Large Molecule Therapeutics

Anetumab Ravtansine: A Novel Mesothelin-Targeting Antibody–Drug Conjugate Cures Tumors with Heterogeneous Target Expression Favored by Bystander Effect

Sven Golffier, Charlotte Kopitz, Antje Kanhert, Iring Heisler, Christoph A. Schatz, Beatrix Stelte-Ludwig, Anke Mayer-Bartschmid, Kerstin Unterschenmann, Sandra Bruder, Lars Linden, Axel Harrenga, Peter Hauff, Frank-Detlef Scholle, Beate Müller-Tiemann, Bertolt Kreft, and Karl Ziegelbauer

Abstract

Mesothelin is a tumor differentiation antigen frequently overexpressed in tumors such as mesothelioma, ovarian, pancreatic, and lung adenocarcinomas while showing limited expression in nonmalignant tissues. Mesothelin is therefore an attractive target for cancer therapy using antibody–drug conjugates (ADC). This study describes the detailed characterization of anetumab ravtansine, here referred to as BAY 94-9343, a novel ADC consisting of a human anti-mesothelin antibody conjugated to the maytansinoid tubulin inhibitor DM4 via a disulfide-containing linker. Binding properties of the anti-mesothelin antibody were analyzed using surface plasmon resonance, immunohistochemistry, flow cytometry, and fluorescence microscopy. Effects of BAY 94-9343 on cell proliferation were first studied in vitro and subsequently in vivo using subcutaneous, orthotopic, and patient-derived xenograft tumor models. The antibody binds to human mesothelin with high affinity and selectivity, thereby inducing efficient antigen internalization. In vitro, BAY 94-9343 demonstrated potent and selective cytotoxicity of mesothelin-expressing cells with an IC50 of 0.72 nmol/L, without affecting mesothelin-negative or nonproliferating cells. In vivo, BAY 94-9343 localized specifically to mesothelin-positive tumors and inhibited tumor growth in both subcutaneous and orthotopic xenograft models. In addition, BAY 94-9343 was able to induce a bystander effect on neighboring mesothelin-negative tumor cells. Antitumor efficacy of BAY 94-9343 correlated with the amount of mesothelin expressed and was generally superior to that of standard-of-care regimen resulting in complete tumor eradication in most of the models. BAY 94-9343 is a selective and highly potent ADC, and our data support its development for the treatment of patients with mesothelin-expressing tumors. Mol Cancer Ther; 13(6); 1537–48. ©2014 AACR.

Introduction

Mesothelin, a glycosylphosphatidylinositol (GPI)-anchored glycoprotein, is highly overexpressed in several human tumors, including the majority of ovarian and pancreatic adenocarcinomas, and in 100% of epithelial mesotheliomas. Moreover, mesothelin expression is found in 50% of lung adenocarcinomas, 60% of gastric cancers, and 67% of triple-negative breast cancers (1–8). Expression in normal tissues is mainly restricted to the single cell layers lining the pleura, pericardium, and peritoneum (1, 9).

There is currently limited information available on the physiologic function of mesothelin. Mesothelin-deficient mice are fertile and do not exhibit any apparent phenotype (10). Mesothelin is known to bind to CA125 (cancer antigen 125, also known as MUC16), and this interaction may be involved in the metastatic spread of CA125-expressing ovarian cancer cells that bind to mesothelin-expressing cells lining the peritoneal cavity (11, 12). Mesothelin may further contribute to metastasis by inducing the expression of matrix metalloproteinases 7 and 9 (13, 14). In pancreatic cancer cells, mesothelin has been shown to contribute to tumorigenesis by inducing interleukin-6 expression and cell proliferation and by promoting resistance to TNF-α (15, 16). Because of high expression in several cancers and a conversely restricted expression in normal tissues, mesothelin is an attractive target for anticancer therapy.

Preclinical and clinical development of naked antibodies and recombinant immunotoxins targeting mesothelin has

Authors' Affiliation: Bayer HealthCare Pharmaceuticals, Berlin/Wuppertal, Germany

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

S. Golffier and C. Kopitz contributed equally to this work.

Corresponding Author: Sven Golffier, Bayer HealthCare Pharmaceuticals, Muellerstr 178, 13353 Berlin, Germany. Phone: 49-30-468192707; Fax: 49-30-468992707; E-mail: sven.golffier@bayer.com

doi: 10.1158/1535-7163.MCT-13-0926

©2014 American Association for Cancer Research.
been reported (17). MORAB-009 (amatuximab), a chimeric anti-mesothelin monoclonal antibody, SS1P, a recombinant anti-mesothelin immunotoxin, and CRS-207, a live-attenuated Listeria vaccine expressing mesothelin engineered for targeted elimination of mesothelin-expressing cells, are all in clinical trials (18–20). The nonhuman origin renders recombinant immunotoxins highly immunogenic inducing the generation of neutralizing antibodies and thereby preventing multiple treatments. For instance, SS1P showed only limited antitumor activity because most patients developed neutralizing antibodies against the drug (21). Therefore, less immunogenic variants are being evaluated (22). Alternatively, mesothelin can be targeted with antibody–drug conjugates (ADC) combining the specificity of an antibody with the potency of a toxophore. Antibodies conjugated to various toxophores have shown potent antitumor activity in preclinical animal models (23). Recently, encouraging clinical efficacy has been observed with SAR3419 targeting CD19 (24). Furthermore, approvals of brentuximab vedotin targeting CD30 (25, 26) and trastuzumab-DM1 in HER2-positive breast cancer (27–29) support the development of ADCs for cancer treatment.

Herein, we introduce a novel ADC, BAY 94-9343, consisting of a fully human anti-mesothelin antibody (MF-T) coupled via a reducible disulfide linker to a microtubule-targeting toxophore DM4. This combination of a linker and toxophore was selected because of its reported potential bystander effect (30). According to detailed preclinical characterization both in vitro and in vivo, BAY 94-9343 is highly selective and shows strong in vitro efficacy in several pancreatic and ovarian cancers and in mesothelioma models, including patient-derived xenografts.

Materials and Methods

Cells

CHO-K1, MIA PaCa-2, and OVCAR-3 cells were obtained from the American Type Culture Collection, NCI-H226 cells from NIH (Rockville, MA), and HT-29 from DSMZ. All cell lines, excluding CHO-K1, were authenticated using PCR fingerprinting by the provider. Cells were maintained in an incubator of 5% CO₂ at 37°C. NCI-H226 cells were cultured in RPMI-1640 with 10% fetal calf serum (FCS) and 2 mmol/L glutamine; OVCAR-3 cells in RPMI-1640 with 20% FCS, 10 μg/mL insulin (bovine), and 2 mmol/L glutamine; HT-29 cells in RPMI-1640 with 10% FCS, 1% sodium bicarbonate, and 2% hygromycin; and MIA PaCa-2 cells in DMEM/HAMS F12 with 10% FCS, 2.5% horse serum, and 2 mmol/L glutamine. Human adult mesothelial cells, obtained from Zen-Bio, were cultured according to the provider’s instructions.

Preparation of mesothelin-expressing cell lines

CHO-K1 cells were stably transected with a pEAK vector (31) encoding the GPI-anchored, N-terminally FLAG-tagged mesothelin protein and selected with puromycin to give a CHO-A9 mesothelin-expressing cell line. HT-29 and MIA PaCa-2 cells were transected with pcDNA3.1Hyg without (vector control) and with human mesothelin cDNA (including flag tag), using Lipofectamine 2000 (Invitrogen). Cells were selected with hygromycin for 2 weeks and subcloned by limiting dilution, and stable transfectants were generated. Mesothelin expression was assessed by fluorescence-activated cell sorting (FACS; FC500, Beckman-Coulter) using MF-T and a fluorescein isothiocyanate (FITC)-conjugated anti-human immunoglobulin G (IgG) secondary antibody (Sigma). The resulting cell lines were HT29/vector, HT29/meso, MIA PaCa-2/vector, and MIA PaCa-2/meso (MIA PaCa-2#37; overexpressing mesothelin).

Preparation and characterization of mesothelin antibody MF-T and ADC BAY 94-9343

Mesothelin antibody discovery using the HuCAL Gold Fab-phage library, the expression, purification, and characterization of Fab and IgG are described in Supplementary Methods. MF-T was conjugated to the maytansinoid DM4 via a hindered disulfide linker (SPDB) at ImmunoGen, following published procedures (32–34). An average drug to antibody ratio of 3.2 was achieved, which is in agreement with the previously described optimal range of 2 to 4 (35). Because different batches of BAY 94-9343 were used, the doses were calculated on the basis of the amount of DM4, the toxic proportion of the antibody. The used doses calculated on the basis of the total ADC are indicated in brackets. Calibrated FACS analysis and immunohistochemistry (IHC) of the cells and tumor sections and the antibody internalization assay are described in Supplementary Methods.

In vivo tumor targeting

MF-T antibody was conjugated with a Bayer Healthcare proprietary near infrared (NIR)-fluorescent tetrastulfonated carbocyanine dye, and fluorescence imaging was performed as previously described (36). Briefly, 12 NMRI nu/nu mice (Taconic M&B A/S Breeding) per group were subcutaneously inoculated with 1 × 10⁶ HT-29/meso or HT29/vector cells suspended in 0.1 mL 50% Matrigel (BD Biosciences). Before imaging, all animals received a single intravenous injection of fluorescent dye-conjugated MF-T.

Proliferation assay

To determine IC₅₀ of cell viability, 0.8 × 10⁵ cells/well were seeded in 384-well plates, incubated for 24 hours, and thereafter washed with PBS°Ca²⁺,Mg²⁺ and treated with 0.01 to 300 mmol/L BAY 94-9343. After 4 or 24 hours, the cells were washed with PBS°Ca²⁺,Mg²⁺, and growth medium was added. The proliferation assay endpoint was selected on the basis of the observation that ADC internalization is saturated by 24 hours (Fig. 2D). The plates were incubated for 72 or 92 hours, and cell proliferation was quantified using the WST-1 assay (Roche) according to the manufacturer’s instructions with the following exception: 5 μL (instead of 10 μL) WST-1 mixture was added into each well. IC₅₀ was assessed using the Prism 4 software (GraphPad Software), setting the positive control (untreated
cells) at 100% and the negative control (without cells) at 0%.

**In vivo tumor models**

All animal experiments were conducted in accordance with the German animal welfare law and approved by local authorities. For subcutaneous tumor models, female NMRI nu/nu mice (18–25 g, 7–10 weeks) from Taconic M&B were implanted on day 0 with either 3 × 10^6 MIA PaCa-2/vector, 3 × 10^6 MIA PaCa-2/meso, 1 × 10^6 HT-29/vector, 1 × 10^6 HT-29/meso, 3 × 10^6 OVCAR-3, or 3 × 10^6 NCI-H226 cells suspended in 0.1 mL 50% Matrigel. Patient-derived pancreatic (PAXF736) model was performed at OncoTest GmbH and ovarian (OVCAR6719) and mesothelioma (Meso7212) models at EPO Berlin-Buch GmbH. Tumor fragments were subcutaneously passaged on naïve female NMRI nu/nu mice (5–7 weeks, Charles River; PAXF736), naïve NOD SCID (8 weeks, Taconic M&B; OVCAR6719), or naïve male NMRI nu/nu (Charles River; Meso7212) mice as previously described (37). Subcutaneous tumor growth was monitored by measuring the tumor volume ([length × width^2]/2). Treatment response was defined using the Response Evaluation Criteria in Solid Tumors (RECIST) criteria. Partial regression (PR) was defined as more than 30% reduction in tumor size and tumor eradication as an absence of any palpable tumor mass. No tumor growth or a slight (<30%) reduction or a slight (<20%) increase in tumor size was defined as a stable disease.

For the orthotopic ovarian cancer model, NMRI nu/nu mice were anesthetized [intraperitoneal (i.p.) administration of 80 mg/kg ketamine (Pfizer) and 16 mg/kg xylazine (Bayer HealthCare AG)]. An incision was made in the peritoneum on the left side near the backbone, and the ovary was exteriorized and injected with 1.5 × 10^6 dermis and peritoneum on the left side near the backbone, (Bayer HealthCare AG)]. An incision was made in the peritoneum on the left side near the backbone. Mice were anesthetized via intraperitoneal (i.p.) injection of 80 mg/kg ketamine (Pfizer) and 16 mg/kg xylazine (Bayer HealthCare AG) for orthotopic tumor injection.

Statistical analyses

Statistical analyses for the comparison of more than two groups were performed by one-way ANOVA, followed by a Dunnett test (comparisons against one group) or Bonferroni test (pairwise comparisons). Two-group comparisons were performed by Student t test. P values less than 0.05 were considered statistically significant.

Results

**Mesothelin expression in cancer**

The eligibility of mesothelin for antibody-directed drug targeting was confirmed in a set of tumor samples with high prevalence of mesothelin overexpression, that is, mesothelioma, ovarian, and pancreatic cancer (Supplementary Fig. S1 and Supplementary Table S1). On the basis of comparative immunohistochemical analyses of primary and metastatic ovarian cancer samples, obtained from the same patient, mesothelin expression remained mostly stable or increased during disease progression in 86% of the cases (Fig. 1A). Concordant with published data (38), the highest endogenous mesothelin expression was found in OVCAR-3 cells (24,000 antibodies bound per cell) as measured by

www.aacrjournals.org Mol Cancer Ther; 13(6) June 2014 1539

Published OnlineFirst April 8, 2014; DOI: 10.1158/1535-7163.MCT-13-0926
Figure 1. Mesothelin expression in clinical samples and cell lines. A, change in mesothelin staining intensity in primary ovarian cancer samples versus matched relapse (n = 50). B, immunohistochemical staining of cell lines for which the number of antibodies bound per cell was determined by quantitative FACS analysis. C, immunohistochemical staining of primary ovarian cancer samples. Quantification of staining (staining score) is based on the staining intensity of the cell lines. Scale bar, 50 μm.
calibrated IHC (Fig. 1B). In comparison with the endogenous expression in cancer cell lines, mesothelin expression was much higher in primary ovarian cancer samples (Fig. 1B and C).

**Preparation and characterization of a mesothelin ADC (BAY 94-9343)**

To generate a mesothelin-specific ADC, 22 unique mesothelin-binding Fab sequences were identified using MorphoSys HuCAL technology (39), and selected Fabs were converted to human IgG1s. The MF-T Fab sequence was selected because of a high binding affinity for human mesothelin (Kd of 10 nmol/L) detected by surface plasmon resonance (SPR) and due to identical staining of HT29/meso xenograft tumor sections (Fig. 2A) and primary human cancer samples (Fig. 2B) compared with mesothelin control antibodies. Moreover, flow-cytometric binding curves for MF-T and endogenous mesothelin-expressing NCI-H226 cells showed a sigmoid increase in the fluorescence signal, characteristic for specific antibody binding (Fig. 2C). MF-T was found not to bind to mouse, rat, or monkey mesothelin, confirmed by SPR (data not shown) and IHC (Supplementary Figs. S2–S4).

A prerequisite for ADC activity is its internalization upon target binding, to effectively deliver the cytotoxic payload into the cells. To study MF-T internalization, MF-T conjugation with DM4 did not alter the binding properties of MF-T as demonstrated by SPR analysis (data not shown) and IHC (Supplementary Figs. S2–S4).

The therapeutic function of an ADC is its internalization upon target binding, to effectively deliver the cytotoxic payload into the cells. To study MF-T internalization, MF-T was labeled with a pH-sensitive fluorescent dye Cy5-Her5E exhibiting fluorescence activity at acidic pH, that is, in late endosomes and lysosomes (40). Consequently, any detected fluorescent signal is derived from internalized antibodies. In OVCAR-3 cells, MF-T internalization was enhanced by >2.5-fold as compared with the unspecific internalization of the isotype control, with maximal differences observed during the first 3 hours of incubation (~4-fold; Fig. 2D).

The therapeutic function of an ADC is highly dependent on the specific intracellular delivery of toxophores to target presenting cells. Therefore, we next studied the ability of MF-T to selectively accumulate in mesothelin-positive tumors in vivo. Because MF-T showed no cross-reactivity with murine mesothelin, the target in this approach is comprised by the inoculated tumor only. Fluorescently-labeled MF-T was injected into mice bearing either HT29/meso or HT29/vector tumors. NIR fluorescence imaging revealed that MF-T accumulation in HT29/meso tumors was 1.4-fold higher than in HT29/vector tumors (Fig. 2E).

As MF-T demonstrated the desired properties of an effective ADC antibody, we subsequently linked MF-T to an average of 3.2 DM4 molecules through a disulfide linker to produce the ADC BAY 94-9343. Importantly, conjugation with DM4 did not alter the binding properties of MF-T as demonstrated by SPR analysis (data not shown) and IHC (Fig. 2A).

**Cytotoxicity of BAY 94-9343 in vitro**

Next, we determined IC_{50} values for BAY 94-9343 in human pancreatic (MIA PaCa-2) and colon carcinoma (HT-29) cell lines, transfected with human mesothelin or the corresponding vector control, and in human ovarian carcinoma (OVCAR-3) and mesothelioma (NCI-H226) cell lines with endogenous mesothelin expression. BAY 94-9343 showed antiproliferative activity with IC_{50} in the low nanomolar range with respect to antibody concentration in all of the mesothelin-expressing tumor cell lines tested (Fig. 3A–D). In contrast, mesothelin-negative tumor cells were not affected by BAY 94-9343 except at very high concentrations approaching the micromolar range (Fig. 3A and B). In accordance with DM4 mode of action, that is, inhibition of microtubule dynamics, BAY 94-9343 did not affect nondividing mesothelin-positive primary peritoneal mesothelial cells (Fig. 3E). Mesothelin expression in these cell types was confirmed by IHC or FACS analysis (Supplementary Fig. S5).

**Antitumor activity of BAY 94-9343 in vivo**

We evaluated the antitumor activity of BAY 94-9343 in vivo. The highest dose and schedule selected for the studies, 0.2 mg/kg based on the amount of DM4 (corresponding to 9.5–14.3 mg/kg ADC, depending on the antibody to drug ratio in the respective ADC batch), did not affect mouse body weight. In the xenogeneic tumor models MIA PaCa-2/meso or HT-29/meso cells, BAY 94-9343 showed dose-dependent efficacy, and treatment with 0.2 mg/kg (10.6 mg/kg ADC) BAY 94-9343 resulted in complete tumor eradication, lasting for at least 17 weeks following the final treatment (Fig. 4A–C and Supplementary Fig. S6A). At a dose of 0.05 mg/kg (2.7 mg/kg ADC), BAY 94-9343 eradicated tumors in 5 out of 6 animals in the MIA PaCa-2/meso pancreatic cancer model (Fig. 4A and B), whereas in the HT-29/meso colon cancer model, tumor regression without complete eradication was observed in 25% of the animals (P < 0.001; Fig. 4C). Treatment with unconjugated MF-T in the pancreatic tumor model (data not shown) or with free S-methyl-DM4 in the colon cancer model did not significantly affect tumor growth (Fig. 4C). Treatment of HT-29/meso tumors with 0.2 mg/kg BAY 86-1899 (corresponding to 9.5 mg/kg ADC), a nontargeted isotype control ADC, reduced tumor size by 73% as compared with the vehicle control. However, in contrast with BAY 94-9343, BAY 86-1899 did not result in tumor eradication and lower doses did not significantly affect tumor growth (Fig. 4C).

Next, we studied whether BAY 94-9343 shows antitumor activity in endogenous mesothelin-expressing tumor models. In the xenogeneic OVCAR-3 ovarian cancer model treated with 0.05 mg/kg (Q3Dx3) BAY 94-9343 (2.8 mg/kg ADC), 100% of mice responded to the treatment, and complete tumor eradication was observed in 4 out of 6 mice lasting for at least 12 weeks following the final treatment. In contrast, the ADC isotype control reduced tumor growth at a dose of 0.2 mg/kg (9.5 mg/kg ADC) compared with the vehicle but did not have any effect at the comparatively lower dose used for BAY 94-9343 (0.05 mg/kg; 2.4 mg/kg ADC; Fig. 4D and Supplementary Fig. S6B).

**Published OnlineFirst April 8, 2014; DOI: 10.1158/1535-7163.MCT-13-0926**
In the NCI-H226 mesothelioma model, two Q3Dx3 cycles of BAY 94-9343 were applied and compared with the standard therapy cisplatin and to the combination of cisplatin and pemetrexed. The dose of 0.2 mg/kg (11.2 mg/kg ADC) BAY 94-9343 inhibited tumor growth by 94% as compared with the vehicle control on day 153.
(P < 0.001) and achieved a 63% response rate (partial regression in 5 out of 8 mice), whereas cisplatin alone and in combination with pemetrexed resulted in 70% reduction of tumor growth (P < 0.01; Fig. 4E and Supplementary Fig. S6C). The treatment groups were also compared by measuring the tumor weight at the end of the study (day 174). Two treatment cycles with 0.2 mg/kg (11.2 mg/kg ADC) BAY 94-9343 resulted in a 14-fold lower tumor weight as compared with the combination of cisplatin and pemetrexed (P < 0.05; Supplementary Fig. S6D).

The inhibitory effect of BAY 94-9343 on tumor growth was also analyzed in an orthotopic ovarian cancer model using endogenous mesothelin-expressing human ovarian cancer cells (OVCAR-3-s-05). Treatment with 0.2 mg/kg (10.6 mg/kg ADC; Q3Dx3) BAY 94-9343 markedly reduced tumor burden as indicated by 81% reduction in ovary weight at the end of the study as compared with the vehicle treatment (P < 0.05). Neither the control ADC BAY 86-1899 (0.2 mg/kg based on DM4, corresponding to 9.5 mg/kg ADC) nor unconjugated S-methyl-DM4 alone or in combination with MF-T significantly affected tumor growth (Fig. 4F and G).

**Antitumor activity of BAY 94-9343 in patient-derived tumor models**

Tumor models based on established tumor cell lines have the disadvantage of low heterogeneity (37). Therefore, we used tumor models based on the transfer and in vivo passage of human patient-derived tumors in immunocompromised mice. BAY 94-9343 administered at 0.2 mg/kg (corresponding to 14.3 mg/kg ADC; Q3Dx3) resulted in transient regression in 6 out of 8 tumors in the pancreatic tumor model PAXF736 (P < 0.001; Fig. 5A), complete eradication of the ovarian tumor OVCAR6719 in all mice (P < 0.001; Fig. 5B), and at least partial tumor regression in all mice in the mesothelioma model Meso7212 (P < 0.001; Fig. 5C). Comparisons of respective standards of care revealed that the antitumor efficacy of BAY 94-9343 was more pronounced than gemcitabine (P < 0.01) in the pancreatic model, cisplatin (P < 0.01) in the ovarian model, and cisplatin (P < 0.05) and pemetrexed (P < 0.001) in the mesothelioma model. The difference between the effects of a microtubule-targeting drug vinorelbine and BAY 94-9343 in the mesothelioma model was not significant (Fig. 5).
Titration of mesothelin-positive tumor cells in vivo

To determine the minimum proportion of mesothelin-positive cells required for BAY 94-9343-mediated tumor response, different ratios of mesothelin-positive HT-29/meso and mesothelin-negative HT-29/vector cells were subcutaneously inoculated into nude mice, resulting in tumors with 100%, 80%, 60%, 20%, and 0% mesothelin-positive cells (Fig. 6A). Treatment of these tumors demonstrated a pronounced mesothelin expression-dependent antitumor efficacy of 0.2 mg/kg BAY 94-9343 (corresponding to 11.2 mg/kg ADC; Fig. 6B). In tumors with 100% mesothelin-positive cells, complete tumor
regression was achieved in 7 out of 8 mice (data not
shown) and in three of the cases, regression was
observed as early as 28 days when the vehicle control
group was sacrificed (Fig. 6B). Furthermore, a 100%
response rate to the BAY 94-9343 treatment was
observed even in tumors with 40% mesothelin expres-
sion. In the 20% mesothelin-positive group, the treat-
ment reduced mean tumor size by 82% compared with
the vehicle, and tumor size remained either the same or
reduced in 7 out of 8 mice (4 stable disease, 3 PR). In
contrast, tumor size was increased by 30% to 300% in 7
out of 8 mice bearing mesothelin-negative tumors trea-
ted with BAY 94-9343. However, tumor size was
approximately 64% smaller in this group as compared
with the vehicle, demonstrating a small target-indepen-
dent antitumor effect at this dose (0.2 mg/kg based on
DM4, corresponding to 11.2 mg/kg ADC; Q3Dx3).

Discussion
A high medical need remains for the treatment of
advanced mesothelioma, pancreatic, and recurrent ovar-
ian cancers. A large proportion of these tumors have been
shown to overexpress the surface protein mesothelin.
Moreover, the limited expression of mesothelin in normal
human tissues qualifies mesothelin as an attractive can-
didate for antibody-based cancer therapy. This study
describes the establishment of human anti-mesothelin
antibody MF-T conjugated to the tubulin inhibitor DM4
and includes detailed preclinical characterization of the
resulting BAY 94-9343, an ADC targeting mesothelin-
expressing tumors.
The overexpression of mesothelin in mesothelioma,
ovidian and pancreatic cancer specimens was confirmed
by detailed immunohistochemical studies. More impor-
tantly, it was demonstrated for the first time that meso-
thelein continues to be expressed in recurrent ovarian cancer
in most patients, suggesting that immunohistochemical
staining of archived primary tumor biopsies could be
used to stratify patients for anti-mesothelin therapy.
The efficacy of unconjugated antibodies relies on the
function of their target antigen as demonstrated by the
growth factor receptor blocking activity exhibited by
antibodies such as cetuximab and trastuzumab for EGF
receptor and HER2/neu, respectively (41, 42). The
unknown biologic function of mesothelin does not, how-
ever, conflict with the development of an ADC. The
efficacy of an ADC predominantly depends on the expres-
sion of the target antigen, ADC-binding affinity and
subsequent internalization within the cells, and the poten-
cy of the conjugated toxophore. Our data demonstrate that
the MF-T antibody possesses a specific nanomolar-bind-
ing affinity for human mesothelin and undergoes subse-
quent internalization. The binding properties are not
altered by the presence of the mesothelin-binding CA125
(43) and are fully retained in the ADC BAY 94-9343. BAY
94-9343 is shown to be both potent and highly selective in
killing mesothelin-expressing tumor cells, whereas the
same dose range does not affect mesothelin-negative cells.
DM4, the tubulin-binding toxophore, mainly acts on
proliferating cells and therefore provides further func-
tional specificity to the ADC beyond the specific target-
binding of the antibody moiety. Indeed, we and others
(44) observed no cytotoxicity upon treatment of mesothe-
lin-expressing primary mesothelial cells with low prolif-
eration rates. In contrast, the bacterial immunotoxin PE38
in SS1P is not only highly immunogenic, preventing read-
ministration, but is also toxic to all antigen-positive cells
due to its mode of action (21). Whole animal imaging
using a fluorescently labeled antibody that is selectively
retained in antigen-positive tumors demonstrates target
selectivity and tumor specificity of the anti-mesothelin
antibody in vivo. Furthermore, treatment of mice,
bearing either mesothelin-negative or mesothelin-posi-
tive tumors, with BAY 94-9343 or a nontargeted ADC
control harboring the same linker toxophore, respectively,
indicates that the target-independent antitumor effects
are only minor when compared with the targeted activity
of BAY 94-9343. The inferior target-independent in vivo
effects of the control ADC at high doses are presumably

Figure 5. Antitumor activity of BAY 94-9343 in patient-derived tumor
models. Fragments of human tumors were passaged in naïve
immunocompromised mice. After randomization into groups, treatments
(BAY 94-9343 and S-methyl-DM4 i.v., gemcitabine i.p., cisplatin i.p.)
were initiated (time points indicated by arrows). Mean ± SD of
tumor volume for the pancreatic cancer model PAXF736 (n = 9; A),
ovidian cancer model OVCAR8719 (n = 8; B), and mesothelioma model
Meso7212 (n = 10; C).
due to enhanced permeability and retention (EPR) in solid tumors. This phenomenon of passive drug targeting of tumors has been widely described for macromolecules and lipids (45).

Once BAY 94-9343 is bound and internalized by a tumor cell, degradation of the BAY 94-9343 disulfide-based linker releases a cell permeable toxophore metabolite with bystander killing potential (33). This bystander effect was demonstrated, for the first time in vivo, using a xenograft model with different proportions of mesothelin-positive and -negative cells within the inoculated tumors. BAY 94-9343 not only inhibited tumor growth, but also more importantly induced tumor regression even when only 20% of the cells within the tumor were mesothelin positive. This strongly suggests that, in addition to the EPR effect observed in the mesothelin-negative cells, the bystander effect contributes to the antitumor activity of BAY 94-9343. Similar results have been reported previously by Kovtun and colleagues (30).

In addition to the subcutaneous and orthotopic xenograft tumor models, we further demonstrated the dose-dependent therapeutic activity of BAY 94-9343 in patient-derived tumors that more closely mimic human tumor characteristics, such as heterogeneity. Treatment with BAY 94-9343 resulted in total eradication of established tumors with no tumor recurrence and was reproducible in several models over a long observational period, further indicating complete tumor cell elimination by the ADC. Importantly, the efficacy of BAY 94-9343 in pancreatic, ovarian, and mesothelioma cancer models was more pronounced as compared with the current respective standard-of-care treatments, suggesting that BAY 94-

Figure 6. Titration of mesothelin-positive tumor cells in vivo. Different ratios of mesothelin- and vector-transfected HT-29 cells were subcutaneously transplanted into nude mice (randomized on day 6 to treatment and vehicle groups). A, immunohistochemical staining of mesothelin in vehicle-treated tumors. B, changes in tumor size from randomization until day 28 (the last time point at which the vehicle groups remained in the study). Each bar represents the change in tumor size during the study of an individual mouse. Positive bars represent growth and negative bars shrinkage of tumors as compared with tumor size at treatment start. The vehicle control was included in all the different study groups but the data are only shown for the group inoculated with 100% mesothelin-positive cells.
9343 is a potential therapeutic option for patients with heavily pretreated and even resistant tumors.

Taken together, our preclinical results validate mesothelin as a cancer antigen for an ADC as a potential therapeutic approach. Future studies determining the threshold of mesothelin expression required to achieve efficient ADC-induced antitumor efficacy are essential. This information is crucial in developing companion diagnostics to stratify patient populations in clinical development. Currently, anetumab ravtansine (BAY 94-9343) is in phase I clinical evaluation about its safety, tolerability, pharmacokinetics, and pharmacodynamics (ClinicalTrials.gov Identifier: NCT01439152). The maximum tolerated dose of anetumab ravtansine will be assessed in several advanced solid tumors with high frequency of mesothelin expression.

Disclosure of Potential Conflicts of Interest

C. Kopitz has ownership interest in the corresponding patent. A. Kahnert has ownership interest in patents and patent. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: S. Golifier, C. Kopitz, A. Kahnert, I. Heisler, B. Stelte-Ludwig, A. Harrenga, B. Müller-Tiemann, B. Kreft

Development of methodology: S. Golifier, C. Kopitz, I. Heisler, B. Stelte-Ludwig, L. Linden, A. Harrenga, P. Hauff, F.-D. Scholle, B. Müller-Tiemann, S. Bruder

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Golifier, C. Kopitz, I. Heisler, C.A. Schatz, B. Stelte-Ludwig, A. Mayer-Bartschmid, K. Unterschemmann, A. Harrenga, P. Hauff, F.-D. Scholle, B. Müller-Tiemann, S. Bruder

Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): S. Golifier, C. Kopitz, A. Kahnert, I. Heisler, B. Stelte-Ludwig, A. Harrenga, P. Hauff, F.-D. Scholle, B. Müller-Tiemann, B. Kreft, S. Bruder

Writing, review, and/or revision of the manuscript: S. Golifier, C. Kopitz, A. Kahnert, I. Heisler, B. Stelte-Ludwig, B. Kreft

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): I. Heisler, B. Müller-Tiemann

Study supervision: B. Müller-Tiemann, B. Kreft, K. Ziegelbauer

Acknowledgments

The authors thank Stephanie Ernests, Karola Henschel, Katrin Jansch, Sabrina Leppin, and Bianka Timper for excellent technical assistance, Aurexel Ltd. (www.aurexel.com) for editorial support funded by Bayer HealthCare Pharmaceuticals, ImmunoGen Inc., for their support and valued advice about maytansinoid-ADCs, and Lauren Clancy of (ImmuNoGen) for skillful preparation of the conjugates studied herein.

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 October 25, 2013; revised March 24, 2014; accepted March 26, 2014; published OnlineFirst April 8, 2014.

References

Molecular Cancer Therapeutics

Anetumab Ravtansine: A Novel Mesothelin-Targeting Antibody–Drug Conjugate Cures Tumors with Heterogeneous Target Expression Favored by Bystander Effect

Sven Golfer, Charlotte Kopitz, Antje Kahnert, et al.


Updated version
Access the most recent version of this article at: doi:10.1158/1535-7163.MCT-13-0926

Supplementary Material
Access the most recent supplemental material at: http://mct.aacrjournals.org/content/suppl/2014/04/09/1535-7163.MCT-13-0926.DC1

Cited articles
This article cites 43 articles, 25 of which you can access for free at: http://mct.aacrjournals.org/content/13/6/1537.full.html#ref-list-1

Citing articles
This article has been cited by 13 HighWire-hosted articles. Access the articles at: /content/13/6/1537.full.html#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.