Long-term tumor regression induced by an antibody-drug conjugate that targets 5T4, an oncofetal antigen expressed on tumor-initiating cells

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Abstract

Antibody-drug conjugates (ADCs) represent a promising therapeutic modality for the clinical management of cancer. We sought to develop a novel ADC that targets 5T4, an oncofetal antigen expressed on tumor-initiating cells (TICs), which comprise the most aggressive cell population in the tumor. We optimized an anti-5T4 ADC (A1mcMMAF) by sulphydryl-based conjugation of the humanized A1 antibody to the tubulin inhibitor monomethylauristatin F (MMAF) via a maleimidocaproyl linker. A1mcMMAF exhibited potent in vivo anti-tumor activity in a variety of tumor models and induced long-term regressions for up to 100 days after the last dose. Strikingly, animals showed pathological complete response in each model with doses as low as 3 mg antibody/kg dosed every 4 days. In a non-small cell lung cancer patient-derived xenograft model, in which 5T4 is preferentially expressed on the less differentiated tumor cells, A1mcMMAF treatment resulted in sustained tumor regressions and reduced TIC frequency. These results highlight the potential of ADCs that target the most aggressive cell populations within tumors, such as TICs. In exploratory safety studies, A1mcMMAF exhibited no overt toxicities when administered to cynomolgus monkeys at doses up to 10 mg antibody/kg/cycle x 2 and displayed a half-life of 5 days. The preclinical efficacy and safety data established a promising therapeutic index that supports clinical testing of A1mcMMAF.
**Introduction**

Antibody-drug conjugates (ADCs) constitute a therapeutic modality in which a cytotoxic agent is chemically linked to an antibody that recognizes a tumor-associated antigen (1). The basic strategy underlying ADCs is to combine the exquisite target selectivity of monoclonal antibodies with the potent cytotoxic activity of certain natural products and synthetic molecules, with the goal of generating agents that are highly efficacious and also safe. The ADC platform currently includes a growing repertoire of cytotoxic payloads, linker technologies and conjugation methods. Key considerations in generating an optimal ADC include target biology, antibody properties, linker chemistry and payload characteristics. Notably, more than half of the ADCs in clinical development are based on auristatin, a synthetic analog of the natural product dolastatin-10 that inhibits tubulin polymerization and ultimately induces G2/M cell cycle arrest and cell death at low picomolar intracellular concentrations (1-4).

While target selection for ADCs is typically based on simple criteria including target internalization and overexpression in tumor versus normal tissues, it is appealing to consider developing ADCs that target TICs (also called cancer stem cells), which comprise a population of tumor cells that drive tumor growth and metastasis (5-7). The TIC model could help explain the high rate of recurrence observed in the clinic, as it postulates that the tumor cells frequently missed by conventional therapies actually drive tumor growth. If TICs could be targeted directly by ADCs, patient survival might be improved dramatically.

We recently demonstrated that the 5T4 oncofetal antigen is expressed on proliferating TICs in NSCLC and is associated with the undifferentiated state and the epithelial-
mesenchymal transition [EMT] (8), which has been linked to TICs and an invasive phenotype (9, 10). 5T4, also known as trophoblast glycoprotein (TPBG), is a cell surface antigen that internalizes rapidly and thus has the potential to efficiently deliver ADCs into tumor cells (11, 12). Expression of 5T4 is observed in a variety of solid carcinomas, while expression in normal adult tissues is limited (13, 14). Expression of 5T4 is associated with advanced disease and/or worse clinical outcome in NSCLC and gastric, colorectal and ovarian carcinomas (8, 15-17). 5T4 was shown to modulate CXCR4 function (18) and Wnt signaling (19), yet its specific function remains unknown. Together these clinical observations suggest that 5T4 is an attractive target for ADC therapeutics.

Our previous work demonstrated that an anti-5T4 ADC bearing the DNA-damaging agent calicheamicin had potent anti-tumor activity (8, 11), but subsequent toxicology studies indicated that it was not well tolerated at exposures required for anti-tumor activity (unpublished data). Therefore we explored new antibody-linker-payload combinations to target TICs via the 5T4 antigen. Here we present the preclinical profile of a novel anti-5T4 ADC termed A1mcMMAF, which is comprised of the humanized anti-5T4 A1 antibody linked to the potent tubulin inhibitor monomethyl auristatin F (MMAF) via a noncleavable maleimidocaproyl (mc) linker. In contrast to calicheamicin which impacts both quiescent and proliferating cells, auristatin preferentially impacts proliferating cells due to its anti-mitotic mechanism of action; thus calicheamicin can elicit broader anti-tumor activity but can also present additional safety liabilities, and the optimal payload for each target could depend on the proliferative status of the tumor and normal cells that express the target. A1mcMMAF was highly potent in a variety of tumor models and did not cause any overt toxicity in non-human primates at comparable exposures. Thus A1mcMMAF is a
promising clinical candidate that targets TICs with the goal of providing long-term therapeutic benefit to cancer patients.

**Materials and Methods**

**Cancer cell lines and PDX:**

A panel of cancer cell lines was chosen to include lines that expressed low, moderate and high expression of 5T4 and exhibited reproducible growth curves as tumor xenografts. MDAMB435/5T4 and control MDAMB435/neo stably transfected cells were used to demonstrate specificity for the antigen as described previously (11). MDAMB361-DYT2 cells were obtained from Dr. D. Yang (Georgetown University). PC14PE6 and PC3MM2 cells were obtained from Dr I. Fidler (11). The other cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Each cell line was cultured in its standard medium as recommended by ATCC, and no further cell line authentication was conducted. LG-0476 was obtained from Jackson Laboratories. The 37622A1 NSCLC PDX, and the establishment and characterization of primary serum-free culture TUM622 from 37622A1, were described (8).

**Immunohistochemistry**

Primary antibodies used were anti-TPBG (5278-1, Epitomics; 0.36 μg/ml), anti-Ki67 (Clone SP6, Labvision; 1:200), anti-human Pan IgG antibody (3443-1, Epitomics; 0.3
μg/ml), anti-Phospho Histone H3 (#9701, Cell Signaling Technologies; 0.13 μg/ml) and control rabbit IgG. See supplement for detailed protocol.

**Preparation of antibody drug conjugate:**

Generation of A1 antibody is detailed in the Supplement. Sulfhydryl-based bioconjugation was performed as described (20) with modifications. Antibody was pretreated with 3 equivalents of tris(2-carboxyethyl)phosphine (TCEP) to liberate the thiol residues, and this partially reduced material was exposed to ~6 equivalents of maleimidocaproyl-MMAF (mMMAF). Isolation and purification was accomplished by size-exclusion chromatography, and the material was characterized by hydrophobic-interaction chromatography and mass spectrometric analysis under denaturing and non-denaturing conditions (Supplemental Figures S1-S3). The in vivo efficacy studies with 37622A1 and MDA MB435/5T4 were performed with A1mMMAF conjugated via lysine instead of cysteine residues. Bridging studies both in vitro and in vivo (MDAMB435/5T4) indicated that there was no difference in activity between these preparations (unpublished data). All safety and toxicokinetic studies were performed with ADC conjugated via cysteine, which is the preparation method for the clinical material.

**In vitro characterization of A1mMMAF:**

Cell binding and internalization protocols are described in the Supplement. Cytotoxicity in vitro was assessed using a cell viability indicator (MTS, Promega, Madison, WI). IC$_{50}$ values (concentrations that reduced cell viability by 50%) were calculated following determination of the number of viable cells after 96 hours of drug exposure. Since TUM622 have a doubling time of 48-72 hours which is about twice as long as the standard
cancer cell lines, TUM622 cells were exposed to drug for 192 hours total (re-fed after 96 hours) before IC\textsubscript{50} values were determined. For the spheroid assay, TUM622 cells were embedded in growth factor reduced Matrigel (BD Biosciences, Bedford, MA) and overlayed with Bronchial Epithelial Cell Growth Medium (BEGM; Lonza, Walkersville, MD). Medium was replaced every 2 days. A1mcMMAF and control ADC were added after 5 days of growth when spheroids were <100 \(\mu\text{m}\) in diameter. After 7 days of treatment, spheroids with a diameter of 125-300 \(\mu\text{m}\) were quantitated on a Gelcount colony counter (Oxford Optronix, Oxford, UK); the assay was ended at that timepoint while the integrity of the Matrigel was still intact.

**In vivo pharmacology:**

All procedures using mice were approved by the Pfizer Institutional Animal Care and Use Committee according to established guidelines. Mice were injected subcutaneously with tumor cells, and animals with staged tumors were administered intravenously with saline (vehicle), A1mcMMAF, or control ADC. See supplement for additional information including the TIC frequency assay.

**Tolerability and toxicokinetics:**

For studies in cynomolgus monkey, A1mcMMAF was administered to male animals (2-6 kg) at a dosage of 1, 3, 10 mg/kg as a slow iv bolus infusion once every other week for 2 cycles. Animals were observed twice daily for 24 days and weighed every 2 to 4 days. Hematology, coagulation and clinical chemistry parameters were evaluated on days 3, 17 and 29. Blood samples were drawn up to 336 h after each dose for Ab, ADC and released payload (cys-mcMMAF) determination. At euthanasia, a panel of 45 tissues, including
bone marrow, brain, eye, heart, kidney, liver and lung, was collected for histological evaluation.

Quantitation of A1 concentrations in mouse or cynomolgus serum was achieved using an enzyme-linked immunosorbant assay (96 well format) with colorimetric detection. Briefly, the capture protein was 5T4 and the detection antibody was a biotinylated goat anti-human kappa chain IgG for the antibody assay and a biotinylated anti-MMAF antibody for the ADC assay. Optical density was measured on a spectrophotometer. To assess the released payload cys-mcMMAF (20), quantitation was achieved using an UPLC-MS/MS system (5500 Qtrap, C18 column and cys-mcMMAD as the internal standard) with a lower limit of quantitation ranging from 0.002 to 0.1 ng/mL in plasma and tumor, respectively. Toxicokinetic analysis was done using the pharmacokinetics module within Watson LIMS v7.4 (Thermo Scientific, Philadelphia PA) using a standard noncompartmental model.

Results

The 5T4 antigen is expressed on proliferating tumor cells:

To inform the design of the anti-5T4 ADC, we sought to determine whether the 5T4-expressing cancer cells were actively proliferating and thus might be susceptible to an anti-mitotic agent. Indeed, when two NSCLC samples were co-stained with antibodies to 5T4 and cell proliferation marker Ki67, considerable overlap was observed (Figure 1A). These results suggested that 5T4-expressing tumor cells would be sensitive to an anti-
mitotic agent such as auristatin. We assembled a panel of preclinical tumor models with a broad range of 5T4 expression (Figure 1B). The panel included traditional cancer cell line xenografts and patient-derived xenograft (PDX) models. PDX models were established by direct implantation of freshly resected human tumor fragments into immune-compromised mice. PDX models preserve the original architecture of human tumors and circumvent selection imposed by tissue culture conditions (21). Notably, two NSCLC PDX lines (37622A1 and LG-0476) displayed marked heterogeneity of 5T4 expression, with highest expression levels in the less differentiated cells located in the periphery of tumor nests, i.e. at the tumor-stroma interface (Figure 1B).

**In vitro properties of A1mcMMAF:**

The A1mcMMAF ADC is comprised of the humanized A1 antibody conjugated to MMAF via a noncleavable maleimidocaproyl linker (20) and contains an average drug:antibody ratio of 4 mol/mol (Supplemental Figures S1-S3). The antibody exhibited an affinity of 0.48 nM to human 5T4 antigen and cross-reacted with cynomolgus monkey antigen but not mouse or rat antigen (Supplemental Figure S4 and Table S1). A1 was specific to 5T4 antigen as indicated by binding to MDAMB435 cells that were stably transfected with 5T4 but not empty vector (Figure 2A).

A1mcMMAF binding to MDAMB435/5T4 cells, incubated on ice to prevent internalization, was comparable to the unconjugated antibody (Figure 2B). In addition, similarly to unconjugated antibody, A1mcMMAF did not bind 5T4-negative Raji cells (Figure 2B). To assess internalization kinetics of the ADC, MDAMB435/5T4 cells were
exposed to A1mcMMAF or unconjugated antibody and subsequently transferred to 37°C to allow internalization. After one hour, 60% of antibody and 62% of ADC had been internalized; after four hours the values were 82% and 85% respectively (Supplemental Table S2). Internalization kinetics in MDAMB361-DYT2 and MDAMB468 cells were also comparable for antibody and ADC and were similar to MDAMB435/5T4 cells (data not shown). Together these results demonstrate that conjugation of A1 antibody to mcMMAF did not significantly alter its binding or internalization properties.

Immunofluorescence microscopy also demonstrated that A1mcMMAF bound to the membrane of MDAMB435/5T4 cells incubated at 4°C, and that 4 hours after transfer of cells to 37°C, A1mcMMAF displayed reduced membrane staining and instead was found in the lysosomes, as evidenced by the overlap of the ADC and the lysosomal marker LAMP-1 (Figure 2C). These results demonstrate that A1mcMMAF binds to 5T4-expressing cells, internalizes and traffics to the lysosomal compartment. The noncleavable maleimidocaproyl linker depends on lysosomal proteases for release of active payload (20).

Binding of A1 antibody to cells was determined in vitro for a panel of cancer cell lines that represented many solid tumor types (Supplemental Figure S5). For five cell lines, the number of antigen-binding sites was quantified by flow cytometry using the murine A1 antibody. The average numbers of binding sites per cell were: MDAMB435/5T4, 197,000; MDAMB468, 35,000; MDAMB361-DYT2, 40,000; PC14PE6, 11,000; and PC3MM2, 27,000.
A1mcMMAF inhibited the growth of 5T4-expressing cell lines (MDAMB435/5T4, MDAMB468 and MDAMB361DYT2) in a concentration-dependent manner but in contrast was not active against 5T4-negative Raji lymphoma cells (Table 1). The control ADC (antibody against a non-human non-mouse antigen, conjugated to mcMMAF) was not active against these cell lines (Table 1). The differences in A1mcMMAF potencies were not attributable to differential sensitivities of the cell lines to auristatin (Supplemental Table S3) and therefore reflected the specificity of the ADC to the 5T4 antigen. To investigate the impact of A1mcMMAF on TICs, a primary culture termed TUM622 was established in serum-free medium from the 37622A1 PDX and shown to be enriched for stem-like cells with high 5T4 expression (8). A1mcMMAF inhibited the growth of TUM622 in monolayer culture (Table 1).

Cells cultured in the presence of an extracellular matrix grow as three-dimensional multicellular spheroids in a microenvironment that mimics native tumor tissue and can respond differently to drugs compared to cells cultured in monolayer cultures (22). TUM622 cells were embedded in growth factor-reduced Matrigel and allowed to form tumor spheroids of approximately 80-100 µm in diameter, and then treated with A1mcMMAF or control ADC. Growth of TUM622 spheroids was inhibited by treatment with A1mcMMAF relative to control ADC in a concentration-dependent manner (Figure 2D and Supplemental Figure S6). We noted that treatment with control ADC at all drug concentrations tested resulted in an approximate 50% reduction in spheroid numbers, compared to untreated samples, which suggested the presence of a non-specific activity in vitro potentially caused by proteolysis of the ADC in the matrix or phagocytosis and degradation of ADC-containing matrix (23). Interestingly, higher concentrations were
required to inhibit spheroid growth compared to monolayer growth (compare data in Figure 2D and Table 1). In summary, A1mcMMAF specifically inhibited growth of the TIC culture TUM622 in 2D and 3D growth conditions.

**Pharmacokinetics**

The specificity of tumor targeting of A1 was investigated in mice bearing H1975 tumor xenografts. Animals were injected with fluorescently labeled A1 or control antibody and subjected to in vivo bioimaging analysis using an IVIS Spectrum. In contrast to the control antibody, the anti-5T4 antibody accumulated rapidly within the tumor (Figure 3A). Given the low level of target expression in H1975 cells (Figures 1B, S5), the data also suggested that A1-based ADCs might be active against tumors with modest expression levels of 5T4.

To determine whether the ADC was delivering active payload to the tumor, the concentrations of the released payload cys-mcMMAF in plasma and tumor homogenates were determined in treated animals bearing MDAMB435/5T4 tumor xenografts. Forty eight hours after a second dose, the concentration of cys-mcMMAF in tumor tissue was 0.0517 μM, substantially (~313-fold) greater than in plasma (Figure 3B). The results indicated a greater propensity for released payload to accumulate in tumor tissue compared to the circulation and suggested that the ADC was effectively concentrating the cytotoxic payload in tumor tissue bearing the target antigen.
To visualize A1mcMMAF pharmacodynamics at the cellular level, animals bearing MDAMB361DYT2 tumor xenografts were injected with A1mcMMAF or control ADC, and tumors were harvested 4 and 24 hours later. Immunohistochemical analysis revealed that A1mcMMAF but not control ADC bound to the cell membrane of tumor cells (Figure 3C, top panel). Cell binding was observed at both the 4 and 24 hour timepoints and appeared marginally greater at the later timepoint (data not shown). Further analysis with an antibody against a mitotic marker, phosphorylated histone H3, demonstrated a significant increase in the proportion of tumor cells in mitosis after treatment with A1mcMMAF but not control ADC (Figure 3C, bottom panel). These results suggested that the tubulin inhibitor payload was selectively delivered to tumor cells by the anti-5T4 antibody and caused mitotic arrest.

**In vivo tumor growth inhibition:**

A1mcMMAF exhibited potent anti-tumor activity in a variety of tumor models. The ADC was administered to mice bearing subcutaneous tumor xenografts established from cancer cell lines or a PDX model. These tumor models represent a broad range of 5T4 expression levels, as determined by IHC staining of formalin fixed, paraffin embedded tumor sections (Figure 1B).

In the 37622A1 PDX, A1mcMMAF caused long term tumor regressions at 10 mg/kg every 4 days for 4 cycles (Q4dx4; Figure 4A). In this model, not all tumor cells express the target (Figure 1B); nevertheless A1mcMMAF displayed dramatic anti-tumor activity, which underscores the promise of targeting antigens expressed on subpopulations of cancer cells, in particular the more tumorigenic and aggressive cells such as TICs. In MDAMB361DYT2 tumors, 3 mg/kg Q4dx4 of A1mcMMAF caused complete inhibition
of tumor growth in all 7 mice treated (Figure 4A). Monitored over the 110 days of the study, 6 out of 7 A1mcMMAF-treated animals showed pathological complete response, as defined by the lack of evidence of tumors by gross pathology examination at the termination of the study. One animal had a single small tumor of 77 mm$^3$ in size.

A1mcMMAF also exhibited strong anti-tumor activity against the MDAMB468 breast xenograft model at dose levels of 3 and 10 mg/kg Q4dx4 (Figure 4A). In the H1975 lung tumor model with low expression of 5T4, A1mcMMAF exhibited dose-dependent anti-tumor activity of moderate duration and magnitude and one mouse showed pathological complete response (Figure 4A). Finally, in the MDAMB435/5T4 tumor model, two doses of A1mcMMAF at 10 mg/kg were sufficient for complete tumor regression, and two doses at 3 mg/kg showed moderate anti-tumor activity (Supplemental Figure S7A).

In contrast to the anti-tumor activity observed with A1mcMMAF, no activity was observed with the control ADC (Figure 4A) or the unconjugated antibody (Supplemental Figure S7B and unpublished data). In addition, A1mcMMAF did not exhibit activity against 5T4-negative tumor xenografts (Supplemental Figure S8). A1 does not recognize murine 5T4 antigen (Supplemental Figure S4) and therefore the anti-tumor activity can be attributed to direct impact on the tumor cells. Together these data demonstrate that the observed efficacy of A1mcMMAF is the result of targeting the 5T4 antigen and delivering the auristatin payload to tumor cells in a highly selective manner.

**Reduction of TIC frequency:**
Based on our work indicating that 5T4 expression is enriched on NSCLC TICs (8), we hypothesized that A1mcMMAF treatment would reduce TIC frequency as measured by tumorigenicity of the remaining cells upon reimplant into naïve animals (Figure 4B). This approach has been used to assess TIC targeting of other compounds (24). We performed the assay in the NSCLC PDX 37622A1 which has heterogeneous expression of 5T4 (Figure 1B). A1mcMMAF treatment resulted in lower TIC frequency (Figure 4C), with \( p = 0.028 \) compared to control ADC and \( p = 0.10 \) compared to vehicle. These results provide direct evidence that A1mcMMAF targets TICs via the 5T4 antigen. Similar experiments are ongoing in additional models and tumor types.

**Tolerability and toxicokinetics:**

We determined that the cynomologus monkey represented a suitable species for toxicology studies since A1 binds to the cynomolgus monkey 5T4 antigen with high affinity (Supplemental Table S1), and our tissue cross-reactivity studies indicated comparable staining patterns in normal tissues from human and cynomolgus monkey (unpublished data).

A1mcMMAF was well tolerated in cynomolgus monkeys after two doses of 10 mg/kg administered every other week. No deaths or significant adverse events were recorded throughout the observation period. With the exception of elevated levels of aspartate transaminase (AST), there were no hematology, coagulation or clinical chemistry parameters that were considered adverse. Histopathological evaluation identified morphologically negligible and minimal glomerulonephropathy at 3 and 10 mg/kg.
dose levels. The highest non-severe toxic dose (HNSTD) in this study was set at 10mg/kg. Toxicokinetic analysis of ADC, total antibody (A1) and free payload (cys-mcMMAF) demonstrated dose dependency with half-life \( t_{1/2} \) values of 5.0 ± 0.6 days for A1mcMMAF, 8.7 ± 2.2 days for total A1 antibody and 3.4 ± 0.2 days for cys-mcMMAF (Figure 5A). A similarity in the \( t_{1/2} \) values of cys-mcMMAF and ADC indicated that the pharmacokinetics of cys-mcMMAF were formation rate limited. At 10 mg/kg/cycle, the AUC after the second cycle of dosing was 24,100 ± 2,540 \( \mu \text{g} \cdot \text{h/mL} \) for A1mcMMAF, which was 63% of the value for total antibody. The \( C_{\text{max}} \) values for ADC and total antibody were 435 ± 52 and 343 ± 28 \( \mu \text{g/mL} \) respectively. The AUC of free payload (cys-mcMMAF) was 0.149 ± 0.013 \( \mu \text{g} \cdot \text{h/mL} \), which is equivalent to 0.0006% of the ADC value and substantially below the threshold of cytotoxic activity of cell-permeable MMAF compounds. Importantly, after treatment with A1mcMMAF, the cys-mcMMAF concentrations remained very low in plasma in monkeys and mice. In contrast, cys-mcMMAF accumulated in tumor tissue in A1mcMMAF-treated mice (Figure 3B). These data suggest that A1mcMMAF provides sufficient ADC exposure in tumor xenografts in mice (including exposure of the released payload to tumor tissue) with limited non-targeted exposure of the cytotoxic payload.

The therapeutic index of A1mcMMAF in the preclinical setting was assessed by comparing drug exposures in the efficacy and safety studies (Figure 5B). For MDAMB435/5T4 and 37622A1 efficacy studies in mice, exposures were taken 48 hours after the last dose. For the MDAMB361-DYT2 pharmacokinetic study, exposures were taken up to 240 hours. At exposures that achieved long-term suppression of tumor
growth, no significant adverse events were observed in the tolerability studies. Therefore A1mcMMAF was safe at doses that demonstrated strong anti-tumor activity.

**Discussion**

In this study we characterized the safety and anti-tumor activity of a novel anti-5T4-ADC that targets the most tumorigenic cell population of human tumors. A1mcMMAF exhibited anti-tumor activity in models that represent a wide range of 5T4 expression levels at doses that did not cause significant adverse effects in cynomologus monkey. In contrast, the anti-5T4 calicheamicin ADCs that we previously investigated (8, 11) were not sufficiently tolerated at efficacious doses, even though calicheamicin has proven suitable for other ADC targets such as CD22 (25).

There are currently two 5T4-targeting agents in clinical development for oncology indications: a vaccine consisting of 5T4-expressing vaccinia virus (26-28) and an anti-5T4 antibody fragment conjugated to superantigen from Staphylococcus enterotoxin A (29, 30). Both of these agents engage the host immune system to attack the 5T4-expressing cells. In contrast, A1mcMMAF delivers an anti-mitotic payload directly to 5T4-expressing cells and thus has a distinct mechanism of action that does not depend on immune cell infiltration or activation; therefore A1mcMMAF could display a distinct efficacy and safety profile in the clinic.

A1mcMMAF was active in vivo against multiple tumor models, including H1975 which exhibited low expression of the target. Interestingly, A1mcMMAF displayed more pronounced anti-tumor activity in vivo compared to in vitro. Potential explanations for
this observation are (1) differential conformation or presentation of the A1 epitope in vivo vs. in vitro, for example due to interactions with the extracellular matrix; (2) differential internalization kinetics or intracellular trafficking in vivo vs. in vitro; (3) differential 5T4 expression in vivo vs. in vitro, for example due to tumor-stromal interaction or microenvironmental stress such as hypoxia. The concordance between our immunohistochemistry data (in vivo) and flow cytometry data (in vitro) argues against the third explanation.

In our preclinical models, we did not see a linear relationship between efficacy of A1-mcMMAF and target expression, as the conjugate was efficacious in models with varied expression of the target; however we did demonstrate specific activity as models not expressing the target failed to respond to the treatment. As the efficacy of antibody drug conjugates is determined by several factors including target biology, target expression, internalization kinetics, intracellular processing of the conjugates, payload efficacy etc. it may not be appropriate to expect a linear relationship between target expression and efficacy and careful consideration for patient selection strategies based on target expression and/or receptor density is warranted.

The therapeutic potential of targeting TICs was highlighted in this study by the sustained tumor regression observed upon A1mcMMAF treatment of 37622A1 tumor xenografts despite heterogeneous expression of the target, and by the reduction in TIC frequency after treatment. A1mcMMAF has exhibited a favorable safety profile and is scheduled to be the first 5T4-targeting ADC tested in the clinical setting, and to our knowledge the first ADC in development that is designed specifically to target TICs. Targeting the most
tumorigenic and aggressive cell populations with ADCs can be sufficient to achieve long-term efficacy in preclinical models and potentially in the clinic.

**Acknowledgments**

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**References**


Table 1. In vitro cytotoxicity of A1mcMMAF (IC\textsubscript{50} values, ng/mL).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MDAMB435/5T4</th>
<th>MDAMB361DYT2</th>
<th>MDAMB468</th>
<th>TUM622</th>
<th>Raji</th>
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<tbody>
<tr>
<td>A1mcMMAF</td>
<td>6.3 ± 1.4</td>
<td>415 ± 177</td>
<td>894 ± 87</td>
<td>275 ± 71</td>
<td>&gt;45,000</td>
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</table>
Increasing concentrations of A1mcMMAF or a nonbinding control ADC were incubated for 96 h with tumor cell lines in monolayer culture. The inhibitory concentration of 50% (IC\textsubscript{50}) was calculated by logistic nonlinear regression and is reported as the mean ± SEM of multiple experiments (n=8 or more experiments with all cell lines except TUM622, for which n=3).

| Control ADC | 21,000 | >45,000 | >45,000 | >45,000 | >45,000 |
Figure Legends

Figure 1. 5T4 expression in clinical samples and a panel of preclinical models.

A, NSCLC clinical samples were co-stained for 5T4 antigen (brown stain at membrane) and Ki67 cell proliferation marker (purple stain in nucleus), and counterstained with hematoxylin (blue).

B, Tumor xenografts from a panel of preclinical models were stained for 5T4 and counterstained with hematoxylin. The models exhibited a range of 5T4 expression levels.

Figure 2. Anti-5T4 ADC binding, internalization and cytotoxicity in vitro.

A, Anti-5T4 A1 antibody binds to MDAMB435 cells stably transfected with 5T4 but not empty vector (neo). Binding was assessed by flow cytometry using a PE-labeled secondary antibody.

B, Comparable cell binding of anti-5T4 ADC and unconjugated antibody to 5T4-positive MDAMB435/5T4 cells and 5T4-negative Raji cells. Cells were incubated at 4°C with ADC or antibody followed by peroxidase-conjugated secondary antibody and then exposed to substrate. Error bars represent standard deviation of the mean (n=3).

C, A1mcMMAF internalizes and traffics to lysosomes in MDAMB435/5T4 cells upon incubation at 37°C. A1mcMMAF is indicated in green, lysosomes (LAMP-1) in red and DNA in blue.
**D.** Concentration-dependent cytotoxic activity of A1mcMMAF on TIC-enriched TUM622 spheroids. Error bars represent standard error of the mean (n=3).

**Figure 3. Pharmacodynamics and mechanism of action of the anti-5T4 ADC.**

**A.** Anti-5T4 antibody accumulates in H1975 tumor xenografts. Bioluminescence images from mice injected with Alexa750-labeled antibody. Images were acquired 24 hours after single administration of antibody at 2 mg/kg.

**B.** Concentrations of cys-mcMMAF released payload were 313-fold higher in tumor samples compared to plasma samples at 48 hours after the second dose in the MDAMB435/5T4 tumor model. *, value of 0.00016 μM.

**C.** A1mcMMAF binds tumor cells in vivo and causes mitotic arrest. Animals bearing MDAMB361DYT2 tumor xenografts were given a single administration of A1mcMMAF or control ADC at 3 mg/kg. The tumors were harvested 24 hours later and stained with anti-human IgG1 to detect the ADC (top row; brown stain at membrane) or with anti-phospho-histone H3 to detect mitotic cells (bottom row; brown stain in nucleus).

**Figure 4. Anti-tumor activity of 5T4-ADC in tumor models with a broad range of 5T4 expression levels.**

**A.** Athymic (nu/nu) mice were treated with vehicle, A1mcMMAF or control ADC on the schedule Q4Dx4. In 37622A1 NSCLC PDX, A1mcMMAF was significantly different than vehicle from day 17 onward; in MDAMB361DYT2 breast cancer, from day 5
onward; in MDAMB468 breast cancer, from day 6 onward. in H1975 NSCLC, from day 7 onward at all dose levels. Statistical significance is based on $p < 0.05$.

**B.** Schematic of the TIC frequency assay. In the first phase, tumor-bearing animals are dosed with TIC-targeting agent or control. Treated tumors are harvested and dissociated to single cells, and after depletion of mouse cells, live human cells are reimplanted into naïve animals. The hypothesis is that TIC-targeting agents will result in reduced tumor incidence upon reimplant.

**C.** A1mcMMAF treatment of 37622A1 PDX resulted in lower tumor incidence of the remaining live human cells upon reimplant into naïve animals. $p = 0.028$ for A1mcMMAF vs. control ADC and $p = 0.10$ for A1mcMMAF vs. vehicle.

**Figure 5.** A1mcMMAF was safe in cynomologus monkey at exposures that demonstrate strong anti-tumor activity in mice.

**A.** Serum concentration ($\mu$g/mL) profiles for Ab (O), ADC (■) and cys-mcMMAF (Δ) in monkeys after the second cycle of a 10 mg/kg dose. Overall exposure of the released payload (cys-mcMMAF) was dramatically lower compared to Ab and ADC exposure.

**B.** ADC serum concentration ($\mu$g/mL) profiles at 1 (●), 3 (■) and 10 (▲) mg/kg in monkeys after the second cycle of dosing showing that concentrations were generally higher than concentrations observed in two efficacy models (at 48 hr after dosing), 37622A1 PDX (O, Δ) and MDAMB435/5T4 (○), and also higher than concentrations from a mouse (MDMMB361-DYT2 (●)) PK study at an efficacious dose (3 mg/kg).
Sapra et al. Fig. 2

A

B

C

D

0 h 4 h

Mean Fluorescence Intensity

Concentration µg/mL

A1 mAb on MDAMB435/NEO

A1 mAb on MDAMB435/5T4

A1mcMMAF on MDAMB435/5T4

A1 mAb on MDAMB435/5T4

A1mcMMAF on Raji

A1 mAb on Raji

0 ug/ml 5 ug/ml

RLU

0 1000 2000 3000

0.3 1.0 3.0 10.0

Control ADC

A1mcMMAF

Spheroid count (+/- S.E.M.)

0.5 µg/ml 1 µg/ml 2.5 µg/ml 5 µg/ml
A1 Ab Control Ab

Sapra et al. Fig. 3

A

B

Concentration (µM) at 48 hr

0.15
0.10
0.05
0.00

Tumor - Payload
Plasma - Payload
Tumor - Ab
Plasma - Ab
Tumor - ADC
Plasma - ADC

B

Vehicle

Control ADC

A1mcMMAF

ADC
(anti-hIgG1)
40x

Mitotic cells
(p-histone H3)
10x
Sapra et al. Fig. 4

A. 37622A1 PDX

- Vehicle
- A1mcMMAF 1 mg/kg
- A1mcMMAF 10 mg/kg

MDAMB468

- Vehicle
- control ADC 10 mg/kg
- A1mcMMAF 3 mg/kg
- A1mcMMAF 10 mg/kg

MDAMB361DYT2

- Vehicle
- control ADC 3 mg/kg
- A1mcMMAF 3 mg/kg

H1975

- Vehicle
- control ADC 10 mg/kg
- A1mcMMAF 1 mg/kg
- A1mcMMAF 3 mg/kg
- A1mcMMAF 10 mg/kg

B. (Diagram of tumor growth and treatment)

- TIC
- Non-TIC

- Control ADC
- New tumors
- Dose
- Reimplant
- Reduced tumor incidence
- Anti-TIC ADC

C. (Graph showing tumor frequency post-treatment)

- Vehicle
- Control ADC
- A1mcMMAF
Sapra et al. Fig 5

A

Concentration (µg/mL)

0 00001
0.0001
0.001
0.01

Time (hr)

0 48 96 144 192 240 288 336

Ab
ADC
Payload

B

ADC Concentrations (µg/mL)

0 0.1
0.1 1 10 100

Time (hr)

0 48 96 144 192 240 288 336

Cyno TK, 1 mg/kg
Cyno TK, 3 mg/kg
Cyno TK, 10 mg/kg
Mouse DYT2, 3 mg/kg
Mouse 37622, 1 mg/kg
Mouse 37622, 10 mg/kg
Mouse MDA435, 10 mg/kg
Molecular Cancer Therapeutics

Long-term tumor regression induced by an antibody-drug conjugate that targets 5T4, an oncofetal antigen expressed on tumor-initiating cells

Puja Sapra, Marc Damelin, John F DiJoseph, et al.

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