Supplemental data

Balancing Selectivity and Efficacy of Bispecific EGFR x c-MET Antibodies and Antibody-Drug Conjugates

Carolin Sellmann[#,$], Achim Doerner[$], Christine Knuehl[§], Nicolas Rasche[$], Vanita Sood[*], Simon Krah[#,$], Laura Rhiel[$], Annika Messemer[#], John Wesolowski[*], Mark Schuette[$], Stefan Becker[$], Lars Toleikis[$], Harald Kolmar[$1], Bjoern Hock[$1]

From the [#]Institute for Organic Chemistry and Biochemistry, Technische Universität Darmstadt, Alarich-Weiss-Strasse 4, D-64287 Darmstadt, Germany, [$]Protein Engineering and Antibody Technologies, Merck KGaA, Frankfurter Strasse 250, D-64293 Darmstadt, Germany, [§]Merck Research and Development, Merck KGaA, Frankfurter Strasse 250, D-64293 Darmstadt, Germany and [*]EMD Serono Research and Development Institute, 45A Middlesex Turnpike, Billerica, MA 01821, USA

Supplemental experimental procedures:

Serum stability - After incubation of 5 µg/ml antibodies in human serum (kindly provided by Merck) in 50 µl aliquots at 37°C with 750 rpm shaking, bridging ELISA was applied. 100 ng human recombinant EGFR ECD were immobilized on MaxiSorp® plates overnight at 4°C. After blocking, bsAbs were incubated in plates (B10v5x225-H and CSx225-H 1:60 dilution, B10v5x225-M and CS06x225-M 1:6 dilution) for 1 h at RT. After washing, 100 ng biotinylated human recombinant c-MET ECD were added and were detected with streptavidin-horse reddish peroxidase conjugate (Merck). Cetuximab samples were subjected to plates with immobilized EGFR ECD (100 ng) and detected with HRP-conjugated goat-anti-human IgG, Fcγ specific (Jackson Immuno Research). The zero time point control served for each antibody as 100% control and based on this, reduction in protein functionality was determined.

Flow cytometry - Cellular binding of mAbs was determined using Guava easyCyte HT cytometer (Millipore) and corresponding software Guava ExpressPro (ver. 2.2.3, Millipore). Briefly, serial dilutions of bsAbs (0.02 – 200 nM) in FACS buffer (1% BSA in DPBS, Life Technologies) were incubated with at 1.0 x 10^5 cells/well on ice and detected via FITC conjugated goat anti-human Fcγ specific pAb (Jackson ImmunoResearch). Propidium iodide (Life Technologies) enabled dead cell staining. 5,000 events were detected at 488 nm and for geometric mean intensity values, background autofluorescence values of cells treated with medium only were subtracted. Means of triplicate mean fluorescence intensity (MFI) values were plotted versus logarithm of mAb concentration and fitted by a 3PL model using GraphPad Prism 5 (GraphPad Software, Inc).

Immunoblotting - Two days prior treatment, cells (1-3 x 10^5 cells per well) were seeded in a 24 well plate in cell line specific medium and changed for serum starvation devoid FBS the following day. For treatment, 300 nM of mAbs were incubated for 3 h prior to stimulation (100 ng/ml EGF and 100 ng/ml HGF; R&D Systems) for 10 min. Cells were lysed with 50 µl RIPA buffer (Cell Signaling Technologies) supplemented with phosphatase and protease inhibitors (Calbiochem) and Benzonase (Novagen). Cell lysates were subjected to immunoblot analysis using antibodies directed against c-MET (Biomol), p-EGFR Y1173 (Abcam), EGFR, AKT, p-AKT, Erk1/2, p-Erk1/2, p-c-MET Y1234/35, GAPDH (all Cell Signaling Technologies), and actin (Sigma-Aldrich). Detection was carried out with Alexa Fluor® 680 goat anti-rabbit IgG (Life Technologies). Band intensities were quantified using the LI-COR immunoblotting system (LI-COR). Antibody VH and VL sequences for DN30 based on patent no. EP 2,500,036 B1 (2014) were subcloned into pTT5 IgG_1 vectors.

ADCC assay - ADCC assays were performed using the ADCC Reporter Bioassay Core Kit (Promega) according to the manufacturer's instruction. Briefly, target cells (e.g. A431 cells) were detached and seeded into the inner wells of opaque white tissue culture treated 96 well plates (Perkin&Elmer) with 12.500 viable cells/well in ADCC buffer (RPMI 1640, 4% low IgG fetal bovine serum (FBS; both Life Technologies))

and allowed to attach overnight in a humidified chamber at 37°C, 5% CO_2. The next day, cells were treated with 5 to 0.0016 nM of mAbs diluted in ADCC buffer and 75,000 recombinant Jurkat cells (Promega) per well as effector cells. After 6 h of incubation, 75 µl per well Bio Glo Luciferase Substrate (Promega) equilibrated RT were added for 10 min at RT avoiding light. Luminescence was measured at a Synergy 5 (Biotek) with a read time of 0.5 seconds per well (sensitivity: 170). Background luminescence in only medium wells was subtracted. Mean relative luminescence units (in triplicates) were plotted against the logarithm of antibody concentration and dose response curves were fitted using 3PL model by GraphPad Prism 5 (GraphPad Software, Inc.).

Internalization - Internalization was either determined by flow cytometry using an anti-Alexa Fluor 488 quenching antibody[1] or by confocal microscopy applying pH stripping[2]. For flow cytometry, cells (1 x 10^5) were incubated with 100 nM bsAbs followed by Alexa Fluor 488 conjugated anti human Fc (Fcγ specific, Jackson Immuno Research). After washing with FACS buffer, cells were incubated for 1 h at either 37 °C (allowing internalization) or 4 °C for 1 h (as reference preventing internalization). Afterwards, residual surface binding of bsAb was quenched by anti-Alexa Fluor 488 IgG (Life Technologies) and cells were fixated with 4% (w/v) formaldehyde (Calbiochem) and subjected to flow cytometric analysis as described before. Internalization was calculated as following:

$$rel.interalization\ [\%] = \frac{\left(37\ °C\ with\ quench\right) - \left(4\ °C\ with\ quench\right)}{\left(37\ °C\ without\ quench\right)} x100$$

For fluorescence microscopy, cells (3 x 10^5) were grown on glass coverslips (Menzel Glaser) placed in 6 well plates. Two days later, cells were kept on ice and treated with 100 nM bsAbs followed by detection with Alexa Fluor 488 conjugated anti human Fc Fab fragment. After washing with 1% BSA in PBS, cells were incubated in respective medium at either 37 °C or 4 °C for 1 h allowing internalization. By addition of ice-cold low pH buffer (50 mM glycine, 150 mM NaCl, pH 2.7 adjusted with HCl), residual bsAbs on the cell surface were removed. Finally, cells were fixated with 4% (w/v) formaldehyde and mounted on object slides with ProLong Diamond Antifade Mountant with DAPI (Life Technologies). Analysis was carried out with a Leica TCS SPS confocal microscope equipped with a 100x objective (Leica Microsystems).

Supplemental figures and tables:

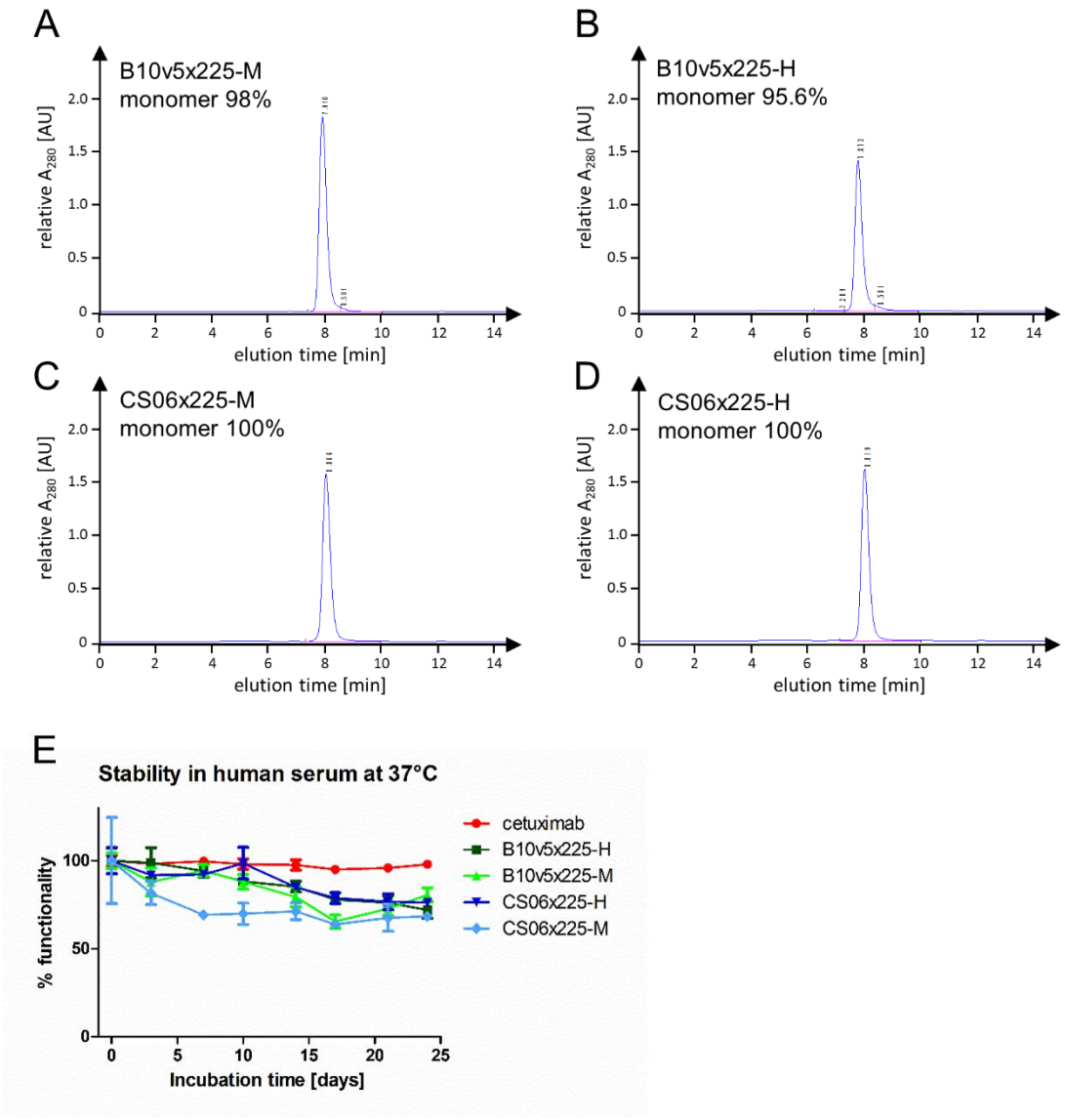

Figure S-1: Analytical SE-HPLC indicated purity greater as 95% of bsAbs B10v5x225-M **(A)**, B10v5x225-H **(B)**, CS06x225-M **(C)** and CS06x225-H **(D)** after purification. **(E)** All four bsAbs demonstrated long term stability in human serum at 37°C for 24 days assessed by ELISA. Briefly, bsAbs were incubated in human serum for defined period of time and subsequently added to immobilized EGFR-ECD. Detection was revealed with biotinylated c-MET ECD and streptavidin-peroxidase conjugate. For cetuximab, detection was carried out with anti-human Fc-peroxidase conjugate.

Table S-1: Mean fluorescence intensity values for binding of bsAb to several cell lines. Cells were incubated with 100 nM bsAbs or control mAbs and subjected to flow cytometric analysis. n.d. = not determined

	EBC-1	MKN-45	A431	A549	HepG2	CHO-S
mAb	c-MET^{+++} EGFR^{++}	c-MET^{+++} EGFR^{++}	c-MET^{+} EGFR^{+++}	c-MET^{+} EGFR^{++}	c-MET^{+} EGFR^{+}	c-MET^{-} EGFR^{-}
B10x225-L	1.10E+03	1.20E+03	9.30E+02	1.80E+02	7.20E+01	3.00E+00
B10x225-M	1.20E+03	1.80E+03	1.50E+03	5.60E+02	7.10E+01	2.30E+01
B10x225-H	1.10E+03	1.30E+03	2.00E+03	4.50E+02	8.70E+01	3.30E+01
B10x425	1.20E+03	1.30E+03	1.10E+03	2.60E+02	8.80E+01	5.00E+00
F06x225-L	9.30E+02	1.20E+03	1.20E+03	2.10E+02	8.50E+01	1.50E+01
F06x225-M	9.00E+02	1.20E+03	1.90E+03	n.d.	6.10E+01	1.00E+00
F06x225-H	8.80E+02	1.20E+03	3.20E+03	2.90E+02	7.40E+01	2.00E+00
F06x425	7.10E+02	1.20E+03	1.30E+03	2.60E+02	6.30E+01	9.00E+00
B10v5x225-L	8.40E+02	1.20E+03	8.30E+02	2.40E+02	4.30E+02	1.20E+02
B10v5x225-M	1.10E+03	1.30E+03	1.30E+03	3.70E+02	7.70E+02	2.70E+02
B10v5x225-H	1.10E+03	1.30E+03	1.70E+03	3.30E+02	3.30E+02	8.30E+01
B10v5x425	1.00E+03	1.30E+03	1.40E+03	3.20E+02	3.30E+02	9.80E+01
CS06x225-L	1.20E+03	1.50E+03	8.70E+02	2.00E+02	2.30E+02	7.00E+00
CS06x225-M	1.20E+03	1.50E+03	1.20E+03	2.60E+02	3.10E+02	3.00E+01
CS06x225-H	1.10E+03	1.50E+03	1.80E+03	2.80E+02	1.80E+02	4.00E+00
CS06x425	9.80E+02	1.40E+03	1.30E+03	2.60E+02	1.40E+02	1.00E+00
oa 225-L	1.40E+02	7.40E+01	9.90E+02	8.10E+01	1.10E+01	3.00E+00
oa225-M	2.70E+02	1.40E+02	1.10E+03	1.30E+02	2.40E+01	8.00E+00
oa 225-H	6.30E+02	1.90E+02	1.70E+03	1.70E+02	3.70E+01	1.00E+00
oa 425	2.40E+02	1.80E+02	8.60E+02	1.60E+02	4.30E+01	6.00E+00
oa F06	1.10E+03	9.10E+02	3.10E+01	8.10E+01	1.50E+01	0.00E+00
oa B10	1.30E+03	1.30E+03	9.40E+01	1.20E+02	5.60E+01	3.40E+01
oa CS06	1.30E+03	1.50E+03	1.80E+01	1.40E+02	2.70E+02	1.00E+00
oa B10v5	1.30E+03	1.50E+03	3.00E+01	1.50E+02	3.80E+02	7.70E+01

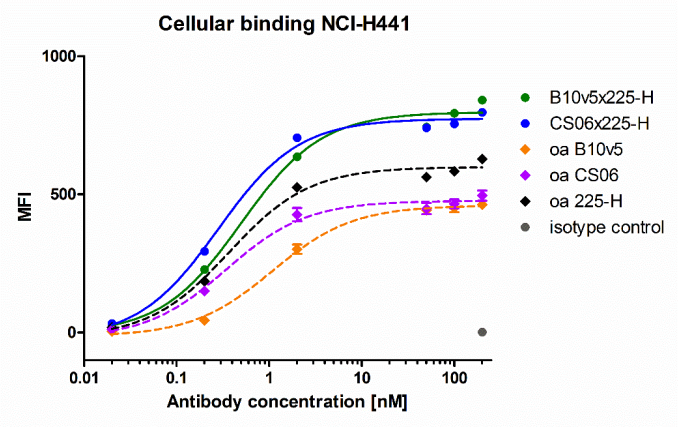

Figure S-2: Cellular binding of bsAb to NCI-H441 cells in comparison to monovalent, one-armed controls. NCI-H441 cells were incubated with varying concentration of bsAbs and control mAbs (two independent experiments with duplicates). Curves were plotted with 3 PL in GraphPad Prism 5 (GraphPad Software, Inc.).

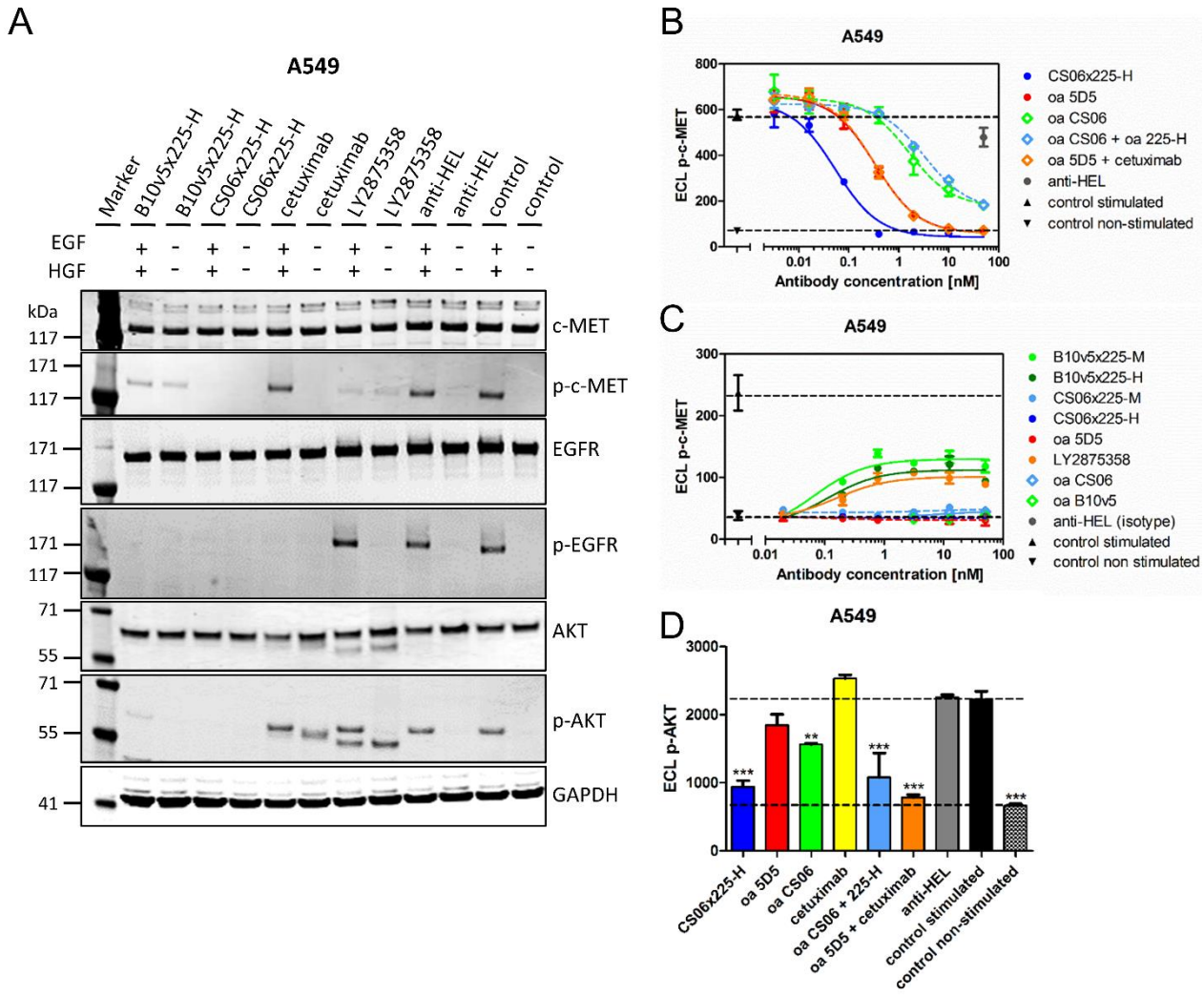

Figure S-3: Synergistic effect of CS06x225-H on inhibition of c-MET, EGFR, and AKT phosphorylation. **(A)** A549 cells were incubated with 300 nM mAbs for 3 h and stimulated with HGF and EGF. Cell lysates were subjected to Western blotting and both phosphorylated and total EGFR, c-MET, and AKT were detected. GAPDH was employed as a loading control. Molecular weights are indicated. **(B)** Electrochemiluminescence (ECL) ELISA of mAbs treated and HGF-stimulated A549 cell lysates for phosphorylated c-MET indicated increased potency of CS06x225-H in comparison to the combination of oa CS06 and oa 225-H. **(C)** A549 cells were treated with varying concentrations of mAbs without stimulation and lysates were subjected to ECL ELISA detecting phosphorylated c-MET levels. B10v5x225-M and B10v5x225-H demonstrated comparable partial agonism to LY2875358. **(D)** Quantification of phospho-AKT levels in A549 cells after treatment with 500 nM mAbs as well as combinations of control mAbs (each 500 nM) and stimulation with HGF and EGF. Cell lysates were subjected to ECL ELISA. Asterisks indicate significant difference in comparison to the stimulated control (** p-value < 0.01; *** p-value < 0.001; determined with one-way ANOVA with Dunnett's test).

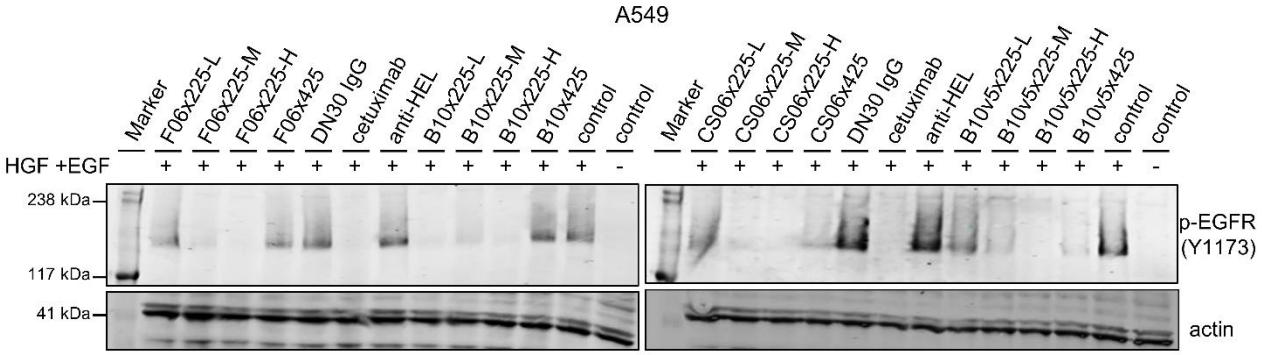

Figure S-4: Inhibition of EGFR phosphorylation. A549 cells were incubated with 300 nM mAbs for 3 h and stimulated with 100 ng/ml HGF and EGF. Cell lysates were subjected to Western blotting and phosphorylated EGFR was detected. Actin was employed as a loading control. Molecular weights are indicated.

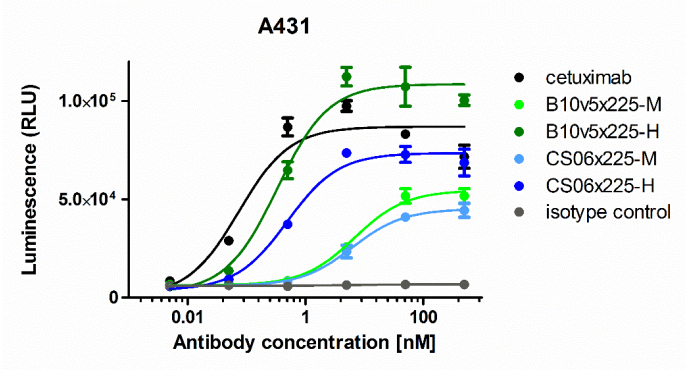

Figure S-5: ADCC mediated by bsAbs. A431 cells were treated with bsAb, cetuximab or anti-HEL SEED as isotype control followed by adding recombinant Jurkat effector cells (in ratio 6:1) with luciferase in the nuclear factor of activated T-cells (NFAT) pathway. Activation of FcγRIIIa receptor by mAbs induced conversion of substrate by luciferase yielding in luminescence signal. As a result, bsAb induced ADCC comparable to cetuximab whereas isotype control. Data of two independent experiments with triplicates were plotted as relative luminescence units versus logarithm of antibody concentration using GraphPad Prism 5 (GraphPad Software, Inc.).

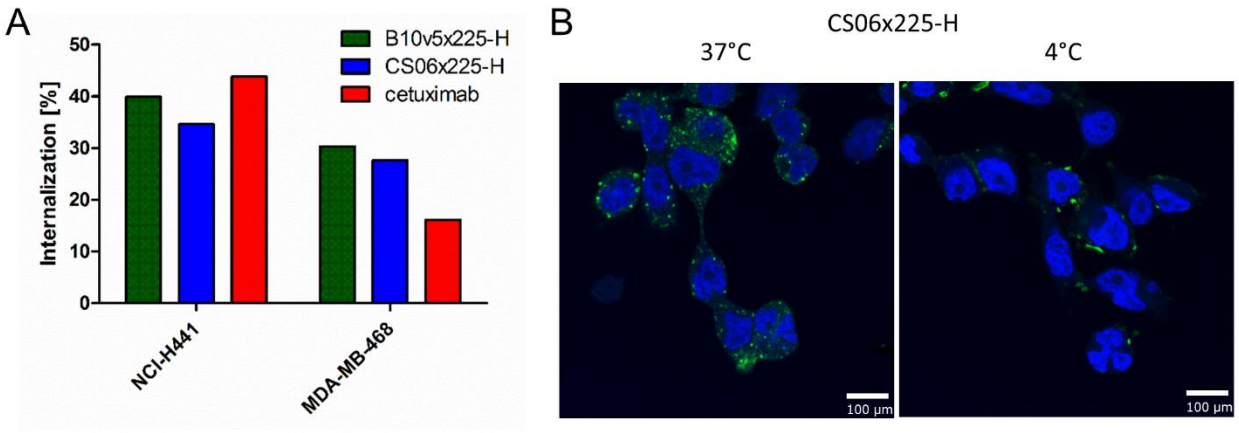

Figure S-6: Internalization of bsAbs determined by flow cytometry and confocal fluorescence microscopy**. (A)** Internalization was quantified by flow cytometric analysis employing 100 nM mAbs detected with anti-human Fc-AlexaFluor488 conjugate at 37 °C for 1 h allowing internalization in comparison to cells incubated at 4 °C at which temperature internalization is prevented. Residual cell surface binding was quenched by anti-AlexaFluor488 antibody. **(B)** EBC-1 cells were incubated with 100 nM CS06x225-H and detected with anti-human Fc-AlexaFluor488 conjugate (green) at 37 °C allowing internalization or at 4 °C preventing internalization. Residual surface staining was removed by acidic wash. The cell nucleus was stained with DAPI (blue).

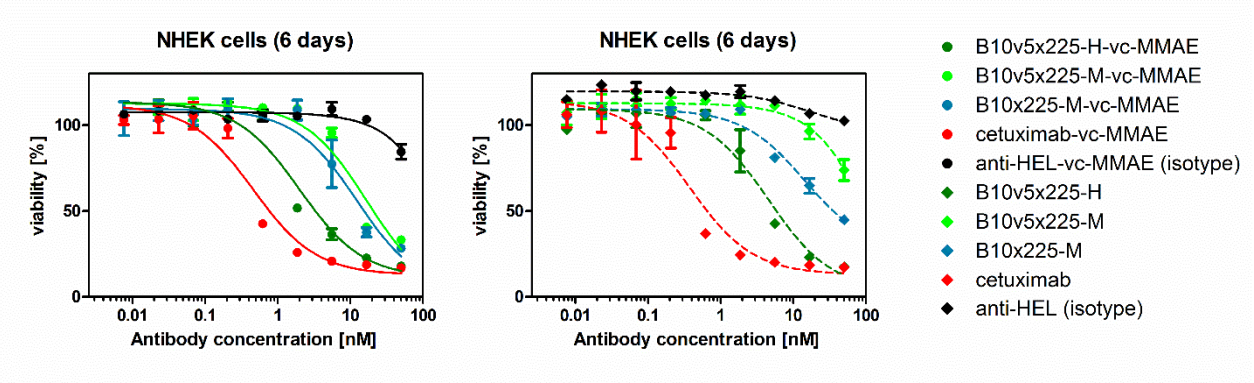

Figure S-7: Cytotoxicity of bispecific ADCs and bsAb on NHEK after 6 days. Primary keratinocytes (NHEK) were incubated with varying concentrations of bispecific ADC or alternatively with bsAbs for 6 days, in order to exclude that slow division rate of keratinocytes in comparison to tumor cells influenced cytotoxicity of the tubulin inhibitor MMAE. Curves were plotted using 3PL fitting in GraphPad Prism 5 (GraphPad Software, Inc.).

Reference List

1. Gostring L, Chew MT, Orlova A, Hoiden-Guthenberg I, Wennborg A, Carlsson J, et al. Quantification of internalization of EGFR-binding Affibody molecules: Methodological aspects. Int.J.Oncol. 2010; 36: 757-63
2. Li N, Hill KS, Elferink LA. Analysis of receptor tyrosine kinase internalization using flow cytometry. Methods Mol.Biol. 2008; 457: 305-17