**RG7386, a novel tetravalent FAP-DR5 antibody, effectively triggers FAP-dependent, avidity-driven DR5 hyperclustering and tumor cell apoptosis**

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- Supplementary Material and Methods

**Supplementary Materials and Methods**

***Cell lines and host strains***

HEK293 EBNA cells were maintained in DMEM (PAN Laboratories), 10% fetal calf serum (FCS) (Gibco) and non-essential amino acids (Sigma # M7145); NIH-3T3 fibroblasts, MDA-MB-231 breast cancer cells, ACHN renal carcinoma cells, and A549 lung carcinoma epithelial cell line in DMEM, 10% FCS and 2 mM L-glutamine (PAA Laboratories); colorectal cancer cell line DLD-1 and LOX-IMVI human desmoplastic melanoma cells in DMEM, 10% FCS and 1 mM L-glutamine; colorectal cancer cell line Colo205 in RPMI, 10% FCS and 2 mM L-glutamine; the human fibroblast cell line GM05389 in MEM + Earle’s medium (Gibco) supplemented with 15% FCS and non-essential amino acids; and 1833-PPOP233 tumor cells representing a bone metastasized subclone of the breast cancer MDA-MB-231 cell line in DMEM, 10% FCS, 2 mM L-Glutamine and 200 µg/ml G418. NIH3T3 cells stably transfected with either full-length murine or human FAP (mu/huFAP) gene (cDNA) under the control of the chimeric MPSV promoter (minimal MPSV promoter with CMV enhancer) were maintained in DMEM High Glucose with 10% CS, 1 mM Sodium Pyruvate and 4 mM L-glutamine. To increase stability of FAP expression and to omit position effects the plasmids contained a scaffold attachment region (from human interferon ) and barrier elements from the murine T-cell receptor locus control region. Positive muFAP or huFAP 3T3 clones were selected using puromycin (1.5 µg/ml) followed by FACS. *E. coli* XL-1 Blue from Invitrogen was used as a host for plasmid amplification and preparation.

***Isolation of novel anti DR5 binders from generic Fab libraries***

Antibodies with specificity for human DR5 were selected from a generic phage-display antibody library in the Fab format. This library was constructed on the basis of human germline genes comprising randomized sequence space in CDR3 of the light chain (L3) and CDR3 of the heavy chain (H3). Selections were carried out against HEK293-expressed monomeric or dimeric human DR5 fused to the Fc-portion of a human IgG1 antibody over three rounds over a constant antigen concentration at 100 nM. Specific binders were identified by ELISA assays and clones exhibiting signals on human DR5 but none on human DcR2 and human IgG1 were short-listed for further analyses.

***Transient antibody production***

All bispecific antibodies and antigens (if not obtained from a commercial source) used herein were transiently produced in HEK293 EBNA cells using polyethylenimine (PEI)-mediated transfection. DNA (200 μg) and PEI solution (540 μl) were incubated in pre-warmed CD CHO medium (Life Technologies) (20 ml), vortexed for 15 s and subsequently incubated for 10 min at room temperature. HEK293-EBNA suspension cells cultivated in CD CHO culture medium were centrifuged for 5 min at 210 x g and supernatant was replaced by the DNA/transfection reagent mixture and incubated for 3 hours at 37 °C, 5% CO2 with agitation. Medium was then replaced with F17 medium and cells were cultivated for 24 h prior to addition of 1 mM valporic acid and 7% Feed 1. Supernatants were collected 7 days post transfection, centrifuged for 15 min at 210 x g, sterile filtered (0.22 μm filter) and stored at 4 °C. All bispecific molecules were purified in two steps using standard protein A affinity purification (Äkta Explorer) and size exclusion chromatography. Fractions containing less than 2% oligomers were pooled and concentrated to final concentration of 1 - 1.5 mg/ml using ultra concentrators (Vivaspin 15R 30.000 MWCO HY, Sartorius). Purified proteins were frozen in liquid N2 and stored at -80 °C.

***Biochemical and biophysical characterization***

The purity and molecular weight of the BsAb preparations were determined by Microchip capillary electrophoresis (CE)-SDS on Caliper`s LabChip GXII using the Protein Express LabChip kit. The procedure was carried out according to the manufacturer`s specifications. The thermal stability of the protein was monitored by Dynamic Light Scattering (DLS). 30 µg of filtered protein sample with a protein concentration of 1 mg/ml was applied in duplicate to a Dynapro plate reader (Wyatt Technology Corporation; USA). The temperature was ramped from 25 to 70 °C at 0.05 °C/min and radius and total scattering intensity was collected. Transient production and purification of the bispecific molecules in HEK293 EBNA cells resulted in up to 30 mg of purified antibody (data not shown) in good quality: The material mostly consists of monomers (> 96%), exhibited thermal stability up to 59 °C and only minor amounts of light chain mispairing were detected.

***Surface plasmon resonance (SPR)***

Simultaneous binding of bispecific antibodies to human DR5 as well as hu or muFAP was assessed by surface plasmon resonance (SPR). All SPR experiments were performed on a Biacore T100 at 25 °C with HBS-EP+ as running buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20 (GE # 100-69). Briefly, purified, recombinant, biotinylated human DR5 was immobilized on a Streptavidin chip (GE # BR-1005-31) with an immobilization level of 1033 RU. The bispecific antibodies were diluted in HBS-EP+ (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20 (GE # 100-69) and injected at a concentration of 300 nM (150 nM for RG7386) for 90 s with a flow rate of 30 µl/min, followed by a dissociation phase of 10 s. As second analyte either hu or muFAP was injected at a concentration of 0, 100 or 500 nM for 90 s at 30 µl/min and the dissociation monitored for 60 s. Regeneration of the chip surface was performed 2 times for 60 s following each cycle using 10 mM glycine pH 1.5.

***BsAb binding to DR5 and FAP - TagLite assay***

***Preparation of cells***

The binding of bispecific antibodies on cells was determined using a time-resolved fluorescence resonance energy transfer (TR-FRET) assay (referred to as TagLite). HEK EBNA cells were grown to 60-80% confluency and transfected with cDNA encoding huDR5 extracellular domain or huFAP fused to a SNAP Tag and either the PDGFR (DR5) or natural TM (FAP) using Lipofectamine 2000 (Invitrogen Cat No. 11668-019) according to manufacturer protocol. Transfected cells were washed with D-PBS, followed by the addition of a mixture of TagLite buffer (Cisbio # LABMED) containing 100 nM SNAP-Lumi4-Tb (Cisbio # SSNPTBG) and 1 h incubation at 37 °C. Cells were plated in a 384 well format were washed and labeling efficiency determined by fluorescence measurements at 620 nm (excitation 343 nm) on a Victor3 1420 Multilable counter (Perkin Elmer).

***Binding of BsAb to huDR5 and huFAP***

TagLite-labeled cells werethawed, washed and 5 x 103 cells per well mixed with pre-labeled (labeled according to manufacturer protocol using d2 labeling kit from Cisbio # 62D2DPEA) Drozitumab-d2 (0.78 nM) and BsAb (0.18 - 750 nM). The fluorescent signal was measured at 620 nm for the fluorescent donor (Terbium) and at 665 nm for the fluorescent acceptor dye after 0, 2.5, and 4 h at room temperature (Reader M1000 Pro, Tecan). The ratio of 665/620\*1000 was calculated, and the reference (cells only) was subtracted. For Ki determination the results were analyzed in Graph Pad Prism5 with one site fit Ki. Binding to FAP was assessed in the same way as DR5 binding but using 1 x 104 cells per well and with labeled mAb007-d2 (12.5 nM).

***Binding of BsAb (RG7386) to hu and cyno DR5 and hu FAP***

For studying the binding of the novel BsAb (RG7386) to huDR5 or cynoDR5 pre-labeled cells were thawed, washed and 1000 cells per well mixed with 5 μl of BsAb (RG7386) (0.09 – 50 nM) and 5 μl of anti-huFc-d2 (150 nM) (61HFCDAA, Cibio). The fluorescent signal was measured at 620 nm for the fluorescent donor (Terbium) and at 665nm for the fluorescent acceptor dye (d2) after 4 h at room temperature (Victor3 1420 Multilable counter, Perkin Elmer). The ratio of 665/620\*1000 was calculated, and the reference (cells mixed with 150 nM anti-huFc-d2) was subtracted. For KD determination the results were analyzed in Graph Pad Prism5 with one site specific binding fit.

Binding to FAP was carried out in the same way but 10000 cells per well were used.

***Flow cytometry***

Cells were detached with Cell Dissociation Buffer, resuspended and washed in FACS buffer (PBS/0.1% BSA), and incubated with primary antibodies (anti-FAP: 0.01 mg/ml; anti-DR5: 0.02 mg/ml) for 30 min at 4 °C. Unbound antibodies were removed by centrifugation and washes with FACS buffer. Cells were then incubated with secondary goat anti-human Fc specific PE-labeled secondary antibody (0.01 mg/ml) (Jackson ImmunoResearch # 109-116-170) for 30 min at 4 °C, washed and resuspended in FACS buffer and fluorescence was measured with BD CantoII at 488 nm.

***Immunohistochemistry***

IHC protocols for cleaved caspase 3 and FAP were performed on Discovery or BenchMark XT automated stainers, using NEXES Version 10.6 software and the Chromomap (for cleaved caspase 3) or UltraView (for the FAP D8 and mAb082 antibodies) DAB Detection Kits (Ventanna #760-4456). All reagents except the antibodies were obtained from Ventana Medical Systems. Briefly, 2.5 μm formalin-fixed, paraffin-embedded tissue sections from various tumor indications were stained with rat anti-human Seprase antibody (Vitatex, MABS1001; IgG2a, anti-FAP clone D8) on the Ventana Benchmark XT. Sections were subjected to standard CC1 (tris-based cell conditioning 1) treatment followed by antibody [5 μg/ml in Dako antibody diluent (S3022)] incubation for 1 h at 37 °C and positive staining was detected using the Ultraview DAB detection system. Matched isotype antibody from Abcam (ab18450) was used as the negative control. The mAb082 was used for FAP staining in the various xenograft models. Fresh frozen xenograft tumors (2.5 µm sections), fixed for 10 min at room temperature in 10% neutralized formalin solution supplemented with 5 mM sucrose, were incubated with the mAb082 antibody at a concentration of 0.453 µg/ml for 12 min at 37 °C and positive staining was detected using the Ultraview DAB detection system.

Cleaved caspase 3 antibody [0.24 µg/ml in discovery antibody diluent (#760-108)] from Cell Signalling (#9661) was used to stain formalin-fixed, paraffin-embedded tissue sections from the different xenograft models. After a standard CC1 pre-treatment, positive immunoreactivity was detected with the Chromomap DAB detection system (#760-159).

Matched isotype antibody from Cell signalling (#3900) was used as the negative control for the mAb082 and cleaved caspase 3 automation.