The search for new treatment strategies for malignant pleural mesothelioma

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Chapter 4

Throphoblast Glycoprotein is Associated With a Favorable Outcome for Mesothelioma and a Target for Antibody Drug Conjugates

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Abstract

Introduction: The prognosis for patients with mesothelioma is poor, which prompts the need for the development of better treatment options. Antibody drug conjugates (ADCs) are gaining interest as a therapeutic strategy in mesothelioma. Throphoblast glycoprotein (5T4) is an oncofetal protein overexpressed in mesothelioma with low expression in normal tissue and therefore a good candidate for ADC treatment. Here, we evaluated and manipulated 5T4 as a suitable antigen for ADC targeted therapy in patients with mesothelioma.

Methods: Expression of the 5T4 antigen is evaluated in (primary) mesothelioma cell lines and biopsy specimens, and correlated with clinical outcome. Internalization was assessed in 5T4 expressing cells. The cytotoxicity of three different 5T4-targeting ADCs was tested on (primary) mesothelioma cells.

Results: 5T4 was expressed in 10 out of 12 (primary) cell lines. Most biopsy specimens stained positive for the 5T4 antigen, with marked differences in staining intensity and percentage of positive cells. High expression correlated with long progression-free survival. Both, free antibody and ADCs targeting 5T4, were internalized and entered lysosomal compartments. Cytotoxicity experiments showed that cell lines with a high expression for 5T4 were sensitive to two out of three ADCs. Lack of efficacy for the third ADC could be restored by neutralizing lysosomal compartments with chloroquine.

Conclusion: The 5T4 antigen is expressed in mesothelioma and 5T4-based ADCs are internalized in lysosomes. Two out of three ADCs were capable of killing the mesothelioma cells; the third ADC required additional lysosomal neutralization for its effect. 5T4-based ADC would be a selective strategy for the treatment of mesothelioma.

Keywords

Malignant pleural mesothelioma, 5T4, Antibody-drug conjugate, treatment, Lysosome

Introduction

Malignant pleural mesothelioma (MPM) is an aggressive tumor from mesothelial cells covering the pleural cavity. The prognosis for MPM is poor and most patients die within 2 years after diagnosis [1-4]. Standard of care chemotherapy, consisting of a platinum-based drug and anti-folate combination, gives a modest median survival benefit of at least 3 months [5, 6]. No further improved second-line therapy has been developed over the last 15 years.

Recently, antibody drug conjugates (ADCs) have gained substantial interest as a new strategy in the treatment of MPM. ADCs consist of monoclonal antibody chemically conjugated to a potent cytotoxic agent. They are developed to selectively target tumor cells, and as a result would minimize the toxicity in normal cells [7-9]. Conceptually, following binding of the antibody to the target antigen, the ADC will be internalized into tumor cells followed by the release of the toxin and elimination of the tumor cell (Fig. 1A). The toxin is released inside the lysosomes of the cells. Either a linker between toxin and antibody is constructed as a substrate for lysosomal proteases (the cleavable mechanism) or the full ADC is degraded (the noncleavable mechanism) to release the toxin. The cytotoxic agents that are mostly used in ADC technology are microtubule disrupting agents and DNA damaging agents (Fig. 1A) [7, 8, 10, 11].

Trophoblast glycoprotein (5T4) is an oncofetal cell surface glycoprotein, which plays a role in cell migration and epithelial-mesenchymal transition, and has been involved in wingless-type mouse mammary tumor virus (Wnt) signaling [12-16]. These functions may link to tumorigenesis, although 5T4 is not described as an oncogenic driver [8, 17]. 5T4 expression is limited in normal tissue, but overexpressed in various solid tumors, including lung, breast, ovarian, endometrial, bladder, pancreatic, colon and gastric cancers [18-22]. Upon antibody binding, 5T4 is rapidly internalized into cells, which is one important factor for ADC based-activity [16]. The first described 5T4 targeting ADC is A1-MMAF, an anti-5T4 immunoglobulin (Ig) G1 antibody that is conjugated by a maleimidocaproyl (mc) linker to monomethyl auristatin F (MMAF), an inhibitor of tubulin polymerization inducing G2/M cell cycle arrest and cell death [8, 10, 23]. A1-MMAF was highly potent in vitro and in vivo, and well tolerated in a phase I study performed in advanced solid tumors [23, 24].

Only one study has evaluated 5T4 expression in MPM cell lines, primary tumor cells in pleural fluid, and mesothelioma biopsy specimen [25]. We report that 5T4 is expressed at different levels in MPM cell lines and biopsy specimen, and internalizes to cathepsin B-positive lysosomes. High expression of 5T4 correlated with longer progression-free survival (PFS). We tested three different 5T4-ADCs and showed that they were cytotoxic in cell lines at a

5T4 antigen-dose-dependent manner. One ADC was lysosomally trapped and neutralization with chloroquine was required to release the drug from this site to induce cytotoxicity. We conclude that 5T4-mediated ADC therapy is a promising novel therapeutic option in a subset of patients with mesothelioma defined by high expression of 5T4.

Materials and methods

Patient samples

Biopsy specimens from patients with MPM were collected between 2009 and 2015 in the Netherlands Cancer Institute. All patients provided written informed consent for use and storage of their tumor biopsy specimens. Diagnosis was determined on available tumor biopsy specimens and confirmed by the Dutch Mesothelioma Panel, a national expertise panel of certified pathologist who evaluate all patient samples suspected of MPM.

Cell lines

NCI-H2052, NCI-H2731, NCI-H2795, NCI-H2810, NCI-H2818 and MeT-5A were a kind gift of Professor McDermott from the Sanger Institute (Cambridgeshire, United Kingdom). Cells were cultured in Dulbecco's Modified Eagle Medium-F12 (Thermo Fisher Scientific, Waltham, Massachusetts) with 10% fetal calf serum (FCS) (Merck, Darmstadt, Germany). Cell lines VAMT and M28 were a kind gift of Professor C. Broaddus from the University of California, San Francisco. Cells were cultured in Dulbecco's Modified Eagle Medium (Thermo Fischer scientific) with 10% FCS. Isolating tumor cells from pleural fluid as described generated early passage cell lines NKI04, PV130913, PV180314, PV170614, and PV041214 [26]. LnCap cells were cultured in Roswell Park Memorial Institute soluation (Life Technologies, Carlsbad, California) with 10% FCS. All cells were maintained at 37°C, 5% CO_2.

Preparation and characterization of ADC

Molecular anti-5T4 antibodies, H8 and A1 are produced in Expi293 cells according to manufactures instructions using commercially available pcDNA-based vector backbones (A14635, Life technologies) [23,27]. MMAF (T1006 LN-T-6871, Levana Biopharma, San Diego, California) is conjugated to H8 and A1 via a noncleavable mc linker as described [28]. Monomethyl auristatin E (MMAE) (T1004 LN-T-1458, Levana Biopharma) was conjugated to H8 via a valine-citrulline (vc) *p*-aminobenzylcarbamate linker that is cleaved by intracellular proteases such as cathepsin B as described [28].

Immunohistochemistry

Immunohistochemistry (IHC) of the formalin-fixed paraffin-embedded biopsy specimens and cell lines was performed using a Discovery Ultra autostainer (Ventana Medical Systems, Oro Valley, Arizona). Paraffin sections were cut at 3 µm, heated at 75°C for 28 minutes and

deparaffinized with EZ prep solution (Ventana Medical Systems). Heat-induced antigen retrieval was performed using Cell Conditioning 1 (CC1, Ventana Medical Systems) for 64 minutes at 95°C. 5T4 was detected using clone EPR5529 (1/400 dilution, 1 hour at 37°C, AbCam, Cambridge, United Kingdom) and visualized using anti-rabbit HQ for 12 minutes at 37°C followed by anti-HQ HRP for 12 minutes at 37°C and the ChromoMap DAB detection kit (Ventana Medical Systems). Slides were counterstained with hematoxylin II and Bluing Reagent (Ventana Medical Systems). Staining was scored by a pathologist using H-score.

Western Blot

Samples were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L Tris-Chloride, 1% nonylphenoxypolyethoxylethanol-40 [Sigma, St. Louis, Missouri], 0.5% sodium-deoxycholate [Sigma], 150 mmol/L sodium chloride, 1mmol/L ethylenediaminetetraacetic acid [Promenga, Madison, Wisconsin], 1mmol/L ethyleneglycol-bis [β-aminoethyl ether] [VWR, Radnor, Pennsylvania] and protease/phosphatase inhibitors[Roche, Basel, Switzerland]), sonicated and centrifuged at 10,000 rpm for 15 minutes at 4°C. Membranes were stained with 5T4 (1:1,000, clone EPR5529, AbCam) and tubulin (1:6000, T9026, Sigma, St. Louis, Missouri) for 1 hour at room temperature (RT) or cathepsin B (1:1,000, clone D1C7Y, Cell Signaling, Danvers, Massachusetts) and actin (1:10,000, MAB1501R, Millipore, Burlington, Massachusetts) overnight at 4°C. Membranes were imaged by the Odyssey Classic imager (Li-Cor, Lincoln, Nebraska). Intensity of band was quantified using Image Studio Software (Li-Cor).

Confocal microscopy

One hundred thousand cells were seeded on glass slides (13 mm diameter) and incubated with anti-5T4 monoclonal antibody (H8) for 1 hour at 4°C. Subsequently, cells were incubated for the indicated time, fixated in 3.7% formaldehyde (Merck) for 10 minutes, and permeabilized using 0.1% Triton X-100 (Merck). Staining was performed in 0.5% bovine serum albumin phosphate-buffered saline with antibodies against CD63 and Phalloidin-Alexa647 (Thermo Fisher scientific, Waltham, Massachusetts) [29]. 4',6-diamidino-2-phenylindole (DAPI) - containing Prolong Gold mounting medium (Thermo Fisher scientific) was used to mount the coverslips and detection of the nucleus. Images were acquired using a Leica TCS SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany) at x63 magnification and quantified using Image J plugin Jacob for Pearson's coefficient calculations and processed using Adobe Photoshop and Illustrator (Adobe, San Jose, California).

Flow cytometry

For expression experiments, 100,000 cells were incubated with 5 µg/ml A1 or H8 antibody for 1 hour at 4°C. Cells were washed with phosphate-buffered saline/ 0.2% bovine serum albumin and incubated with secondary AlexaFluor488 (AF488) antibody (AffiniPure F(ab)2 fragment

goat anti human IgG-APC, 1:600, Jackson ImmunoResearch, West Grove, Pennsylvania) for 30 minutes at 4°C. Fluorescence intensities were measured by flow cytometry (BD Calibur, BD Bioscience, San Jose, California). To determine antibody binding capacity, human IgG calibrator (BioCytex, Marseille, France) was used according to manufactures protocol. For internalization experiments, H8 antibody was used with AF488 secondary antibody (1:8,000). Each antibody sample was prepared twice for total and quenched measurement. Cells were incubated at either 37°C to assess internalization or 4°C as control. After indicated incubation times, one sample was fixed in 3.7% formaldehyde. The other sample was quenched with anti-AF488 rabbit IgGAb (1:30 diluted Thermo Fisher scientific) for 30 minutes at 4°C after which it was fixed in 3.7% formaldehyde. Fluorescence intensity was determined by flow cytometry (BD Calibur, BD Bioscience, San Jose, California). Total signal was determined by the median fluorescence intensity of the unquenched sample corrected for the untreated cells (only AF488). Internalization signal was determined by the median fluorescence intensity of the quenched sample corrected for the untreated cells.

Cytotoxicity assay

Cells were seeded in a flat bottom 96-well plate at appropriate cell density. After overnight incubation, ADC or MMAE were added in a concentration range of 1 ng/mL – 10 µg/mL and 0.001- 0.1 nmol/L, respectively. After 9 days of incubation, cytotoxicity was measured using a metabolic activity assay (Cell Titer blue G8081, Promenga, Madison, Wisconsin). Fluorescent readout was performed with the Envision Multilabel reader (Perkin Elmer, Waltham, Massachusetts). Percentage survival was calculated by dividing the fluorescent signal with the average mean fluorescence of control cells (0.1% dimethyl sulfoxide for ADC and 1% dimethylsulfoxide for MMAE).

Chloroquine reconstitution of drug activity

Lysosomal trapping assay was performed with H8-mcMMAF and H8-vcMMAE as described in the cytotoxicity assay at concentration range between 1 ng/mL and 3.33 µg/mL. Each condition was prepared twice to compare cytotoxicity of ADC by itself with the cytotoxicity of ADC in combination with chloroquine. After 5 hours of incubation with the ADC, 10 µmol/L of chloroquine was added to the cells for 7 days. Cells were washed and incubated for an additional 48 hours after which cytotoxicity was measured.

Statistics

Overall survival (OS) was defines as time from diagnosis until death of any cause. PFS was defined as time from start of firs-line treatment until radiological progression or death of any cause. Both are analyzed with the Kaplan-Meyer method and compared with the log-rank test. Patients not having the event of interest were right-censored at their day of last follow-up. Prognostic value for 5T4 H-score, age, sex, MPM subtype and disease control at 6 weeks

was assessed using Cox models, where disease control (defined as absence of progression) was not used in the model for PFS because of the overlap in definitions between disease control and PFS. 5T4 and age were continuous used in the multivariate analysis, whereas the hazard rate for 5T4 was determined per steps of 10 and age per 1 year.

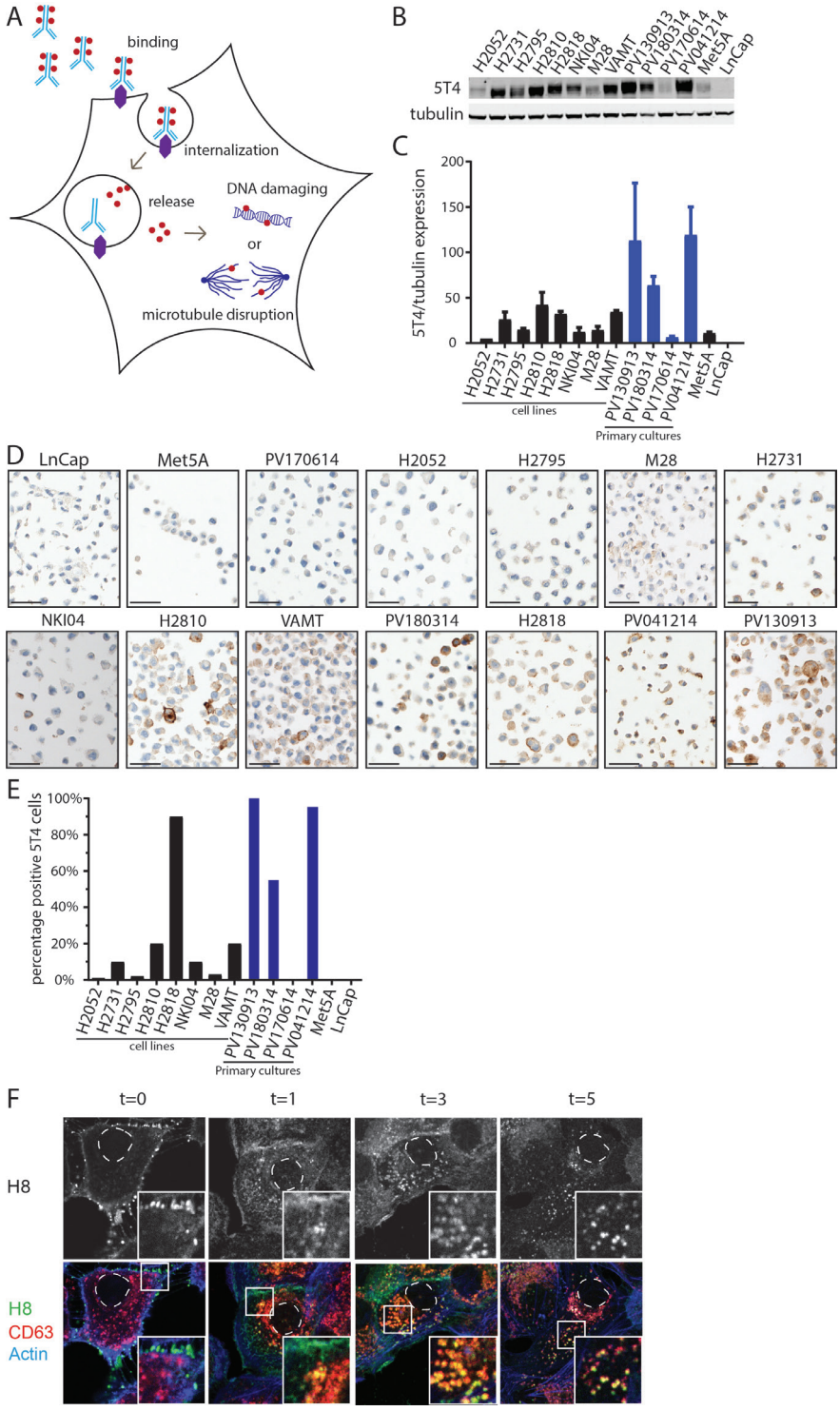
Results

Expression of 5T4 antigen in mesothelioma cell lines

To evaluate whether 5T4 is a suitable antigen for ADC-targeted therapy, we first analyzed 5T4 expression in eight human MPM tumor cell lines, four primary MPM tumor cell lines, and one human normal mesothelial cell line. As negative controls, the human prostate cancer LNCap and the melanoma SK-MEL30 cell lines were used. Seven of eight cell lines and three of four primary cell lines stained positive for 5T4 by Western blotting, including the sarcomatoid mesothelioma cell line VAMT. The normal mesothelial cell line, Met5A, expressed 5T4 at only very low levels (Fig. 1B and C). 5T4 expression in these cell lines was further confirmed using IHC, revealing strong plasma membrane and diffuse cytoplasmic expression for all cell lines that were positive based on Western blot analysis (Fig. 1D and E). The percentage of positive cells was variable between cell lines and stronger in primary MPM cultures as compared to the immortalized cell lines (Figure. 1D and E). To analyze 5T4 cell surface expression, the cells were analyzed by flow cytometry with two independent antibodies (also used in ADC technology): A1 and H8 [32, 27]. These data further confirmed 5T4 cell surface expression in the cell lines which were positive by Western blotting and IHC. Antibody binding capacity was stronger for the H8 antibody compared to the A1 antibody (Supplementary Fig. 1A). In summary, these data show that almost all MPM cell lines express 5T4 at the cell membrane albeit at different levels.

Antibody-induced internalization of the 5T4 antigen in mesothelioma cell lines

5T4 is observed at the cell surface of MPM cell lines. Altough this is a prerequisite for detection by the ADC, the antigen-antibody complex subsequently must internalize to release the associated toxin. To assess this, internalization of 5T4 upon binding by antibody H8 was determined in high 5T4 expressing cell lines: the epithelial cell line H2810, the sarcomatoid cell line VAMT and the primary cell line PV130913. Cells were incubated with AF488 (Jackson ImmunoResearch, West Grove, Pennsylvania) -labeled H8 antibody for 1 hour, after which internalization at 37°C was determined at different times. Quenching of the surface-bound AF488 labeled H8, with an anti-AF488 antibody, resulted in the fluorescence signal of the internalized H8-AF488 as quantified by flow cytometry. Quenching of the surface bound AF488 labeled H8 is incomplete as is evident from the 4°C controls (Supplementary Fig. 1B). This signal remained constant over time and represents the background of this assay. Internalization of the H8 antibody was observed in all three cell lines. Internalization is time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dependent and reached a maximum of 80% to 90% after 12 hours incubation for H2810 and VAMT cells, and a maximum of 100% internalization after 24 hours incubation for the primary cell line PV130913 (Supplementary Fig. 1B). As the cytotoxic drug will be activated in the lysosome, we next determined whether the ADCs ended up in the lysosome [8-11, 27]. After internalization of the H8-vcMMAE ADC, cells were stained for actin and the lysosomal marker CD63. Before internalization (0 hour) H8 is localized at the cell membrane, confirming the IHC results (Fig. 1D). After 1 hour, H8 is partly internalized into the cells and partly located at the cell surface. Over time, increasing internalization and colocalization of H8 with the lysosomal marker CD63 is observed, reaching complete H8 internalization after 5 hours of culture (Fig. 1F). These results indicate that H8 ADCs bind to the cell surface of mesothelioma cells followed by internalization and transport to the lysosomes.

Expression of the 5T4 antigen in MPM biopsies is associated with longer survival.
For 5T4 to function as potential ADC-based drug target, there are two prerequisites: 1) expression in MPM; and 2) no expression in normal tissue. Therefore, we assessed the expression of the 5T4 antigen on 49 MPM biopsy specimens (34 epithelial, 8 mixed, and 7 sarcomatoid) and one tissue microarray containing different normal tissues including intestine, liver, kidney, prostate, and lung by IHC. Placenta was used as a positive control [16, 18, 22]. In line with cell line-based results (Fig. 1D), 5T4 localized to the plasma membrane in these biopsy specimens (Fig. 2A). 5T4 was not expressed in any of the normal tissues (Supplementary Fig. 2). For scoring the 5T4 expression, the H-score was used, in which staining intensity was scaled 0 to 3 for negative, weak positive, moderate positive, or strongly positive staining, respectively. The percentage of positive tumor cells was multiplied with the staining intensity giving an H-score between 0 and 300. 5T4 expression was observed in epithelial, mixed, as well as sarcomatoid mesothelioma (Fig. 2B). This indicates that 5T4 -based ADC therapy is not restricted to one of the MPM subtypes. The median H-score in our patient cohort was 60. Based on this, two groups were differentiated, biopsy specimens with no to low expression of 5T4 (H-score ≤60) (23 of 49), and biopsy specimens with a medium to high expression of 5T4 (H-score >60) (26 of 49). Two examples of each group

Figure 1. Expression and internalization of the 5T4 antigen in mesothelioma cells. A) Model of the working mechanism of ADCs. When the antibody binds to the target it is internalized and the cytotoxic compound is released from the lysosomes. **B)** Expression of 5T4 by Western Blot in 12 mesothelioma cell lines and the normal mesothelial cell line Met5A. Prostate cancer cell line LnCap was used as negative control. **C)** Quantification of the Western Blot analysis, with 7 out of 8 cell lines (black) and 3 out of 4 primary cell lines (blue) staining positive for 5T4. Error bars indicate standard deviation from two independent experiments. **D)** Analysis of 5T4 expression using IHC showing strong plasma membrane and diffuse cytoplasmic expression (magnification: 20x, scalebar: 50 µm). **E)** Quantification of IHC images. **F)** Internalization of H8-vcMMAE ADC (green) in the high 5T4 expressing cell line PV130913. At 0h, H8-vcMMAE is only expressed at the cell membrane, over time H8 is internalized and co-localization with lysosomal marker CD63 (red) is observed (Pearson's coefficient 0h: 0.19, 1h: 0.41, 3h: 0.55, 6h 0.56). Actin is depicted in blue and the dashed line indicates the nucleus.

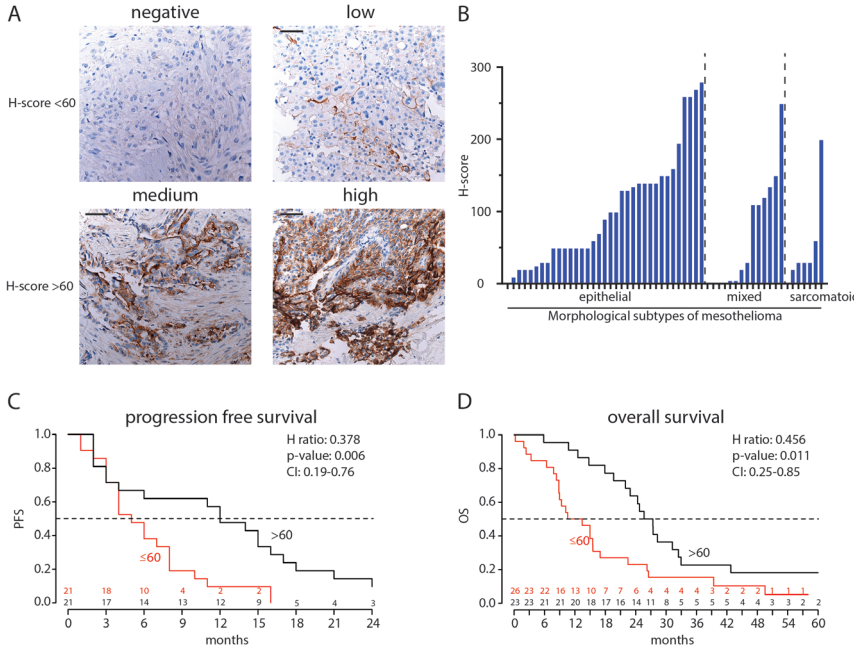

Figure 2. Expression of the 5T4 antigen in mesothelioma biopsies correlates with survival. A) Expression of 5T4 in mesothelioma biopsies (magnification: 40x, scalebar: 50 µm). **B)** H-scores for all biopsies classified to the epithelial, mixed and sarcomatoid subtypes. **C)** Progression free survival analysis in patients with mesothelioma, with low (H-score ≤60) or high (H-score >60) 5T4 expression. P-value, Hazard rate and 95% confidence interval (CI) are indicated. **D)** Similar as C, but now with overall survival as event.

are depicted in Figure 2A. PFS and OS were determined for 42 and 49 patients, respectively. Median PFS for the patients with high 5T4 expression was 12 months (95% confidence interval [CI]: 4-18 months) versus 5 months (95% CI: 4-8 months) for the patients with low 5T4 expression. Median OS for the patients with high 5T4 expression was 26.5 months (95% CI: 22.9-33 months) versus 12 months (95% CI: 8.84-22.5 months) for patients with low 5T4 expression. Therefor, high 5T4 expression (H-score >60) was associated with longer PFS (p = 0.006, hazard ratio = 0.38, 95% CI: 0.19-0.76) and OS (p = 0.011, hazard ratio = 0.46, 95% CI: 0.25-0.85) in univariate analysis (Fig. 2 C and D) as well as in multivariate analysis using age, sex, histological subtype (epithelial versus nonepithelial) and (only in the case of OS) disease control (defined as absence of progression) after 6 weeks as covariates (Supplementary Table 1). Independent of 5T4, our analysis showed that the nonepithelial subtype is associated with shorter PFS and OS which was expected [30, 31]. Disease control at 6 weeks was also associated with longer OS.

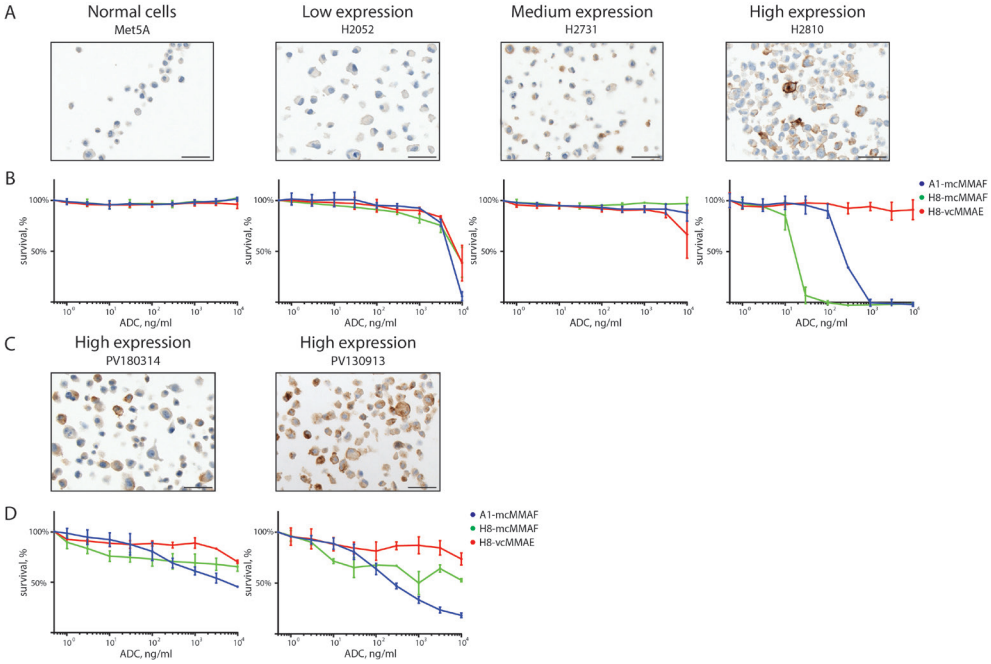

Figure 3. Efficacy of 5T4 targeting ADCs in killing mesothelioma cells. A) 5T4 expression by IHC for the different cell lines that were screened with ADCs. Cell lines are classified as normal mesothelial cells and low, medium or high 5T4 expression (magnification: 20x, scalebar: 50 µm). **B)** Response curves indicating percentage of survival for cell lines Met5A, H2052, H2731 and H2810. Survival is depicted in relation to increasing concentration (ng/ml) of ADC; A1-mcMMAF (blue), H8-mcMMAF (green), H8-vcMMAE (red). Error bar indicates standard deviation from three independent measurements. **C)** 5T4 expression by IHC for two primary cell lines with high 5T4 expression. **D)** Response curves indicating percentage survival for cell lines PV180314 and PV130913. Survival is depicted in relation to increasing concentration (ng/ml) of ADC. Error bar indicates standard deviation from three independent measurements.

Efficacy of 5T4 ADC-treatment in tumor cell killing

5T4 is expressed at the cell membrane of MPM cell lines and tumors, and high expression is associated with a favorable OS. To test whether 5T4 targeting ADC treatment is effective in MPM cell killing, we performed cytotoxicity experiments. To relate 5T4 ADC efficiency to 5T4 expression, four MPM cell lines and two primary cell lines were selected based on their 5T4 expression: normal mesothelial cells Met5A (no 5T4 expression), H2052 (low 5T4), H2731 (medium 5T4) and H2810, PV130913 and PV180314 (high 5T4 expression). We first tested the separate components of the ADC for their effect on toxicity. All cell lines were highly sensitive for the free toxin MMAE, a tubulin polymerization inhibitor and an uncharged analog of MMAF (Supplementary Fig 3A) [32]. The unconjugated 5T4 antibody did not affect cell survival, as was observed for the high expressing cell lines H2810 and PV130913 (Supplementary Fig. 3B). In total, three ADCs were tested. Two 5T4 antibodies

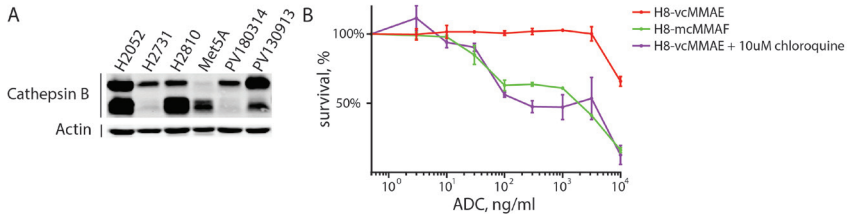

Figure 4. Neutralizing lysosomal pH by chloroquine rescues ADC H8-vcMMAE mediated cell killing. A) Western Blot analysis showing all cell lines express cathepsin B. **B)** Response curves of the high 5T4 expressing cell line H2810. Survival is depicted in relation to increasing concentration (ng/ml) ADC; H8-mcMMAF (green), H8-vcMMAE (red), H8-vcMMAE with 10 µmol/L chloroquine (purple). Error bars indicate standard deviation from three independent measurements.

(A1 and H8) with the same linker and toxin were tested. A1-mcMMAF and H8-mcMMAF were conjugated by the same mc linker to MMAF [23]. Both ADCs must undergo complete proteolytic degradation to release the toxin [7, 10]. In addition, the H8 antibody was also coupled to a different linker-toxin combination. In this combination, H8 has a cleavable dipeptide vc linker that requires cleavage by the lysosomal protease cathepsin B to release the toxin [10, 28]. The normal mesothelial cell line Met5A, which does not express 5T4, was insensitive to any of the ADCs. Also the cell lines H2052 (low 5T4) and H2731 (medium 5T4) were insensitive to all ADCs. All high 5T4 expressing cell lines were sensitive to A1-mcMMAF and H8-mcMMAF, but not to H8-vcMMAE (Fig. 3). H8-mcMMAF performed superior over A1-mcMMAF, which is in concordance with 5T4 binding affinities of these antibodies, as deducted by flow cytometry (Supplementary Fig. 1A). Surprisingly, H8-vcMMAE was not active on these cells. We considered two options to explain this phenomenon: 1) the MPM cells did not express the lysosomal protease cathepsin B; or 2) the active drug is sequestrated in lysosomes. Lysosomal sequestration is the accumulation of the active cytotoxic drug in acidic lysosomes, which is due to protonation of the toxin that as a result may not pass the lipid membrane of the lysosome and - as a consequence - fails to enter the cytosol where the drug is active [33]. Both options were tested. Western blot analysis showed that all cell lines express cathepsin B (Fig. 4), indicating that proteolytic cleavage of the drug, resulting in activation of the cytotoxic drug MMAE, could be achieved. Lysosomal sequestration was assessed by neutralizing acidic lysosomes in H8-vcMMAE-treated cells with 10 µmol/L chloroquine (Fig. 4). MPM cells incubated with H8-vcMMAE were now efficiently killed in the same range as the H8-mcMMAE, indicating that lysosomal trapping of MMAE limits the effects of this ADC. In summary, both A1-mcMMAF and H8-mcMMAF are effective treatments in high-5T4 tumor cell killing, whereas H8-vcMMAE treatment requires addition of chloroquine for full potency.

Discussion

Because of the poor prognosis of MPM, a pressing need exists for developing new treatment options. No druggable driver mutations have been identified, making targeted therapies difficult for patients with mesothelioma [34-36]. ADCs represent an interesting treatment option in which a cytotoxic agent is delivered to tumor cells via a cell-specific antibody based delivery [7, 8, 10, 11]. ADC therapy has been successfully applied in lymphoma and erb-b2 receptor tyrosine kinase 2 (Her2)-positive breast cancer [7, 11, 37, 38]. In mesothelioma, mesothelin has been evaluated as a target for ADC-based therapy, but the primary end point of PFS was not met in a phase II clinical trial [39, 40].

The cell surface protein 5T4 is highly expressed in MPM with limited expression in normal tissue, making this protein a suitable alternative candidate for ADC-based treatment [25]. In this report, we evaluated the potential use of 5T4-targeting ADC treatment in MPM by evaluating the expression and internalization of 5T4, determining correlations of 5T4 with patient prognostics and testing the efficacy of 5T4-targeting ADCs on MPM cells. We considered three different ADCs, in which we varied both the antibody as well as the linker-toxin combination.

Most cell lines and biopsy specimens stained positive for 5T4 regardless of MPM subtype, as reported before, indicating that 5T4-targeting ADCs could be of value for patients with MPM [25]. In our patient cohort, high 5T4 expression correlated with good survival, which is opposite to observations made in lung, colorectal, ovarian and gastric cancer [19-22]. This observation indicates that associations of 5T4 levels with outcome are tumor-type specific, which render it unlikely as a general driver of disease progression. However, most relevant is that 5T4 can be selectively targeted using ADCs, effectively yielding a targeted therapy for tumor types that would otherwise lack targeted therapeutics. As in lung cancer, 5T4-recognizing antibodies as well as ADCs rapidly internalize in MPM cells for intracellular transport to lysosomes [24]. The conjugation of H8 antibody to vcMMAE apparently did not alter its binding and internalization properties, consistent with the previously reported conjugation of A1 to mcMMAF [24]. This is not unexpected as the antigen recognition sites are not close to the cysteines to which the linker toxin is conjugated. The two Fab parts of the antibodies cluster the antigen and this likely accelerates endocytosis and lysosomal delivery [41].

We next tested the A1-mcMMAF ADC in MPM, which was previously reported to have antitumor activity in NSCLC and mammary cell line models. This ADC was active in various in vivo models and well tolerated in a phase I study [23, 24]. Next to this ADC, we generated two other ADCs by conjugating MMAF and MMAE to a second anti-5T4 antibody, H8. This

allowed us to assess the effect of different antibodies and the effect of different linker toxin conjugates on effectivity of the ADCs in eliminating 5T4-positive MPM cells. Two of the three ADCs were effective in high 5T4-expressing MPM cells, implying that sufficient quantities of drugs should be delivered to induce toxicity in the tumor cell. The current ADC tested will be of interest for MPM tumors expressing high amounts of 5T4, and this may be a biomarker for these ADCs.

We scored expression of 5T4 in biopsy specimens with the H-score because both percentage of positive cells as well as intensity of the staining were variable among tumors, and we believed that these variables were best-appreciated by using the H-index. Clearly, cell lines are more homogeneous in expression patterns, explaining why intensity of staining was similar among positive cells. These intrinsic differences render it challenging to compare expression of cells lines with biopsy specimens. Based on the responsiveness of 3 of 12 cell lines with high expression of 5T4, we expect that at least 25% of the tumors with the highest H-score will be responsive. However, further analyses using larger numbers of cell lines or primary tumor cultures are required to identify the optimal cut-off of 5T4 expression required for drug efficacy.

Changing the linker and drug used in ADC technology can also significantly alter the threshold of expression necessary for ADC efficacy, suggesting other 5T4 targeting ADCs could be effective in lower 5T4 expressing MPM cells [8]. Also, ADCs containing antibodies with a higher affinity than the ones we tested could be effective in lower 5T4-expressing MPM cells, although this could lead to unwanted killing of normal tissues that express low amounts of 5T4. We also noted effects of linkers on ADC activity. H8-vcMMAE was inactive in MPM cells. H8-vcMMAE was binding the target, and internalized into the cells, but sequestrated into the lysosome which prevented release of the cytotoxic compound into the cytosol and abrogated cell killing. Neutralization of the lysosomes by chloroquine alleviated lysosomal trapping and facilitated the cytotoxic potential of the compound. Lysosomal sequestration of weak base chemotherapy is a known phenomenon in multi-drug resistance [33, 42]. Adding chloroquine to the treatment schedule after the toxin is released in the lysosomes could provide opportunities for ADC treatment; however, further research is required to assess the impact of lysosomal sequestration in more physiologically relevant systems.

An advantage of ADC-based treatment is the focused drug delivery limiting the toxicity to the target cell and minimizing side effects, which makes it possible to combine this treatment with other treatments. Especially combining ADCs with immune checkpoint inhibitors, which are under investigation in MPM, are expected to sustain the antitumor effect [7, 43]. In this study, we provide a first proof-of-concept of 5T4-based ADC monotherapy in MPM, in which 5T4 expression levels may be used as companion diagnostic tool to identify patient

populations who would be most eligible for this treatment. This ADC treatment targeting the 5T4 antigen could be a promising novel strategy in the treatment of MPM with high 5T4 expression.

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Supplementary figures and tables

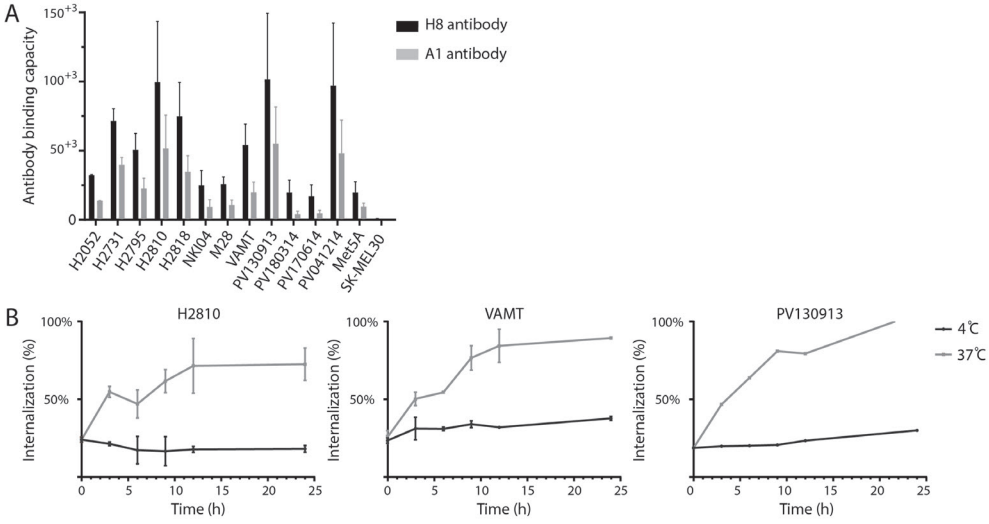

Fig. S1. Expression and internalization of 5T4 by FACS analysis. A) Antibody binding capacity of two anti-5T4 antibodies, H8 (black) and A1 (gray), in mesothelioma cells. The melanoma cell line SK-MEL30 was used as negative control. Error bars indicate standard deviation from two independent measurements. **B)** Internalization of the H8 antibody at 37°C (gray) in cell lines H2810, VAMT and PV130913. As control, cells were incubated at 4°C (black). Error bars indicate standard deviation from two (H2810) or three (VAMT) independent experiments.

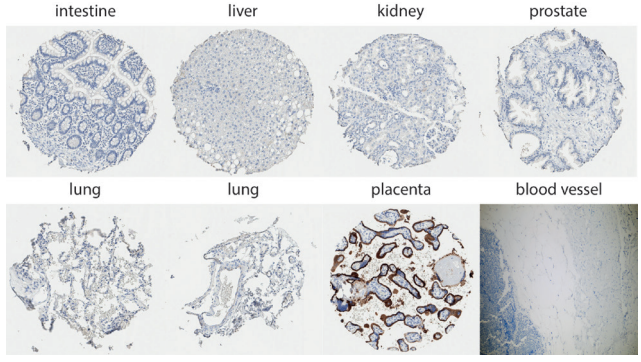

Fig. S2. Expression of 5T4 in normal tissues. 5T4 expression by IHC for normal tissues including intestine, liver, kidney, prostate, lung and a blood vessel. Placenta was used as a positive control (magnification 10x).

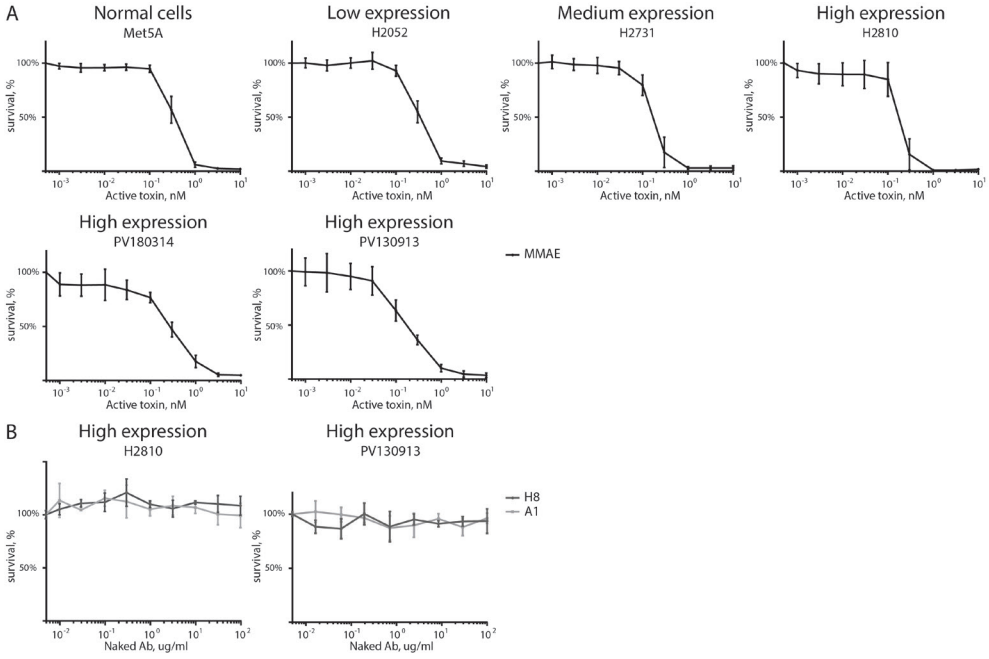

Fig. S3. Efficacy of the free toxin and the unconjugated antibody. A) Response to free toxin MMAE is depicted for all cell lines. Survival in relation to increasing concentrations (nmol/L) free toxin is depicted. Error bars indicate standard deviation from three independent measurements. **B)** Response curves for high 5T4 expressing cell lines H2810 and PV130913 are shown. Survival in relation to increasing concentrations (µg/ml) unconjugated antibody is depicted. Error bars indicate standard deviation from two independent measurements.

Table S1. Multivariate analysis

A) Multivariate analysis for progression free survival

	Hazard rate	Confidence interval	P-value
H-score of 5T4	0.40	0.19-0.82	0.012
Age	1.00	0.96-1.04	0.957
Gender (male)	1.64	0.70-3.86	0.254
Non-epithelial subtype	2.18	0.97-4.89	0.058

B) Multivariate analysis for overall survival

	Hazard rate	Confidence interval	P-value
H-score of 5T4	0.53	0.25-1.14	0.104
Age	0.99	0.94-1.04	0.723
Gender (male)	4.13	1.39-12.30	0.011
Non-epithelial subtype	5.06	2.19-11.72	0.0002
Disease control rate	0.38	0.17-0.86	0.020

H-score of 5T4 and age were continuous used in the analysis, hazard rate, 95% confidence interval and P-value were determined per steps of 10 for 5T4 and per one year for age.