CHAPTER 3

Melanoma-directed activation of apoptosis using a novel bispecific antibody directed at MCSP and TRAIL receptor 2/Death Receptor 5

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Abstract
Agnostic anti-DR5 antibodies, alone or in combination with other treatment modalities, show promising activity towards a variety of cancer types, including malignant melanoma. However, the therapeutic efficacy of current anti-DR5 antibodies is potentially limited as they indiscriminately interact with DR5 that is broadly present on normal cells. Therefore, we developed a novel bispecific antibody-based approach that promotes melanoma-directed pro-apoptotic activation of DR5. We engineered a novel recombinant bispecific antibody, designated MCSPxDR5, which combines high binding affinity for the melanoma-associated antigen MCSP with potent agonistic activity towards DR5. The mode of action of MCSPxDR5 involves high-affinity binding to tumor cell surface-expressed MCSP with concomitant locally enhanced cross-linking of DR5. MCSPxDR5 showed potent MCSP-directed pro-apoptotic activity towards MCSP-positive melanoma cells with essentially no or minimal toxicity towards normal cells. The antitumor activity by MCSPxDR5 was enhanced after secondary cross-linking of its IgG domain by either an artificial cross-linker or by Fc receptors present on myeloid immune effector cells. Importantly, MCSPxDR5 potently induced apoptosis in primary patient-derived melanoma cells that was further enhanced after secondary cross-linking of its human IgG1 Fc domain. Taken together, we present a melanoma-directed DR5 agonistic bispecific antibody in which high-affinity binding to MCSP results in melanoma cell-localized activation of pro-apoptotic DR5 signaling. This novel antibody-based approach may provide a new avenue to unlock the therapeutic potential of DR5-targeted cancer therapy, in particular for targeted treatment of melanoma and other MCSP-expressing malignancies.

Introduction
Malignant melanoma is the most lethal type of skin cancer and its incidence continues to increase at an alarming rate. When diagnosed at an early stage, localized malignant melanoma can be cured by radical removal of the lesion, resulting in excellent survival rates. However, once progressed to the metastatic stage, melanoma is extremely difficult to cure. Currently, ipilimumab (CTLA4 blockade), BRAF inhibitors (vemurafenib and dabrafenib) and high-dose IL-2 are used as first line agents for stage IV melanoma. Newly emerging treatment options include antibodies that target the PD-1/PD-L1 checkpoint axis (e.g. nivolumab), oncolytic viruses, adoptive T cell transfer and dendritic cell vaccines.

In this respect, Tumor Necrosis Factor-related apoptosis-inducing ligand (TRAIL) appears to be a promising anticancer agent as it induces apoptosis in a wide range of refractory malignancies including melanoma. TRAIL is an immune effector protein that induces apoptosis in virus-infected cells and cancer cells by activating death receptor-4 (DR4) and/or death receptor-5 (DR5) without deleterious activity towards DR4/DR5-expressing normal cells. Consequently, DR4/DR5 agonists have been regarded as promising anticancer agents. Indeed, treatment with “first-generation” DR4/DR5-targeted therapeutics, such as recombinant human soluble TRAIL (rhTRAIL) and agonistic DR4/DR5 antibodies was well tolerated, but had disappointing clinical efficacy. For instance, in a phase I dose-escalation study in patients with relapsed or refractory carcinoma, the DR5-agonistic antibody tigatuzumab only induced stable disease in selected patients. Similarly, in a phase II trial in non-small cell lung cancer patients, combined treatment with rhTRAIL and chemotherapy had no added benefit compared to chemotherapy treatment alone.

However, advances in the understanding of death receptor signaling revealed that first-generation DR4/DR5 agonists do not fully exploit the unique signaling characteristics of TRAIL receptor-mediated cancer cell death. Specifically, DR4 and DR5 have distinct cross-linking requirements for the induction of apoptosis. DR4 is activated upon binding of rhTRAIL (or conventional DR4 antibodies), whereas apoptotic DR5 signaling requires membrane-bound TRAIL or secondarily cross-linked rhTRAIL. Indeed, to gain therapeutic activity, agonistic DR5 antibodies such as tigatuzumab appear to require cross-linking by Fc-receptors as present on myeloid effector cells. Further, rhTRAIL and conventional agonistic DR4/5 antibodies have no tumor-selective binding activity, since TRAIL receptors are ubiquitously expressed on normal tissue. Consequently, a massive target antigen sink formed by DR5 expressed on normal cells may hamper the efficacy of agonistic anti-DR5 antibodies.

To overcome these limitations, we engineered a recombinant bispecific antibody, designated MCSPxDR5, with high binding affinity for Melanoma-associated Chondroitin Sulfate Proteoglycan (MCSP) and potent agonistic activity towards DR5, complemented with a human IgG1 Fc domain. MCSP is highly overexpressed on the cell surface of over 90% of cutaneous and uveal melanomas and is a well-established target for melanoma immunotherapy. MCSP expression in normal tissues is largely restricted to cells of the melano-
cyte lineage. MCSPxDR5 was designed to selectively bind with high affinity to tumor cell surface-expressed MCSP and concomitantly trigger localized activation of apoptosis by DR5 cross-linking.

**Material and Methods**

**Antibodies and reagents**

mAb 9.2.27, a high-affinity murine IgG2a antibody directed against an extracellular epitope on MCSP was purchased from Abcam. Anti-DR5 antibody DJR2-4 was purchased from Enzo Life Sciences. Recombinant TRAILR2:Fc protein was from ALEXIS. Goat-anti-human IgG (Southern Biotech); goat anti-human IgG-PE (Santa Cruz biotechnology); mouse anti-human IgG-488 and mouse anti-human IgG-647 (ImmunoTools), and FITC-conjugated goat anti-mouse (Invitrogen) were used for flow cytometry. BRAF inhibitor vemurafenib (APEXBio) and caspase inhibitor zVAD-fmk (Calbiochem) were dissolved at 10 mM in DMSO. Histone deacetylase inhibitor valproic acid (VPA) (Sigma-Aldrich) was dissolved at 2.5 mM in DMSO. The proteasome inhibitor Bortezomib (Millennium Pharmaceuticals) was dissolved at 10 mM in DMSO; Vybrant DiD cell-labeling reagent (Invitrogen); Easy-Titer Human (gamma chain) kit (Thermo Scientific), were used according to the manufacturer’s recommendations.

**Cell lines**

Human cell lines A375M, MM-RU, SK-MEL-28 (MCSPpos cutaneous melanomas); DLD-1, HCT 116 (colorectal adenocarcinomas) and Jurkat (T-ALL leukemia) were purchased from American Type Culture Collection (ATCC, Rockville, MD). Human MCSPpos uveal melanoma cell line OCM3 was kindly provided by Prof. G.P.M. Luysten, Department of Ophthalmology, LUMC, Leiden, Netherlands. The MCSPpos human melanoma parental cell line M14 and the MCSPpos transfectant cell line M14.MCSP were kindly provided by Prof. James McCarthy, University of Minnesota, Minneapolis, USA. Cell line HEK293.CD64 stably expressing human CD64 (high-affinity IgG Fc-receptor) was generated by transfecting parental HEK293 cells with pCMV/hygroCD64 (Sino Biological Inc). HEK293.CD64 cells were cultured in DMEM plus 10% dialyzed fetal calf serum (dFCS), supplemented with 500 μg/ml Hygromycin B (Life technologies). Human cells were cultured in either RPMI 1640 or DMEM culture medium with 10% FCS at 37°C in a humidified 5% CO2 atmosphere. Cells lines were authenticated by commercial Short Tandem Repeat analysis and routinely tested for mycoplasma infection.

**Primary patient-derived melanoma cells and primary human hepatocytes (PHH)**

Tumor samples were collected during conventional surgical resection procedure after informed consent. Tumor samples were minced and cultured in RPMI 1640 supplemented with 10% FCS and allowed to adhere to culture flasks. Non-adherent cells were discarded and phenotyping of remaining adherent cells was performed by flow cytometry using antibodies directed against CD14, DR5 and MCSP, respectively. Primary patient-derived melanoma cells used in this study were CD14 negative, DR5 and MCSP positive and were used before passage 4.

**Isolation of white blood cells and lymphocytes**

White blood cells (WBCs) were obtained from venous blood from healthy volunteers after informed written consent. Briefly, blood was diluted ten times in cold isotonic ammonium chloride lysis buffer and incubated at 4°C for 10-15 min until red blood cells had lysed. Leukocytes were harvested by centrifugation (1000g, 10 min), washed with PBS and re-suspended in RPMI 1640/10% FCS. Peripheral blood lymphocytes (PBLs) were obtained from venous blood of healthy volunteers after informed written consent using standard density gradient centrifugation (Lymphoprep).

**Analysis of cell surface expression of DR5 and MCSP**

Cell surface expression levels of DR5 and MCSP were assessed by flow cytometry. In short, cells were harvested and washed with PBS and re-suspended at 1×10⁸ cells in 200 μl fresh culture medium containing anti-DR5 mAb (DJR2-4) or mAb 9.2.27 according to the manufacturer’s recommendations. Specific binding of these antibodies was detected using secondary conjugated antibodies (FITC-conjugated for DR5 staining and APC-conjugated for MCSP staining). All antibody incubations were carried out for 45 min at 0°C followed by 3 washes with PBS.

**Construction of MCSPxDR5**

Antibody fragment scFv-MCSP was constructed using published VH and VL sequence data of mAb 9.2.27 by applying standard antibody engineering technologies. Similarly, scFv-DR5 was constructed using published VH and VL sequence data of agonistic anti-DR5 antibody tigatuzumab. For construction and production of MCSPxDR5 we used eukaryotic expression plasmid pEE14-bsAb, which is equipped with an enhanced CMV promoter to drive recombinant protein expression and an N-terminal leader peptide for excretion of MCSPxDR5 protein in the culture medium. The pEE14-bsAb contains 3 consecutive multiple cloning sites (MSC#1, MSC#2 and MSC#3, respectively). MSC#1 and MSC#2 are interspersed by a 22 amino acid flexible linker derived from a CH1 domain. MSC#1 was used for directional and in-frame insertion of the scFv-DR5 encoding DNA fragment. MSC#2 was used for directional and in-frame insertion of the scFv-MCSP encoding DNA fragment. MSC#3 of pEE14-bsAb was used for in-frame insertion of DNA fragments encoding either a human IgG1 or IgG4 domain. Of note, hinge regions and flexible spacers were inserted between the different antigen recognizing parts of the bispecific antibody (bsAb) to allow each part of the molecule to function independently. Moreover, the codon usage of the various protein encoding DNA sequences mentioned above were optimized for expression in human (HEK) and hamster (CHO) production cell lines and were ordered from a certified gene synthesis services (Genscript). This procedure yielded plasmids pEE14-MCSPxDR5-IgG1 and the corresponding IgG4 isotype variant pEE14-MCSPxDR5-IgG4.
Production of MCSPxDR5 in CHO cells

Plasmids pEE14-MCSPxDR5-IgG1 and pEE14-MCSPxDR5-IgG4 were stably transfected in CHO production cells using the FuGene-HD reagent (Promega), after which clones with amplified recombinant protein production (10 µg/ml and 7 µg/ml for MCSPxDR5-IgG1 and MCSPxDR5-IgG4, respectively) were selected by the glutamine synthetase selection method as described previously.16

Concentrations of MCSPxDR5 and MCSPxDR5-IgG4 were determined using the Human Easy-Titer kit (Thermo Scientific) according to manufacturer’s recommendations.

MCSP-selective binding of MCSPxDR5

MCSP-selective binding of MCSPxDR5 was assessed by flow cytometry using CHO.MCSP cells and MCSP-negative parental CHO cells. In short, cells were incubated with MCSPxDR5 (1 µg/ml) in the presence or absence of an excess amount of the competing parental anti-MCSP antibody mAb 9.2.27 (10 µg/ml). Detection of cell surface-bound MCSPxDR5 was analyzed by flow cytometry using anti-human IgG1-PE.

MCSP-directed induction of apoptosis by MCSPxDR5

A panel of MCSPpos and MCSPneg melanoma cell lines (A375M, SK-MEL-28, MM-RU and HT1080) were selected by the glutamine synthetase selection method as described previously.16

Concentrations of MCSPxDR5 and MCSPxDR5-IgG4 were determined using the Human Easy-Titer kit (Thermo Scientific) according to manufacturer’s recommendations.

Assessment of synergy between MCSPxDR5 and clinical antitumor drugs

MCSPpos/D5pos melanoma cell lines (A375M, MM-RU and OCM-3) were treated with MCSPxDR5 (0.25 µg/ml) in the presence or absence of proteasome inhibitor velcade (1 nM), HDAC inhibitor VPA (2.5 µM) or BRAF-inhibitor vemurafenib (10 µM). After 18h, apoptosis was assessed using Annexin-V staining. Briefly, cells were washed once with cold binding buffer (140 mmol/L NaCl, 2.5mmol/L CaCl2 and 10 mmol/L HEPES, pH 7.4) and resuspended in binding buffer containing Annexin-V-FITC. After incubation for 10-15 min at 4°C, apoptosis was assessed by flow cytometry. Synergy was determined using the cooperativity index (CI), in which the sum of apoptosis induced by single-agent treatment is divided by apoptosis induced by combination-treatment. When CI<1, treatment was termed synergistic.

Colonial forming assay

In short, a single-cell suspension of tumor cells was prepared in 0.6% low gelling temperature (LGT) agarose dissolved in pre-warmed complete medium (RPMI 1640 with 20% FCS). Tumor cells (1.0x10⁴ cells per well) were placed in 24-well plates containing a bottom layer of solidified 4% LGT agarose in complete medium. Once the tumor cell-containing agarose top layer had solidified, a final 0.5 ml of complete medium was added. In this assay, MCSPpos melanoma cells A375M, SK-MEL-28, MM-RU and HT1080 were treated with MCSPxDR5 (final concentration up to 250 ng/ml) for 14 days, after which colony formation was evaluated by light microscopy. In control experiments, identical treatment was performed in the presence of an excess amount of TRAIL-R2-Fc (5 µg/ml) or the pan-caspase inhibitor zVAD-fmk (10 µM). Colony forming assays were performed in quadruplicates. The number and size of colonies were quantified using image processing software (ImageJ). The percentage of colony formation was calculated according to the formula: percentage of colonies formed = (number of colonies in experimental condition) / (number of colonies in medium control) x 100%.

siRNA-mediated knockdown of DR5 expression

SK-MEL-28 cells were pre-seeded at a concentration of 1x10⁴ cells/well in 12-wells plates, 24h before treatment with either DR5-selective siRNA or scrambled siRNA according to manufacturer’s recommendations (DharmaFECT 2 transfection reagent (Thermo Scientific)). siRNA knockdown DR5 protein expression was evaluated after 72h by flow cytometry using anti-DR5 antibodies. Antibody incubations were carried out for 45 min at 0°C and were followed by 3 washes with PBS. Subsequently, DR5 silenced SK-MEL-28 cells were seeded and subjected to treatment as indicated.

Assessment of MCSPxDR5 antitumor activity upon cross-linking

The following assays were used to assess whether MCSP-directed pro-apoptotic activity of MCSPxDR5 towards cancer cell lines and primary patient-derived melanoma cells could be further enhanced by secondary cross-linking of its IgG1 Fc domain.

Artificial cross-linking: In short, cancer cells were seeded at 3x10⁴/well in 96-well micro-culture plates and subsequently treated with increasing doses of MCSPxDR5 in the presence or absence of an Fc cross-linker (0.5 µg/ml of goat-anti-human IgG), hereafter indicated as CL. Apoptosis and cell viability were evaluated after 24h and 72h, respectively. To investigate whether the observed apoptotic activity of MCSPxDR5 was dependent on DR5 signaling, MM-RU cells (3x10⁴/well) were co-treated with MCSPxDR5 and CL in the presence or absence of recombinant TRAILR2-Fc (5 µg/ml) or pan-caspase inhibitor zVAD-fmk (5 µM) for 24h, after which cell viability was assessed by MTS.
we used MCSP\textsuperscript{*}{\*}{\*{*}} SK-MEL-28 melanoma cells in which DR5 expression was knocked-down using DR5-specific siRNA silencing technology as described previously.\textsuperscript{17} Subsequently, DR5-silenced SK-MEL-28 cells and wt SK-MEL28 cells were treated with MCSPxDR5 (1 µg/ml) or anti-TRAILR2 monoclonal antibody HGT-ETR2 (1 µg/ml), after which apoptosis was assessed using Annexin-V staining.

**Cell-based cross-linking:** To mimic cell-based Fc cross-linking of MCSPxDR5, we used HEK293.CD64 cells ectopically expressing human CD64, also known as the high-affinity IgG receptor FcγRI. In short, DiD-labeled MM-RU cells were co-cultured with HEK293.CD64 cells at a cellular ratio of 5:1 in the presence or absence of MCSPxDR5 (250 ng/ml). After 24h, apoptosis in DiD-labeled MM-RU cells was evaluated. Analogously, various effector cells (PBLs, leukocytes (WBCs) or NK cells) were used as natural cell-based Fc cross-linkers\textsuperscript{18} of MCSPxDR5. In short, DiD-labeled MM-RU target cells were treated with MCSPxDR5 in the presence of various ratios of myeloid effector cells (E:T cell ratios were 20:1, 5:1 and 20:1 for PBLs, WBCs and NK cells, respectively). To discriminate ADCC from DR5-cross-linking antitumor activity we used MCSPxDR5-IgG4, a human IgG4-isotype variant of MCSPxDR5 that has only marginal ADCC activity.\textsuperscript{19}

**Statistical analysis**

The results reported in this study are mean values ± standard deviation of the mean of at least three independent experiments. Statistical analysis was performed by one-way ANOVA followed by Tukey-Kramer post-test or by two-sided unpaired Student’s t-test. p<0.05 was defined as a statistically significant difference.

**Results**

MCSPxDR5 has MCSP-directed pro-apoptotic activity

BsAb MCSPxDR5 (Fig. 1A) was designed to bind to MCSP on cancer cells and subsequently induce apoptosis in targeted cancer cells by local ligation of DR5. In line with this, flow cytometric analysis showed that MCSPxDR5 strongly bound to MCSP\textsuperscript{*}{\*}{\*{*}}/DR5\textsuperscript{**{**}**} SK-MEL-28 melanoma cells (Fig. 1B), but not to MCSP\textsuperscript{*}{\*}{\*}+/DR5\textsuperscript{**} DLD-1 carcinoma cells (Fig. 1C). Binding of MCSPxDR5 to SK-MEL-28 cells was blocked by co-incubation with excess amounts of epitope-competing anti-MCSP mAb 9.9.27 (Fig. 1B), indicating that MCSPxDR5 indeed selectively binds to cell surface-expressed MCSP. Correspondingly, treatment of tumor cells incubated with MCSPxDR5 for 40 min at 4°C, after which any unbound antibody was removed by washing, selectively induced apoptosis in a panel of MCSP\textsuperscript{*}{\*}{\*}/DR5\textsuperscript{**} melanoma cells, but not in MCSP\textsuperscript{*}{\*}/DR5\textsuperscript{**} HCT116 carcinoma cells (Fig. 1D). Treatment in the continued presence of MCSPxDR5 (no washing) induced apoptosis in both MCSP\textsuperscript{*}{\*}{\*}/DR5\textsuperscript{**} and MCSP\textsuperscript{*}/DR5\textsuperscript{**} cancer cells (Fig. 1E), albeit in the latter cancer cells at much lower levels. Moreover, MCSPxDR5 treatment was associated with reduced tumor cell viability in MCSP\textsuperscript{*}{\*}{\*}/DR5\textsuperscript{**} MM-RU, SK-MEL-28 and OCM3 cells, but not in MCSP\textsuperscript{*}/DR5\textsuperscript{**} DLD-1 cells (Fig. 1F).

MCSP-selective pro-apoptotic activity of MCSPxDR5 was also evaluated using primary patient-derived melanoma cells that expressed MCSP and DR5 (Fig. 1G). These primary tumor cells were sensitive to MCSPxDR5 treatment, with an >40% increase in apoptosis compared to medium control (Fig. 1H). Thus, MCSPxDR5 selectively binds to MCSP and has MCSP-directed pro-apoptotic activity towards MCSP\textsuperscript{*}/DR5\textsuperscript{**} cancer cell lines and primary patient-derived melanoma cells.
MCSPxDR5 inhibits of colony formation of MCSPpos/DR5pos cancer cells

Next, the effect of MCSPxDR5 on anchorage-independent growth of MCSPpos melanoma was evaluated using a soft agar colony forming assay. Treatment with MCSPxDR5 for 14 days led to a significant reduction in colony number in MCSPpos melanoma cell lines (Fig. 2A). Further, as illustrated for A375M, residual colonies after MCSPxDR5 treatment were reduced in size by >90% (Fig. 2B). This MCSPxDR5-mediated effect was completely blocked by either co-treatment with recombinant anti-TRAILR2-Fc or by pan-caspase inhibitor zVADfmk, indicating that the inhibitory effect of MCSPxDR5 on colony formation is caspase-mediated and dependent on DR5 (Fig. 2C). Of note, MCSPxDR5 also reduced colony number of MCSPpos/DR5pos HT1080 sarcoma cells by ~90%, whereas control non-targeted anti-DR5 antibody HGS-ETR2 only reduced colony number by ~50% (Fig. 2D). Taken together, MCSPxDR5 selectively inhibits colony forming capacity of MCSPpos/DR5pos cancer cells.

Efficacy of MCSPxDR5 is augmented by FcR-mediated cross-linking

Next, the effect of MCSPxDR5 by surface expressed Fc receptors was evaluated using HEK293.CD64 cells ectopically expressing the high-affinity Fc receptor CD64. HEK293 cells are resistant to TRAIL receptor-mediated apoptosis and lack intrinsic cytolytic activity (data not shown). Treatment of mixed cultures of MM-RU target cells and parental HEK293 cells with an increasing dose of MCSPxDR5 only induced apoptosis in ~30% of MM-RU cells. However, in mixed cultures of MM-RU with HEK293.CD64, MCSPxDR5 treatment resulted in >70% apoptosis (Fig. 4A). To further confirm this cellular FcR-mediated cross-linking effect and exclude contributions of antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) to MCSPxDR5 activity, experiments were performed with isotype variant MCSPXDR5-IgG4, containing the human IgG4 Fc domain that is known to be largely devoid of ADCC and CDC activity. Treatment of MM-RU/HEK293.CD64 mixed cultures with MCSPxDR5-IgG4 induced up to 60% apoptosis, similar to MCSPxDR5-IgG1 treatment (Fig. 4B).

Importantly, Fc receptor cross-linking also induced MCSP-independent DR5-mediated activation in ~30% of MM-RU cells. However, in mixed cultures of MM-RU with HEK293.CD64, MCSPxDR5 treatment resulted in >70% apoptosis (Fig. 4A). To further confirm this cellular FcR-mediated cross-linking effect and exclude contributions of antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) to MCSPxDR5 activity, experiments were performed with isotype variant MCSPXDR5-IgG4, containing the human IgG4 Fc domain that is known to be largely devoid of ADCC and CDC activity. Treatment of MM-RU/HEK293.CD64 mixed cultures with MCSPxDR5-IgG4 induced up to 60% apoptosis, similar to MCSPxDR5-IgG1 treatment (Fig. 4B).

To investigate this in more detail, we exploited MCSPpos/DR5pos Jurkat cells as indicator cells for effective DR5 cross-linking. Treatment of Jurkat cells with MCSPxDR5 alone did not induce apoptosis. However, in the presence of a human IgG cross-linking reagent, MCSPxDR5 strongly induced apoptosis in Jurkat cell up to almost 100%, with apoptotic activity of MCSPxDR5 already apparent at doses as low as 5 ng/ml (Fig. 3A). Similarly, secondary cross-linking of MCSPxDR5 enhanced its pro-apoptotic antitumor activity in a panel of MCSPpos/DR5pos melanoma cell lines (Fig. 3B and 3C). Furthermore, secondary cross-linking of MCSPxDR5 also increased the level of apoptosis induced in primary patient-derived melanoma cells, with a mean increase in apoptosis from 48% to 70% (Fig. 3D). Although the apoptotic activity of MCSPxDR5 was enhanced by cross-linking, its activity was still abrogated by co-treatment with recombinant DR5-Fc or pan-caspase inhibitor zVADfmk (Fig. 3E). Moreover, siRNA-mediated DR5 silencing in A375M cells significantly decreased DR5 expression (Fig. 3F) and strongly reduced the apoptotic activity of both MCSPxDR5 and the DR5-agonistic antibody HGS-ETR2 (Fig. 3G). Thus, Fc-mediated cross-linking augments the pro-apoptotic antitumor activity of MCSPxDR5 through enhanced DR5 signaling.

**Figure 2: MCSPxDR5 inhibits colony formation of melanoma cells.**

A) MCSPpos/DR5pos cell lines were treated with MCSPxDR5 (250 ng/ml) or left untreated in colony-forming assays for 14 days, after which the number of colonies was determined by counting three fields of view per condition. Number of colonies was represented as percentage of colonies compared to medium control. B) Representative light microscopic images of colony size of A375M cells in medium control versus MCSPxDR5-treated conditions in colony-forming assay and dose-response curve of colony size upon MCSPxDR5 treatment. C) MCSPpos/DR5pos A375M cells were treated with MCSPxDR5 (50 ng/ml) and parental HEK293 cells with an increasing dose of MCSPxDR5 only induced apoptosis in ~30% of MM-RU cells. However, in mixed cultures of MM-RU with HEK293.CD64, MCSPxDR5 treatment resulted in >70% apoptosis (Fig. 4A). To further confirm this cellular FcR-mediated cross-linking effect and exclude contributions of antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) to MCSPxDR5 activity, experiments were performed with isotype variant MCSPXDR5-IgG4, containing the human IgG4 Fc domain that is known to be largely devoid of ADCC and CDC activity. Treatment of MM-RU/HEK293.CD64 mixed cultures with MCSPxDR5-IgG4 induced up to 60% apoptosis, similar to MCSPxDR5-IgG1 treatment (Fig. 4B).

Efficacy of MCSPxDR5 is enhanced by cross-linking of its Fc domain

MCSPxDR5 was designed to trigger apoptosis through MCSP-directed cross-linking of DR5. Since the antitumor activity of tigatuzumab appears to requires cross-linking of its IgG1 Fc domain through binding to FcγRs on immune effector cells.10,12 Therefore, cross-linking of tumor cell-bound MCSPxDR5 via its IgG domain may further augment its pro-apoptotic activity.
Figure 3: Cross-linking of its Fc domain enhances antitumor activity of MCSPxDR5. A) MCSP+/DR5- Jurkat cells were treated with MCSPxDR5 in the presence or absence of goat-anti-human IgG (cross-linker [CL]; 0.5 mg/ml). Apoptosis was measured after 18h by flow cytometry using Annexin-V. B) MCSP+/DR5- melanoma cell lines were treated with MCSPxDR5 (250 ng/ml) in the presence or absence of CL for 18h, after which apoptosis was measured by flow cytometry using Annexin-V. C) MCSP+/DR5- melanoma MM-RU cells were treated with an increasing dose of MCSPxDR5 in the presence or absence of CL for 18h, after which apoptosis was determined by MTS assay. D) Primary patient-derived melanoma cells were co-treated with MCSPxDR5 (0.25 mg/ml) and CL. Apoptosis was measured after 18h. E) MM-RU cells were treated with MCSPxDR5 in the presence or absence of pan-caspase inhibitor (zVAD-fmk, 5 µM) or recombinant human DR5:Fc (5 µg/ml) for 18h, after which apoptosis was evaluated by flow cytometry using Annexin-V/PI staining. F) RNA silencing was used to selectively knock down DR5 expression in SK-MEL-28 cells, which was confirmed by flow cytometry using an anti-DR5 mAb. G) SK-MEL-28 cells were treated with either CL alone, MCSPxDR5 plus CL, or DR5 agonistic antibody HGS-ETR2 for 18h, after which apoptosis was evaluated by flow cytometry using Annexin V/PI staining.
MCSPxDR5 shows no or limited toxicity toward normal cells
Treatment of DR5-expressing normal primary human hepatocytes and immortal human hepatocytes (IHH) with MCSPxDR5 induced no or minimal apoptosis (Fig. 5A). Moreover, treatment of HUVECs and normal lymphocytes with MCSPxDR5 (2.5 µg/ml) did not induce apoptosis (Fig. 5B). This indicates that MCSPxDR5 has selective tumoricidal activity with no or only limited toxicity towards normal cells. This is in line with a previous phase I clinical study that showed that tigatuzumab was well-tolerated with dose-limiting toxicity (DLT) reached and no drug-related grade 3 or 4 hepatic or hematological toxicity observed.20

Anticancer drugs synergize with anticancer activity of MCSPxDR5
Melanoma-relevant drugs (bortezomib, valproic acid and vemurafenib) were evaluated for their ability to synergize the antitumor activity of MCSPxDR5. Co-treatment of MCSPBv/DR5 melanoma cells with MCSPxDR5 and proteasome inhibitor velcade triggered synergistic induction of apoptosis in A375M cells and MM-RU cells but not in OCM-3 cells (Fig. 6A). MCSPxDR5-mediated apoptosis showed strong synergy with HDAC inhibitor VPA in A375M cells, as apoptosis increased up to 60% compared to 20% for single treatment with either MCSPxDR5 or VPA alone. A synergistic treatment effect with VPA was also observed in MM-RU and SK-MEL-28 cells (Fig. 6B). Interestingly, we found that vemurafenib not only enhanced MCSPxDR5-mediated apoptosis in V600-mutant cell lines OCM-3 and SK-MEL-28, but also induced apoptosis up to 60% in V600-wt melanoma cell line MM-RU compared to single treatment with MCSPxDR5 (Fig. 6C).

Discussion
Recombinant TRAIL formulations (e.g. dulanermin) and agonistic anti-DR4/5 antibodies (e.g. conatumumab and tigatuzumab) have been extensively evaluated in pre-clinical models and in a number of clinical studies.6-7,21 Results from early-stage clinical studies indicated that both recombinant TRAIL and agonistic anti-DR antibodies are well tolerated in patients with various cancers. However, the therapeutic efficacy of current DR agonists is rather disappointing. This appears to be related to the fact that these agonists indiscriminately interact with DRs that are broadly present on normal cells,13,22 thereby precluding sufficient accumulation of such agonists in malignant lesions. Moreover, current DR agonists have limited capacity to activate pro-apoptotic DR5 signaling in cancer cells in the absence of secondary cross-linking.12
To overcome these limitations, we developed a novel bispecific antibody-based approach that promotes melanoma-directed pro-apoptotic activation of DR5. Hereeto, we engineered bispecific antibody MCSPxDR5 that combines high binding affinity for the melanoma-associated antigen MCSP with potent agonistic activity towards DR5. The mode-of-action of MCSPxDR5 involves high-affinity binding to tumor cell surface-expressed MCSP with concomitant localized enhanced cross-linking of DR5. The antitumor activity of tumor-bond MCSPxDR5 was further enhanced by secondary cross-linking of its human IgG Fc domain by either an artificial cross-linker or by Fc receptors on myeloid immune effector cells. Moreover, various melanoma-relevant drugs (bortezomib, valproic acid and vemurafenib) synergistically enhanced the antitumor activity of MCSPxDR5. Importantly, MCSPxDR5 potently induced apoptosis in primary patient-derived melanoma cells, which was further enhanced after secondary cross-linking of its IgG1 Fc domain. The anti-DR5 antibody fragment we used in bsAb MCSPxDR5 is derived from the agonistic DR5 antibody tigatuzumab. The in vivo therapeutic activity of tigatuzumab appears to fully rely on cross-linking of its Fc domain that may occur after binding to FcyRs as expressed on myeloid effector cells.10-12 In contrast, selective binding of MCSPxDR5 to MCSP already triggered effective activation of membrane DR5 in melanoma cells, indicating that on MCSP+/+ target cells MCSPxDR5 can at least partially circumvent the need for FcR-mediated cross-linking. Importantly, the antitumor activity of MCSPxDR5 could be further enhanced by FcR-expressing immune effector cells, via FcR-mediated cross-linking as well as by induction of ADCC.
Recently, a similar DR5-targeted bispecific antibody, RG7386, targeting the fibroblast-associated protein (FAP) on cancer-associated fibroblasts was described.\textsuperscript{22} RG7386 induced apoptosis in FAP-positive cells and, when combined with irinotecan or doxorubicin, induced tumor regression in patient-derived xenograft models.\textsuperscript{23} Our \textit{in vitro} data largely corroborates these observations, indicating a common mode-of-action for RG7386 and MCSPxDR5.

MCSP is expressed in 90% of melanoma lesions\textsuperscript{15} and other malignancies, e.g. sarcomas and gliomas.\textsuperscript{24-25} MCSP overexpression plays a significant role in melanoma progression by influencing tumor cell adhesion/spreading, migration, invasion, and metastasis. It has been reported that binding of mAb 9.2.2.7 to MCSP already in its own right can partially inhibit various oncogenic features of MCSP expression.\textsuperscript{26} Indeed, we previously reported on a MCSP-targeting TRAIL fusion protein that not only induced MCSP-mediated TRAIL-mediated apoptosis in melanoma cells, but also inhibited MCSP-mediated tumorigenic signaling.\textsuperscript{27} The MCSP-directed antibody fragment used in bsAb MCSPxDR5 is derived from mAb 9.2.2.7. Although not formally studied here, it is tentative to speculate that bsAb MCSPxDR5 may have retained the potential to (partially) inhibited MCSP-tumorigenic signaling that may add to or even synergize with its pro-apoptotic anticancer activity.

Co-treatment of agonistic anti-DR5 antibodies or soluble TRAIL with various chemotherapy agents or (experimental) small inhibitory molecules has been evaluated in order to partially augment their antitumor activities towards various forms of cancer, including carcinoma, breast cancer, lung cancer and metastatic melanoma.\textsuperscript{28-30} Here, we found that co-treatment of HDAC inhibitor VPA synergistically enhanced the pro-apoptotic anticancer activity of MCSP in MCSP\textsuperscript{pos}/DR5\textsuperscript{pos} melanoma cells. This synergy is likely attributable to the fact that HDAC inhibitors are known to down-regulate c-FLIP and thereby TRAIL-mediated apoptosis in melanoma cells, but also inhibited MCSP-mediated tumorigenic signaling that may add to or even synergize with its pro-apoptotic anticancer activity.

Conflict of interest

The authors state no conflict of interest.

References