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

Suzie J. Scales1, Nidhi Gupta1, Glenn Pacheco2, Ron Firestein3, Dorothy M. French3, Hartmut Koeppen3, Linda Rangell3, Vivian Barry-Hamilton1, Elizabeth Luis4, Josefa Chuh5, Yin Zhang6, Gladys S. Ingle1, Aimee Fourie-O’Donohue5, Katherine R. Kozak5, Sarajane Ross2, Mark S. Dennis6, and Susan D. Spencer2

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 (h7D9.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 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 immunohistchemistry. 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 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 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: antimitotic 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 generated OVCAR3-x2.1 and H226-x2, which grew reproducibly in athymic nude mice, respectively) and cultivated 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 mudes 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), 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 phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L sodium fluoride, and 1× Complete Protease Inhibitor tablet (Roche). Lysates of flash-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
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.

IHC

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 horse antimouse 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; 39005) 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 (Novoceastra) 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 x (length x 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 OXVF1023 and PAXF165 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 in H226, OVCAR3, 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-aminobenzyloxycarbonyl (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 (158% inhibition) and mean tumor doubling delayed from 6.5 (vehicle) to more than 91 days (Supplementary Table S2). The relapsed tumors retained their original levels of mesothelin expression (Fig. 2B, D–G).
suggesting target 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 inter-
mediate activity of the mc-vc-PAB-MMAF conjugate
(110% inhibition) probably reflects more efficient dif-
fusion 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; 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 charac-
terized 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 bind-
ing, nor introduced nonspecific binding, as was also the
case for the αgD 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 αgD ADCs (Scales and
colleagues, unpublished data), were assessed for via-
ability 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
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 αRagweed-vc-MMAE–treated (day 74; F and G) OVCAR3 tumors. Scale bars, 200 μm. C, growth rate [calculated by linear mixed effects (LME) modeling] of OVCAR3 tumor transplants in the mammary fat pad following one intravenous dose of 3 mg/kg chimeric 7D9 (black) or negative control antiragweed (αRW, gray) conjugates. Raw mean tumor volumes are shown in Supplementary Fig. S3A. H, FACS with 0.5 μg/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 antihuman alone; gray, cells alone.

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 2. 7D9 is more efficacious conjugated as MMAE than MMAF conjugates in an OVCAR3 xenograft model. Antimesothelin 19C3 IHC staining of OVCAR3 cells (A) and untreated (B, 10×; B', 2.5×), 7D9-vc-MMAE-treated relapsed (day 91; D and E, tumors from two representative animals), or αRagweed-vc-MMAE–treated (day 74; F and G) OVCAR3 tumors. Scale bars, 200 μm. C, growth rate [calculated by linear mixed effects (LME) modeling] of OVCAR3 tumor transplants in the mammary fat pad following one intravenous dose of 3 mg/kg chimeric 7D9 (black) or negative control antiragweed (αRW, gray) conjugates. Raw mean tumor volumes are shown in Supplementary Fig. S3A. H, FACS with 0.5 μg/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 antihuman alone; gray, cells alone.
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 μg/mL MTO-211H, 2.5 μg/mL HPAC, 2.5 μg/mL OVCAR3-x2.1, and 3.0 μg/mL 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),
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 (−9×, 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 approximated 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...
4.5 (vehicle) to 83 days (Fig. 6A and Supplementary Fig. S3B). Activity was specific because a

\[ \text{gD-MMAE} \] had little activity at this dose, and naked a

\[ \text{MSLN} \] 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).

a

MSLN-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 \( \geq \)74 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, a

MSLN-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 \( \text{gD-MMAE} \) as well (Fig. 6F and data not shown), suggesting that 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 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 (H126-v2) 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 vitro 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 less active in OVCAR3 and MSTO-211H cells (44), which upregulated as much as the others. Cell–cell interactions might play a role, as 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 was 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
Development of methodology: D.M. French, L. Rangell, Y. Zhang
Writing, review, and/or revision of the manuscript: S.J. Scales, L. Rangell, S. Ross, S.D. Spencer
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Rangell, A. Fourie-O’Donohue, K.R. Kozak
Study supervision: S.J. Scales, M.S. Dennis
Other (conducing animal research, and providing raw data and some graphed figures that may have been changed): G. Pacheco

Acknowledgments
The authors thank Seattle Genetics for the ADC technology license and are indebted to Jennifer Batson, Suzy Clark, Eric Cheng, Kathryn Parsons-Reponte, Lisa Crocker, Nika Polevaya, Randy Musterer, Gail Colburn, Klara Tolpal, Allison Cordrey, and Inna Bailer for xenograft development; Leanne Ross, Jenny Bostrom, and Oncotest GmbH for primary xenograft studies; Fred de Sauvage, Paul Polakis, Jay Tibbitts, Michel duPage, Brigitte deVaux, Yvonne Chen, Seereehi Chalsasni, Caroline Meloty-Kapela, Danielle Pasquale, and Genentech core services for excellent support.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 6, 2014; revised August 1, 2014; accepted September 5, 2014; published OnlineFirst September 23, 2014.
Molecular Cancer Therapeutics

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


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-14-0487-T

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2014/09/24/1535-7163.MCT-14-0487-T.DC1

Cited articles
This article cites 43 articles, 27 of which you can access for free at:
http://mct.aacrjournals.org/content/13/11/2630.full#ref-list-1

Citing articles
This article has been cited by 8 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/13/11/2630.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.