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Targeting MHC-I related proteins for cancer diagnosis and therapy

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Citation

Verhaar, E. R. (2024, July 4). *Targeting MHC-I related proteins for cancer diagnosis and therapy*. Retrieved from <https://hdl.handle.net/1887/3766089>

Version: Publisher's Version

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Note: To cite this publication please use the final published version (if applicable).

Chapter 4:

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

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Unpublished data

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 $\gamma\delta$ 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 transformation²²⁴. MICA/B act as ligands for the NKG2D activating receptor found on NK cells, CD8⁺ T cells, and $\gamma\delta$ T cells²¹⁸, engagement of which activates these cytotoxic cells to eradicate MICA/B-positive targets by secretion of granzymes, perforins, and cytokines²¹⁹⁻²²¹. High levels of MICA/B are found in hematopoietic malignancies, as well as in many solid tumors of epithelial origin²³⁵. 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 immunoglobulins³⁰¹. 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 cells⁵⁷⁵. 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 matrix⁵⁷⁵. Nanobodies were expressed in WK6 *E.Coli* in terrific broth by periplasmic protein expression, activated with isopropyl β -thiogalactopyroniside (mM) 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 μ M 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-DM_{1/4} was removed by desalting on a PD-10 desalting column (Cytiva). Fractions were eluted in 500 μ L PBS. To prevent inclusion of free GGG-DM_{1/4}, 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% CO₂ 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 0.5×10^6 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 200mm^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-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-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-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-II}-DM₄ (Figure 1C).

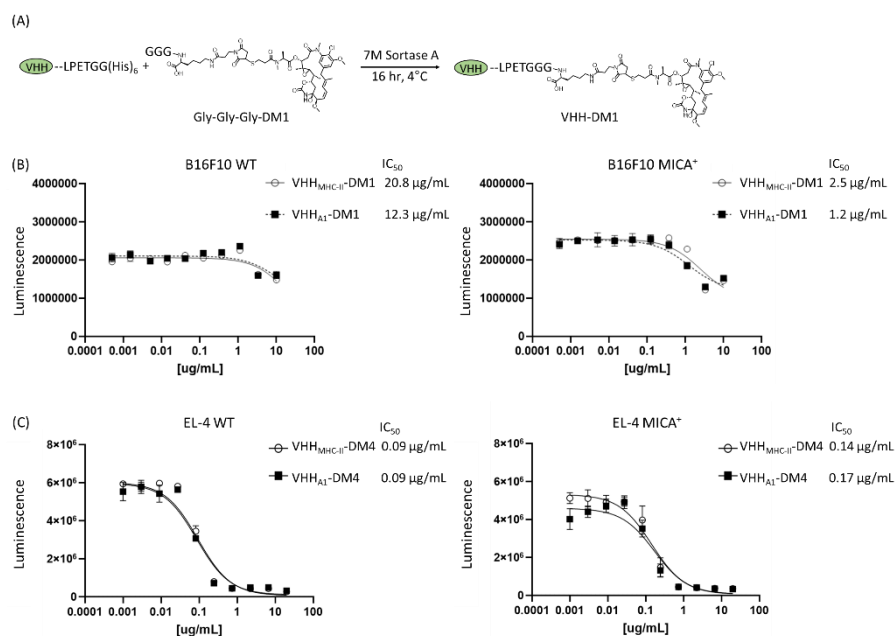


Figure 1. Anti-MICA VHHs as nanobody-drug conjugate with the Maytansine derivative DM1. (A) We ligated the microtubule-inhibitor GGG-DM1 to VHH A1 or VHH_{MHC-II} as a non-targeting control through sortase-mediated transpeptidation reaction. (B) We performed an *in vitro* cytotoxicity assay by incubating 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 hours⁵⁷⁶. 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 immunoglobulins⁵⁷⁷. 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.

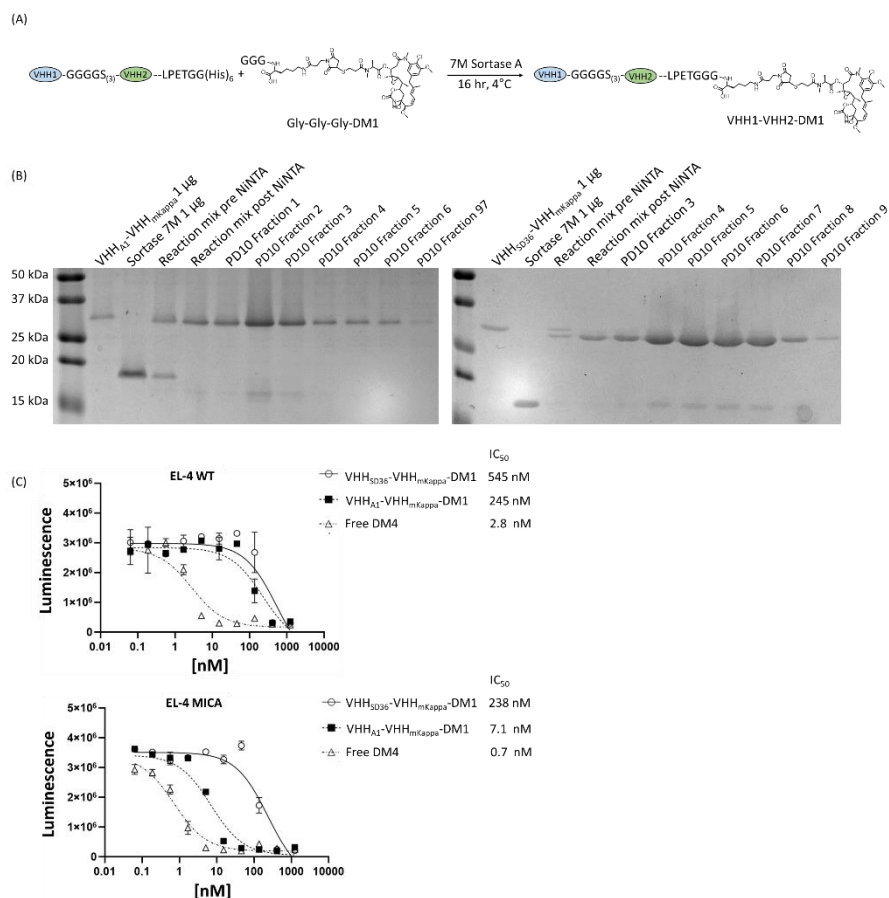


Figure 2. Production of nanobody-drug conjugate with the Maytansine DM₁ and calculation of IC₅₀. (A) We ligated DM₁ to this fusion by sortase-mediated transpeptidation reaction to create VHH_{Ar}-VHH_{mKappa}-DM₁ or VHH_{SD36}-VHH_{mKappa}-DM₁. (B) After the sortase reaction, unreacted VHH and Sortase, 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 μ L PBS. We selected and combined fractions 1 – 6 for VHH_{Ar}-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_{Ar}-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 Glo™ assay. EL-4 MICA⁺ cells treated with VHH_{Ar}-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.

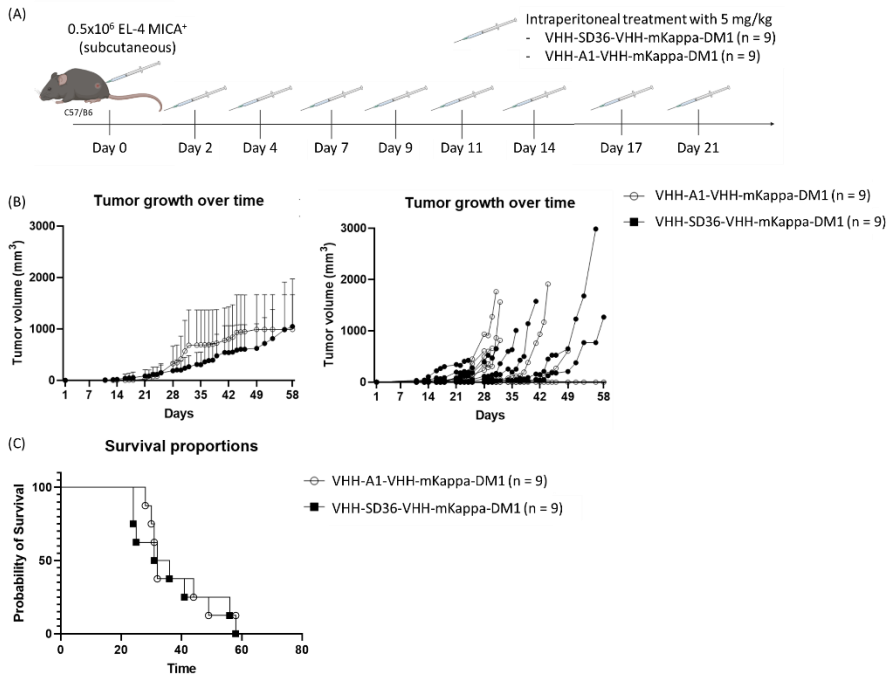


Figure 3. In vivo cytotoxicity of nanobody-drug conjugate in MICA⁺ tumor-bearing mice. (A) We subcutaneously grafted 0.5×10^6 EL-4 MICA⁺ cells in C57/B6 mice. Treatment with 5 mg/kg of VHH_{A1}-VHH_{mKappa}-DM1 (n = 9) or VHH_{SD36}-VHH_{mKappa}-DM1 (n = 9) started on day 2. Treatments were administered 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^{57,8}. 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.