Targeting MHC-I related proteins for cancer diagnosis and therapy

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Chapter 4:

MICA-specific nanobody-drug conjugate for in vivo treatment of MICA^{+} EL-4 tumors

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Abstract

MICA and MICB are MHC-I related glycoproteins, upregulated on the surface of cells in response to stress, for instance when a cell is infected or malignantly transformed. MICA/B act as ligands for NKG2D, the activating receptor on NK cells, $CD8^+$ T cells, and γδ T cells. Upon engagement of MICA/B with NKG2D, these cytotoxic cells get activated and can eradicate MICA/B-positive targets. We have created nanobodies that specifically target MICA on the surface of cancer cells. We have shown that these nanobodies, when fused to the Maytansine derivative DM1, selectively kill MICA positive EL-4 T cell lymphoma cells *in vitro*. Here, we describe the results of an *in vitro* study in which we treated $MICA^+$ B16F10 melanoma cells with nanobody-DM1 adducts. We next performed *in vivo* experiments, attempting treatment of $MICA^+$ EL-4 tumor-bearing mice with the MICA nanobody-DM1 conjugate.

Introduction

The MHC Class I-associated glycoproteins MICA and MICB (MICA/B) are upregulated on the surface of human cells under stress, for instance due to viral infection or malignant transformation[224]. MICA/B act as ligands for the NKG2D activating receptor found on NK cells, $CD8^+$ T cells, and γδ T cells[218], engagement of which activates these cytotoxic cells to eradicate MICA/B-positive targets by secretion of granzymes, perforins, and cytokines[219–221]. High levels of MICA/B are found in hematopoietic malignancies, as well as in many solid tumors of epithelial origin[235]. MICA/B are thus considered possible targets for immunotherapy.

Nanobodies, also referred to as VHHs, are the recombinantly expressed variable regions of camelid heavy chain-only immunoglobulins[301]. Nanobodies retain excellent antigen-binding capabilities and are characterized by their small size, short circulatory half-life, and excellent tissue penetration compared to conventional full-sized immunoglobulins[312,313]. Nanobodies have proven valuable for the construction of nanobody-drug conjugates[315,386,436]. We have developed nanobodies, VHH A1 and VHH H3, that recognize surface-bound MICA with high affinity. When fused to the microtubule inhibitor Maytansine (DM1), we showed that VHH A1 can be used therapeutically as a nanobody-drug conjugate in an *in vitro* study in which we targeted $MICA^+$ EL-4 T cell lymphoma cells[575]. Here, we use the nanobody-drug conjugate to test its *in vitro* cytotoxicity of B16F10 $MICA^+$ melanoma cells. Furthermore, we describe the results of an *in vivo* experiment to treat mice bearing $MICA^+$ EL-4 primary tumors with the VHH A1-based nanobody drug conjugate.

Materials and methods

Production of recombinant nanobodies and sortase reactions

Nanobody sequences were subcloned into a pHen6 expression vector, including C-terminal modifications of an LPETG motif recognized by Sortase A, and a $(His)_6$-tag for recovery and purification on a NiNTA matrix[575]. Nanobodies were expressed in WK6 *E.Coli* in terrific broth by periplasmic protein expression, activated with isopropyl β-thiogalactopyroniside (1mM) once an OD600 of 0.6 was reached. Nanobodies were harvested from the periplasm by osmotic shock. The $(His)_6$-tag allows purification of nanobodies with NiNTA Agarose beads (Qiagen). Nanobodies were purified on an S75 column by FPLC (ÄKTA, Cytiva Life Sciences). GGG-DM1 and GGG-DM4 were produced in-house by modifying a GGG-peptide linker to contain a maleimide group and allowing it to react with the thiol group on DM1 or DM4 (Broadpharm) as described (Chapter 3, supplementary figure 2). For sortase reactions, nanobodies were incubated with a 10-fold molar excess of GGG-DM1 or GGG-DM4 and incubated with 25 μM Sortase for 16 hours at 4°C. Unreacted VHH and Sortase, both containing a $(His)_6$-tag, were depleted by incubation with NiNTA agarose (Qiagen or Prometheus). Excess free GGG-DM1/4 was removed by desalting on a PD-10 desalting column (Cytiva). Fractions were eluted in 500 μL PBS. To prevent inclusion of free GGG-DM1/4, only the fractions eluting early were selected and combined for downstream further applications.

Cell culture

MICA-expressing mouse-derived EL-4 T cell lymphoma cells or B16F10 melanoma cells, and their wild type (WT) counterparts, were a gift from K. Wucherpfennig (Dana Farber Cancer Institute). EL-4 cells were cultured in complete RPMI 1640 (RPMI 1640 supplemented with 10% fetal bovine serum (FBS) + 100 U/mL penicillin/streptomycin (pen/strep)). B16F10 cells were cultured in complete DMEM (DMEM with 4.5 g/L glucose, supplemented with 10% FBS + 100 U/mL pen/strep)

Nanobody-drug conjugate treatment *in vitro*

We plated 4000 B16F10 or EL-4 WT or $MICA^+$ cells per well in a 96-well plate. We incubated the cells with serial 3-fold dilutions of VHH-drug adduct at 37°C in a humidified 5% CO_2 atmosphere. After 72 hours, we measured cell viability by CellTiter Glo™ assay according to the manufacturer's directions (Promega).

Nanobody-drug conjugate treatment *in vivo*

C57/B6 mice were injected subcutaneously in the right flank with $0.5x10^6$ EL-4 MICA$^+$ cells in PBS. On day 2 after injection, intraperitoneal injections of 100 µg (~5 mg/kg) per mouse were given every 2 or 3 days until day 21. Tumor size was measured by calipers and tumor volume was calculated using the following formula: $V = 0.5 \times L \times W^2$. Mice were sacrificed when the tumor volume exceeded 2000mm^3 or when ulcerations were observed.

Mice

C57BL/6J mice were purchased from the Jackson Laboratory or bred in-house. Mice were used at 8-12 weeks of age. Experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Boston Children's Hospital.

Results

Nanobody-drug conjugate fails to induce cytotoxicity of B16F10 MICA$^+$ tumor cells *in vitro*

Because of the promising results in *in vitro* cytotoxicity of VHH_{A1}-DM1 on EL-4 MICA$^+$ cells, we tested the efficacy of this nanobody drug conjugate on a different MICA$^+$ cancer cell line. We used the aggressive mouse-derived B16F10 melanoma line, transfected to stably express MICA on the cell surface. We used a VHH that targets mouse MHC-II ($VHH_{MHC\text{-}II}$)[558] as a negative control. We ligated the microtubule inhibitor Maytansine (DM1) to the nanobodies by a sortase-mediated transpeptidation reaction (Figure 1A). After the sortase reaction, unreacted VHH and Sortase, both containing a $(His)_6$-tag, were depleted by incubation with NiNTA agarose. We performed an in vitro cytotoxicity assay by titration of $VHH_{MHC\text{-}II}$-DM1 or VHH_{A1}-DM1 on B16F10 WT and MICA$^+$ cells. We did not observe an increased sensitivity, measured by IC50, to VHH_{A1}-DM1 by the MICA$^+$ cells compared to $VHH_{MHC\text{-}II}$-DM1. We also did not observe a significant difference in IC50 between WT and MICA$^+$ B16F10 cells treated with either nanobody (Figure 1B). These results indicate that the VHH A1-based nanobody-drug conjugate is ineffective in treating the aggressive B16F10 MICA$^+$ melanoma line *in vitro*.

Using the same strategy, we evaluated the efficacy of VHH A1 conjugated to DM4, the functional analog of DM1 with as the only difference the presence of a cleavable linker (Chapter 3, Supplementary figure 2B) in killing EL-4 WT or MICA$^+$ cells. We did not observe an increased sensitivity, measured by IC50, to VHH_{A1}-DM4 by the MICA$^+$ cells compared to $VHH_{MHC\text{-}II}$-DM4 (Figure 1C).

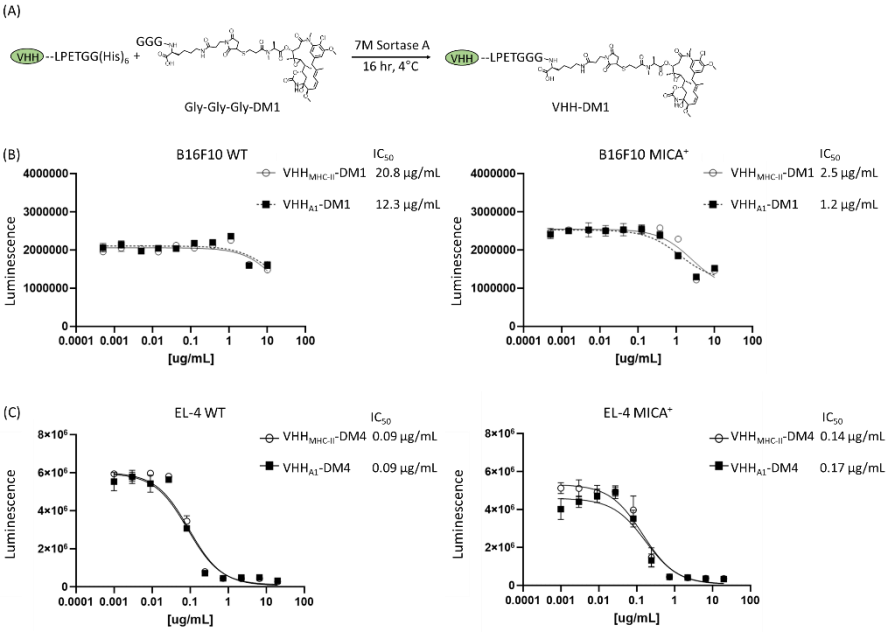

Figure 1. Anti-MICA VHHs as nanobody-drug conjugate with the Maytansine derivative DM1. *(A) We ligated the microtubule-inhibitor GGG-DM1 to VHH A1 or VHH_{MHC-II} as a non-targeting control through sortase-mediated transpeptidation reaction. (B) We performed an in vitro cytotoxicity assay by incubating 4000 B16F10 WT or MICA⁺ cells with VHH_{MHC-II}-DM1 or VHH_{A1}-DM1 at 3-fold serial dilutions. After 72 hours, we measured proliferation by CellTiter Glo™ assay. We observed a similar IC50 in cells incubated with either non-targeting or MICA-targeting nanobody-drug conjugate, thus there is no effect on proliferation of MICA⁺ cells treated with VHH_{A1}-DM1.*

Half-life extension of nanobody-drug conjugate for *in vivo* cytotoxicity of EL-4 MICA⁺ tumor cells

Despite the resistance of B16F10 cells to treatment with the nanobody-drug conjugate, we previously had striking results in treating EL-4 MICA⁺ cells with the VHH A1-based nanobody drug conjugate). The efficacy of treatment of EL-4 MICA⁺ cells with VHH_{A1}-DM1 was comparable to that of cells treated with free DM4, a functional analog of DM1. Because of their small size, unbound nanobody is rapidly cleared from the circulation, with an *in vivo* half-life of less than 2 hours[576]. Thus, to use the VHH A1-based nanobody-drug conjugate for treatment of MICA⁺ tumors *in vivo*, we reasoned that half-life extension of the nanobody might be useful.

To this end, we created a genetic C-C fusion of VHH-A1 to a mouse immunoglobulin kappa-light chain targeting nanobody (VHH_{mKappa}). This nanobody recognizes the κ light chains of mouse immunoglobulins[577]. As a negative control, we used a genetic C-C fusion of VHH_{mKappa} to a nanobody that targets influenza virus hemagglutinin (VHH_{SD36}). We created VHH_{A1}-VHH_{mKappa}-DM1 or VHH_{SD36}-VHH_{mKappa}-DM1 using sortase-mediated transpeptidation (Figure 2A). We combined fractions 1–6 for VHH_{A1}-VHH_{mKappa}-DM1 and fractions 3-6 for VHH_{SD36}-VHH_{mKappa}-DM1. We confirmed successful ligation by SDS-PAGE (Figure 2B).

To test the efficacy of VHH_{A1}-VHH_{mKappa}-DM1, we performed an *in vitro* cytotoxicity assay by titration of VHH_{A1}-VHH_{mKappa}-DM1, VHH_{SD36}-VHH_{mKappa}-DM1, or free DM4 on EL-4 WT and $MICA^+$ cells. 72 hours after co-culture, we measured proliferation by CellTiter Glo assay. EL-4 $MICA^+$ cells were sensitive to VHH_{A1}-VHH_{mKappa}-DM1 with a stronger cytotoxic effect at lower doses of the VHH-drug conjugate compared to VHH_{SD36}-VHH_{mKappa}-DM1, as estimated by IC50. Despite the reduction in IC50, the sensitivity of EL-4 $MICA^+$ cells was lower for treatment with VHH_{A1}-VHH_{mKappa}-DM1 compared to free DM4. Similarly treated WT cells showed no obvious reduction in proliferation with either nanobody-drug conjugate (Figure 2C).

Nanobody-drug conjugates fail to reduce growth of $MICA^+$ tumors *in vivo*

Mice bearing subcutaneously grafted EL-4 $MICA^+$ tumors were treated every 2 or 3 days until day 21 with an intraperitoneal injection of 5 mg/kg of VHH_{A1}-VHH_{mKappa}-DM1 or VHH_{SD36}-VHH_{mKappa}-DM1 (Figure 3A). Although tumor growth in the treated mice was delayed relative to mice treated with a non-targeting nanobody-drug conjugate, once treatment was stopped this delay no longer applied. In fact, treated mice showed accelerated tumor growth upon cessation of treatment (Figure 3B). We also did not observe a significant difference in survival probability between the mice treated with VHH_{A1}-VHH_{mKappa}-DM1 and VHH_{SD36}-VHH_{mKappa}-DM1 (Figure 3C).

Discussion

MICA/B are MHC-I related proteins expressed on stressed and malignant cells. Their presence can serve as a target for therapy. We produced the MICA-targeting nanobody (VHH A1) and conjugated it to the Maytansinoid DM1, a microtubule inhibitor. We observed increased, specific cytotoxicity *in vitro* of VHH_{A1}-DM1 on $MICA^+$ EL-4 T cell lymphoma tumor cells, compared to WT EL-4 cells.

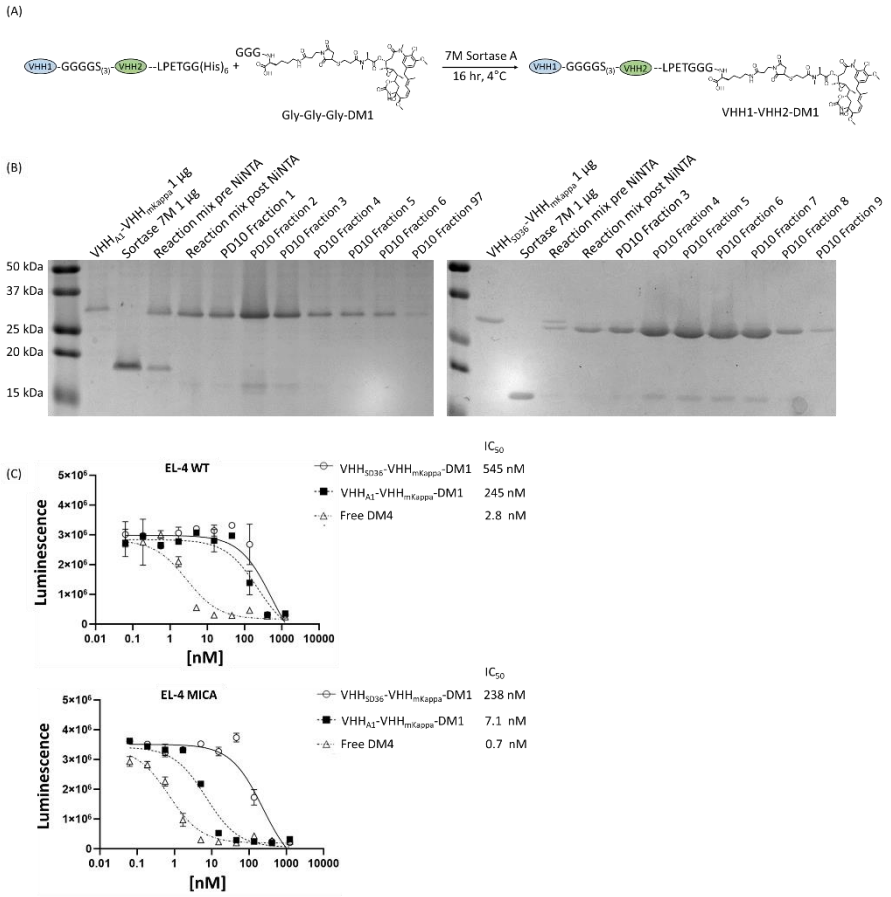

Figure 2. Production of nanobody-drug conjugate with the Maytansine DM1 and calculation of IC50. *(A) We ligated DM1 to this fusion by sortase-mediated transpeptidation reaction to create VHH_{A1}-VHH_{mKappa}-DM1 or VHH_{SD36}-VHH_{mKappa}-DM1. (B) After the sortase reaction, unreacted VHH and Sortase, both containing a $(His)_6$-tag, were depleted by incubation with NiNTA agarose. Excess free GGG-DM1 was removed by desalting on a PD-10 desalting column, eluting in fractions of 500 µL PBS. We selected and combined fractions 1 – 6 for VHH_{A1}-VHH_{mKappa}-DM1 and fractions 3-6 for VHH_{SD36}-VHH_{mKappa}-DM1. We confirmed successful ligation by SDS-PAGE. (C) We performed an in vitro cytotoxicity assay by titration of VHH_{A1}-VHH_{mKappa}-DM1, VHH_{SD36}-VHH_{mKappa}-DM1, or free DM4 on EL-4 WT and MICA$^+$ cells. After incubation for 72 hours, we measured cell viability by CellTiter GloTM assay. EL-4 MICA$^+$ cells treated with VHH_{A1}-VHH_{mKappa}-DM1 showed a significant reduction in IC50, and thus a decrease in viability with a lower concentration of drug added, compared to similarly treated EL-4 WT cells, or EL-4 cells treated with the non-targeting VHH_{SD36}-VHH_{mKappa}-DM1.*

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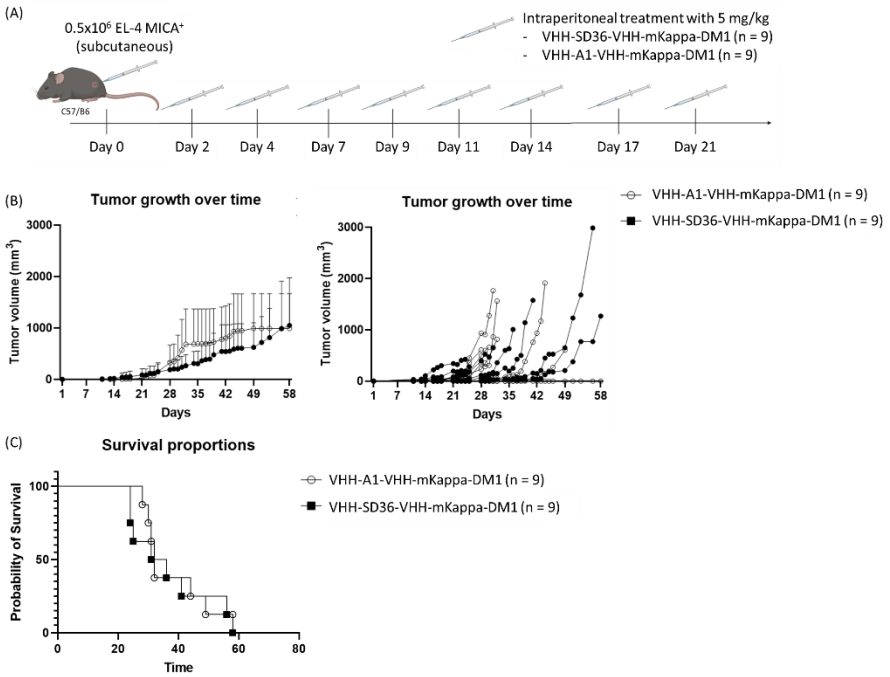

Figure 3. In vivo cytotoxicity of nanobody-drug conjugate in MICA⁺ tumor-bearing mice. *(A) We subcutaneously grafted 0.5x10⁶ EL-4 MICA⁺ cells in C57/B6 mice. Treatment with 5 mg/kg of VHH_{A1}-VHH_{mKappa}-DM1 (n = 9) or VHH_{SD36}-VHH_{mKappa}-DM1 (n = 9) started on day 2. Treatments were administered intraperitoneal every 2-3 days until day 21. (B) Tumors were measured daily by calipers. The average tumor volumes with standard deviations are plotted in the left graph. The measurements of each mouse individually are depicted in the right graph. We did not see a significant reduction in tumor growth in the mice treated with VHH_{A1}-VHH_{mKappa}-DM1 compared to the mice treated with VHH_{SD36}-VHH_{mKappa}-DM1. (C) We did not observe a significant difference in survival probability between the mice treated with VHH_{A1}-VHH_{mKappa}-DM1 or VHH_{SD36}-VHH_{mKappa}-DM1.*

Here, we tested the efficacy of VHH A1-based nanobody-drug conjugate on MICA⁺ B16F10 cells, a highly aggressive mouse-derived melanoma cell line. The VHH A1-based nanobody-drug conjugate was ineffective in treating B16F10 MICA⁺ cells *in vitro*. Published literature suggests a certain resistance of B16F10 cells to DM1 treatment[578]. Because of the promising results obtained when using the EL-4 cell line, we suggest inclusion of more cell lines that represent different tumor types to determine the extent of resistance to VHH_{A1}-DM1 across a broader spectrum of malignancies.

For our *in vivo* model, we extended the half-life of the nanobody drug conjugate by creating a genetic C-C fusion of VHH A1 to an anti-mouse kappa light chain nanobody (VHH_{mKappa}). Using a sortase reaction, we ligated DM1 to this fusion and created VHH_{A1}-VHH_{mKappa}-DM1. We used VHH_{SD36}, a nanobody that targets the influenza virus hemagglutinin, fused to VHH_{mKappa} and DM1, as a negative control. We treated mice bearing subcutaneous EL-4 $MICA^+$ cells 3x weekly with an intraperitoneal injection of 5 mg/kg of the fusions and showed that the VHH_{A1}-VHH_{mKappa}-DM1 was ineffective in treating the EL-4 $MICA^+$ tumors. Possibly, intravenous administration of the drug might improve delivery to the tumor, but this was not tested by experiment.

The creation of different VHH-drug combinations, for example with other tubulin inhibitors like Auristatins, immunomodulators like STING agonists, or DNA damaging agents like Exatecans, deserves further research.