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
Agonistic 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.1 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.2 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.3

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.4 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.5 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.4 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.6 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.7

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 cell death.4,8 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.8,9 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.8,9 Further, rhTRAIL and conventional agonistic DR4/5 antibodies have no tumor-selective binding activity, since TRAIL receptors are ubiquitous expressed on normal tissue.10,11 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 (MCSP<sup>+</sup> cutaneous melanomas); DLD-1, HCT 116 (colorectal adenocarcinomas) and Jurkat (T-ALL leukemia) were purchased from American Tissue Culture Collection (ATCC, Rockville, MD). Human MCSP<sup>+</sup> uveal melanoma cell line OCM3 was kindly provided by Prof. G.P.M. Luyten, Department of Ophthalmology, LUMC, Leiden, Netherlands. The MCSP<sup>+</sup> human melanoma parental cell line M14 and the MCSP<sup>+</sup> 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.

Cryopreserved human hepatocytes were purchased from Tebu-bio bv. Hepatocytes were cultured in a 48 well plate to a density of 2.5x10<sup>4</sup> cells per well before use.

**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 1x10<sup>6</sup> 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 scFvMCSP 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 (MCS<sup>1</sup>, MCS<sup>2</sup> and MCS<sup>3</sup>, respectively). MCS<sup>1</sup> and MCS<sup>2</sup> are interspersed by a 22 amino acid flexible linker derived from a CH1 domain. MCS<sup>1</sup> was used for directional and in-frame insertion of the scFvDR5 encoding DNA fragment. MCS<sup>2</sup> was used for directional and in-frame insertion of the scFvMCSP encoding DNA fragment. MCS<sup>3</sup> 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. Concentrations of MCSPxDR5 and MCSPxDR5-IgG4 were determined using the Human Easy-Titer kit (Thermo Scientific) according the 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 tumor cell lines was treated with MCSPxDR5. Briefly, tumor cells were seeded in 48-well plates at a concentration of 2x10^4 cells/well and treated with MCSPxDR5 (1 μg/ml) overnight, after which apoptosis was assessed using Annexin-V staining according to manufacturer's instructions (Immunoools). Briefly, cells were washed once with cold binding buffer (140 mM NaCl, 2.5 mM CaCl_2 and 10 mM 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. Alternatively, tumor cells were treated with MCSPxDR5 (2.5 μg/ml) on ice for 40 min, after which unbound MCSPxDR5 was removed by washing twice with cold PBS. After overnight incubation, apoptosis was assessed using Annexin-V staining. Finally, where indicated, tumor cell lines were treated with MCSPxDR5 for 24-72h, after which cell viability was assessed using an MTS-based calorimetric assay (CellTiter 96 AQueous One Solution, Promega) at 490 nm using a Victor V3 multi-label plate counter (Perkin Elmer). Absorbance of maximum death (treatment with 70% ethanol for 15 min) was subtracted from all values, after which cell viability was calculated as percentage of medium control.

To determine toxicity of MCSPxDR5 to normal cells, human hepatocytes, lymphocytes and HUVECs were treated with MCSPxDR5 (2.5 μg/ml) overnight, after which apoptosis was assessed using Annexin-V staining.

**Assessment of synergy between MCSPxDR5 and clinical antitumor drugs**

MCSPpos/DROS 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 mM NaCl, 2.5 mM CaCl_2 and 10 mM 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.

**Colonizing 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 (1.0x10^4 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 1x10^4 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 3x10^4/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 (3x10^4/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. Additionally,
we used MCSPpos SK-MEL-28 melanoma cells in which DR5 expression was knocked-down using DR5-specific siRNA silencing technology as described previously.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-linkers18 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.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 MCSPpos/DR5pos SK-MEL-28 melanoma cells (Fig. 1B), but not to MCSPpos/DR5neg DLD-1 carcinoma cells (Fig. 1C). Binding of MCSPxDR5 to SK-MEL-28 cells was blocked by co-incubation with excess amounts of epitope-competiting 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 MCSPpos/DR5pos melanoma cells, but not in MCSPpos/DR5neg HCT116 carcinoma cells (Fig. 1D). Treatment in the continued presence of MCSPxDR5 (no washing) induced apoptosis in both MCSPpos/DR5pos and MCSPpos/DR5neg 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 MCSPpos/DR5pos MM-RU, SK-MEL-28 and OCM3 cells, but not in MCSPpos/DR5neg 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 MCSPpos/DR5neg cancer cell lines and primary patient-derived melanoma cells.

![Figure 1: MCSP-directed apoptotic activity of MCSPxDR5 toward melanoma cells.](image)
MCSPxDR5 inhibits of colony formation of MCSP\textsuperscript{pos}/DR5\textsuperscript{pos} cancer cells

Next, the effect of MCSPxDR5 on anchorage-independent growth of MCSP\textsuperscript{pos} 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 MCSP\textsuperscript{pos} 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 medium MCSP\textsuperscript{pos}/DR5\textsuperscript{pos} 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 MCSP\textsuperscript{pos}/DR5\textsuperscript{pos} cancer cells.

**Figure 2:** MCSPxDR5 inhibits colony formation of melanoma cells. A) MCSP\textsuperscript{pos}/DR5\textsuperscript{pos} 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) MCSP\textsuperscript{pos}/DR5\textsuperscript{pos} A375M cells were treated with MCSPxDR5, MCSP:sTRAIL or HSG-ETR2 (250 ng/ml) or left untreated in colony-forming agar assays for 14 days, after which the number of colonies was determined. D) Colony diameter (µM) of MCSP\textsuperscript{pos}/DR5\textsuperscript{pos} HT1080 cells were treated with MCSPxDR5, MCSP:TRAIL or HSG-ETR2 (250 ng/ml) or left untreated in colony-forming agar assays for 14 days, after which the number of colonies was determined. Statistical analysis was performed using two-sided unpaired Student \( t \) test. *\( P < 0.05; ** P < 0.01; *** P < 0.001.

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 Fc\( \gamma \)Rs on immune effector cells.\textsuperscript{10,11} Therefore, cross-linking of tumor cell-bound MCSPxDR5 via its IgG domain may further augment its pro-apoptotic activity.

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

Next, the effect of Fc cross-linking 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.\textsuperscript{19} 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 MCSP\textsuperscript{pos}/DR5\textsuperscript{pos} M14 melanoma cells (Fig. 4C). However, Fc receptor-mediated cross-linking on MCSP\textsuperscript{pos} cells was abrogated when treatment was performed in the presence of MCSP-competing mAb 9.9.27 (Fig. 4D). In mixed culture experiments with freshly isolated PBLs, MCSPxDR5 dose-dependently reduced the viability of MM-RU cells to down to ~40% (Fig. 4E). Moreover, treatment with an increasing dose of MCSPxDR5 reduced MM-RU cell viability down to ~20% in the presence of leukocytes (Fig. 4F). Similarly, MCSPxDR5 significantly enhanced NK cell-mediated killing of MCSP\textsuperscript{pos}/DR5\textsuperscript{pos} A375M cells, but not of MCSP\textsuperscript{pos}/DR5\textsuperscript{pos} DLD1 cells (Fig. 4G). Thus, the MCSP-direct- ed pro-apoptotic activity of MCSPxDR5 is further augmented by FcR-mediated cellular cross-linking and is able to induce ADCC through interaction with Fc receptor-expressing immune effector cells.
Apoptosis was measured after 18h by flow cytometry using Annexin-V/PI staining.

**Figure 3:** Cross-linking of its Fc domain enhances antitumor activity of MCSPxDR5. A) MCSP<sup>+/−</sup>DR5<sup>−/−</sup> 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<sup>+/−</sup>DR5<sup>−/−</sup> 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<sup>+/−</sup>DR5<sup>−/−</sup> melanoma MM-RU cells were treated with an increasing dose of MCSPxDR5 in the presence or absence of CL for 72h, after which cell viability was determined by MTS assay. D) Primary patient-derived melanoma cells were co-cultured 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, 5µM) 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.

**Figure 4:** FcR-mediated cross-linking augments MCSPxDR5 efficacy. A) Pre-seeded MM-RU cells were co-cultured with parental HEK293 cells or HEK293.CD64 cells and treated with an increasing dose of MCSPxDR5. Apoptosis in MM-RU cells was assessed by flow cytometry using Annexin-V/PI. B) Pre-seeded MM-RU cells were co-cultured with parental HEK293 cells or HEK293.CD64 cells and subsequently treated with either medium only, MCSPxDR5 (50 ng/ml), or its IgG4 isotype variant MCSPxDR5-4 for 24h. Apoptosis in melanoma cells was assessed by flow cytometry using Annexin-V/PI. C) Pre-seeded M14 cells (MCSP<sup>neg/DR5pos</sup>) were co-cultured with parental HEK293 cells or HEK293.CD64 cells and subsequently treated with either medium only, MCSPxDR5 (50 ng/ml), or its IgG4 isotype variant MCSPxDR5-4 for 4h. Apoptosis in melanoma cells was assessed by flow cytometry using Annexin-V/PI. D) (DiD)-labeled A375M cells were treated with either culture medium or MCSPxDR5 (1.5 µg/ml) for 6h after which cell viability of MM-RU cells was assessed by MTS assay. E) Peripheral blood lymphocytes (E) were added to pre-seeded MM-RU target cells (T) at an E:T ratio of 20:1 and treated with an increasing dose of MCSPxDR5. Apoptosis in MM-RU cells was assessed by flow cytometry using Annexin V/PI staining. F) As in E, WBCs (E) were added to pre-seeded MM-RU target cells (T) at an E:T ratio of 5:1. G) Pre-seeded MM-RU cells (T) were co-cultured with NK cells of healthy donors (E) at E:T ratio of 20:1 in the presence or absence of MCSPxDR5 (250 ng/ml) for 6h after which cell viability of MM-RU cells was assessed by MTS assay. Statistical analysis was performed using two-sided unpaired Student t test. *P < 0.05; **P < 0.01; ***P < 0.001.

**Figure 3**

**Figure 4**
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 MCSP<sup>pos</sup>/DR5<sup>pos</sup> 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-DR5/5 antibodies (e.g. conatumumab and tigatuzumab) have been extensively evaluated in pre-clinical models and in a number of clinical studies. 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, thereby precluding sufficient accumulation of such agonists in malignant lesions. Moreover, current DR agonists have limited capacity to activate pro-apoptotic DR 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. Hereto, 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-bound 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. In contrast, selective binding of MCSPxDR5 to MCSP already triggered effective activation of membrane DR5 in melanoma cells, indicating that on MCSP<sup>pos</sup> 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.22 RG7386 induced apoptosis in FAP-positive cells and, when combined with irinotecan or doxorubicin, induced tumor regression in patient-derived xenograft models.23 Our in vitro data largely corroborates these observations, indicating a common mode-of-action for RG7386 and MCSPxDR5.

MCSP is expressed in 90% of melanoma lesions15 and other malignancies, e.g. sarcomas and gliomas.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.26 Indeed, we previously reported on a MCSP-targeting TRAIL fusion protein that not only induced direct MCSP-mediated TRAIL-mediated apoptosis in melanoma cells, but also inhibited MCSP-mediated tumorigenic signaling.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 chemotherapeutics or (experimental) small inhibitory molecules has been evaluated in order to mutually augment their antitumor activities towards various forms of cancer, including carcinoma, breast cancer, lung cancer and metastatic melanoma.

BRAFV600E inhibitor vemurafenib enhanced MCSPxDR5-induced apoptosis in both wild-type and V600E-mutant melanoma cells. This non-specific synergy with vemurafenib and TRAIL was also published by others28 but the underlying mechanism remains unclear.

Our current data indicates that MCSPxDR5 has potent MCSP-directed pro-apoptotic activity towards MCSP+/DR5+ melanoma cells with essentially no or minimal toxicity towards various normal cells. However, more in-depth studies are needed to establish whether this will ultimately also translate in a safe toxicity profile when applied in melanoma patients. Taken together, we present a novel bispecific antibody-based approach that promotes melanoma-directed pro-apoptotic activation of DR5. This novel approach may be of value for the targeted treatment of melanoma and other MCSP-expressing malignancies.

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Conflict of interest

The authors state no conflict of interest.

References