An anti-mesothelin-monomethyl auristatin E conjugate with potent anti-tumor activity in ovarian, pancreatic and mesothelioma models.

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Abbreviations: ADC, antibody-drug conjugate; αMSLN, humanized anti-mesothelin antibody h7D9.v3; gD, glycoprotein D; MSLN, Mesothelin; MPF, megakaryocyte promoting factor; MMAE, monomethyl auristatin E; MMAF, monomethyl auristatin F; mc, maleimidocaproyl; vc, valine-citrulline; PAB, p-aminobenzyloxycarbonyl; IHC, immunohistochemistry; TMA, tissue microarray; FACS, fluorescence activated cell sorting; LME, linear mixed effects; TGI, tumor growth inhibition.

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Abstract

Mesothelin (MSLN) is an attractive target for antibody-drug conjugate therapy because it is highly expressed in various epithelial cancers, with normal expression limited to non-dividing mesothelia. We generated novel anti-mesothelin antibodies and conjugated an internalizing one (7D9) to the microtubule-disrupting drugs monomethyl auristatin E (MMAE) and MMAF, finding the most effective to be MMAE with a lysosomal protease-cleavable valine-citrulline linker. The humanized (h7D9.v3) version, αMSLN-MMAE, specifically targeted mesothelin-expressing cells and inhibited their proliferation with an IC50 of 0.3nM. Because the anti-tumor activity of an anti-mesothelin immunotoxin (SS1P) in transfected mesothelin models did not translate to the clinic, we carefully selected in vivo efficacy models endogenously expressing clinically relevant levels of mesothelin, after scoring mesothelin levels in ovarian, pancreatic and mesothelioma tumors by immunohistochemistry. We found that endogenous mesothelin in cancer cells is upregulated in vivo and identified two suitable xenograft models for each of these three indications. A single dose of αMSLN-MMAE profoundly inhibited or regressed tumor growth in a dose-dependent manner in all six models, including two patient-derived tumor xenografts. The robust and durable efficacy of αMSLN-MMAE in preclinical models of ovarian, mesothelioma and pancreatic cancers justifies the ongoing phase I clinical trial.
Introduction

Mesothelin (MSLN) is a glycoprotein limited to the surface of the single mesothelial cell layer lining the pericardia, pleura and peritoneum (1). It is expressed as a ~75 kDa fusion protein with the so-called megakaryocyte promoting factor (MPF), which is removed by furin cleavage, leaving a ~50kDa glycosylphosphatidylinositol-anchored mature mesothelin on the cell surface (2). Mesothelin knockout mice exhibit no discernible phenotype, thus the normal biological functions of MPF and mesothelin are unknown (3). Mesothelin is also overexpressed in a high percentage of various epithelial cancers, including ovarian, pancreatic and mesothelioma (see references in Supplemental Table 1).

The high tumor versus normal expression makes mesothelin an attractive target for cancer therapy. Indeed, besides vaccine (4) and chimeric antigen receptor T-cell immunotherapy (5), a murine dsFv to mesothelin has been fused to the protein synthesis inhibiting (PE38) domain of pseudomonas exotoxin to generate SS1P (CAT-5001) (6); (1). This immunotoxin exhibits marked anti-tumor activity against xenografts of mesothelin-transfected A431 cells, but was not tested in endogenously expressing xenografts due to lack of suitable models. In a phase I trial SS1P produced only 4/34 minor responses in mesothelin-selected cancers, and was dose limited by pleuritis (7), likely because PE38 inhibited protein synthesis in the mesothelin-positive pleura. Immunogenicity prevented further cycles of treatment even following lymphocyte depletion (8), so a deimmunized version (RG7787) is currently under investigation (9).

Chimeric SS1 IgG without PE38 (MORAb-009, Amatuximab) exerts modest preclinical
activity via antibody-dependent cell-mediated cytotoxicity (10), but no clinical activity (11).

Recent advances in antibody drug-conjugate (ADC) technology suggest anti-mitotic ADCs with human or humanized antibodies to mesothelin could be a more promising approach. ADCs comprise whole IgGs covalently linked to cytotoxic drugs that are orders of magnitude more potent than traditional chemotherapeutics (12,13). ADCs are essentially pro-drugs, optimally requiring internalization so the antibody and/or linker is digested to release active drug (12,14). The recent approval of two microtubule-inhibiting ADCs with different linker-drug platforms – anti-CD30-valine-citrulline-monomethyl auristatin E (SGN-35)(15) and anti-HER2-MCC-DM1 (T-DM1) (16) – offers hope for these technologies with other tumor antigens. Indeed, several other ADCs are at various stages of clinical development, including an anti-mesothelin-DM4 conjugate (BAY-94-9343) with a reducible SPDB linker (17). Anti-mesothelin ADCs have three major potential advantages over SS1P: anti-mitotic activity conferring inherent specificity for rapidly dividing cancerous over non-dividing mesothelial cells; less immunogenicity, permitting multiple dosing cycles; and longer serum half-life to increase therapeutic exposure.

Given the association of DM4 with ocular toxicity (18,19), we chose to test auristatin-based anti-mesothelin ADCs using novel monoclonals to mesothelin. The most efficacious was an internalizing protease-sensitive valine-citrulline linked monomethyl auristatin E (vcMMAE) conjugate. In addition, because SS1P preclinical activity in highly overexpressing MSLN-A431 xenografts did not translate to the clinic, we conducted comprehensive IHC of mesothelin expression levels in human tumors and
xenografts to identify the most representative models for efficacy studies. We used these to demonstrate that a single dose of humanized anti-MSLN-vcMMAE could regress ovarian, pancreatic and mesothelioma xenografts.

Materials and Methods

Antibody production. Two human mesothelin antigens were expressed in E.coli 58F3: unizyme His (HQ)-tagged MPF-MSLN propeptide (aa 34–580 of GenBank NM_005823) and HQ-mature MSLN extracellular domain (aa 296–580). Both were purified on a Ni-NTA column (Qiagen; Valencia, CA) followed by Superdex 200 gel filtration in 20mM MES pH 6.0, 6M Guanidine-HCl, then dialyzed into 1mM HCl. Antibodies were produced as described (20), screened by ELISA (21) (see supplemental) then FACS on MSLN-transfected SV-T2 cells.

Cell lines. Cell lines originating from ATCC (acquisition year in brackets) included NCI-H226 (2009), NIH:OVCAR3 (2006), Caov-3 (2009), HPAC (2010), MSTO-211H (2010), PC3 (2006), 293 (2007) and SV-T2 (before 2002). All except SV-T2 were obtained through in-house repositories, which authenticated the lines by Short Tandem Repeat profiling prior to banking. MSTO-211H was instead completely sequenced by Next Generation Sequencing. No further authentication by our lab was performed, but only SV-T2 were not authenticated and cells were not passaged beyond 2 months. gD-MSLN- PC3, SV-T2, HT1080 and 293 cells were generated by stable transfection with Herpes Simplex Virus glycoprotein D (gD)-tagged MSLN or gD-MPF-MSLN (GenBank
NM_005823), using the gD signal sequence in a puromycin-resistant pCMV vector. For efficacy studies, NIH:OVCAR3 and NCI-H226 cells were adapted for in vivo growth by dissociating the best growing tumor (in the SCID beige mammary fat pad and subcutaneously in athymic nude mice, respectively) and cultivating the cells in vitro. These x1 cells were retransplanted into mice, and resultant tumor growth was improved but variable, so the entire procedure was repeated (except orthotopically in the lung of NCr nudes for H226-x1) to generate OVCAR3-x2.1 and H226-x2, which grew reproducible tumors.

**Humanization and conjugation.** 7D9 hybridoma was chimerized onto a human IgG1 backbone as reported (22) then conjugated to mc-MMAF (3.8 drugs/Ab), mc-vc-PAB-MMAE (3.8/Ab) and mc-vc-PAB-MMAF (3.9/Ab) via cysteines of the interchain disulfide bonds at Seattle Genetics as described (23,24). Negative control ADCs were anti-Ragweed (αRW) with 2.8, 3.2 and 3.0 drugs/Ab, respectively. See Supplemental Methods for h7D9.v3 humanization. h7D9.v3 was protein A-purified from Chinese Hamster Ovary cells as described (22) and conjugated to an average of 3.5 vc-PAB-MMAE per antibody at Genentech. The isotype control was humanized anti-gD-tag 5B6 (Genentech, averaging 3.3 MMAE/Ab), which doubled as a positive control on gD-MSLN cells.

**Internalization** was performed as described (20) except using 1µg/ml anti-mesothelin antibodies and 0.4µg/ml Alexa488-EGF (Molecular Probes).
FACS was as published (25).

qRT-PCR was as reported (26), using human mesothelin probe set Hs00245879_m1, spanning exons 16-17 (Applied Biosystems) and RPL19 as the reference gene.

Western Blotting. Cells were lysed (in 1% NP-40, 150mM NaCl, 50mM Tris pH 8.0, 1mM EDTA, 1mM phenylmethylsulfonyl fluoride (PMSF), 1mM sodium fluoride and 1x Complete Protease Inhibitor tablet (Roche)). Lysates of flash frozen xenografts were prepared using Tissue Lyzer (Qiagen). 30 µg lysates (measured by BCA, Pierce) were separated by SDS-PAGE, transferred to nitrocellulose and blocked in 5% w/v non-fat milk, 0.1% Tween-20 in PBS. Primary antibodies were used at 1-10 µg/ml overnight, detected with horseradish peroxidase-conjugated secondaries and ECL (enhanced chemiluminescence) or ECL prime (all from Amersham) and exposed to BioMaxMR film (Kodak). Films were scanned on an HP ScanJet 8200 and figures made with Adobe Photoshop (CS5.1).

Scatchard analysis was as described (27) except ^125^I-h7D9.v3 was incubated for 2h at room temperature.

Immunohistochemistry. Formalin-fixed paraffin embedded (FFPE) human tumor microarrays (Supplemental Table 1A) were stained with 10 µg/ml 19C3 or IgG2b isotype control MPC-11 (BD Pharmingen) at room temperature for 60 minutes on a Dako autostainer following TARGET retrieval (Dako). Detection employed horse anti-mouse biotinylated secondary (Vector), ABC complex (ABC Elite Kit, Vector) and DAB visualization (Pierce). To avoid mouse-on-mouse issues with xenografts, rabbit monoclonal anti-human mesothelin SP74 (Spring Bioscience M3744) or isotype control
DA1E (Cell Signaling Technologies 3900S) were used at 1µg/ml for 16 minutes at 37°C on the Ventana platform, with CC1 digestion, Omnimap detection and DAB visualization. 19C3 and SP74 were first validated for IHC (Supplemental Figures 1,2).

Mesothelin staining was scored by a Board Certified pathologist, noting the distribution and intensity of staining, with a 10% cutoff as follows: 0 (negative) indicates very weak or no staining in >90% of tumor cells; 1+ indicates predominantly weak staining (i.e. >50% of the stained cells are weak); 2+, predominantly moderate; 3+, predominantly strong. This differs from the published 5B2 (Novocastra) scoring system, which typically uses a 1% cutoff and scores percentages of cells only, ignoring intensity.

In vitro efficacy was assayed with CellTiterGlo II (Promega) as described {Chen, 2007 #187}, except with 2000 gD-MSLN-HT1080 cells per well.

In vivo efficacy. All animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals (28). Cells or tumor fragments were implanted subcutaneously into female mice as summarized in Supplemental Table 2, except OVCAR3 were in the mammary fat pad (27). Mice were randomized into groups of 8-11 with tumors averaging ~ 150 mm³. Tumors were measured with UltraCal-IV calipers and volumes calculated as 0.5(length × width²). Growth curves were fitted by linear mixed effect modeling and analyzed using the nlme package of R software (R Foundation for Statistical Computing; Vienna, Austria) (29). Percentage tumor growth inhibition was calculated relative to the vehicle on day 21 or day 22 after dosing. As per IACUC guidelines, animals were euthanized before tumor volumes reached 3000 mm³ or...
ulcerated, or body weight loss exceeded 20%. Primary OVXF1023 and PAXF1657 xenograft studies were conducted at Oncotest GmbH (Freiburg, Germany) (30,31).

Results

Antibody, linker and drug selection.

We began by generating novel antibodies to mesothelin. In contrast to published anti-mesothelin antibodies (10,32-35), our mouse monoclonals were raised to unfolded, non-glycosylated mesothelin extracellular domain (with and without MPF). Hybridoma supernatants were screened by ELISA, followed by FACS and immunohistochemistry (IHC) analysis of cells stably transfected with mesothelin to select those capable of detecting native glycosylated and formalin-fixed mature mesothelin, respectively (data not shown).

The two highest affinity antibodies, 19C3 (0.06nM by surface plasmon resonance analysis) and 7D9 (0.23nM), which bound non-competing epitopes, were further characterized. Both were specific by FACS and Western blot, only recognizing transfected and endogenously mesothelin-expressing cells, but 19C3 was more sensitive on low expressers (Fig 1A-C), possibly reflecting its higher affinity. However, because internalizing ADCs are generally more efficacious due to greater drug release from lysosomes (12,13,36), we selected 7D9 for conjugation because it internalized to lysosomes to a greater extent in H226, OVCAR3 and MSLN-PC3 cells (Figure 1D and data not shown), all known to express mesothelin (34,37). Even so, internalization was relatively slow, with little uptake before 6 hours and significant surface signal remaining after overnight incubation.
Chimeric 7D9 antibody was conjugated via the protease-sensitive linker maleimidocaproyl-valine-citrulline-p-aminobenzyloxycarbonyl (mc-vc-PAB) to the auristatin MMAE and the relatively impermeable MMAF, and also to uncleavable maleimidocaproyl-MMAF (mc-MMAF (23,24)). Conjugation did not affect 7D9 binding, and all three conjugates, but not the naked antibody, exhibited anti-proliferative activity \textit{in vitro} against OVCAR3 cells (not shown). By IHC, monoclonal 19C3 specifically stained mesothelin-positive cells, including normal mesothelia (Supplemental Fig 1), and detected mesothelin on OVCAR3 cells at 2+ by both IHC (Figure 2A) and FACS (red in Figure 1B). Serially transplanted OVCAR3 tumors exhibited stronger (IHC 3+) apical mesothelin staining (Figure 2B), similar to many ovarian tumors. In this model a single 3mg/kg dose of cleavable 7D9-mc-vc-PAB-MMAE was clearly more efficacious than both the MMAF conjugates (Figures 2C, S3A), with robust tumor regressions (138% inhibition) and mean tumor doubling delayed from 6.5 (vehicle) to over 91 days (Supplementary Table 2). The relapsed tumors retained their original levels of mesothelin expression (Figure 2B’,D-G), suggesting target down-regulation did not account for recurrence and raising the possibility of re-treatment. We speculate that the lower efficacy of the uncleavable mc-MMAF conjugate (74% inhibition) may be due to slower diffusion than free MMAE/F into the cytoplasm of its lysosomal metabolite, cys-mc-MMAF (cys being the conjugated cysteine of the antibody that remains following its lysosomal degradation (24)). The intermediate activity of the mc-vc-PAB-MMAF conjugate (110% inhibition) probably reflects more efficient diffusion of free MMAF (than cys-mc-MMAF) following val-cit cleavage and PAB self-immolation, but lack of a bystander effect (the ability to intoxicate neighboring cells (38)) compared to free
MMAE; MMAF has poor membrane permeability unless protonated in low pH environments such as lysosomes (24). Humanized 7D9 (h7D9.v3) was therefore conjugated to mc-vc-PAB-MMAE, henceforth called αMSLN-MMAE, and characterized in detail.

In vitro characterization of αMSLN-MMAE ADC.

h7D9.v3 (hereafter named αMSLN) retained high affinity for mesothelin, ranging from 0.4 to 3nM as measured by Scatchard analysis of endogenous mesothelin expressed from 0.5+ on MSTO-211H to 2+ on HPAC and OVCAR3-x2.1 [in vivo growth-adapted OVCAR3 cells (see Methods), which express equivalent mesothelin levels to the parental line, Figure 2H] and 3+ on H226-x2 cells (Figure 3A). MMAE conjugation neither inhibited antigen binding, nor introduced nonspecific binding, as was also the case for the αgD control ADC (Figure 3A-C). To verify cytotoxic activity, gD-MSLN-HT1080 cells (expressing 130,000 copies of mesothelin, Kd 0.97nM), which internalize both αMSLN and αgD ADCs (Scales et al., in preparation), were assessed for viability following 3 days’ incubation with these ADCs in vitro. αMSLN-MMAE exhibited more potent killing (average IC₅₀ of 0.32 ± 0.1nM (n=3 independent experiments)) than the positive control αgD-MMAE (IC₅₀ 2.2 ± 0.9 nM) despite similar drug loading (Figure 3D), likely reflecting its greater binding capacity since their EC₅₀s were similar (Figure 3B). Cytotoxicity was specific as there was no detectable killing except at the highest concentration on untransfected HT1080 cells (with comparable sensitivity to free MMAE: IC₅₀ 0.17 ± 0.01 nM versus 0.13 ± 0.02 nM for MSLN-HT1080, Figure 3E), nor with αTENB2-vc-MMAE, whose antigen is absent from these cells (Figure 3D).
expected, on lower expressing (40,000 copies) OVCAR3-x2.1 cells with similar MMAE sensitivity (0.2nM), the IC\textsubscript{50} of αMSLN-MMAE was substantially higher (20.3nM, data not shown). Naked αMSLN had no cytotoxic activity up to 100nM on any cell line tested (Figure 3E and data not shown).

*Mesothelin prevalence and intensity in human tumors*

It is important to demonstrate *in vivo* efficacy in models expressing mesothelin at levels representative of human tumors; however, typical levels are unknown since most of the anti-mesothelin IHC (5B2 antibody) literature scores only percentages (not intensities) of positive cells (summarized in Supplemental Table 1). We therefore scored mesothelin staining by 19C3 in ovarian, pancreatic and mesothelioma tissue microarrays (TMAs) using intensity thresholds of no staining (0), weak (1+), moderate (2+) and strong (3+) (Figure 4). We used a 10% cutoff (instead of the 1% cutoff used in the 5B2 literature), hypothesizing that tumors would need ≥ 10% positive cells for meaningful anti-tumor responses with our bystander-capable ADC, but the actual threshold and intensity remain to be determined.

Of 86 unspecified ovarian adenocarcinomas 85% were mesothelin-positive, with a membranous pattern and diffuse distribution. Staining intensity was mostly moderate (2+, 35% of the total) or strong (3+, 36%), with only 14% weak (1+, Figure 4A). In a separate TMA, mesothelin prevalence was 75%, including 98% (42/43) serous adenocarcinomas, 23% of which were 1+, 44% 2+, and 33% 3+. 26/33 (79%) endometrioid, 5/13 clear cell and 1/10 mucinous ovarian tumors were also positive. These prevalence data are
consistent with the 5B2 literature (Supplemental Table 1B) and further validate 19C3 for IHC.

In contrast, only 75% of 196 pancreatic ductal adenocarcinomas were mesothelin-positive. The staining was usually weaker than in ovarian tumors (36% were 1+, 27% 2+ and only 11% 3+) and often restricted to the apical membrane of the ducts (Figure 4B). This is lower than the reported 95% prevalence (Supplemental Table 1C), likely because staining is focal in up to 20% cases (39,40) and would have missed our 10% cutoff and/or not been captured on the TMA.

Unexpectedly, only 45% of 29 mesotheliomas (4/12 pleural, 6/13 peritoneal and 3/4 pericardial) were positive, far fewer than the 86% for pleural (95% for epithelioid pleural) in the literature (see Supplemental Table 1D,E). Staining was mostly diffuse and membranous, as reported, and was of variable intensity: 10% 1+, 14% 2+ and 21% 3+ (Figure 4C). Our lower prevalence is probably not due to lower 19C3 sensitivity, because in a head-to-head comparison 19C3 detected more ovarian samples than 5B2 (not shown). It is possible that this mesothelioma TMA was simply not representative, as histological subtypes were not specified and sarcomatous mesotheliomas usually lack mesothelin. These data suggested mesothelin 2-3+ ovarian, 1-2+ pancreatic and 3+ mesothelioma efficacy models would be the most representative of human tumors.

Identification of suitable efficacy models

To identify such xenograft models, we screened cell lines for mesothelin expression by FACS and selected ovarian OVCAR3-x2.1 (2+), pancreatic HPAC (2+) and mesothelioma H226-x2 (3+). Mesothelioma MSTO-211H (0.5+, with only 136
copies/cell) was additionally chosen as a low expressing comparator (Figure 3A). IHC scores for cell pellets were all consistent with FACS, but unexpectedly all cell lines tested substantially upregulated mesothelin in vivo: the 2+ OVCAR3-x2.1 cells (Figure 2H) increased ~6.5-fold at both mRNA and protein levels to 3+ expressers in vivo (Figure 5A-D; as did their parental OVCAR3 cells, Figure 2A,B,H). Unfortunately, four pancreatic cell lines with 2+ mesothelin by FACS/IHC also upregulated to 3+ in vivo, including HPAC cells (~9x, Figure 5E-G). We were unable to identify a suitable 1+ cell line that remained ≤2+ in vivo, thus selected 2+ primary pancreatic PAXF1657 transplants as the most representative pancreatic model (Figure 5H). Two mesothelioma models similarly increased mesothelin expression in xenografts: H226-x2 (Figure 5I-K) and MSTO-211H (from 0.5 to 1+, Figure 5L-O). Ovarian OVXF1023 (3+) primary human tumors were identified by IHC (Figure 5P).

αMSLN-MMAE is highly efficacious in multiple xenograft models

To determine the preclinical activity of humanized αMSLN-MMAE ADC, we measured the anti-tumor effects of single doses from 1 to 15 mg/kg in the identified models compared to the non-targeted αgD-MMAE ADC. Efficacy parameters for the 10mg/kg dose used in all models are shown in Supplemental Table 2. The mesothelin 3+ ovarian OVCAR3-x2.1 model was the most sensitive to αMSLN-MMAE, with an approximated IC₅₀ for tumor growth inhibition (TGI) of 2.7mg/kg and almost complete regressions (108% TGI) at 10mg/kg, which increased the mean tumor doubling time from 4.5 (vehicle) to 83 days (Figures 6A and S3B). Activity was specific because αgD-MMAE had little activity at this dose, and naked αMSLN had no activity at 15mg/kg in
any of the models tested, indicating the importance of conjugating MMAE to a mesothelin-targeting antibody. Despite similar mesothelin levels, the primary ovarian model OVXF1023 was less sensitive, with 10mg/kg ADC achieving only 53% TGI after 3 weeks, necessitating a second dose that achieved 78% TGI by 84 days and slowed tumor doubling from 8 to 45 days (Figures 6B, S3C).

αMSLN-MMAE was also active in pancreatic models: HPAC xenografts strongly regressed at 10-15 mg/kg (Figure 6C) with an IC$_{50}$ of 3.4 mg/kg and a tumor doubling delay to $\geq$74 days. The 2+ primary pancreatic PAXF1657 model was more sensitive than OVXF1023, with tumor shrinkage (104% TGI) at 10mg/kg and doubling delayed to 37 days. While 20mg/kg was more effective (107% TGI), with 9/10 complete responses and doubling delayed beyond 81 days (Figures 6D, S3E), significant nonspecific activity occurred at this dose. Similar efficacy was observed in the higher (IHC 3+) expressing PAXF736 primary pancreatic model (data not shown).

Additionally, αMSLN-MMAE inhibited the growth of the 3+ mesothelioma H226-x2 model, albeit only resulting in stasis (102% TGI) for 3 weeks and delaying tumor doubling to 34 days at 10mg/kg (Figure 6E). While αMSLN-MMAE also caused stasis (96% TGI) in the low (1+) mesothelin-expressing MSTO-211H xenografts, the duration of the response was shorter and the high permeability of these tumors resulted in some activity with αgD-MMAE as well (Figure 6F and data not shown), suggesting mesothelin targeting played less of a role in this model. Pharmacokinetic analysis confirmed similar exposure in all models (data not shown).

To summarize, potent dose-dependent anti-tumor activity by a single systemic injection of αMSLN-MMAE was achieved in each of the three indications.
Discussion

We have demonstrated strong and sustained anti-tumor activity by αMSLN-MMAE ADC in one mesothelioma, two ovarian and two pancreatic xenograft models, including two primary tumor explants. In these five models, significant activity was seen at 5 mg/kg and tumor regressions at 10 mg/kg following a single dose. Similar results were obtained in OVCAR3, PAXF736 and H226 models with 10 mg/kg anti-mesothelin-DM4 conjugate (17), except with 3-6 repeated doses. Importantly, the models in which αMSLN-MMAE was highly active expressed clinically relevant levels of mesothelin (2-3+ by tumor IHC). Modest activity was seen in the 1+ mesothelioma MSTO-211H model. While further work is required to determine the minimum mesothelin expression level required for activity, the differential responses of the 3+ models indicate that other factors such as internalization rate, MMAE-sensitivity, tumor accessibility and growth rate likely also play a role.

We took pains to undertake systematic scoring of mesothelin expression intensities in clinical samples in order to identify relevant xenografts, with the goal of achieving better predictive power for clinical activity than over-expressing models. Notably the A431-K5 cells used to demonstrate SS1P preclinical activity express 2.5 million copies of mesothelin per cell (41), 10-fold more than the highest endogenous line (H226-x2) and likely more than the patients who failed to respond to SS1P (7). Additionally αMSLN-MMAE internalizes consistently better in transfected than endogenous lines, even with matching expression levels (data not shown), supporting the superiority of endogenous models for assessing ADC activity. Nonetheless, in all lines
αMSLN accumulation in lysosomes was far slower than most other ADC targets (25,36,42), likely explaining the higher activity with cleavable vcMMAE than mc- or vc-MMAF, and also the lower (by an order of magnitude) in vitro potency on OVCAR3 cells (20nM) compared to other vcMMAE conjugates to similarly expressed endogenous targets in other adherent cell lines. Our efficacy data in OVCAR3 cells and xenografts are similar to those of αMuc16-aauristatin conjugates, which also internalize slowly (27,43).

Unexpectedly, endogenous (but not exogenous) mesothelin was upregulated up to 10-fold in vivo by an unknown mechanism in all lines tested (including others not shown). This enabled robust ADC activity even in HPAC cells with only 2600 copies of mesothelin, suggesting many other tumorigenic cell lines could be potential efficacy models. It is unclear if upregulation is mediated by the CanScript promoter, since this is reportedly less active in OVCAR3 and MSTO-211H cells (44), which upregulated as much as the others. Cell-cell interactions might play a role, since we observed higher mesothelin in confluent cells (which may mimic the in vivo situation), but are likely independent of mesothelin-Muc16 binding (45) because Muc16 is not expressed in MSTO-211H. This would be consistent with the converse finding that mesothelin is lost from patient-derived mesotheliomas cultured in vitro (46).

Overall αMSLN-MMAE is highly active at inhibiting or even shrinking tumor growth in mesothelin-positive mouse models. It is also well tolerated, and did not exhibit pleuritis in non-human primates (Scales et al., manuscript in preparation). Furthermore, in phase I trials (NCT01469793) it (DMOT4039A) is showing activity in both ovarian
and pancreatic cancers (47), suggesting our preclinical models do indeed have predictive value.

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References

Figure Legends

Figure 1. 7D9 has lower affinity but internalizes better than 19C3. FACS of MPF-MSLN-transfected PC3 stable cells (numbered subclones in different colors, A) or endogenously expressing ovarian cells (B) OVCAR5 (blue), OVCAR4 (green), Caov-3 (purple) and OVCAR3 (red), with PC3 vector (grey) as a negative control in all panels, using 1 µg/ml 19C3 (upper) or 7D9 (lower) detected with Alexa488-anti-mouse. (C) Western blot (enhanced chemiluminescence detection) showing specificity of 19C3 (left) and 7D9 (right) for mesothelin. Arrowhead, ~75kDa MPF-MSLN; arrow, mature ~50kDa mesothelin. (D) H226 cells after 20h co-internalization of 1 µg/ml 19C3 or 7D9 (top and red channel in merge) and the lysosomal pathway marker Alexa488-EGF (middle and green channel). 7D9 internalizes to lysosomes (arrows). Insets (50% magnification) show lack of isotype control uptake. Scale bars=20µm.

Figure 2. 7D9 is more efficacious conjugated to MMAE than MMAF conjugates in an OVCAR3 xenograft model. Anti-mesothelin 19C3 IHC staining of OVCAR3 cells (A) and untreated (B, 10x; B’ 2.5x), 7D9-vc-MMAE-treated relapsed (day 91; D,E, tumors from two representative animals) or αRagweed-vc-MMAE-treated (day 74; F,G) OVCAR3 tumors. Scale bars= 200µm. (C) Growth rate (calculated by linear mixed effects (LME) modeling) of OVCAR3 tumor transplants in the mammary fat pad following one intravenous dose of 3mg/kg chimeric 7D9 (black) or negative control anti-ragweed (αRW, grey) conjugates. Raw mean tumor volumes are shown in Supplemental Figure 3A. (H) FACS with 0.5µg/ml h7D9.v3 showing ex vivo-derived OVCAR3-x2.1
(blue) express similar levels of mesothelin to parental OVCAR3 (red) cells by FACS. Dashed lines, Alexa488 anti-human alone; grey, cells alone.

**Figure 3. In vitro characterization of αMSLN-MMAE ADC.** (A) FACS binding of naked αMSLN (black bars) and αMSLN-MMAE (grey) incubated at 0.05-50µg/ml with mesothelin 0.5+ MSTO-211H, 2+ HPAC, 2.5+ OVCAR3-x2.1 and 3+ H226-x2 cells (detected with Alexa647-anti-human). Negative control αgD (white) and αgD-MMAE (striped) were only tested at 50µg/ml, showing minimal background binding. Inset, FACS histograms of 0.5µg/ml naked αMSLN (cells in same order); grey is secondary alone on MSTO-211H. Scatchard results for mesothelin copy number and affinity are shown below (mean of 2 experiments or mean of 3 ± SD). (B) gD-MSLN-HT1080 stable cells were incubated as in (A) with naked (closed symbols) or MMAE-conjugated (open symbols, dashed lines) αMSLN (black triangles) or αgD control (grey squares) at concentrations spanning 5 logs. Mean fluorescence intensity versus concentration plots reveal sigmoidal dose-dependent binding with an EC50 of 0.46 ± 0.11 µg/ml for αMSLN-MMAE binding versus 0.5 ± 0.17 for naked h7D9.v3 αMSLN; and 0.54±0.13 for αgD-MMAE versus 0.24±0.04 for αgD. (C) There was no detectable binding to untransfected HT1080 cells. (D) Cell viability of gD-MSLN-HT1080 (closed symbols) or untransfected HT1080 (open symbols and dashed lines) with αMSLN-MMAE (triangles), αgD-MMAE (grey squares) or αTenB2-MMAE (circles), normalized to untreated cells. (E) As (D) except with naked αMSLN (triangles) and free MMAE (diamonds). The means and SDs of three independent duplicate experiments are shown in all 5 panels.
Figure 4. Representative mesothelin expression levels in human tumors by IHC.

Typical anti-mesothelin 19C3 signal intensities on 86 ovarian adenocarcinomas (A), 196 pancreatic ductal adenocarcinomas (B) and 29 mesotheliomas (C). The percentage of tumors with each IHC score is indicated (0+ is negative or less than 10% cells stained; 1+ is predominantly weak; 2+ is moderate and 3+ is strong staining). Scale bars=50µm.

Figure 5. Efficacy model selection and upregulation of mesothelin in vivo. Top row: expression level of mesothelin in OVCAR3-x2.1 (A), HPAC (E), H226-x2 (I) and MST0-211H (L) by qRT-PCR (black, normalized to RPL19) and western blot (grey; Supplemental Figure 4) of the same samples with rabbit monoclonal anti-mesothelin SP74 in cells and xenografts tumors (X1, X2, X3), with cells normalized to 1. Error bars are SD of triplicate PCR reactions for individual samples or SD of the mean of all xenograft samples for “Ave X”. n.a., insufficient tumor for Western. 2nd row: SP74 IHC of cell pellets of OVCAR3-x2.1 (B), HPAC (F), H226-x2 (J) and MST0-211H (M) showing IHC scores consistent with the respective FACS shifts (Figure 3A). 3rd row: SP74 IHC of xenografts of OVCAR3-x2.1 (C), HPAC (G), H226-x2 (K) and MST0-211H (N), confirming upregulation of mesothelin compared to parental cell lines. Bottom row: adjacent section isotype control IHC of OVCAR3-x2.1 (D) and MST0-211H (O) (rest in Figure S2) showing no signal; or SP74 IHC of primary xenografts PAXF1657 (H, 3/4 were 2+) and OVXF1023 (P, 7/7 were 3+). Scale bar=50µm (20x, all panels).

Figure 6. αMSLN-MMAE is highly efficacious in multiple xenografts of each indication. Xenografts (~150 mm³, Supplemental Table 2) of ovarian OVCAR3-x2.1
(A), primary ovarian papillary serous adenomatous carcinoma OVXF1023 (B), HPAC (C), primary pancreatic ductal adenocarcinoma PAXF1657 (D), and mesotheliomas H226-x2 (E) and MSTO-211H (F) with 8-11 mice/group were dosed on day 0 (and day 21 for OVXF1023 only, arrow). Tumor growth curves were fitted using Linear Mixed Effects modeling. All models were dosed with vehicle (dashed line), αMSLN-MMAE (αMSLN, black) and control αgD-MMAE (αgD, grey) at 5mg/kg (circles) and 10mg/kg (triangles, dashed line), and some models also received 1mg/kg (crosses), 2.5mg/kg (open diamonds, dashed line), 15mg/kg (squares) and/or 20mg/kg (inverted triangles); see (B) for legend. For clarity inefficacious 1 and 2.5 mg/kg groups are omitted in F. Naked αMSLN (hatched squares) had no effect at 15mg/kg (A,C,F). Graphs A-D are truncated at day 60 to better reveal the effects of lower doses. Later time points are shown in Supplemental Figure 3 as mean tumor volumes (± SEM), with efficacy measures in Supplemental Table 2.
Figure 1

A

MSLN-PC3 cells

19C3

7D9

# events

Mean Fluorescence Intensity

B

Ovarian cells

19C3

7D9

C

|            | 19C3        | 7D9
|------------|-------------|-------------
|            | clone 3     | clone 5     | OVCAR3 vector | clone 3 | clone 5 | OVCAR3 vector |
|            |             |             |              |          |         |              |
| Mean Fluorescence Intensity | 98 | 64 | 50 | 36 |

D

19C3

7D9

α-Mesothelin

Alexa488-EGF

Merge + DAPI

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Figure 2

(A) Untreated
(B) 7D9-vc-MMAE
(C) αRagweed-vc-MMAE

Fitted tumor volume (mm$^3$)

Days since dosing

% of max

Mean Fluorescence Intensity
**Figure 3**

**A**

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<tr>
<th>Antibody</th>
<th>MSTO-211H</th>
<th>HPAC</th>
<th>OVCAR3-x2.1</th>
<th>H226-x2</th>
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**B**

- **gD-MSLN-HT1080**

**C**

- **HT1080**

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<td>αgD-MMAE</td>
<td>216,000 ± 34,400</td>
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**D**

- **% Viability**

**E**

- **% Viability**
Figure 4

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<tr>
<td><strong>C</strong> Mesothelioma</td>
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<td>14%</td>
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Figure 5

**A** OVCAR3-x2.1  
**B** 2+  
**C** 3+  
**D** 0

**E** HPAC  
**F** 2+  
**G** 3+  
**H** 2+ PAXF1657

**I** H226-x2  
**J** 3+  
**K** 3+  
**L** MSTO-211H  
**M** 0.5+  
**N** 1+  
**O** 0

**Relative mesothelin**

Cells X1 X2 X3 Ave X

1° Xenografts or isotype

50µm
Figure 6

(A) OVCAR3-x2.1
(B) OVXF1023
(C) HPAC
(D) PAXF1657
(E) H226-x2
(F) MSTO-211H

Fitted tumor volume (mm³)

Days since dosing

MSLN-MMAE: αgD-MMAE

- 1.0
- 2.5
- 5.0
- 10
- 15
- 20
- 15 naked
- Vehicle

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Molecular Cancer Therapeutics

An anti-mesothelin-monomethyl auristatin E conjugate with potent anti-tumor activity in ovarian, pancreatic and mesothelioma models.


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