Supplementary Information

Pavet *et al.* "Multivalent DR5-selective peptides activate the TRAIL-death pathway and exert tumoricidal activity *in vivo*"

A) Legends to the Supplementary Figures

Figure S1: Binding of mono, di and trivalent TRAIL$^{mim/DR5}$ peptides to death receptors. Curves obtained from Surface Plasmon Resonance (SPR) experiments in which TRAIL-receptors (DR4, DR5, DcR1, DcR2) as well as anti-CD95 and TNF-alpha were immobilized on the sensor chip and either rhTRAIL or TRAIL$^{mim/DR5}$ divalent molecules were injected. Whereas CD95, TNF and rhTRAIL bind to their cognate receptors, 1d, 2d and 3d only bind to DR5.

Figure S2: Binding efficiencies of TRAIL$^{mim/DR5}$ peptides to DR5. SPR curves showing the direct interaction of rhTRAIL, 1 (m, d and t), 2 (m, d and t), 3 (m, d and t) (analytes) with immobilized recombinant protein DR5 (ligand).

Figure S3: Dose-dependent increase of apoptosis induction by TRAIL$^{mim/DR5}$ peptide 1d. BJAB DR5-DEF+DR5 cells were treated with increasing concentration of **1d** peptide and incubated during 16hrs. Apoptosis was determined as percentage of Annexin V positive cells by FACS analysis.

Figure S4: Monovalent TRAIL$^{mim/DR5}$ peptides do not induce significant apoptosis. BJAB DR5-DEF+DR5 cells were treated with monovalent peptides at 15µM and 30µM as well as with trivalent 3t at 5µM during 16hrs. Apoptosis was determined as percentage of Annexin V positive cells by FACS analysis.

Figure S5: Anti-cancer activity of TRAIL$^{mim/DR5}$ peptide 2d in xenograft experiments with human HCT116 colon carcinoma cells. Curves represent the mean+/- SEM of normalized tumor volume obtained from control (top panel) and **2d** (8mpk; bottom panel) groups treated during 14 days and followed during 6 days after treatment.

B) Supplementary Materials and Methods:

Antibodies. Antibodies for Western blotting were purchased from Cell Signalling, Beverly, MA (anti-caspase-8 1C12, anti-cleaved caspase-3 Asp175, anti-caspase-9 and anti-PARP Asp214) and Santa Cruz Biotechnology, California, USA (anti-actin clone sc-1615); for FACS analyses Annexin V-FITC was obtained from BD Pharmingen (San Jose, CA USA), APO2.7-PE from Beckman Coulter (Fullerton, USA). For immunoprecipitation analysis, anti-caspase-8 antibodies were purchased from Santa Cruz Biotechnology (clone C20) or from Medical & Biological Laboratories (Nagoya, Japan) (clone 5F7). Anti-DR4 and anti-DR5 were from Chemicon (Temecula, CA), anti-FADD was from Transduction Laboratories (Lewington, KY). Recombinant human TRAIL (R&D Systems, Minneapolis, MN).

Cell Lines: The Burkitt lymphoma BJAB DR5 deficient cells (BJAB DR5-DEF) as well BJAB DR5-DEF cells in which the expression of DR5 was recovered by stable expressing DR5 (BJAB DR5-DEF+DR5) were kindly provided by Andrew Thorburn (Department of Pharmacology, University of Colorado Denver School of Medicine, USA) and were maintained as previously described (1). Briefly, cells were cultured in RPMI 1640 + 10% FCS (Foetal Calf Serum). The identity of the cells was confirmed by (i) antibiotic resistance (BJAB DR5-DEF+DR5 are resistant to puromycin 0.5µg/ml) ; (ii) the presence of DR5 and DR4 receptor in DISC induced by rhTRAIL in BJAB DR5-DEF+DR5 and only DR4 receptor in the case of BJAB DR5-DEF cells; wild-type and $p53^{-/-}$ HCT116 (2) and cells were kindly provided by Bert Vogelstein (Johns Hopkins Kimmel Cancer Center, Baltimore, USA) and were maintained in McCoy 5A + 10% FCS. Stepwise systems (HEK and BJ derived) were kindly provided by William C. Hahn (Department of Medical Oncology, Department of Medicine, Dana-Farber Cancer Institute, Brigham and Women's Hospital, Boston, USA) and were cultured as previously described (3). Cells were cultured in DMEM (1g/l glucose) + Med 199 (4:1) + 15% FCS heat inactivated. HA1ER and BJELR culture medium was

supplemented with 50mg/ml hygromycin (hTERT selection), 50mg/ml G418 (SV40 selection) and 1mg/ml puromycin (RAS selection). The presence of SV40 and RAS in normal and tumorigenic cells was confirmed by Western Blot.

Western blotting: After transfer membranes were blocked with 5% non-fat dry milk and 0.1% Tween 20 in PBS for 1hr at room temperature and incubated overnight at 4°C with primary antibodies directed against caspase-3, caspase-8, caspase-9 and PARP according to manufacturer's instructions, followed by incubation with corresponding secondary peroxidase-conjugated antibodies. Actin was used as an internal standard for protein loading. Immunoreactive bands were visualized by enhanced chemiluminescence and subsequent exposure to hyperfilm (Amersham). Images were scanned and processed by Adobe Photoshop CS2 software. Full length blots are presented in supplementary figures 6, 7 and 8.

Surface Plasmon Resonance analysis. A BIAcore® 3000 (BIAcore, Uppsala, Sweden) instrument was used in all the different experiments.

Binding kinetics of the various peptides to DR5: DR5, the other TRAIL receptors (DR4, DcR1, DcR2) and three other receptors of the TNF superfamily (RANK, CD95 and TNFR1) were immobilized on a CM5 sensor chip (Research Grade, Biacore AB) using amine coupling at 5µg/mL in 10mM acetate buffer (pH 5.0) according to the manufacturer's instructions. The chip was then flushed with 1M ethanolamine hydrochloride (pH 8.5) (Biacore AB) and 25mM HCl to eliminate unbound protein. Biosensor assays were performed at 25°C in HBS-EP buffer [10mM HEPES (pH 7.4) containing 0.15M NaCl, 3.4mM EDTA, and 0.005% (v/v) surfactant P20] as running buffer. The peptides were injected (kinetic mode) at a flow rate of 30µL/min for 8 min and allowed to dissociate for an additional 10 min. The channels were then regenerated for 45 s with 25mM HCl. The RANK protein was considered as negative control, thus the control sensograms of RANK channel were substracted from the other ones and analyzed with BIAevaluation version 4.1 using the

simple 1:1 Langmuir binding model. As positive control, natural ligand for each receptor was injected on the sensor chip following the protocol described for the peptides.

Inhibition of TRAIL binding on DR5: To assess the ability of the various peptides to inhibit the binding of rhTRAIL to DR5, His-TRAIL was immobilized on a NTA sensor chip previously washed with regeneration buffer [10mM HEPES, 0.15M NaCl, 0.35M EDTA and 0.005% surfactant P20 (pH 8.3)] and regenerated with a $NiCl_2$ solution (500µM) at a flow rate of 10µL/min for 1 min (as prescribed by the purveyors). The DR5 protein was preincubated with different concentrations of the peptides and then injected on the sensor chip containing His-TRAIL or an empty channel (as subtracted control) at a flow rate of 30µL/min for 3 min and allowed to dissociate for an additional 3 min. After each cycle the chip was regenerated with regeneration buffer (1 min) followed by $NiCl_2$ solution injection (1min). Under this mass transfer condition, the slope of the curves is directly correlated with the active concentration of DR5. The IC_{50} (peptide concentration that inhibits 50% of the TRAIL-DR5 binding) was calculated from a logit plot (Sigmaplot). The sensorgrams were analysed by global fitting using the Biaeval 1.4 program.

Active concentration measurements of synthetic peptides using Biacore technology: Measurement of active concentration was assessed using Surface Plasmon Resonance (Biacore) under conditions of mass transport limitation (4). Briefly, high concentration (4000RU) of rhDR5 was covalently immobilized on a CM5 chip and 10nM of 2d was injected for 2 min at flow rates of 2, 5, 10, 25, 50 and 100 µl/min. For data analysis, the sensorgrams were analyzed with the BIAevaluation 2.1 software (BIAcore) and then exported as a text file and opened in the BIA-CONC program (http://www.be.dtu.dk/.lc/). The analysis determined a concentration of 8,047+/-0,843nM representing that 80.5+/-8.5% of the peptide is active.

Peptide stability analysis: Divalent **1d** and **2d** were dissolved in phosphate buffer saline (PBS) at 10 mg/mL concentration, an equal volume of freshly prepared mouse serum was added and time count was started. The solutions were incubated at 37°C in a thermostatic bath and sampled immediately after serum addition (as a control) and 1, 2, 4, 8 and 24 hrs thereafter. For each time-point 20µL solution were taken, added with 2 µL TFA, with precipitation of serum proteins, diluted with 80µL PBS, then centrifugated (10 min at 5000 rpm). The supernatant was diluted with 120µL H_2O milliQ and analyzed by HPLC (Varian, 5-65% B in 10 min). The results of these assays reveal a half-life of the intact divalent peptide of $t_{1/2}$ = 4hrs, with a 10% intact divalent molecules present after 8hrs (data not shown).

References

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3. Hahn WC, Counter CM, Lundberg AS, Beijersbergen RL, Brooks MW and Weinberg RA. Creation of human tumour cells with defined genetic elements. Nature 1999; 400: 464-468.

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