Supplementary Methods

Antibody discovery using the HuCAL Gold Fab-phage library

MF-T was identified by panning the HuCAL Gold Fab-phage library (MorphoSys AG), comprising 10^{10} different monovalent phage, encoded within phagemid vector pMORPH23 and allowing for monovalent CysDisplay™ of Fab fragments as previously described (43). Dual-alternating cell surface panning was performed with CHO-A9 and the endogenous mesothelin-expressing mesothelioma cell line NCI-H226 as previously described (40). Pre-absorption with CHO-K1 cells was performed and repeated three times to avoid selection of Fab fragments that bind to epitopes of these parental cells. Pre-absorbed phages were then subjected to three rounds of alternating whole cell panning on mesothelin-expressing CHO-A9 (first and third panning) and NCI-H226 (second panning) cells followed by pH elution. Eluted phages were transfected into *E. coli* TG1 according to the HuCAL instructions, and the polyclonal DNA preparation was digested with *XbaI/EcoRI* thereby excising the Fab-encoding insert. The insert was cloned into the *XbaI/EcoRI* digested HuCAL Fab expression vector pMORPH®x9 and electroporated into *E. coli* TG1 F^-. A total of 1,760 single clones were picked and grown. Expressed Fabs were screened using two cell-based ELISAs in which CHO-A9 and NCI-H226 cells were coated and corresponding negative cell lines (CHO-K1 and A431) were used for counter screens. 214 clones developed a positive signal in the cell-based ELISA. After sequence analysis of positive clones, 22 novel unique sequences were obtained.

Expression and purification of Fab and IgG

Fab fragments in pMORPHX9_MH were transformed in *E coli* TG1-F. Single clone expression and preparation of *E. coli* lysates containing HuCAL-Fab fragments were performed as previously described (44). For expression of huIgG1, HKB11 cells were

transfected with IgG1 heavy and light chain pMorph or pMorph2 expression vectors. 3 or 7 days after transfection, the cell culture supernatant was harvested and cleared by centrifugation. After adjusting the pH of the supernatant to 7.0–8.0 and sterile filtration (0.2 µm), the solution was subjected to standard protein affinity chromatography (HiTrap rProtein A FF columns, GE Healthcare). The IgG was eluted at pH 3.0 and neutralized with 3 M TRIS (pH 8.0). After buffer exchange to 1x Dulbecco's PBS (Gibco) and sterile filtration (0.2 µm), the yield and purity of the IgG were determined using an Agilent BioAnalyzer Protein 200 Plus chip for electrophoresis under denaturing, reducing, and non-reducing conditions. In order to express full length IgG1s from the Fabs obtained from the pannings with HUCAL GOLD, variable domain fragments of heavy (VH) and light chains (VL) were subcloned from Fab expression vectors into the appropriate pMorph_hIg vector for human IgG1. Restriction enzymes *EcoRI*, *MfeI*, and *BlpI* were used for subcloning of the VH domain fragment into pMorph_hIgG1.1, while *EcoRV* and *BsiWI* were used for subcloning of the Vkappa domain fragment into pMorph_hIgκ_1 and *EcoRV* and *HpaI* for subcloning of the Vlambda domain fragment into pMorph_hIgλ_1.

For large scale IgG expression , stable CHO cells were generated using pUCOE plasmid (Millipore). Both Fabs and IgGs of the new mesothelin-binding sequences were further characterized in multiple assays, including binding kinetics using SPR, the ability to bind murine and rat mesothelin, the titration profiles in flow cytometry on mesothelin-expressing cells and in cellular ELISAs, the ability to internalize into mesothelin-expressing cells, and the competitiveness of the epitopes for the mesothelin antigen.

Determination of antibody binding affinities and kinetics

Binding affinities of BAY 94-9343 and its unconjugated antibody MF-T were determined by SPR analysis using a Biacore T100 instrument (GEHealthcare),

Monovalent affinities were measured with an indirect capturing protocol. The Human Antibody Capture Kit (GEHealthcare) was used according to the manufacturer's instructions. Mouse anti-human IgG (Fc) antibody was immobilized onto a CM5 sensor chip. Antibodies were injected at a concentration of 1 µg/mL at 10 µL/min for 20 seconds. Various concentrations (200 nM–1.56 nM) of recombinant mesothelin in HEPES-EP buffer (GEHealthcare) were injected over immobilized BAY 94-9343 and MF-T at a flow rate of 60 µL/min for 3 min, and dissociation was allowed for 10 min. Sensograms were generated after in-line reference cell correction followed by buffer sample subtraction. Dissociation equilibrium constant (K_D) was calculated based on the ratio of association and dissociation rated constants, obtained by fitting sensograms with a first order 1:1 binding model using the BiaEvaluation Software.

Calibrated FACS analysis and IHC of cells

Antibody MF-T was stoichiometrically labeled with a single molecule phycoerythrobilin (PE, custom conjugate) by Becton Dickinson (BD Biosciences). AsPC-1, HT-29, HT-29/meso, and OVCAR-3 cells were stained with PE-labeled MF-T. The number of labeled antibodies bound per cell was determined using FACSCalibur (BD Biosciences) and Phycoerythrin Fluorescence Quantitation Kit according to manufacturer's instructions. The cells were formalin-fixed and paraffin-embedded (FFPE) using a standard protocol. The FFPE sections of cells were demasked in a vegetable steamer in TBS (pH 9) for 17 min, incubated with anti-mesothelin mouse monoclonal antibody MB-G10 (Novus Biologicals) and subsequently the EnVision system HRP labeled polymer anti-mouse (Dako). DAB was used for visualization.

IHC of the tumor tissues

Frozen tissue sections were fixed in acetone at room temperature (RT) for 10 min, rinsed in Tris-buffered saline with 0.05% Tween80 (TBS-T), incubated with 1%

hydrogen peroxide in methanol, rinsed with TBS-T and blocked with the Avidin/Biotin Blocking Kit (Zymed). Next, the sections were blocked with Serum-free Proteinblock (Dako) for 10 min, incubated with primary anti-mesothelin antibodies (MF-T, biotinylated MF-T, BAY 94-9343, mouse monoclonal antibody clone K1 (Abcam)) at RT for 1 h, and rinsed with TBS-T. The primary antibodies were detected by incubation with an alkaline phosphatase coupled goat anti-human IgG antibody (Abcam), Extravidin Peroxidase (Sigma), or EnVision system HRP labeled polymer anti-mouse (Dako), respectively. For visualization, the sections were incubated with either Fast Red Substrate System (Dako) or 3,3'-diaminobenzidine chromogen solution (DAB, Dako) at RT for 1 min. The sections were counterstained with hematoxylin.

Antibody internalization assay

Antibodies were conjugated with CypHer5E dye (GEHealthcare) at pH 8.3 according to the manufacturer's instructions. After conjugation, the reaction mixture was dialyzed (Slide-A-Lyzer Dialysis Cassettes MWCD 10 kD, Pierce) at 4°C for 16 h to eliminate the unconjugated dye. The protein solution was concentrated using VIVASPIN 500 (Sartorius). Dye load of antibodies was determined spectrometrically (NanoDrop) and calculated with the formula D: $P=A_{dye}\ \varepsilon_{protein}: (A_{280}–0.16A_{dye})\ \varepsilon_{\delta\psi\varepsilon}$. Prior to testing antibody internalization, $2x10^4$ cells/well were seeded in 96-well plates, incubated for 18 h, medium was changed and labeled antibodies were added in various concentrations (0.1–10 µg/mL). Internalization was determined using the In Cell Analyzer 1000 (GEHealthcare). Granule counts and total fluorescence intensity were measured for up to 24 h.

Supplementary Data

Supplementary Figure S1. Mesothelin is overexpressed in various human tumor specimens. Immunohistochemical detection of mesothelin expression in formalin-fixed, paraffin-embedded (FFPE) tissues of human primary ovarian cancer (A), pancreatic cancer (B), and mesothelioma (C) using monoclonal murine anti mesothelin antibody MB-G10. Scale bar 50 µm. The samples were scored 1–3 based on the staining intensity.

Supplementary Table S1. Mesothelin is overexpressed in various human tumor specimens. Mesothelin staining intensity in FFPE tissues of human primary ovarian cancer, pancreatic cancer and mesothelioma. Monoclonal murine anti-mesothelin antibody MB-G10 was used, and the samples were scored 1–3 based on the staining intensity.

Supplementary Figure S2. MF-T shows no cross-reactivity with murine mesothelin. Immunohistochemical detection of mouse mesothelin on Cryo embedded peritoneum sections using biotinylated MF-T (A) and biotinylated cross reactive anti-mesothelin antibody BAY 1132694 (B). Scale bar 200 µm.

Supplementary Figure S3. MF-T shows no cross-reactivity with rat mesothelin. Immunohistochemical detection of rat mesothelin on Cryo embedded tissue sections: pericardium (A, B, C) and peritoneum (D, E, F) using biotinylated MF-T (A, D), biotinylated cross reactive anti-mesothelin antibody BAY 1132694 (B, E), or biotinylated isotype control BAY 86-1906 (C, F). Scale bar 200 µm.

Supplementary Figure S4. MF-T shows no cross-reactivity with monkey mesothelin. Immunohistochemical detection of cynomolgus monkey mesothelin on Cryo embedded tissue sections: pericardium (A, B, C), peritoneum (D, E, F), and pleura (G, H, I) using biotinylated MF-T (A, D, G), biotinylated cross reactive anti-mesothelin antibody BAY 1132694 (B, E, H), or biotinylated isotype control BAY 86-1906 (C, F, I). Scale bar 200 µm.

Supplementary Figure S5. Mesothelin is expressed in the cell models used *in vitro* and *in vivo*. Confirmation of mesothelin expression by IHC (scale bar 50 µm) in vector or mesothelin-transfected tumor cell lines HT-29 (A) and MIA PaCa-2 (B), endogenous mesothelin-expressing NCI-H226 (C) and OVCAR-3 (D) cells, or by FACS analysis in primary adult mesothelial cells (E).

Supplementary Figure S6. Anti-tumor activity of BAY 94-9343 *in vivo*. Mean +/- SD of tumor volume in HT-29/meso (A), OVCAR-3 (B), and NCI-H226 (C) subcutaneous tumor models over time. D, mean +/- SD of NCI-H226 tumor weight on study day 174. The vehicle control group had already been sacrificed before this time point.