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Targeted and armed oncolytic adenovirus via chemoselective modification

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ABSTRACT

Oncolytic adenoviruses (Ads) are an emerging alternative therapy for cancer; however, clinical trial have not yet demonstrated sufficient efficacy. When oncolytic Ads are used in combination with taxoids a synergistic increase in both cytotoxicity and viral replication is observed. In order to generate a next generation oncolytic adenovirus, virion were physically conjugated to a highly potent taxoid, SB-T-1214, and a folate targeting motif. Conjugation was enabled via the metabolic incorporation of non-canonical monosaccharides (*O*-GlcNAz) and amino acids (homopropargylglycine), which served as sites for chemoselective modification.

Despite substantial progress in understanding the molecular underpinnings of cancer, current chemotherapeutic options are limited and often unsuccessful. One promising alternative strategy is the use of conditionally replicative oncolytic vectors. Such viruses are designed to preferentially replicate in cancerous cells, such as those lacking common tumor suppressors (e.g., p53), leading to partially selective tumor toxicity. In addition, many carry a toxic transgene designed to amplify the inherent cytotoxic nature, which results from viral protein expression and immune stimulation.[1] Despite this multifaceted cytotoxicity, the major limitation for oncolytic viruses in clinical trials has been efficacy.[2] In an effort to increase potency, a number of oncolytic viruses have been used in combination with traditional chemotherapeutics.[3–5] In particular, conditionally replicative adenoviruses (Ads) have demonstrated significant synergism when used in combination with a number of different chemotherapeutics including doxorubicin, paclitaxel/docetaxel, cisplatin and histone deacetylase inhibitors.[6] In the case of taxoid/oncolytic Ad combination therapy, an increase in viral replication is seen in addition to synergistic cytotoxicity.[7–12] While the mechanistic origin of synergism is not well understood, it is clearly a general and significant phenomenon.

Paclitaxel treatment of cancer cells results in the upregulation of TNF related apoptosis inducing ligand (TRAIL) receptors.[13] Notably, one of the most promising oncolytic Ads in clinical trials bears the cytotoxic TRAIL transgene, which induces apoptosis in the infected cell and mediates substantial bystander cytotoxicity. As a result, taxoid/AdTRAIL would be expected to have an additional source of synergism. SB-T-1214 is a next generation taxoid that exhibits significantly improved cytotoxicity, against a number of drug resistant cancer cell lines.[14–16] In addition, this taxoid exhibited substantial inhibition of cancer stem cell related genes (Oct4, Sox2, Nanog, and c-Myc) when screened against 3 unrelated invasive colon cancer cell lines.[17] These results indicate that SB-T-1214 has significant potential, particularly with respect to cancer stem cells and cancers that are resistant to traditional chemotherapeutics.

While combination therapy demonstrates significant promise, it holds that efficiently targeted Ad particles bearing a therapeutic payload would provide an additional boost in efficacy. This would be a result of spatially and temporally concerted delivery of cytotoxicity, and may have the added benefit of reducing systemic toxicity. In order to achieve this goal, selective chemical modification routes for adenovirus are required, particularly those that allow the generation of multifunctional particles. Previously we reported the incorporation and modification of a non-canonical sugar residue, *O*-GlcNAz on serine 109 of the fiber protein, as a means of chemoselectively tailoring Ad particles.[18] The specificity of this strategy, derived from the fidelity of the biosynthetic machinery and the highly selective chemistries developed for azide modification, allowed folate modification without compromising virus infectivity. Folate decorated Ad exhibited substantial (~20 fold) increase transgene delivery to breast cancer cells.[18]

Here, we extend this approach towards multimodal adenovirus particles (Fig. 1) via the simultaneous metabolic labeling with

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O-GlcNAz and an alkyne bearing non-canonical amino acid, homopropargylglycine (HPG). Introduction of these surrogates into Ad particles was envisioned to allow sequential Staudinger ligation of *O*-GlcNAz followed by copper assisted 'click' modification of homopropargylglycine (HPG).

In order to explore this potential, adenovirus type 5 particles were produced in the presence of a metabolic precursor of GlcNAz, peracetylated *N*-azidoacetylgalactosamine (Ac_4GalNAz), and HPG.[19,20] Azido-sugar incorporation was accomplished by supplementing media with 50 μM Ac_4GalNAz for the entire duration of virus production (48 h). Introduction of the alkyne-amino acid was mediated by exposure of producer cells to 4 mM HPG during a 6 h window (18–24 h post infection), in a pulse chase format with minus methionine containing media. 48 h post infection, the cells were harvested, lysed and viruses were purified via a standard two-step ultracentrifugation procedure in CsCl gradients.[21]

Purified *O*-GlcNAc and HPG bearing virion were treated with 300 μM of Staudinger probe bearing a FLAG epitope (PhosFLAG) (3 h, rt).[22] Reaction mixtures were subsequently exposed to tetramethylrhodamine 5-carboxamido-(6-azidohexanyl) (az-TAMRA) dye (500 μM) using copper assisted 'click' reaction conditions under deoxygenating conditions in the presence of bathophenanthroline disodium salt (3 mM) and CuBr (1 mM) (rt, 12 h).[23] Particles were purified by size exclusion (Sephadex G-25) and interrogated by western blot and fluorescent gel imaging. Western analysis demonstrated that virus particles produced in the presence of both Ac_4GalNAz and HPG and only Ac_4GalNAz are labeled on a single coat protein occurring at 62 kD by PhosFLAG (Fig. 2A). No PhosFLAG labeling is seen on particles produced in the absence of Ac_4GalNAz, consistent with previous studies demonstrating the specific labeling of the fiber protein via *O*-GlcNAz. Fluorescent gel imaging of az-TAMRA labeled HPG-Ad and HPG/*O*-GlcNAz-Ad demonstrated labeling of a number of different proteins, consistent with the presence of solvent exposed methionine sites (Fig. 2B).[24]

Previous characterization of *O*-GlcNAz labeled Ad particles demonstrated 22 ± 1.5 chemically addressable azides per particle.[18] In order to quantitate HPG incorporation, TAMRA labeled virion were quantified via fluorescent gel imaging against a free az-TAMRA standard addition curve (Supplementary Fig. 2),

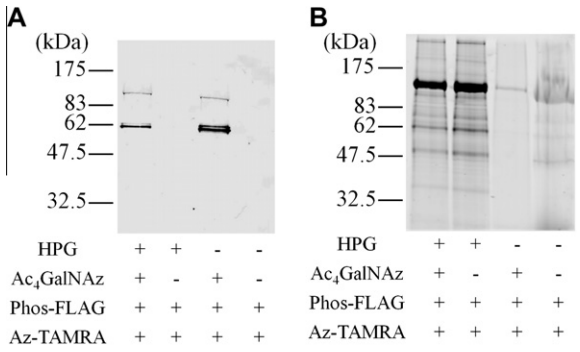

Figure 2. Chemoselective modification of GalNAz and HPG labeled adenovirus with Phosphine-FLAG and az-TAMRA. (A) Anti-FLAG western blot of peptide and dye labeled Ad5, demonstrating incorporation of azido sugar onto adenoviral fiber. (B) Same samples from A, analyzed for fluorescence as a reporter of HPG incorporation onto virus capsid.

demonstrating an attachment of 193 ± 12 dyes per virion (Supplementary Table 1). Vector production and infectivity are often compromised during genetic engineering of Ad particles, which has slowed the pace of vector development and effectively limited the production of multifunctional particles. While previous results indicate that *O*-GlcNAz incorporation does not impact either particle production or infectivity, the incorporation of HPG into the protein backbone at significantly higher incorporation levels was a concern. Surprisingly, no significant loss in either particle production or infectivity was observable for either of the singly modified Ad particles or particle bearing both *O*-GlcNAz and HPG (Fig 3).

In order to generate a chemotherapeutically 'armed' Ad particle, an azido derivative of SB-T-1214 (az-SB-T-1214) was synthesized that included a reductively self-immolative linker, designed to release the taxoid after Ad particle endocytosis (Scheme 1B). This linker has demonstrated efficient endosomal release of SBT-1214 in cell culture studies.[16,25] Modification of AdTRAIL with az-SB-T-1214 was accomplished in an identical manner to az-TAMRA modification of Ad described above. Chemically modified virion (SB-T-1214AdTRAIL) were purified by size exclusion and used to

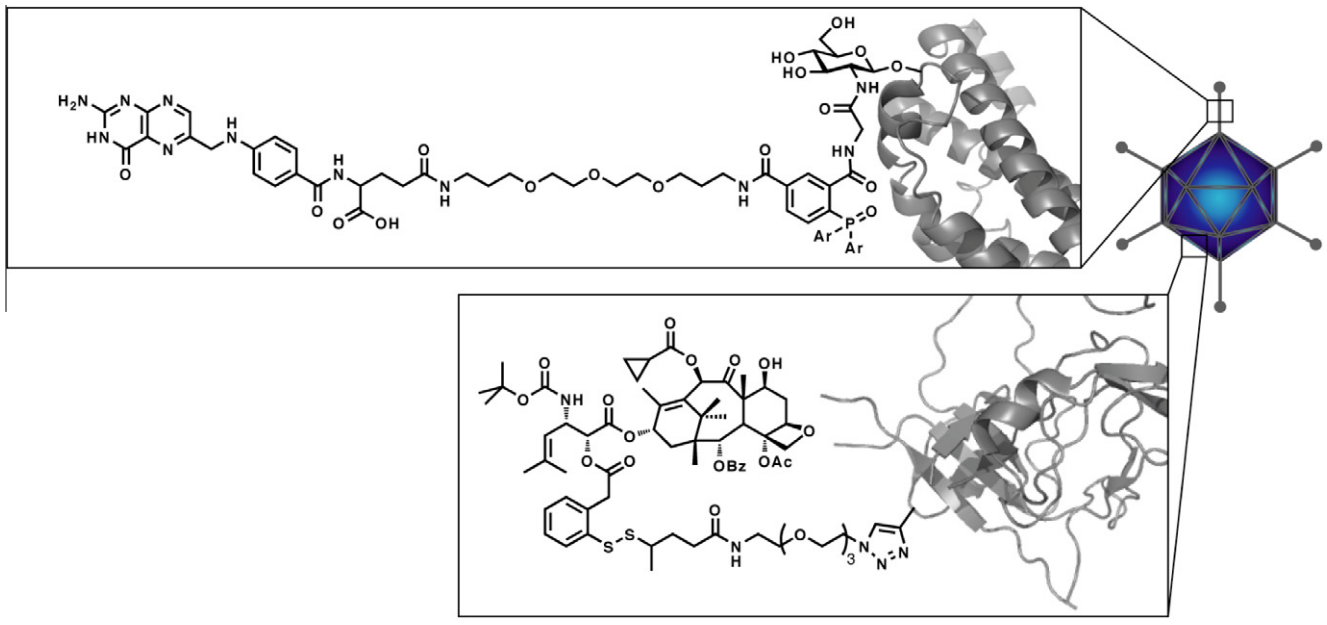

Figure 1. A cartoon illustrating adenovirus particles chemically modified with both a folate moiety, via Staudinger conjugation with an introduced *O*-GlcNAz, and SB-T-1214, via 'click' modification of metabolically introduced homopropargylglycine