Brief Report | Basic and Mechanism Sciences

Cell type-specific upregulation of NKG2D ligand MICA in response to APTO253

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Abstract: One of the most important targets for natural killer (NK) cell-mediated therapy is the induction of natural killer group 2D ligand (NKG2D-L) expression. APTO253 is a small molecule that selectively kills acute myeloid leukemia (AML) cells, and it has been reported that APTO253 can induce Krüppel-like factor 4 (KLF4) expression and downregulate c-MYC expression. Recently, we discovered a novel role of APTO253 in modulating the NK cell response by inducing surface expression of NKG2D-Ls, especially MHC class I polypeptide-related sequence A (MICA), in AML cells. In this study, we extended the research to validate the effect of APTO253 in other cancer cell lines and found that the enhanced expression of NKG2D-Ls in response to APTO253 is limited in a tumor cell-specific manner. Here, we show that MICA induction upon treatment with APTO253 not only varies between ovarian and pancreatic cancer cell lines but also differs in two ovarian cancer cell lines for an unknown reason. Additionally, our data suggest a link between the induced expression of *MICA* and the regulation of both, *KLF4* and *c-MYC*, which might represent a mechanism underlying the induction of NKG2D-L expression upon treatment with APTO253. These results may contribute to the potential use of APTO253 as a treatment to improve tumor cell-mediated NK cell cytotoxicity in various cancers.

Keywords: APTO253; natural killer group 2D (NKG2D); MHC class I polypeptide-related sequence A induction (MICA induction); natural killer cell-mediated therapy (NK cell-mediated therapy)

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Immunotherapy is considered a milestone in cancer treatment. At present, the main focus is on targeting the adaptive immune system, however, several clinical studies have reported natural killer (NK) cell-based immunotherapy to be a promising treatment for cancer (1,2). NK cells are tightly regulated by a series of inhibitory and activating receptors expressed on their surface and do not require any antigen-specific activation. One of the major activating receptors is natural killer group 2D (NKG2D) which recognizes and binds ligands [namely, MHC class I polypeptide-related sequence A and B (MICA/B) and unique long protein 16 binding protein 1 to 6 (ULBP1 to 6)] induced on the surface of tumor cells. This recognition and engagement play a crucial role in NK cell-mediated cytotoxicity (3). A decreased expression of NKG2D-ligands (NKG2D-Ls) on tumor cells is observed in many malignancies including acute myeloid leukemia (AML) and pancreatic cancer (4-6). Moreover, the use of NKG2D knockout NK cells or antibodies to disrupt the interaction between NKG2D and its ligands impairs NK cell antitumor

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activity and promotes tumor progression, highlighting the role of NKG2D in tumor immune surveillance (7,8). Therefore, the NKG2D/NKG2D-L axis has emerged in recent years as a promising target for immunotherapy to treat cancers, including AML (9-11). Of note, recent studies report a pro-tumorigenic role of NKG2D reflecting the paradoxical role of NKG2D/NKG2D-L in cancer immunity (12-14).

Several mechanisms regulating NKG2D-L expression are described (15,16), however, the mechanisms driving the induction of NKG2D-Ls in response to therapeutics are not well studied. NKG2D can recognize tumor cells after exposure to different drugs, e.g., histone deacetylase inhibitors (HDACi), which induce the expression of NKG2D-Ls in several solid tumors as well as AML (17-19).

To develop promising NKG2D-based immunotherapies aiming at enhanced expression of NKG2D-Ls on the surface of tumor cells it is important to identify transcription factors that induce the expression of NKG2D-Ls on the tumor cell surface.

Recently, we reported that the transcription factor Krüppel-like factor 4 (KLF4) is involved in the upregulation of *MICA* gene expression in AML (20,21) rendering these cells prone to NK cell-mediated recognition and killing. KLF4 is a transcription factor that has a dual effect as tumor suppressor or oncogene in cancer depending on the cellular context (22). In addition, KLF4 is one of the Yamanaka factors required for the induction of pluripotent stem cells, which in AML are characterized by a suppression of MICA expression (23). Of note, a small molecule, APTO253, known to induce KLF4 expression leads also to MICA expression in AML cells, linking this novel drug to innate immune surveillance (20,24). Moreover, it was reported that APTO253 suppresses the expression of the tumor promoter c-MYC (25), which is also involved in the regulation of MICA in AML (26,27). Here, we investigated whether this novel APTO253-KLF4/c-MYC/MICA axis is AML-specific or observed in tumor cells more generally. The potential role of KLF4 as a tumor suppressor in ovarian cancer has already been demonstrated and is linked to patient survival (28). It was reported that APTO253 induced cell cycle arrest in ovarian cancer cell lines (OVCAR3 and SKOV3) and this was dependent on the induction of KLF4 (28). So far, a potential link to innate immune alert was not investigated. We demonstrate that APTO253 at sublethal, non-toxic concentrations can induce the expression of both *KLF4* and *MICA* in ovarian cancer OVCAR8 cells (*Figure 1A*,*1B*, left panel). In contrast, OVCAR4 cells showed induced expression of *KLF4*, without induction of *MICA* (*Figure 1A*,*1B*, middle panel). Similar to OVCAR4, the pancreatic cancer cell line PANC1 showed no upregulation of *MICA* expression in response to APTO253 and very weak *KLF4* induction only at a high dose, indicating that the link of APTO253 to *KLF4* and *MICA* is not a general, but a cell type-specific mechanism (*Figure 1A*,*1B*, right panel). The high degree of *MICA* polymorphisms (29) may further contribute to the differences in cellular responses. Measuring the surface expression of MICA/B using flow cytometry revealed an increase for OVCAR8 cells, but not for OVCAR4 and PANC1, thereby reflecting the regulation on the transcriptional level (*Figure 2*, for the staining procedure see Appendix 1).

It is known that certain stimuli regulate specifically one ligand, whereas other molecular pathways such as DNA-damage-dependent cellular stress induce the expression of all ligands (30). However, other ligands than MICA were only moderately affected by APTO253. The expression of *ULBP2*, *5*, and *6* was not significantly up-regulated in OVCAR8 or OVCAR4 cells, and only a moderately induced expression of *MICB* was detectable in OVCAR8 and for all ligands in PANC1 cells (*Figure 3*) excluding a general regulation of NKG2D-Ls by APTO253.

The positive effect of HDACi including LBH589 on the expression of MICA was reported for several tumor entities (19) and was here observed in ovarian as well as pancreatic cancer cells. This correlates strongly with the LBH589-dependent expression of *KLF4*, whereas the expression of *c-MYC* was repressed or unaffected. In contrast, APTO253 treatment led to a strong increase of *c-MYC* in OVCAR4 and to a lesser extent in PANC1 cells, which correlated in this cell line with the failure to express *MICA* (*Figure 4*).

The induction of *KLF4* and *MICA* by APTO253 observed in OVCAR8 cells but not in OVCAR4 cells is in line with our previous results in AML cell lines. Of note, *c-MYC* expression was specifically induced in OVCAR4 cells but was low and remained unaffected in OVCAR8 cells. Expression of c-MYC, a transcription factor and oncogene, was also inhibited in AML cells in response to APTO253 treatment (25). Therefore, we speculate that induction of *KLF4* expression alone is not sufficient to upregulate *MICA*, but suppression of *c-MYC* is also required. Thus, both induction of KLF4 and down-regulation of c-MYC are important for the induction of *MICA* expression. Besides the availability of transcription factors in a given cell (see model *Figure 5*), we anticipate that cell type-specific epigenetic regulatory mechanisms may contribute

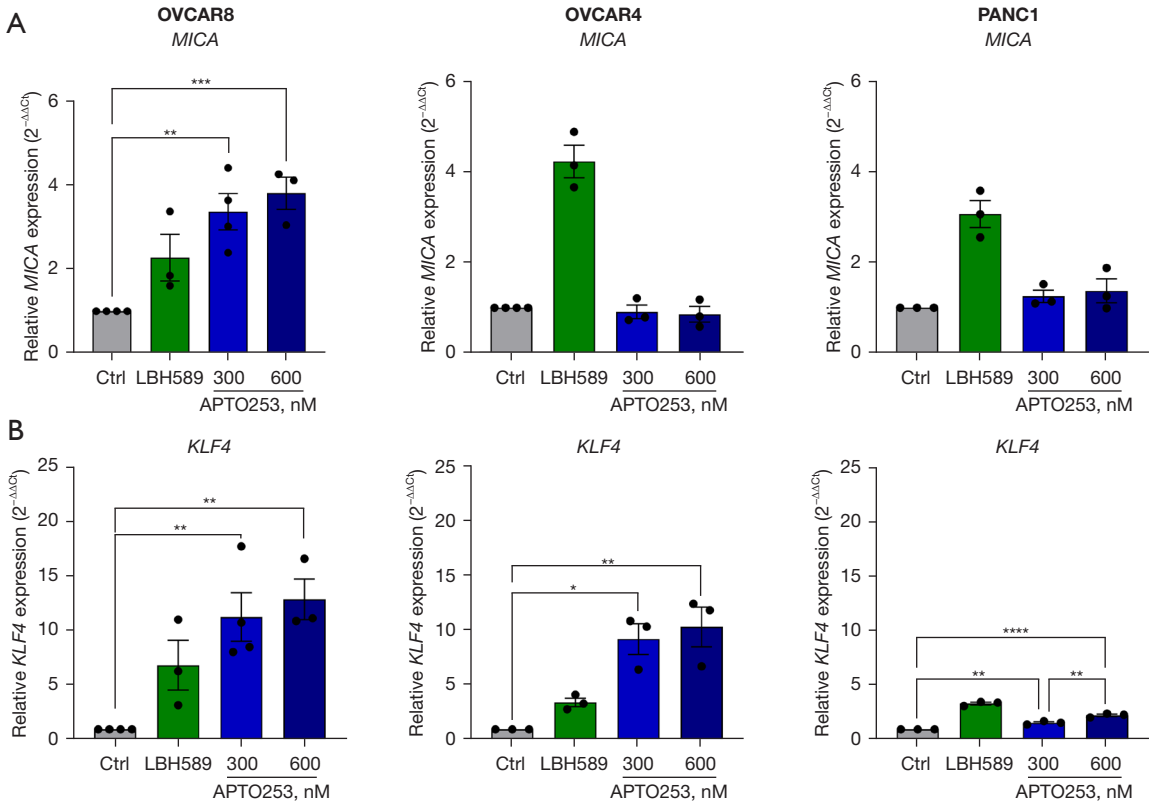

Figure 1 The effect of LBH589 and APTO253 on the expression of *MICA* and *KLF4* in different tumor cell lines. Ovarian and pancreatic cell lines were treated with 100 nM LBH589, 300 or 600 nM APTO253 for 24 hours. Then, cells were subjected to RT-qPCR analysis to evaluate the gene expression of (A) *MICA* and (B) *KLF4*. Data are presented as mean ± standard error of the mean of three to four independent experiments, performed in three technical replicates. Statistical significance was calculated using one-way analysis of variance followed by Tukey's multiple comparison test. *, P≤0.05; **, P≤0.01; ***, P≤0.001; ****, P≤0.0001. Primer sequences are indicated in the Appendix 1. RT-qPCR, reverse transcription quantitative polymerase chain reaction.

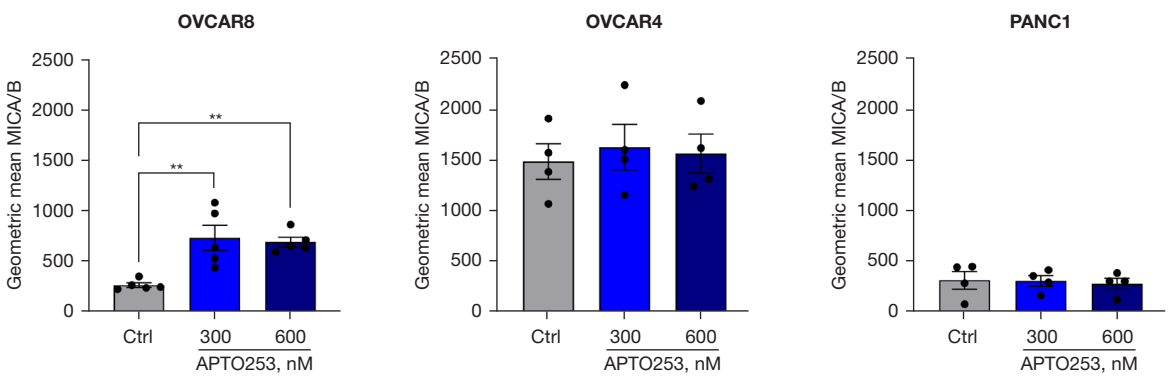

Figure 2 The effect of APTO253 on the protein expression of MICA/B in different tumor cell lines. Cells were treated with 300 or 600 nM APTO253 for 24 hours. Then, the cells were harvested, incubated with MICA/B antibody, and subsequently analyzed by flow cytometry. MIC protein expression is depicted. Data are presented as mean ± standard error of the mean of four to five independent experiments. Statistical significance was calculated using one-way analysis of variance followed by Tukey's multiple comparison test. **, P≤0.01. For detailed methods see Appendix 1.

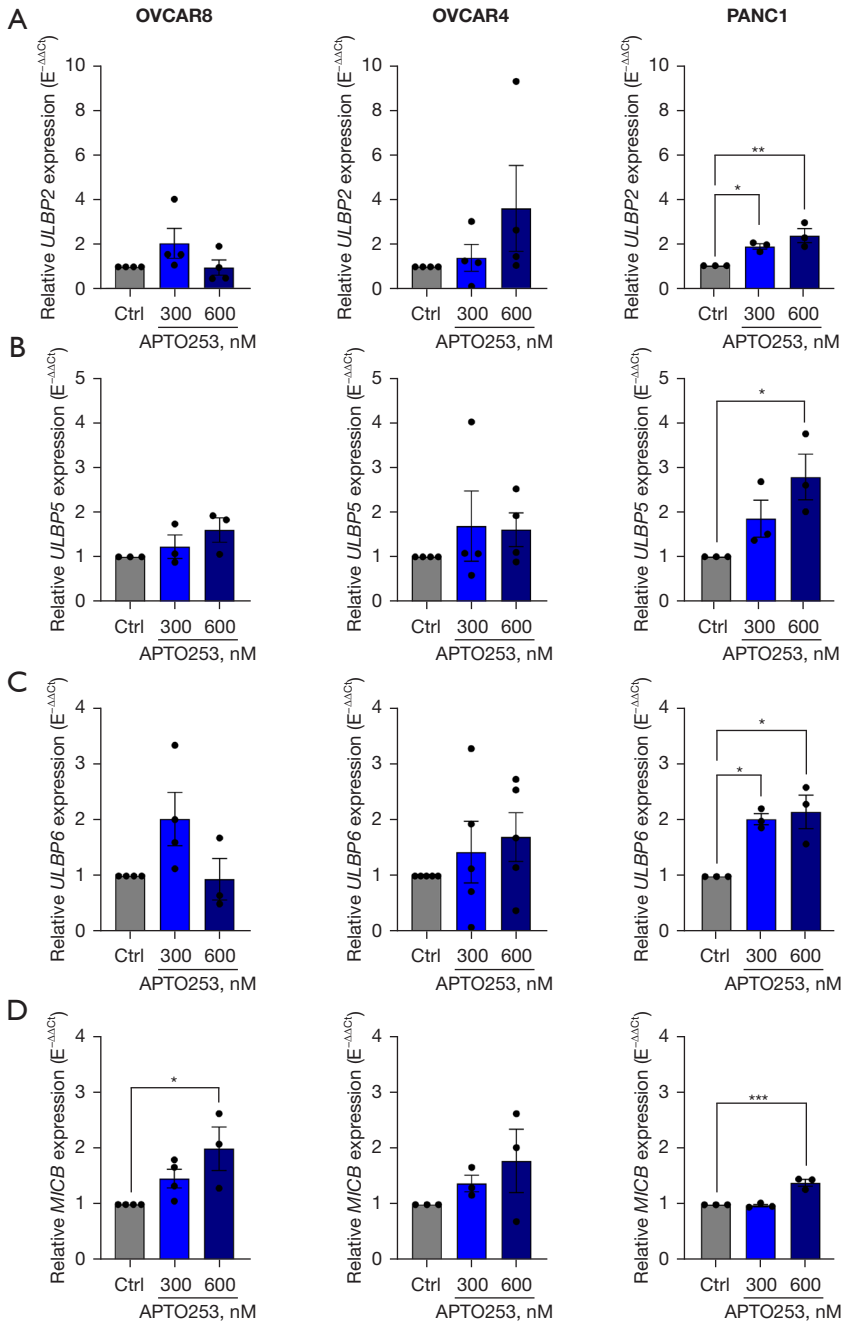

Figure 3 The effect of APTO253 on the expression of *MICB*, *ULBP2*, *5*, and *6* in different tumor cell lines. Ovarian cancer cells OVCAR8 and OVCAR4 were obtained from the National Institute of General Medical Sciences, Human Genetic Cell Repository of the National Institutes of Health, Bethesda, Maryland, USA, and kindly provided by Rolf Müller, Marburg, Germany and the pancreatic cell line PANC1 was obtained from the Leibniz Institute, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany (number ACC 783). The cell lines were treated with 300 or 600 nM APTO253 for 24 hours. Then, cells were subjected to RT-qPCR analysis to evaluate the gene expression of (A) *ULBP2*, (B) *ULBP5*, (C) *ULBP6*, and (D) *MICB*. Data are presented as mean ± standard of the mean of three to five independent experiments, performed in three technical replicates. Statistical significance was calculated using one-way analysis of variance followed by Tukey's multiple comparison test. *, P≤0.05; **, P≤0.01; ***, P≤0.001. RT-qPCR, reverse transcription quantitative polymerase chain reaction.

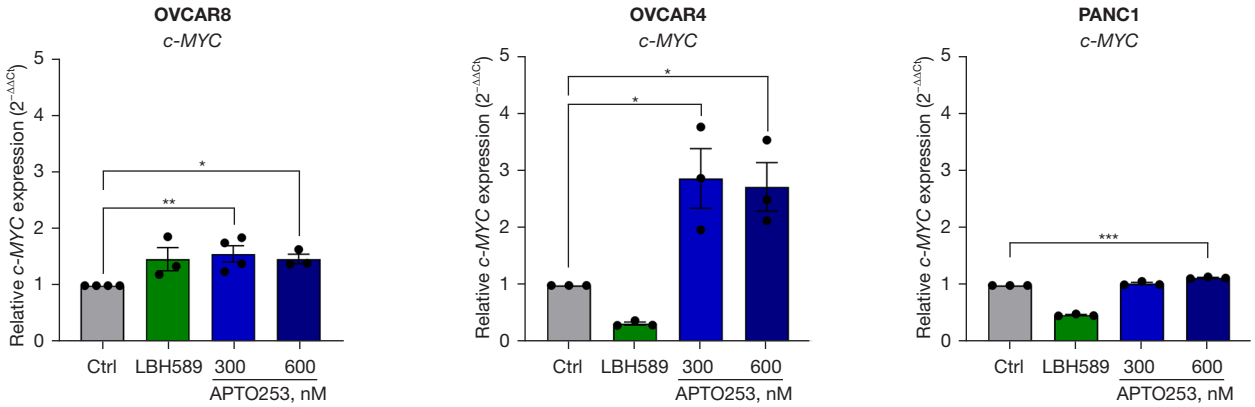

Figure 4 The effect of LBH589 and APTO253 on the expression of *c-MYC* in different tumor cell lines. Ovarian and pancreatic cell lines were treated with 100 nM LBH589, 300 or 600 nM APTO253 for 24 hours. Then, cells were subjected to RT-qPCR analysis to evaluate the gene expression of *c-MYC*. Data are presented as mean ± standard error of the mean of three to four independent experiments, performed in three technical replicates. Statistical significance was calculated using one-way analysis of variance followed by Tukey's multiple comparison test. *, P≤0.05; **, P≤0.01; ***, P≤0.001. Primer sequences are indicated in the Appendix 1. RT-qPCR, reverse transcription quantitative polymerase chain reaction.

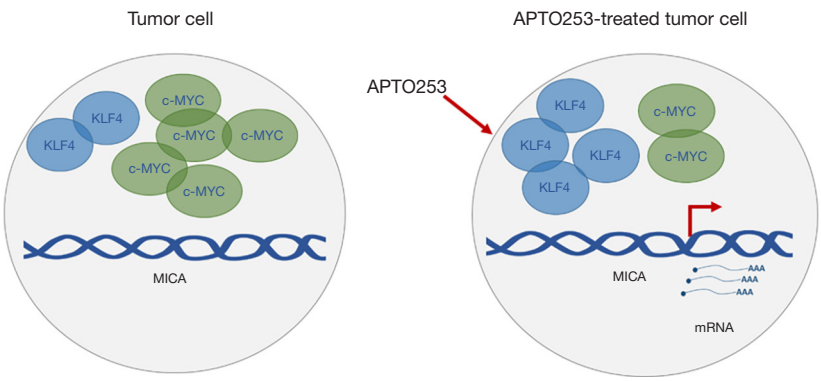

Figure 5 Schematic diagram of our hypothesis. The figure illustrates the mechanism of MICA-induced transcription in response to APTO253 through the upregulation of KLF4 associated with the downregulation of c-MYC.

to the expression of *MICA* and these mechanisms still need to be investigated. However, the *in vitro* data for AML are promising and show increased *MICA* expression and improved NK cell-dependent killing (20). We believe that studies in animal models and humans are warranted to test whether APTO253 can improve NK cell-mediated immune surveillance for a better therapy of AML.

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Footnote

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Ethical Statement: The authors are accountable for all

aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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