Preclinical evaluation of MEDI0641, a pyrrolobenzodiazepine-conjugated antibody-drug conjugate targeting 5T4

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Abstract

Antibody-drug conjugates (ADCs) are used to selectively deliver cytotoxic agents to tumors and have the potential for increased clinical benefit to cancer patients. 5T4 is an oncofetal antigen overexpressed on the cell surface in many carcinomas on both bulk tumor cells as well as cancer stem cells (CSCs), has very limited normal tissue expression, and can internalize when bound by an antibody. An anti-5T4 antibody was identified and optimized for efficient binding and internalization in a target-specific manner, and engineered cysteines were incorporated into the molecule for site-specific conjugation. ADCs targeting 5T4 were constructed by site-specifically conjugating the antibody with payloads that possess different mechanisms of action, either a DNA cross-linking pyrrolobenzodiazepine (PBD) dimer or a microtubule-destabilizing tubulysin, so that each ADC had a drug:antibody ratio of 2. The resulting ADCs demonstrated significant target-dependent activity in vitro and in vivo, however the ADC conjugated with a PBD payload (5T4-PBD) elicited more durable anti-tumor responses in vivo than the tubulysin conjugate in xenograft models. Likewise, the 5T4-PBD more potently inhibited the growth of 5T4-positive CSCs in vivo, which likely contributed to its superior anti-tumor activity. Given that the 5T4-PBD possessed both potent anti-tumor activity as well as anti-CSC activity, and thus could potentially target bulk tumor cells and CSCs in target-positive indications, it was further evaluated in non-GLP rat toxicology studies that demonstrated excellent in vivo stability with an acceptable safety profile. Taken together, these preclinical data support further development of 5T4-PBD, also known as MEDI0641, against 5T4+ cancer indications.
Introduction

Antibody-drug conjugates (ADCs) represent a promising therapeutic approach to effectively treat cancer while reducing drug-related toxicities by combining the specificity of an antibody with the potency of cytotoxic agents (Reviewed in 1-3). An ADC consists of an antibody that is conjugated with a potent warhead, typically a cytotoxic small molecule, which has been chemically modified with a linker used to conjugate the drug to the antibody. The ADC binds to an antigen on the surface of a cancer cell and the ADC/antigen complex is internalized and trafficked to the lysosome where the cytotoxic agent is released through cleavage of a linker or through proteolytic degradation of the antibody itself if a non-cleavable linker is used to conjugate the warhead to the antibody. The warhead exits the lysosome where it can then bind to its target; typically either microtubules or DNA, depending on its mechanism of action. The binding of these cytotoxic agents to their targets results in cell cycle arrest that subsequently leads to cell death. Given the generally high potency of cytotoxics most commonly used in ADCs, it is important to identify targets exhibiting both good expression in tumors and limited normal tissue expression in order to reduce the possibility of on-target toxicity.

The oncofetal antigen 5T4, also known as trophoblast glycoprotein (TPBG), is a 72 kDa glycoprotein that is typically only expressed during embryonic development, whereas expression in normal adult tissues is very limited (4,5). Expression of 5T4, however, is reported to be significantly upregulated in many types of carcinomas including, but not limited to cancers of the lung, breast, stomach, prostate, colon, and ovaries, and its expression has been correlated with poor prognosis in multiple indications (6-13).
The biology of 5T4 in normal and pathologic tissues is not well understood. The extracellular domain of 5T4 contains several leucine-rich repeats that have been implicated in protein-binding that can promote cell-matrix and/or cell-cell interactions (14). However, no ligand or co-receptor has been identified for 5T4 to date. During embryonic development, 5T4 appears to regulate CXCR4-mediated chemotaxis (15) and modulates dendritic branching of granule cells in the developing olfactory bulb (16). The extracellular domain is critical for 5T4-mediated inhibition of Wnt/β-catennin signaling (17). 5T4 has a short cytoplasmic domain consisting of 44 amino acids and aside from a serine-asparagine-valine motif that is implicated in binding PDZ-containing molecules, there are no classical signal transduction motifs contained in the sequence (14,18). Studies with overexpression or knockdown of 5T4 have demonstrated that it likely plays a role in adhesion, cytoskeletal organization and motility, and epithelial-to-mesenchymal transition associated with metastasis (18-20). 5T4 knockout mice are viable, though adults have a number of structural defects in the brain as a result of changes that occur during brain development in utero likely caused by the role of 5T4 in dendritic branching (15).

5T4 is also expressed on cancer stem cells (CSCs) which are hypothesized to be responsible for chemotherapeutic resistance and recurrence of cancer (21,22). Targeting both 5T4-expressing bulk tumor cells and CSCs could, theoretically, produce a more durable clinical response. Previous reports have separately shown that 5T4 targeted ADCs armed with either a microtubule–targeting auristatin payload or a DNA cross-linking pyrrolobenzodiazepine (PBD) payload can inhibit both bulk and CSC tumor
cell populations (22-28). Given the differences in mechanisms of action between these two payloads we wanted to compare the activities of these two drugs side-by-side. We report data from in vitro and in vivo preclinical tumor models comparing the activity of the site-specifically conjugated ADCs 5T4-PBD and 5T4-Tub. The 5T4-PBD, subsequently labeled as MEDI0641, elicited superior anti-tumor activity than 5T4-Tub and reduced the CSC population in vivo unlike ADCs conjugated with either tubulysin or auristatin payloads. Following these observations, the preclinical safety and pharmacokinetic profile of MEDI0641 was evaluated in rats to assess its in vivo stability and off-target toxicity profile.
Materials & Methods

Generation of the lead anti-5T4 antibody, 5T4_0108

The parental antibody, A07, was derived by immunization of VeloImmune II mice (Regeneron, 29) with recombinant human 5T4 using a 48-day rest/boost immunization protocol (30) and was identified from a biochemical assay of hybridoma supernatants for binding to 5T4. This antibody underwent affinity optimization by CDR-directed mutagenesis (31) and phage display selection (32,33). The lead antibody, 5T4_0108, was identified in a biochemical assay where it demonstrated improved binding to 5T4 over the parental A07. This antibody was then expressed and purified as a full length, human IgG1 antibody containing the mutations L234F to significantly reduce FcγR binding, and S239C to provide engineered cysteines for site-specific conjugation of payloads, in the CH2 domain (34) based on EU numbering (35).

Site-specific conjugation of warheads

Site-specific conjugates were generated as previously described (34) and see Supplementary Data for a detailed protocol.

Cell lines

All cell lines (MDA-MB-361, HCC1937, SUM159 and T47D breast carcinoma cells; DU 145 prostate cancer cells, NCI-N87 gastric cancer cells, DMS114 lung carcinoma cells; Panc-1 and HPAC pancreatic carcinoma cells; HCT-116 colorectal carcinoma cells; HepG2 hepatocellular carcinoma cells) were obtained from American Type Culture Collection (ATCC) in the following years: MDA-MB-361 - November 2011; HCC1937 – November 2008; SUM159 – January 2012; T47D – September 2012; DU 145 – October 2011; NCI-N87 – August 2012; DMS114 – July 2008; Panc-1 – January 2012; HPAC –
April 2008; HCT-116 – January 2013; HepG2 – February 2012. Upon arrival, stock vials undergo authenticity and IMPACT testing (for murine viruses) by IDEXX Bioresearch using real-time PCR analyses. Cells are also designated mycoplasma-negative after undergoing internal PCR assays. Master cell banks are made from these stock vials and each time a working cell bank is created from these master banks, the cells are retested at IDEXX and internally. Cells used for all described experiments were derived from these working cell banks and were used within ten passages following thaw. After ten passages (typically within 2-3 months following thaw), cells were discarded and new cell vials were thawed as needed. All cell lines were cultured using ATCC-recommended media at 37°C in a humidified incubator with 5% CO₂ except for the MDA-MB-361 breast carcinoma cells that were cultured in atmospheric air at 37°C.

**In vitro cytotoxicity**

The cells were plated in culture media at a density of 2,000 to 5,000 per well (depending on the growth kinetics of each cell line) of tissue culture-treated 96-well plates in a volume of 80 μL and allowed to adhere overnight. A 5X stock of each concentration of antibody or ADC to be tested was prepared by diluting the test articles in culture medium. Twenty μL of each test article was added to cells in duplicate or triplicate such that the final dose curve ranged from 4 μg/mL down to 61 pg/mL in a stepwise 1:4 serial dilution series. The treated cells were cultured at 37°C / 5% CO₂ for 72 to 144 hours (depending on the growth kinetics of each particular cell line). The CellTiter-Glo® (CTG) Luminescent Viability Assay (Promega) was used to determine relative cytotoxicity. Briefly, 100 μL of CTG reagent was added to each well, allowed to incubate for 10
minutes at room temperature with mild shaking and then the absorbance of each sample at 560nM was read using an EnVision luminometer (Perkin Elmer). The percent cell viability was calculated by the following formula: (average luminescence of treated samples/average luminescence of control samples) x 100. IC50 values were determined using logistic non-linear regression analysis with GraphPad Prism software.

**In vivo efficacy studies**

All *in vivo* studies were carried out in compliance with the guidelines published by the Association for Assessment and Accreditation of Laboratory Care (AALAC). The MedImmune Institutional Animal Care and Use Committee (IACUC) approved all study protocols. For cell line-derived xenograft models, tumor cells were harvested from culture flasks, mixed with Matrigel in a 1:1 ratio and implanted subcutaneously or orthotopically into 5-6 week old athymic nude mice (Envigo). Resulting tumors were measured twice a week throughout the course of the experiments with tumor volume calculated using the following formula: tumor volume (mm$^3$) = (length x width$^2$)/2. Test articles were administered intravenously. See Supplemental Data for additional information.

**Evaluating effects on CSCs in tumors**

Mice bearing NCI-N87 and MDA-MB-361 xenografts were treated with the indicated ADC and upon initial signs of tumor regression, tumors were excised, cut into 4 mm pieces and cryopreserved using Cryostor. Frozen tumor pieces were thawed at 37°C, washed twice in HBSS and further minced using sterile scalpel blades. To obtain single-cell suspensions, the tumor pieces were then mixed with 200 units/mL of ultrapure collagenase III in DMEM/F12 medium. The tumor suspension was incubated
at 37°C for approximately 1 hour, with mechanical disruption every 30 minutes by pipetting with a 5-mL pipette. At the end of the incubation, cells were filtered through a 70-μm nylon mesh and centrifuged at 1200 rpm for 5 minutes. Cells were washed twice with HBSS. Following the last wash, cells were put through a 40-μm cell strainer and counted using a Vi-Cell XR Cell Viability Analyzer. Cells were assayed for aldehyde dehydrogenase activity as a measure of CSCs using the Stemcell Technologies Aldefluor kit following manufacturer’s instructions. The cells were run on an LSRII flow cytometer and analyzed with FlowJo software. Statistical significance of differences between groups was based on P values determined through unpaired t-tests.

The methods used to confirm the CSC phenotype of Aldefluor cells from NCI-N87 and MDA-MB-361 tumors can be found in Supplementary Data.

**Rat toxicology studies with MEDI0641**

Male Sprague Dawley rats (12 per group) were administered a single IV injection (Day 1) of 1 or 2 mg/kg anti-5T4 antibody conjugated to SG3249. Control rats (12 per group) were administered a single IV injection of vehicle control on Day 1. Animals were necropsied on Days 8 and 29 (6 per time point/group) to evaluate acute and delayed effects, respectively. All main study animals were evaluated for clinical signs, changes in body weight, clinical pathology, gross pathology with organ weights, and microscopic observations. In addition, groups of toxicokinetic (TK) satellite animals (12 per group) were included in each treatment arm to measure plasma concentration of total antibody and ADC. All TK satellite animals were evaluated for clinical signs, changes in body weight, and pharmacokinetic analysis.
Hematology and serum chemistry samples were collected and analyzed on Days 8, 14, and 29. Blood samples for pharmacokinetic analysis were collected in K2 EDTA tubes at multiple time points on Days 1, 2, 3, 8, 15, 16, 22, and 29.

A gross necropsy was performed on all main study animals and a standard list of organs, including brain, lung, liver, kidney, spleen, thymus, testes, heart, and bone, were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically by a board certified veterinary pathologist.

Additional Materials and Methods can be found in the Supplementary Data.
Results

Expression of 5T4 in clinical tumor samples

Previous reports have indicated that 5T4 is overexpressed in a variety of human carcinomas (6-13). To confirm these findings and to focus on cancer types with the highest incidence of 5T4 expression, extensive IHC of tumor microarrays and tumor samples as well as normal tissue arrays was conducted. 5T4 was broadly expressed in tumor microarrays and clinical tumor samples representing a number of cancer types, with the highest incidence in gastric cancer, non-small cell lung cancer (NSCLC), and HNSCC (Fig. 1A). At least 90% of tumors analyzed in each of these cancers had detectable levels of 5T4 expression by IHC, and in NSCLC samples, the prevalence of 5T4 was slightly higher in squamous NSCLC compared to adenocarcinoma. Greater than 60% of breast cancer specimens, including triple-negative samples, were 5T4-positive. Clear membrane localization of 5T4 immunostaining was prevalent in various types of cancers (Fig. 1B). Although gastric cancers had the highest incidence of staining, only about 20% of samples could be classified as high/positive with a pathology score of 2+ or 3+ observed in greater than 50% of the tumor cells. In contrast, roughly 70% of all NSCLC and HNSCC samples exhibited high/positive staining.

Similar to previous findings (6,9), 5T4 expression in normal tissues was extremely limited with minimal expression observed in epithelial cells of the renal tubules, gastric glands, skin, adrenal gland, urinary bladder, gall bladder, uterus, and cervix. Collectively, these data confirmed 5T4 would be a good tumor-associated antigen for a
targeted approach such as an ADC, and identified potential indications to target in the clinic due to prevalent expression of 5T4.

**Generation and in vitro characterization of 5T4_0108 antibody**

A 5T4-specific antibody, A07, was isolated from transgenic mice expressing human antibody variable genes immunized with recombinant human 5T4. The $K_D$ of this antibody was measured to be approximately 100 nM as determined via Biacore analysis, therefore *in vitro* affinity optimization using phage display was conducted and led to the generation of 5T4_0108. 5T4_0108 is a human IgG1 with the following mutations engineered into the heavy chain CH2 domain based on EU numbering (35): S239C to introduce a free cysteine for site-specific conjugation of payloads, and L234F to reduce engagement of FcγR and activation of immune effector cells in conjunction with conjugation of payload at S239C (34). The 5T4_0108 antibody has a $K_D$ of 6.5 nM for 5T4 with on- and off-rates of $3.3 \times 10^5$ (1/Ms) and $2.1 \times 10^3$ (1/s) respectively. It binds to and is internalized by 5T4-expressing cells (Figs. 2A and Supplementary Fig. S1), and conjugation of a payload at the S239C site did not affect binding or the internalization kinetics (Supplementary Fig. S1).

The improved affinity translated into superior efficacy. 5T4-Tub which consists of 5T4_0108 conjugated with a tubulysin payload $x$, was 14-fold more potent than an ADC consisting of a site-specific variant of the parental antibody (ssA07) conjugated with the same payload (ssA07-Tub) (Supplementary Fig. S2, Supplementary Table S1).

**In vitro efficacy of anti-5T4 ADCs conjugated with tubulysin and PBD payloads**
As described above, the initial studies to evaluate a candidate anti-5T4 ADC were conducted with 5T4-Tub. However, the activity of cytotoxics can be context dependent, where certain classes of drugs are more effective than others depending on the type of cancer. Therefore studies were conducted to determine the relative activity of 5T4-Tub and 5T4-PBD, an ADC composed of 5T4_0108 conjugated with SG3249, a DNA cross-linking PBD payload (27).

Comprehensive *in vitro* cytotoxicity assays were then conducted as an initial screen to compare relative activities of 5T4-PBD and 5T4-Tub. To determine if 5T4 expression levels impact ADC activity, these ADCs were tested against MDA-MB-361 breast cancer cells representing high 5T4 expression (~65,000 5T4 molecules/cell), DU 145 prostate cancer cells representing moderate 5T4 expression (~30,000 5T4 molecules/cell) and NCI-N87 gastric carcinoma cells representing low 5T4 expression levels (~4,000 5T4 molecules per cell) (Supplementary Table S2).

While the relative activities of 5T4-PBD and 5T4-Tub were comparable in each cell line, there was a clear correlation between the level of 5T4 expression and *in vitro* potency for both 5T4-ADCs in general (Fig. 2). 5T4-PBD and 5T4-Tub were most potent against 5T4-high MDA-MB-361 cells generating IC$_{50}$ values in the pg/mL range based on antibody concentration (Table 1). In addition a greater maximum cytotoxicity was achieved at doses that are logs lower than what was needed in the other two cell lines. The 5T4-ADCs induced potent cytotoxicity (IC$_{50}$ values in the single-digit ng/mL range) of DU 145 cells with moderate 5T4 expression and had the weakest activity against NCI-N87 cells with very low levels of 5T4 *in vitro*. 
Both the PBD and tubulysin warheads should elicit apoptosis, and this was confirmed using companion *in vitro* assays. In one assay the % viability was assessed and in a second assay cleaved-caspase activity was used as an indicator of apoptosis (36). Following treatment with 5T4-PBD or 5T4-Tub (Supplementary Fig. S3), decreased viability is directly correlated with increased caspase 3/7 activity in various cell lines, confirming that both 5T4-PBD and 5T4-Tub induce an apoptotic cell death pathway.

**In vivo anti-tumor activity of 5T4-PBD and 5T4-Tub**

Collectively, the *in vitro* data suggest that both 5T4-PBD and 5T4-Tub were able to induce potent cytotoxicity via apoptosis in a target-dependent manner. To confirm that the *in vitro* activity of 5T4-PBD and 5T4-Tub could translate to potent anti-tumor activity *in vivo*, these ADCs were tested against tumors with varying levels of 5T4 expression as determined by IHC: NCI-N87 xenografts with low/moderate *in vivo* 5T4 expression, DU 145 xenografts with moderate levels of 5T4 expression and MDA-MB-361 xenografts with high 5T4 levels.

In the NCI-N87 model with low/moderate 5T4 levels, 5T4-PBD was able to achieve durable regression with a single administration of a 1 mg/kg dose, whereas 5T4-Tub achieved a period of regression with a 5 mg/kg dose administered QWx4, but then the tumors began to regrow shortly after cessation of treatment (Fig. 3A). Initially, tumor regression was observed in the DU 145 model following QWx5 administration of either a 1 mg/kg dose of 5T4-PBD or a 5 mg/kg dose of 5T4-Tub (Fig. 3B). However, the 5T4-PBD-treated tumors remained regressed for approximately 70 days following cessation of therapy, whereas tumor growth in the 5T4-Tub-treated group appears to resume even before completion of the full course of therapy. In fact the duration of the anti-
tumor response of the 0.3 mg/kg dose of 5T4-PBD was superior to the 5 mg/kg dose of 5T4-Tub. The 1 mg/kg dose of 5T4-Tub only resulted in slight tumor growth inhibition. In the MDA-MB-361 xenograft model expressing high levels of 5T4, both 5T4-Tub and 5T4-PBD achieved durable regressions, though a lower dose and less frequent dosing of 5T4-PBD was required to achieve such anti-tumor activity (Fig. 3C). Statistical significance (P < 0.05 based on two-way ANOVA) was demonstrated for the 5T4-ADCs compared to controls. It should also be noted that no overt signs of toxicity such as significant body weight loss were observed with either ADC at the doses administered in these studies.

**Anti-CSC activity of 5T4-PBD**

To determine if the enhanced *in vivo* efficacy with the PBD-conjugated 5T4-PBD compared to the tubulysin-conjugated 5T4-Tub could be due to differential effects of their warheads on the CSC populations, studies were carried out to determine 5T4 expression on CSCs in cultured tumor cell lines and the effects of these ADCs and their respective warheads on CSCs *in vitro* and *in vivo*. Cell lines of various cancer types were tested for 5T4 expression on both total tumor cells as well as CSCs. CSCs were defined as Aldefluor⁺ or CD44⁺CD24⁻ cells in breast cancer (37,38), CD44⁺CD24⁻ cells in prostate cancer (39), CD90⁺ cells in hepatocellular carcinoma (HCC) (40), Aldefluor⁺ cells in gastric cancer (41) and CD24⁺CD44⁺ cells in pancreatic cancer (42). In all cases, 5T4 expression on the CSC population was equal to or higher than that on non-CSCs (Fig. 4A), even when the cell line had little to no 5T4 expression as a whole (Supplementary Fig. S4).
We first determined the effects of the various warheads on CSCs \textit{in vitro}. CSC-enriched spheroids derived from several of these cell lines were treated with PBD, tubulysin or auristatin warheads to determine if these warheads could inhibit the growth of CSCs \textit{in vitro}. The auristatin MMAE was included since an auristatin-conjugated 5T4-ADC has been reported to have activity against CSCs (23). In our studies only the PBD dimer warhead potently inhibited CSC sphere formation in all lines tested with an IC$_{50}$ range of 0.18 - 0.78 nM whereas the tubulysin or auristatin warheads had little to no effect (Supplementary Fig. S5). This data suggest that the DNA cross-linking PBD dimer warhead could potently and specifically inhibit CSC proliferation whereas tubulin inhibitors may not.

Next, we addressed if 5T4-ADCs conjugated with various payloads had differential effects on CSCs \textit{in vivo} from MDA-MB-361 breast carcinoma xenografts and NCI-N87 xenografts. We first confirmed the CSC phenotype in each of the cell lines by determining tumorigenic potential of each of the putative CSC populations by performing limiting dilution assays. In this manner, we confirmed that Aldefluor$^+$ cells represented the tumorigenic population in both the MDA-MB-361 and NCI-N87 cell lines (Supplementary Table S3) and effects of these ADCs on \textit{in vivo} CSCs were evaluated. Mice were inoculated with either MDA-MB-361 or NCI-N87 cells and when the average tumor volume reached $\sim$500mm$^3$, tumor-bearing mice were treated with a single dose of 5T4-PBD, 5T4-Tub, or 5T4-mcMMAF (an anti-5T4 antibody site-specifically conjugated with the auristatin mcMMAF with a DAR of 2). Once tumors began to regress (approximately 5-7 days in this model) the tumors were harvested and the CSC populations were quantified. The PBD-conjugated 5T4-PBD significantly reduced the
number of CSCs by approximately 50% in both models and was more effective than either the tubulysin-conjugated 5T4-Tub or the auristatin-conjugated 5T4-mcMMAF (Fig. 4B).

The data suggest that the superior anti-tumor activity of 5T4-PBD could be due to the ability of this ADC to target 5T4+ CSCs in addition to the 5T4+ bulk tumor cells, whereas ADCs with tubulin inhibitors, such as 5T4-Tub, are ineffective at inhibiting CSCs. Since 5T4 is a target that is expressed on both CSCs as well as the non-CSCs within a tumor, it is critical to develop a 5T4-ADC conjugated with a payload capable of effectively targeting CSCs to produce the most durable response.

**Tolerability of MEDI0641 in rats**

Given the greater *in vivo* activity and anti-CSC activity observed with 5T4-PBD, this ADC was selected for further development and was designated as MEDI0641. The safety profile of the ADC was subsequently evaluated in a non-GLP rat toxicology study. Although the 5T4 antibody does not cross-react with rodent 5T4, the rat was chosen as the initial toxicity species to evaluate the off-target toxicity profile and assess the *in vivo* stability of the molecule. Male rats were administered a single IV dose of 1 or 2 mg/kg of MEDI0641 on day 1 and necropsied on days 8 and 29 to evaluate acute and delayed toxicities. All animals survived until scheduled necropsy. Major findings were dose-dependent decrease in body weight gain (up to 10%) and dose-dependent bone marrow suppression, with major effects on reticulocytes, platelets, and white blood cells (Fig. 5). All findings were reversible or trending towards reversible by Day 29 and are consistent with the known off-target effects of PBD-conjugated ADCs in rats (43).
Discussion

MEDI0641 is a 5T4-targeting ADC conjugated with a PBD payload that is capable of inducing significant anti-tumor activity, including durable regressions in tumor models with varying intensity and heterogeneity of 5T4 expression. Though 5T4-Tub also demonstrated significant in vivo anti-tumor activity, MEDI0641 produced a more durable response possibly due to the fact that it had a more pronounced effect on CSCs than 5T4-Tub. We and others have shown that 5T4 is expressed on CSCs which are hypothesized to be responsible for chemotherapeutic resistance and recurrence of cancer (21,22). Using multiple HNSCC patient-derived xenograft (PDX) models, Kerk et al. reports that MEDI0641 was able to reduce, and in one model ablate, CSCs in these models, corroborating and extending the anti-CSC properties of MEDI0641 that we describe here. Like us, they also observed that 5T4 expression was increased in the CSC population of these PDX tumors compared to 5T4 expression in non-CSC tumor cells. Here we extend these findings into additional tumor types with significant prevalence and intensity of 5T4 expression in clinical samples, including breast cancer, gastric cancer, pancreatic cancer and prostate cancer. Collectively the strong expression and/or upregulation of 5T4 on CSCs suggest that perhaps 5T4 may have some functional relevance to CSC biology. Further studies are warranted to determine such a role for 5T4 in CSCs.

It has been hypothesized that tubulin inhibitors, such as the tubulysins, auristatins and maytansinoids, are most effective against tumor cells with high proliferative rates and are less effective against relatively quiescent cells such as CSCs (23,44). DNA targeting agents on the other hand, particularly those that form cross-links or other DNA
lesions that are not easily repaired could more effectively target CSCs. The data presented here support this hypothesis. 5T4-mcMMAF conjugated with an auristatin payload had no effect on CSC populations in vivo in either xenograft model and 5T4-Tub was either ineffective or less effective against CSCs compared to MEDI0641 in the MDA-MB-361 and NCI-N87 xenograft models, respectively. Previously, an anti-5T4 ADC conjugated with the auristatin payload mcMMAF (A1-mcMMAF; PF-06263507) was reported to have potent anti-tumor activity in vivo and also was reported to inhibit CSC-like tumor-initiating cells in a NSCLC PDX model (23). There could be several explanations for the conflicting data with the MMAF-conjugated ADCs: 1) The 5T4-mcMMAF ADC used in the present study was a site-specific conjugate with a DAR of 2, as opposed to the classically-conjugated A1-mcMMAF that has a DAR of ~4, 2) differences in dosing schedule (single administration here, Q4Dx4 reported), 3) different CSC+ tumor models were used in each set of studies and each could respond to auristatins differently, and 4) different methodologies were used to assess anti-CSC activity in vivo in each report. Testing our 5T4-ADCs in models and assays similar to those reported where A1-mcMMAF demonstrated anti-CSC activity, and vice versa, could help resolve the contrary observations. Regardless, the present data clearly demonstrates that the PBD-conjugated MEDI0641 had superior anti-CSC effects over either tubulysin- or auristatin-conjugated ADCs.

The different anti-CSC activities observed between MEDI0641 and 5T4-Tub could potentially account for the different anti-tumor responses elicited by these ADCs. MEDI0641 significantly decreased the CSC population in both the MDA-MB-361 and the NCI-N87 models, and in each model, durable regressions were observed following
MEDI0641 treatment. In the NCI-N87 model, even though higher doses and frequency were utilized, 5T4-Tub had inferior anti-CSC activity and less durable anti-tumor activity compared to MEDI0641. Interestingly each ADC was capable of eliciting regressions in the MDA-MB-361 model even though only MEDI0641 was able to inhibit the in vivo CSC population. This could be attributed to the fact that 5T4 expression was significantly higher in both bulk tumor cells and CSCs in the MDA-MB-361 model compared to NCI-N87, suggesting that, hypothetically, more of each ADC would be delivered to MDA-MB-361 tumors compared to NCI-N87 tumors. It could also be due to dosing; a single administration in the CSC experiment while 5T4-Tub was administered QWx4 in the efficacy study. Taken together, these data suggest that greater success of a 5T4-ADC in tumors with lower levels of 5T4 expression may be dependent on the ability of that ADC to deliver a payload that effectively targets the 5T4-positive CSC population.

Evaluating ADCs that target the same surface antigen but deliver warheads with different mechanisms of action helped to potentially identify which ADC could produce a more durable response. To our knowledge, this is the first report where a head-to-head comparison was made between two ADCs comprised of the same antibody but conjugated with payloads from different drug classes with different mechanisms of action: PBDs that form interstrand DNA cross-links and tubulysins that depolymerize microtubules. Others have reported comparing cleavable vs. non-cleavable drug linkers, but typically these comparisons have included the same warhead class; for example comparing conjugates with either cleavable mc-VC-PABC-MMAE or non-cleavable mc-MMAF (45-46), or cleavable SPDB-DM4 versus non-cleavable SMCC-
DM1 (47,48). There have also been reports of comparing conjugates with auristatins and maytansinoids (49) but these are both inherently two classes of drugs with a similar mechanism of action, namely, microtubule inhibition.

Prior to developing A1-mcMMAF, the same research group had evaluated a calicheamicin-conjugated 5T4-ADC, however composed of a different antibody against 5T4. They found that the ADC bearing the calicheamicin payload was not tolerated at efficacious exposure levels in cynomolgus monkeys (23,50). Though also a DNA-targeting agent, the mechanism of action of calicheamicin is different from that of PBDs, and the drivers of the toxicity with the 5T4-calicheamicin were not reported so it’s not clear if they could be attributed to the different antibody and/or the different payload. Non-GLP rat toxicology studies with MEDI0641 demonstrated a reasonable safety profile. Regardless, close attention to the toxicological findings with MEDI0641, particularly in a species where our anti-5T4 antibody cross-reacts is warranted as safety studies with this ADC proceed.

In conclusion, our generation and testing of the PBD-conjugated MEDI0641 and 5T4-Tub permitted a direct comparison of payloads with different mechanisms of action conjugated to the same antibody against 5T4. Our results demonstrated MEDI0641 had overall superior activity and in particular, is more effective at targeting 5T4-positive CSCs which may prevent or reduce tumor recurrence. These data provide motivation for further development of this promising ADC.
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References


Tables

Table 1. Relative activity of 5T4-ADCs against cell lines with varying levels of cell surface 5T4 expression.

The table represents average IC\textsubscript{50} values and the maximum cytotoxicity (Max Kill percentages) from multiple assays run for each ADC against each cell line.

<table>
<thead>
<tr>
<th>ADC</th>
<th>MDA-MB-361 (~65K 5T4/cell)</th>
<th>DU 145 (~30K 5T4/cell)</th>
<th>NCI-N87 (~4K 5T4/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC\textsubscript{50} (ng/ml)</td>
<td>Max Kill (%)</td>
<td>IC\textsubscript{50} (ng/ml)</td>
</tr>
<tr>
<td>5T4-Tub</td>
<td>0.85</td>
<td>78.8</td>
<td>2.67</td>
</tr>
<tr>
<td>5T4-PBD</td>
<td>0.39</td>
<td>90.7</td>
<td>2.47</td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1. Relative prevalence of 5T4 expression in clinical tumor samples.
A, Immunohistochemistry for 5T4 expression was conducted to determine the prevalence of 5T4 expression in tumor samples from patients with various cancers. The values represent the percentage of tumor samples that have detectable levels of 5T4 immunostaining. The number in parentheses represents the number of tumor samples tested for each cancer type. B, Representative images of 5T4 immunostaining in various cancer types.

Figure 2. In vitro characterization of 5T4_0108 and ADCs comprised of this antibody
A, Target-specific binding of 5T4_0108 to human breast cancer (MDA-MB-361), prostate cancer (DU 145) gastric cancer (NCI-N87) cell lines that express 5T4 or to 5T4-negative DMS 114 lung carcinoma cell lines was determined by flow cytometry. B, Structures of 5T4-PBD consisting of the 5T4_0108 antibody site-specifically conjugated to the PBD payload SG3249, and 5T4-Tub consisting of the 5T4_0108 antibody conjugated to the tubulysin payload mc-Lys-MMETA. C, The 5T4-PBD and 5T4-Tub ADCs were tested against tumor cells with different levels of 5T4 expression on the cell surface. A correlation between target expression and cytotoxicity was observed by testing the ADCs against MDA-MB-361 breast carcinoma cells with high levels of 5T4 cell surface expression, DU 145 prostate cancer cells with moderate levels of 5T4 cell surface expression, and NCI-N87 gastric carcinoma cells with low levels of 5T4 cell surface expression. Representative experiments are shown and the values indicate the mean ± SEM. Control ADCs consisting of the site-specific variant of the R347 IgG1
isotype control antibody conjugated to either PBD (Ctrl-PBD) or tubulysin (Ctrl-Tub) were also included to determine target-specific binding.

**Figure 3. 5T4-PBD produces more durable responses in xenograft tumor models with varying levels 5T4 expression**

5T4-PBD and 5T4-Tub were tested in athymic nude mice bearing subcutaneous NCI-N87 (A), DU 145 (B), or MDA-MB-361 (C) xenografts. Following tumor cell inoculation, mice were size-matched into treatment groups and therapeutic administration was initiated when the average tumor volume reached approximately 200mm³. The administration of therapeutics in the study is indicated by the arrowheads, and the dotted lines represent average tumor volume at the time of treatment initiation; tumor volumes below this line are indicative of tumor regression. Values indicate the mean ± SEM. Representative experiments are shown and demonstrate that while 5T4-PBD and 5T4-Tub are able to achieve tumor regressions in the MDA-MB-361 model with high 5T4 expression, 5T4-PBD produces more durable in vivo tumor regression than 5T4-Tub in the NCI-N87 and DU 145 models. Statistical significance of 5T4-ADC treatment groups (P < 0.05) was demonstrated via 2-way ANOVA analysis.

**Figure 4. 5T4-PBD effectively targets CSCs in vivo**

A, 5T4 is expressed on CSC populations of various cancer cell lines. CSC markers are as follows: MDA-MB-361 breast cancer: Aldefluor⁺; NCI-N87 gastric cancer: Aldefluor⁺; DU 145 prostate cancer: CD44⁺CD24⁻. B, ADC-treated tumors were harvested and the CD44⁺CD24⁻ breast cancer CSCs and Aldefluor⁺ gastric carcinoma CSCs were counted. The number of CSCs found in the vehicle-treated control tumors was
normalized to 100% and the data for other treatment groups are reported as a percent of the CSC population found in control tumors. 5T4-PBD decreased the CSC population by approximately 50% in both models. 5T4-Tub had no effect on the MDA-MB-361 CSCs but had some effect on NCI-N87 CSCs though this effect was not as potent as that with 5T4-PBD. Neither 5T4-mcMMAF nor controls had any effect on CSCs in either model. The 5T4-PBD-mediated decrease in CSCs was statistically significant compared to all groups in both models except the 5T4-Tub-treated group in the NCI-N87 model (P values < 0.05 based on unpaired T tests).

**Figure 5. Safety profile of MEDI0641 in rats**

Rats (n=12) were treated with a single IV injection of 1 or 2 mg/kg MEDI0641. Control animals were treated with a single IV injection of vehicle control. A, Tolerability was assessed by measuring body weight changes over a 29-day period. Data points represent the mean value of each group at individual time points; error bars, mean ± SEM. B-D, MEDI0641 is associated with dose-dependent bone marrow suppression. Hematological analyses were conducted on peripheral blood samples collected on Days 8, 14 and 29 post-treatment to enumerate platelet counts (B), reticulocytes (C) and white blood cells (D). Bars represent the mean value of each group; error bars, mean ± SEM.
Figure 1 - Harper et al.

A

Gastric (88)
NSCLC - Squam (62)
NSCLC - Mixed (123)
Head & Neck (95)
NSCLC - Adeno (35)
Renal Cell (9)
Ovarian (11)
Breast - Mixed (122)
Breast - TNBC (32)
Pancreatic (9)
Prostate (12)

B

Gastric
NSCLC
HNSCC
TNBC

Figure 1 - Harper et al.
Figure 2 - Harper et al.

(A) Flow cytometry histograms showing the binding of 5T4 PBD and Ctrl IgG to different cell lines. The histograms are color-coded as follows:

- Red: Unstained
- Blue: 5T4_0108-AF647
- Green: Ctrl IgG-AF647

(B) Structures of 5T4-PBD and 5T4-Tub. 5T4-PBD contains a dPEG5 spacer and a Val-Ala trigger, along with a Maleimide Conjugation Unit and a SG3199 Warhead. 5T4-Tub includes a Maleimide Conjugation Unit and an MMTA Warhead. The latter is linked to mc-Lys-MMTA Payload.

(C) Viability curves for MDA-MB-361 (~65K 5T4/cell), DU 145 (~30K 5T4/cell), and NCI-N87 (~4K 5T4/cell) after treatment with different ADC concentrations. The graphs show the percentage of viability (%) against the ADC concentration (ng/ml).
Figure 4 - Harper et al.
Figure 5 - Harper et al.
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