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 (ADC) 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 (TIC), which comprise the most aggressive cell population in the tumor. We optimized an anti-5T4 ADC (A1mcMMAF) by sulfydryl-based conjugation of the humanized A1 antibody to the tubulin inhibitor monomethylauristatin F (MMAF) via a maleimidocaproyl linker. A1mcMMAF exhibited potent antitumor activity in a variety of tumor models and induced long-term regressions for up to 100 days after the last dose. Strikingly, animals showed pathologic 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 × 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. Mol Cancer Ther; 12(1); 38–47. ©2012 AACR.

Introduction

Antibody–drug conjugates (ADC) 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 over-expression in tumor versus normal tissues, it is appealing to consider developing ADCs that target tumor-initiating cells (TIC; 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 have shown 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), which has been linked to TICs and an invasive phenotype (8–10). 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, whereas 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 has shown that an anti-5T4 ADC bearing the DNA-damaging agent calicheamicin had potent antitumor activity (8, 11), but subsequent toxicology studies indicated that it was not well tolerated at exposures required for antitumor 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 comprises the humanized anti-5T4 A1 antibody linked to the potent tubulin inhibitor mono-methylauristatin F (MMAF) via a noncleavable maleimido-caproyl (mc) linker. In contrast to calicheamicin, which impacts both quiescent and proliferating cells, auristatin preferentially impacts proliferating cells due to its anti-tumototic mechanism of action; thus calicheamicin can elicit broader antitumor activity but can also present additional safety liabilities, and the optimal payload for each target remains unknown. Together these clinical observations suggest that 5T4 is an attractive target for ADC therapeutics.

Preparation of antibody–drug conjugate

Generation of A1 antibody is detailed in the Supplement. Sulphhydryl-based bioconjugation was conducted 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 approximately 6 equivalents of maleimidocaproyl-MMAF (mcMMAF). 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 (Supplementary Fig. S1–S3). The in vivo efficacy studies with 37622A1 and MDAMB435/5T4 were conducted with A1mcMMAF 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 conducted with ADC conjugated via cysteine, which is the preparation method for the clinical material.

In vitro characterization of A1mcMMAF

Cell binding and internalization protocols are described in the Supplement. Cytotoxicity in vitro was assessed using a cell viability indicator (MTS, Promega). IC₅₀ values (concentrations that inhibited cell growth by 50%) were calculated following determination of the number of viable cells after 96 hours of drug exposure. Because TUM622 has a doubling time of 48 to 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 IC₅₀ values were determined. For the spheroid assay, TUM622 cells were embedded in growth factor reduced Matrigel (BD Biosciences) and overlaid with Bronchial Epithelial Cell Growth Medium (BEGM; Lonza). Medium was replaced every 2 days. A1mcMMAF and control ADC were added after 5 days of growth when spheroids were less than 100 μm in diameter. After 7 days of treatment, spheroids with a diameter of 125 to 300 μm were quantitated on a Gelcount colony counter (Oxford Optronix); 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 with tumor cells subcutaneously or into mammary fat pad (MDAMB468), 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 intravenous 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 hours 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 histologic 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) 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 antimitotic agent. Indeed, when 2 NSCLC samples were costained with antibodies to 5T4 and cell proliferation marker Ki67, considerable overlap was observed (Fig. 1A). These results suggested that 5T4-expressing tumor cells would be sensitive to an antimitotic agent such as auristatin. We assembled a panel of preclinical tumor models with a broad range of 5T4 expression (Fig. 1B). The panel included traditional cancer cell line xenografts and 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, 2 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, that is, at the tumor–stroma interface (Fig. 1B).
In vitro properties of A1mcMMAF

The A1mcMMAF ADC comprises the humanized A1 antibody conjugated to MMAF via a noncleavable maleimidocaproyl linker (20) and contains an average drug:antibody ratio of 4 mol/mol (Supplementary Figs. S1–S3). The antibody exhibited an affinity of 0.48 nmol/L to human 5T4 antigen and cross-reacted with cynomolgus monkey antigen but not mouse or rat antigen (Supplementary Fig. 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 (Fig. 2A).

A1mcMMAF binding to MDAMB435/5T4 cells, incubated on ice to prevent internalization, was comparable to the unconjugated antibody (Fig. 2B). To assess internalization to unconjugated antibody, A1mcMMAF did not bind 5T4-negative Raji cells (Fig. 2B). In addition, similarly bated on ice to prevent internalization, was comparable to (Fig. 2A).

MDAMB435/5T4 cells were exposed to ADC and unconjugated antibody to 5T4-positive MDAMB435/5T4 cells upon incubation at 37°C, and 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 (Fig. 2C). These results have shown 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 (Supplementary Fig. S5). For 5 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; MDAMB361DYT2, 40,000; PC14PE6, 11,000; and PC3MM2, 27,000.

A1mcMMAF inhibited the growth of 5T4-expressing cell lines (MDAMB435/5T4, MDAMB468, and MDAMB361-DYT2) 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 nonhuman nonmouse 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 (Supplementary 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
nonspecific activity untreated samples, which suggested the presence of a 50% reduction in spheroid numbers, compared with Fig. S6. We noted that treatment with control ADC at all concentration-dependent manner (Fig. 2D and Supplementary growth factor–reduced Matrigel and allowed to form tumor spheroids of approximately 80 to 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 (Fig. 2D and Supplementary Fig. S6). We noted that treatment with control ADC at all drug concentrations tested resulted in an approximate 50% reduction in spheroid numbers, compared with untreated samples, which suggested the presence of a nonspecific 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 with monolayer growth (compare data in Fig. 2D and Table 1). In summary, A1mcMMAF specifically inhibited growth of the TIC culture TUM622 in 2D and 3D growth conditions.

### Pharmacokinetics/Pharmacodynamics

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 (Fig. 3A). Given the low level of target expression in H1975 cells (Fig. 1B and Supplementary Fig. 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 μmol/L, substantially (~313-fold) greater than in plasma (Fig. 3B). The results indicated a greater propensity for released payload to accumulate in tumor tissue compared with 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 (Fig. 3C, top). 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, has shown a significant increase in the proportion of tumor cells in mitosis after treatment with A1mcMMAF but not control ADC (Fig. 3C, bottom). 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 antitumor 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 (Fig. 1B).

In the 37622A1 PDX, A1mcMMAF caused long-term tumor regressions at 10 mg/kg every 4 days for 4 cycles (Q4dx4; Fig. 4A). In this model, not all tumor cells express the target (Fig. 1B); nevertheless A1mcMMAF displayed dramatic antitumor 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 (Fig. 4A). Monitored over the 110 days of the study, 6 of 7 A1mcMMAF-treated animals showed pathologic 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 mm³ in size. A1mcMMAF also exhibited strong antitumor

### Table 1. In vitro cytotoxicity of A1mcMMAF (IC₅₀ 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>
</tr>
</thead>
<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>
</tr>
<tr>
<td>Control ADC</td>
<td>21,000</td>
<td>&gt;45,000</td>
<td>&gt;45,000</td>
<td>&gt;45,000</td>
<td>&gt;45,000</td>
</tr>
</tbody>
</table>

NOTE: Increasing concentrations of A1mcMMAF or a nonbinding control ADC were incubated for 96 hours with tumor cell lines in monolayer culture. The inhibitory concentration of 50% (IC₅₀) 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).
activity against the MDAMB468 orthotopic breast model at dose levels of 3 and 10 mg/kg Q4dx4 (Fig. 4A). In the H1975 lung tumor model with low expression of 5T4, A1mcMMAF exhibited dose-dependent antitumor activity of moderate duration and magnitude and one mouse showed pathologic complete response (Fig. 4A). Finally, in the MDAMB435/5T4 tumor model, 2 doses of A1mcMMAF at 10 mg/kg were sufficient for complete tumor regression, and 2 doses at 3 mg/kg showed moderate antitumor activity (Supplementary Fig. S7A).

In contrast to the antitumor activity observed with A1mcMMAF, no activity was observed with the control ADC (Fig. 4A) or the unconjugated antibody (Supplementary Fig. S7B and unpublished data). In addition, A1mcMMAF treatment would reduce TIC frequency as measured by tumorigenicity of the remaining cells upon reimplant into naïve animals (Fig. 4B). This approach has been used to assess TIC targeting of other compounds (24). We conducted the assay in the NSCLC PDX 37622A1, which has heterogeneous expression of 5T4 (Fig. 1B). A1mcMMAF treatment resulted in lower TIC frequency ($P = 0.028$ compared with control ADC and $P = 0.10$ compared with 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 cynomolgus monkey represented a suitable species for toxicology studies as A1 binds to the cynomolgus monkey 5T4 antigen with high affinity (Supplementary 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 2 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 hematologic, coagulation, or clinical changes.

**Reduction of TIC frequency**

On the basis of our work indicating that 5T4 expression is enriched on NSCLC TICs (8), we hypothesized that A1mcMMAF binding to 5T4 antigen on TICs would reduce TIC frequency.
chemistry parameters that were considered adverse. Histopathologic evaluation identified morphologically negligible and minimal glomerulonephropathy at 3 and 10 mg/kg dose levels. The highest nonsevere toxic dose in this study was set at 10 mg/kg. Toxicokinetic analysis of ADC, total antibody (A1), and free payload (cys-mcMMAF) shows dose dependence 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 (Fig. 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 µg·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 µg/mL, respectively. The AUC of free payload (cys-mcMMAF) was 0.149 ± 0.013 µg·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

Figure 4. Antitumor 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; and 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 were dosed with TIC-targeting agent or control. Treated tumors were harvested and dissociated to single cells, and after depletion of mouse cells, live human cells were 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.
very low in plasma in monkeys and mice. In contrast, cys-mcMMAF accumulated in tumor tissue in A1mcM-MAF-treated mice (Fig. 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 nontargeted 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 (Fig. 5B). For MDAMB435/5T4 and 37622A1 PDX (O, △) and MDAMB435/5T4 (○), and also higher than concentrations from a mouse [MDM8361-DYT2 (□)] PK study at an efficacious dose (3 mg/kg).

Discussion

In this study, we characterized the safety and antitumor activity of a novel anti-5T4-ADC that targets the most tumorigenic cell population of human tumors. A1mcMMAF exhibited antitumor activity in models that represent a wide range of 5T4 expression levels at doses that did not cause significant adverse effects in cynomolgus 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 2 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 Staphylococcal enterotoxin A (29, 30). Both of these agents engage the host immune system to attack the 5T4-expressing cells. In contrast, A1mcMMAF delivers an antimitotic 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 with in vitro. Potential explanations for this observation are (1) differential conformation or presentation of the A1 epitope in vivo versus in vitro, for example, because of interactions with the extracellular matrix; (2) differential internalization kinetics or intracellular trafficking in vivo.
versus in vitro; (3) differential 5T4 expression in vitro versus in vivo, for example, because of 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 A1mcMMAF and target expression, as the conjugate was efficacious in models with varied expression of the target; however, we did show specific activity as models not expressing the target failed to respond to the treatment. As the efficacy of ADCs 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.

**Disclosure of Potential Conflicts of Interest**

All authors are/or were full time employees of Pfizer Inc. when research was conducted and own company's stock options and/or units.

**Authors' Contributions**


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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Giannakou, K. Khandke

Writing, review, and/or revision of the manuscript: P. Sapra, M. Damelin, J.F. DiJoseph, K. Marquette, J. Golas, A. Giannakou, K. Khandke, R. Dushin, M. Leal, C.J. O’Donnell, R.T. Abraham, H. Gerber

Study supervision: P. Sapra, M. Damelin, J. Lucas

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