁰ both of which are popular targets for immunotherapy. The 14-mer sequence from GCN4 was inserted into the loop of the antibody to replace Lys169 at the CL constant region of the light chain (LC) or Ser180–Gly181 at the CH1 constant region of the heavy chain (HC), following the reported procedures^{3,19,21} for direct peptide–antibody loop fusion. The peptide was flanked with a (Gly)₄Ser linker to afford the desired flexibility. The resulting fusion constructs were expressed in HEK293F cells by transient transfection, with yields of more than 10 mg/L after column purification. SDS/ PAGE analysis revealed that all four fusion antibodies migrated as a single band, with >95% purity and a molecular mass of ~165 kDa (Supporting Information Figure S1). After DTT reduction, the light chains migrated close to ~25 kDa, whereas the heavy chains migrated at ~50 kDa, matching the expected molecular weights. Their identities were further confirmed by ESI-MS analysis (Figure S2).

Design and Synthesis of Anti-GCN4 Fab

Among all the available scaffolds that can be used to create an α GCN4 antibody, we are especially interested in Fab fragments, which have much shorter circulation half-lives than a full-length IgG and may incur less off-target-related systematic toxicity for therapeutic treatment or background signals for imaging diagnosis.²² Compared to other small sized antibody fragments, Fab still has constant regions that are distal to antigen-binding sites to allow for point mutation and conjugation. On the basis of the sequence of an α GCN4 single-chain variable fragment (ScFv) that has a binding affinity of about 5 pM,²³ we constructed a plasmid (pBAD_ α GCN4) harboring the heavy- and lambda light-chain genes of the Fab fragment following an stII signal peptide.²⁴ The wild type α GCN4 Fab was expressed in *E. coli* in shake flask cultures, yielding 7.4 mg/L after purification.

Optimization of Labeling Sites

The genetic incorporation of UAAs such as pAcF not only allows us to site-specifically modify the protein surface but also facilitates our exploration of various Fab conjugates with different geometries and stabilities.²⁴ For example, previous studies on a Fab fragment bearing a kappa light chain revealed solvent-exposed sites such as HC-K129 that has a high expression yield and coupling efficiency, as well as little interference with antigen-binding.^{15,24} Nonetheless, a mutation site on the lambda light chain has not been explored yet. In a search for an optimal conjugation site, we selected three additional sites (LC-S155X, LC-S193X, LC-G202X) on the lambda light chain of α GCN4, which are surface-exposed, in

flexible loops, and distal to binding sites (Figure 1A). Briefly, the pBAD plasmid that encodes the α GCN4 Fab with double mutations to the TAG codon on HC-K129X, and one of the sites from LC-S155X, LC-S193X, or LC-G202X, was cotransformed into the DH10B strain with a plasmid (pUltra_pAcF)²⁴ that encodes the Mj-tRNA/tyrosyl-tRNA synthetase pair evolved to incorporate the pAcF UAA. The shake-flask expression yield for α GCN4-Fab (LC-S155X, HC-K129X, X = pAcF) was 3.4 mg/L, while the yields for α GCN4-Fab (LC-S193X, HC-K129X, X = pAcF) and α GCN4-Fab (LC-G202X, HC-K129X, X = pAcF) were 0.68 mg/L and 0.34 mg/L, respectively. The varying yields for these Fab mutants confirmed that antibody stability depends on the site of mutation, which may also suggest that the closer the amber suppression is toward the N-terminus, the more stable the mutant is. Nevertheless, all the aforementioned α GCN4 Fab antibodies migrated as a single band on SDS/PAGE analysis, with >95% purity and a molecular mass of ~46 kDa (Figure S3). Follow-up ESI-MS analysis further confirmed their identities, with the results correlating well to the theoretical molecular weights (Figure S4). Through an ELISA assay, we also assessed the affinity of these mutants. As shown in Figure S5, the mutated α GCN4 Fab fragments have an affinity indistinguishable from the wild-type Fab, with a half-maximal binding constant (EC_{50}) of ~1 nM.

Next, we conjugated all the purified α GCN4 Fab mutants with a noncleavable auristatin F linker compound (MMAF)^{15,18} (Scheme 1, Figure S6). Each pAcF-containing α GCN4-Fab was reacted with a 10-fold excess of aminooxy-derived MMAF in the presence of acetic hydrazide at 37 °C for 24 h and purified following the reported procedures.^{3,18} SDS/PAGE (Figure S7) and ESI-MS analysis (Figure S6) showed that the final α GCN4 ADCs are >95% pure, and all have the desired molecular weight with a drug-to-antibody ratio (DAR) of 2 (Figure S6). Their binding affinity with GCN4 was assessed by ELISA (Figure 1B). All the conjugates have a similar affinity (EC_{50} ~ 1 nM) to the wild-type Fab, and also the mutant Fab's before conjugation, demonstrating that the binding of the α GCN4 Fab is not affected by payload conjugation at the three selected sites.

On the basis of the expression yield (3.4 mg/L) of the mutant (LC-S155X, HC-K129X, X = pAcF), which is significantly higher than the other two α GCN4 Fab mutants and also higher than most reported UAA mutant antibodies (1–2 mg/L by shake flask),^{15,18,24} we decided to choose α GCN4-Fab (LC-S155X, HC-K129X, X = pAcF) for follow-up experiments.

***In Vitro* Cytotoxicities of Switchable Antibody–Drug Conjugates**

To utilize GCN4 as a switch for the treatment of different cancers, we evaluated the *in vitro* cytotoxicity of the α GCN4-Fab-MMAF conjugate (LC-S155X, HC-K129X, X = pAcF) and/or GCN4-tagged primary IgGs at the end of their 72 h incubation with a CD19-positive Burkitt's lymphoma cell line Ramos or a HER2-positive breast cancer cell line SK-BR-3 (Figure 2). The MMAF compound was used as a positive control and displayed medium cytotoxicity (EC_{50} 's ~ 50–89 nM, Supporting Information Table S1) due to its limited cell permeability.¹⁸ When mixed with α CD19-GCN4 fusions at a molar ratio of 2:1, α GCN4-Fab-MMAF showed significant toxicity toward the Ramos cell line (EC_{50} = 12 ± 7 nM with α CD19-LC-GCN4; EC_{50} = 7 ± 4 nM with α CD19-HC-GCN4; Figure 2A, Table S1). Similarly, the mixture of α GCN4-Fab-MMAF and α HER2-GCN4 fusions potentially killed

SK-BR-3 breast cancer cells ($EC_{50} = 23 \pm 11$ nM with α HER2-LC-GCN4; $EC_{50} = 1.2 \pm 0.6$ nM with α HER2-HC-GCN4; Figure 2B, Table S1). Notably, the most potent mixture (α HER2-HC-GCN4: α GCN4-Fab-MMAF) is similar in efficacy to that of the previously reported Herceptin-MMAF conjugate ($EC_{50} = 0.9 \pm 0.2$ nM when tested in parallel; Figure 2B, Table S1).¹⁵ On the other hand, the α GCN4-Fab-MMAF conjugate or any of the GCN4-tagged primary antibodies, when administered alone, turned out ineffective toward either cancer cell line ($EC_{50}s > 100$ nM), indicating that the cytotoxic effects require the activation of the α GCN4-based ADC by GCN4. To examine the selectivity of GCN4-mediated ADCs, we also tested the cytotoxicity of α HER2-HC-GCN4: α GCN4-Fab-MMAF on Ramos cells that are HER2-negative (Figure S8A), and the cytotoxicity of α CD19-HC-GCN4: α GCN4-Fab-MMAF on SK-BR-3 cells that are CD19-negative (Figure S8B). In both cases, the combination failed to show any activity ($EC_{50} > 100$ nM), while the MMAF small molecule control worked well.

The fact that the GCN4 peptide fused at the heavy chain (CH1) of the primary IgG led to more significant cytotoxicity compared to fusion at the light chain (CL) could be a pattern to possibly guide future antibody tagging with the GCN4 peptide. There could be more space surrounding the CH1 region for binding by α GCN4-Fab. Protein binding to the CH1 region may also interfere less with the primary IgGs' interaction with antigens and their follow-up internalization. For more direct evidence, we also performed ELISA analysis to investigate the protein complexes' antigen binding affinity (Figure S9). The α Her2-GCN4 fusion: α GCN4-Fab-MMAF complexes bound significantly to HER2 antigen but not to CD19 antigen, similarly to the Herceptin-MMAF conjugate. On the other hand, the α CD19-GCN4 fusion: α GCN4-Fab-MMAF complexes bound well to CD19 antigen but not HER2. Although the bindings toward cognate antigens are similar, the α GCN4 conjugate complexed with the GCN4-antibody heavy chain fusion seems to bind slightly better than the conjugate complexed with the GCN4-antibody light chain fusion, in both α HER2 and α CD19 affinity tests. These affinity data are highly consistent with the observed cytotoxicity results and suggest that GCN4 fusion at the heavy chain may eventually bring in less interference to the primary IgGs antigen binding.

Taken together, these results demonstrate that with a GCN4 switch, the site-specific α GCN4-Fab-MMAF conjugate can be directed by primary antibodies to selectively and tightly bind cognate biomarkers, thereby potentially inhibiting the corresponding tumor cells' proliferation. Further evaluation of the conjugate's *in vivo* efficacy on tumor growth inhibition is currently ongoing to determine its therapeutic potential.

Detection and Imaging Using Switchable Antibody-Dye Conjugates

Next, we wanted to test if GCN4 can mediate the detection of different cancer cell lines. Alexa Fluor 488 (AF488) has been commonly used for flow cytometry assays and was conjugated to α GCN4-Fab (LC-S155X, HC-K129X, X = pAcF) through oxime coupling. The identity and purity of the final α GCN4-Fab-AF488 conjugate was confirmed by ESI-MS (Figure S10) and SDS/PAGE analysis (Figure S7). As shown in Figure 3, the AF488 fluorescence channel can selectively detect SK-BR-3 cells in a dose-responsive manner, when tumor cells were prestained by α GCN4-Fab-AF488 and α HER2-HC-GCN4 fusion

rather than α GCN4-Fab-AF488 and α CD19-HC-GCN4 fusion. Similarly, Ramos cells can be detected only when they were preincubated with α CD19-HC-GCN4 and α GCN4-Fab-AF488. Moreover, the shifts of Ramos cells were weaker than that of SK-BR-3 when both were treated with the same amount of antibody mixtures. This indicates that the CD19 receptor on the Ramos surface may be less intensive than the HER2 receptor on SK-BR-3, which is also consistent with the weaker potencies of antibody conjugates on Ramos cells as revealed by cytotoxicity studies.

Encouraged by these *in vitro* results, we next performed *in vivo* imaging experiments to further gauge if the GCN4-mediated α GCN4-Fab conjugate can be developed as a switchable imaging agent to efficiently guide cancer therapy. Noninvasive molecular imaging emerges as a powerful tool to accurately and selectively evaluate receptor expression levels, which is crucial for the prediction and timely assessment of the effectiveness of biomarker-targeted cancer therapy.²⁵ In particular, near-infrared fluorescent (NIRF) imaging allows for deep-penetration and hazard-free imaging of tumor tissues and has been routinely used to delineate tumor margins during surgery.^{26,27} Given that the commonly used NIRF dye Cyanine 7 (Cy7) is not compatible with the aminooxyl functional group (Cy7 has an intrinsic imine functionality), we developed a two-step coupling procedure for the synthesis of α GCN4-Fab-Cy7 (Scheme 1, Figure S11). The first step involved the modification of pAcF on α GCN4-Fab (LC-S155X, HC-K129X, X = pAcF) with an aminooxy-derived BCN linker,²⁸ followed by desalting chromatography to introduce the azide-reactive BCN moiety at a stoichiometry of two per Fab. The modified α GCN4-Fab (LC-S155X, HC-K129X, X = pAcF-BCN) was then reacted with a 10-fold excess of Cy7-azide (Lumiprobe) at 37 °C for 12 h. After size-exclusion chromatography purification, the α GCN4-Fab-Cy7 was confirmed by ESI-MS (Figure S11C) and SDS/PAGE (Figure S11D) to be >95% pure and has the desired molecular weight with a DAR ~ 2.

For *in vivo* NIRF imaging, we incubated α GCN4-Fab-Cy7 (1.75 mg/kg) with either an α HER2-HC-GCN4 fusion or an α CD19-HC-GCN4 fusion (3 mg/kg) at a molar ratio of 2:1 and then injected them intravenously into mouse xenografts of SK-BR-3, and Ramos, respectively. The fluorescence signals in tumors increased and became saturated 4 h post injection (p.i.; Figure 4). The whole-body background fluorescence gradually reduced, while the tumor signals remained high until the end of the NIRF scan (72 h p.i.). The total radiant efficiency of the targeted antibody complex versus that of the nontargeted antibody complex on SKBR3 and Ramos-bearing mice were plotted, respectively (Figure S12). For both xenografts, the best signal ratios (>3) were achieved at 72 h. As expected, α GCN4-Fab-Cy7 was directed by α HER2-HC-GCN4 to selectively accumulate in the SK-BR-3 tumor instead of Ramos. On the other hand, α CD19-HC-GCN4 effectively directed α GCN4-Fab-Cy7 to the Ramos tumor but not SK-BR-3. Following the last NIRF scan at 72 h p.i., mice were sacrificed, with tumors and major organs resected for *ex vivo* NIRF imaging (Figure S13). The biodistribution data are highly consistent with the *in vivo* NIRF observations, indicating that the antibody probe was selectively enriched in tumors compared to the rest of tissues. Importantly, the stronger fluorescence in breast tumor SK-BR-3 compared to that of Ramos was consistent with the trends observed in the cytotoxicity assay and flow cytometry analysis. Thus, our switchable antibody conjugates may ubiquitously probe and distinguish

the expression levels of cell-surface biomarkers, thereby serving as a convenient imaging tool.

Pretargeted *in vivo* Imaging Using Switchable Antibody-Dye Conjugates

Molecular imaging is also used to directly visualize the targeting process, including biodistribution and pharmacokinetics.^{25,29,30} The common approach relies on the direct labeling of immunoconjugates (IgGs) which requires a large dosage to overcome issues with the probe including biostability, photobleaching, and radiodecay (for nuclear imaging).^{26,27,31} Pretargeted imaging was recently proposed to improve tumor signal-to-background ratios and to reduce the dosage requirement, so that there is limited systemic exposure to harmful effects of the agent. We reasoned that our Fab-based α GCN4 conjugates could serve as a secondary diagnostic agent while the GCN4-tagged primary IgG could be an ideal pretargeting agent. In a pilot experiment with NIRF imaging, 3 mg (18 nmol)/kg of α HER2-HC-GCN4 or α CD19-HC-GCN4 was injected as a pretargeting agent to SK-BR-3 breast cancer bearing mouse xenografts, which showed better imaging signals than Ramos mouse xenografts. After ~4 h, during which the tumor could be maximally bound by the primary IgG, we began the injection of α GCN4-Fab-Cy7 at 1.75 mg (36 nmol)/ kg and monitored the NIRF thereafter. For the control group that was injected with only α GCN4-Fab-Cy7, the background fluorescence quickly cleared out and became negligible at the end of 48 h (Figure 5), which could be due to the short half-life of Fab fragments. With the pretargeting of α CD19-HC-GCN4, the fluorescence signal was initially strong (1–8 h post injection of Fab), thereby reflecting the GCN4-mediated recognition of α CD19 IgG by α GCN4-Fab-Cy7 in the circulating blood. However, the signal eventually decreased to a negligible level at 48 h p.i., presumably because the α CD19 IgG failed to specifically recognize the breast cancer tumor. Only with pretargeted recognition of the HER2-positive SK-BR-3 by α HER2-HC-GCN4 can α GCN4-Fab-Cy7 be enriched in the tumor region, which displayed a strong fluorescence signal from 16 h to 48 h p.i. (Figure 5). Our results showed that the GCN4-mediated site-specific antibody conjugates can also be used for pretargeted tumor imaging. For future work, we are preparing a radiolabeled α GCN4 Fab-NOTA conjugate which could be an ideal fit for pretargeted positron emission tomography (PET) imaging.³¹

Conclusion

In summary, we have developed a switchable site-specific antibody conjugate based on the genetically encoded unnatural amino acid p-acetylphenylalanine (pAcF). This antibody conjugate was optimized at fixed sites and stoichiometry and specifically recognizes a GCN4 peptide tag that is orthogonal to human proteins. Using GCN4 as a switch, the antibody conjugate can be directed by different primary IgGs to target the cognate biomarkers unique to different tumors. With various payloads such as a toxin drug or imaging probes, this switchable approach demonstrated promising effects and flexibility in therapeutic treatment and imaging-guided diagnosis of breast cancer and B-cell lymphoma. The technology described here represents a significant step toward the field of protein conjugation. For future applications to different targeting proteins, one will only need to generate the GCN4-fusion protein accordingly, thereby facilitating a wide range of diagnosis and/or therapeutic treatment, especially when large scale production is needed. Finally, this work suggests that with an orthogonal switch, a fixed antibody conjugate can be used to

rapidly deliver small molecule drugs, oligonucleotides, peptides, or proteins in the future to a diverse set of tumors or other disease-related target tissues.

MATERIALS AND METHODS

Please see the Supporting Information for experimental details.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) Sites of mutation in α GCN4 Fab based on the crystal structure of a Fab with a lambda light chain (PDB: 4FQH). (B) ELISA analysis of site-specific α GCN4-Fab-MMAF conjugates captured by GCN4 antigen and detected with anti-human λ -HRP. Error bar represents the standard deviation of three replicates.

Figure 2.

In vitro cytotoxicity of α GCN4-Fab MMAF (LC-S155X, HC-K129X) toward (A) the Ramos cancer cell line when mixed with or without α CD19-GCN4 fusions and (B) the SK-BR-3 cancer cell line when mixed with or without α HER2-GCN4 fusions. MMAF or GCN4-fused primary antibodies were also administered alone as controls.

Figure 3.

In vitro detection of cancer cells by flow cytometry using α GCN4-Fab AF488 (LC-S155X, HC-K129X) switched with GCN4-fused α HER2 or α CD19 IgG (CH1 fusion).

Figure 4.

In vivo near-infrared fluorescence (NIRF) imaging of SK-BR-3 and Ramos tumor bearing mice after administration of α HER2-HC-GCN4: α GCN4-Cy7 (LC-S155X, HC-K129X) or α CD19-HC-GCN4: α GCN4-Cy7 (LC-S155X, HC-K129X). Tumor regions were highlighted by circles.

Figure 5.

In vivo pretargeted NIRF imaging of SK-BR-3 tumor bearing mice after injection with only α GCN4-Cy7 (LC-S155X, HC-K129X) or after preinjection with α HER2-HC-GCN4 or α CD19-HC-GCN4 followed by (after 4 h) injection with α GCN4-Cy7 (LC-S155X, HC-K129X). Tumor regions were highlighted by circles.

Scheme 1.
Schematic Representation of the Switch-Mediated Antibody Conjugate (SAC)