An Antimesothelin-Monomethyl Auristatin E Conjugate with Potent Antitumor Activity in Ovarian, Pancreatic, and Mesothelioma Models

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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 nondividing mesothelia. We generated novel antimesothelin 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 (b7D9.v3) version, αMSLN-MMAE, specifically targeted mesothelin-expressing cells and inhibited their proliferation with an IC50 of 0.3 nmol/L. Because the antitumor activity of an antimesothelin immunotoxin (SS1P) in transfectected 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 immunohistochchemistry. 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. Mol Cancer Ther; 13(11); 2630–40. ©2014 AACR.

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 an approximately 75 kDa fusion protein with the so-called megakaryocyte-promoting factor (MPF), which is removed by furin cleavage, leaving an approximately 50 kDa glycosylphosphatidylinositol-anchored mature mesothelin on the cell surface (2). Mesothelin-knockout mice exhibit no discernible phenotype, thus the normal biologic 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 Supplementary Table S1). 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; refs. 1, 6). This immunotoxin exhibits marked antitumor 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 of 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 that antimitotic 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 prodrugs, optimally requiring internalization so the antibody and/or linker is digested to release the 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; ref. 15) and anti-HER2-MCC-DM1 (T-DM1; ref. 16)—offers hope for these technologies with other tumor antigens. Indeed, several other ADCs are at various stages of clinical development, including an antimesothelin–DM4 conjugate (BAY-94-9343) with a reducible SPDB linker (17). Antimesothelin ADCs have three major potential advantages over SS1P: antimitic activity conferring inherent specificity for rapidly dividing cancerous over nondividing 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 antimesothelin 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 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) followed by Superdex 200 gel filtration in 20 mmol/L MES pH 6.0, 6 mol/L Guanidine-HCl, and then dialyzed into 1 mmol/L HCl. Antibodies were produced as described (20), and screened by ELISA (21; see Supplementary), then by 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), MSTD-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 (except the 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 nude for H226-x1) to generate OVCAR3-x2.1 and H226-x2, which grew reproducibly in vivo.

Humanization and conjugation
7D9 hybridoma was chimerized onto a human IgG1 backbone as reported (22), and 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 antiragweed (uRW) with 2.8, 3.2, and 3.0 drugs/Ab, respectively. See Supplementary 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
Internalization was performed as described (20) except using 1 μg/mL antimesothelin antibodies and 0.4 μg/mL Alexa488-EGF (Molecular Probes).

FACS
FACS was as published (25).

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

Western blotting
Cells were lysed in 1% NP-40, 150 mmol/L NaCl, 50 mmol/L Tris pH 8.0, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonfyl fluoride (PMSF), 1 mmol/L sodium fluoride, and 1× Complete Protease Inhibitor tablet (Roche). Lysates of fresh-frozen xenografts were prepared using Tissue Lyzer (Qiagen). Thirty micrograms of lysates (measured by BCA; Pierce) were separated by SDS-PAGE, transferred to nitrocellulose, and blocked in 5% w/v nonfat milk, 0.1% Tween-20 in PBS. Primary antibodies were used at 1 to 10 μg/mL overnight, detected
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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

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

HIC

Formalin-fixed paraffin-embedded (FFPE) human tumor microarrays (Supplementary Table S1A) 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 used horseresine mouse anti-tissue biotinylated secondary (Vector), ABC complex (ABC Elite Kit; Vector Laboratories) and DAB visualization (Pierce). To avoid mouse-on-mouse issues with xenografts, rabbit monoclonal antihuman mesothelin SP74 (Spring Bioscience; M3744) or isotype control DA1E (Cell Signaling Technology; 3900S) were used at 1 μg/mL for 16 minutes at 37°C on the Ventana platform, with CC1 digestion, OmmiMap detection, and DAB visualization. 19C3 and SP74 were first validated for IHC (Supplementary Figs. S1 and S2).

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; and 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

In vitro efficacy was assayed with CellTiterGlo II (Promega) as described (27), except with 2,000 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 Supplementary Table S2, except OVCAR3 were in the mammary fat pad (27). Mice were randomized into groups of 8 to 11 with tumors averaging approximately 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; ref. 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 3,000 mm³ or ulcerated, or body weight loss exceeded 20%. Primary OVXF1023 and PAXF1657 xenograft studies were conducted at Oncotest GmbH; refs. 30, 31).

Results

Antibody, linker, and drug selection

We began by generating novel antibodies to mesothelin. In contrast with published antimesothelin antibodies (10, 32–35), our mouse monoclonals were raised to unfolded, nonglycosylated mesothelin extracellular domain (with and without MPF). Hybridoma supernatants were screened by ELISA, followed by FACS and 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 (0.06 nmol/L by surface plasmon resonance analysis) and 7D9 (0.23 nmol/L), which bound noncompeting epitopes, were further characterized. Both were specific by FACS and Western blotting, 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 than 19C3 (H226, OVXFC3, and MSLN-PC3 cells (Fig. 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-aminobenzoxycarbonyl (mc-vc-PAB) to the auristatin MMAE and the relatively impermeable MMAF, and also to uncleavable maleimidocaproyl-MMAF (mc-MMAF; refs. 23, 24). Conjugation did not affect 7D9 binding, and all three conjugates, but not the naked antibody, exhibited antiproliferative activity in vitro against OVCAR3 cells (not shown). By IHC, monoclonal 19C3 specifically stained mesothelin-positive cells, including normal mesothelia (Supplementary Fig. S1), and detected mesothelin on OVCAR3 cells at 2+ by both IHC (Fig. 2A) and FACS (Fig. 1B, red). Serially transplanted OVCAR3 tumors exhibited stronger (IHC 3+) apical mesothelin staining (Fig. 2B), similar to many ovarian tumors. In this model, a single 3 mg/kg dose of cleavable 7D9-mc-vc-PAB-MMAE was clearly more efficacious than both the MMAF conjugates (Fig. 2C and Supplementary Fig. S3A), with robust tumor regressions of cleavable 7D9-mc-vc-PAB-MMAE was clearly more efficacious than both the MMAF conjugates (Fig. 2C and Supplementary Fig. S3A), with robust tumor regressions of cleavable 7D9-mc-vc-PAB-MMAE was clearly more efficacious than both the MMAF conjugates (Fig. 2C and Supplementary Fig. S3A), with robust tumor regressions of
suggesting that downregulation 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; ref. 24). The intermediate activity of the mc-vc-PAB-MMAF conjugate (110% inhibition) probably reflects more efficient diffusion of free MMAE (than cys-mc-MMAF) following val-cit cleavage and PAB self-immolation, but lack of a bystander effect (the ability to intoxicate neighboring cells; ref. 38) compared with 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 3 nmol/L 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 Materials and Methods), which express equivalent mesothelin levels to the parental line, Fig. 2H] and 3 + on H226-x2 cells (Fig. 3A). MMAE conjugation neither inhibited antigen binding, nor introduced nonspecific binding, as was also the case for the gD control ADC (Fig. 3A–C). To verify cytotoxic activity, gD-MSLN-HT1080 cells (expressing 130,000 copies of mesothelin; Kd, 0.97 nmol/L), which internalize both αMSLN and gD ADCs (Scales and colleagues, unpublished data), were assessed for viability following 3 days’ incubation with these ADCs *in vitro*. αMSLN-MMAE exhibited more potent killing [average IC50 of 0.32 ± 0.1 nmol/L (n = 3 independent

![Image of FACS analysis 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 (gray) as a negative control in all panels, using 1 μg/mL 19C3 (top) or 7D9 (bottom) detected with Alexa488-antimouse. C, Western blot (enhanced chemiluminescence detection) analysis showing specificity of 19C3 (left) and 7D9 (right) for mesothelin. Arrowhead, ~75 kDa MPF-MSLN; arrow, mature ~50 kDa mesothelin. D, H226 cells after 20 hours of cointernalization 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, 20 μm.]
experiments)] than the positive control αgD-MMAE (IC₅₀ 2.2 ± 0.9 nmol/L) despite similar drug loading (Fig. 3D), likely reflecting its greater binding capacity as their EC₅₀s were similar (Fig. 3B). Cytotoxicity was specific as there was no detectable killing except at the highest concentration on untransfected HT1080 cells (with comparable sensitivity with free MMAE: IC₅₀ 0.17 ± 0.01 nmol/L versus 0.13 ± 0.02 nmol/L for MSLN-HT1080, Fig. 3E), nor with αTENB2-vc-MMAE, whose antigen is absent from these cells (Fig. 3D). As expected, on lower expressing (40,000 copies) OVCAR3-x2.1 cells with similar MMAE sensitivity (0.2 nmol/L), the IC₅₀ of αMSLN-MMAE was substantially higher (20.3 nmol/L, data not shown). Naked αMSLN had no cytotoxic activity up to 100 nmol/L on any cell line tested (Fig. 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 as most of the antimesothelin IHC (5B2 antibody) literature scores only percentages (not intensities) of positive cells (summarized in Supplementary Table S1). We, therefore, scored mesothelin staining by 19C3 in ovarian, pancreatic, and mesothelioma tissue microarrays (TMA) using intensity thresholds of no staining (0), weak (1+), moderate
Figure 3. In vitro characterization of αMSLN-MMAE ADC. A, FACS binding of naked αMSLN (black bars) and αMSLN-MMAE (gray) incubated at 0.05–50 μg/mL with mesothelin 0.5 × MSTO-211H, 2 × HPAC, 2.5 × OVCAR3-x2.1, and 3 × H226-x2 cells (detected with Alexa647-antihuman). Negative control αgD (white) and αgD-MMAE (striped) were only tested at 50 μg/mL, showing minimal background binding. Inset, FACS histograms of 0.5 μg/mL naked αMSLN (cells in same order); gray is secondary alone on MSTO-211H. Scatchard results for mesothelin copy number and affinity are shown below (mean of two experiments or mean of three ± 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 (gray 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 A–E.

(2+), and strong (3+; Fig. 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 antitumor 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+, Fig. 4A). In a separate TMA, mesothelin prevalence was 75%, including 98% (42 of 43) serous adenocarcinomas, 23% of which were 1+, 44% 2+, and 33% 3+. Twenty-six of 33 (79%) endometrioid, 5 of 13 clear cell, and 1 of 10 mucinous ovarian tumors were also positive. These prevalence data are consistent with the 5B2 literature (Supplementary Table S1B) 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 (Fig. 4B). This is lower than the reported 95% prevalence (Supplementary Table S1C),

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Preclinically Efficacious Antimesothelin–MMAE Conjugate
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 of 12 pleural, 6 of 13 peritoneal and 3 of 4 pericardial) were positive, far fewer than the 86% for pleural (95% for epithelioid pleural) in the literature (see Supplementary Table S1D and S1E). Staining was mostly diffuse and membranous, as reported, and was of variable intensity: 10% 1+, 14% 2+, and 21% 3+ (Fig. 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 histologic 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 per cell) was additionally chosen as a low-expressing comparator (Fig. 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 (Fig. 2H) increased approximately 6.5-fold at both mRNA and protein levels to 3+ expressers in vivo (Fig. 5A–D), as did their parental OVCAR3 cells, Fig. 2A, B and H). Unfortunately, four pancreatic cell lines with 2+ mesothelin by FACS/IHC also upregulated to 3+ in vivo, including HPAC cells (Fig. 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 (Fig. 5H). Two mesothelioma models similarly increased mesothelin expression in xenografts: H226-x2 (Fig. 5I–K) and MSTO-211H (from 0.5 to 1+, Fig. 5L–O). Ovarian OVXF1023 (3+) primary human tumors were identified by IHC (Fig. 5P).

αMSLN-MMAE is highly efficacious in multiple xenograft models

To determine the preclinical activity of humanized αMSLN-MMAE ADC, we measured the antitumor effects of single doses from 1 to 20 mg/kg in the identified models compared with the nontargeted αgD-MMAE ADC. Efficacy parameters for the 10 mg/kg dose used in all models are shown in Supplementary Table S2. The mesothelin 3+ ovarian OVCAR3-x2.1 model was the most sensitive to αMSLN-MMAE, with an approximately IC50 for tumor growth inhibition (TGI) of 2.7 mg/kg and almost complete regressions (108% TGI) at 10 mg/kg, which increased the mean tumor-doubling time from

![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, 50 μm.](https://example.com/figure4.png)
4.5 (vehicle) to 83 days (Fig. 6A and Supplementary Fig. S3B). Activity was specific because a

gD-MMAE had little activity at this dose, and naked a

MSLN had no activity at 15 mg/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 10 mg/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 (Fig. 6B and Supplementary Fig. S3C).

aMSLN-MMAE was also active in pancreatic models: HPAC xenografts strongly regressed at 10 to 15 mg/kg (Fig. 6C) with an IC_{50} of 3.4 mg/kg and a tumor-doubling delay to \geq74 days. The 2+ primary pancreatic PAXF1657 model was more sensitive than OVXF1023, with tumor shrinkage (104% TGI) at 10 mg/kg and doubling delayed to 37 days. Although 20 mg/kg was more effective (107% TGI), with 9 of 10 complete responses and doubling delayed beyond 81 days (Fig. 6D and Supplementary Fig. 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).

In addition, aMSLN-MMAE also active in pancreatic models: HPAC xenografts strongly regressed at 10 to 15 mg/kg (Fig. 6C) with an IC_{50} of 3.4 mg/kg and a tumor-doubling delay to \geq74 days. The 2+ primary pancreatic PAXF1657 model was more sensitive than OVXF1023, with tumor shrinkage (104% TGI) at 10 mg/kg and doubling delayed to 37 days. Although 20 mg/kg was more effective (107% TGI), with 9 of 10 complete responses and doubling delayed beyond 81 days (Fig. 6D and Supplementary Fig. 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).
To summarize, potent dose-dependent antitumor activity by a single systemic injection of αMSLN-MMAE was achieved in each of the three indications.

Discussion

We have demonstrated strong and sustained antitumor 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 antimesothelin–DM4 conjugate (17), except with 3 to 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. Although 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 to identify relevant xenografts, with the goal of achieving better predictive power for clinical activity than overexpressing 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). In addition, 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 (20 nmol/L) compared with 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-auristatin 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 the lines tested (including others not shown). This enabled robust ADC activity even in HPAC cells with only 2,600 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, as this is reportedly far slower than most other ADC targets (25, 36, 42), likely explaining the higher activity with cleavable vc-MMAE (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 and colleagues, manuscript in preparation). Furthermore, in phase I trials (NCT01469793), it (DMOT4039A) is showing activity in both ovarian and pancreatic cancers (47), suggesting that our preclinical models do indeed have predictive value.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S.J. Scales, S. Ross, M.S. Dennis, S.D. Spencer
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