Peer Review File

Article information: https://dx.doi.org/10.21037/atm-24-20

Reviewer A

Comment 1: Given that APTO253 induces DNA damage (i.e. a stressful cellular event), it is expected that other NKG2D ligands will be upregulated in addition to MICA (i.e. MICB, ULBP1-6). Currently, it is misleading to the reader that this response is solely by MICA. Is there a way to show that this is specifically MICA? Can you exclude that no other NKG2D ligands respond in the same manner?

Reply: This is a valid question, since it is known that certain stimuli regulate specifically one ligand, whereas other molecular pathways induce the expression of different or all ligands (1). Therefore, we tested the impact of APTO253 on the expression of *MICB, ULBP2, ULBP5* and *ULBP6* in OVCAR8 and OVCAR4 cells. We did not observe any significant regulation of *ULBP2,5 and 6*, however a moderate induction of *MICB* was detectable in OVCAR8 cells. We present these data in a supplementary figure (Fig. 3).

Changes in the text: 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(1). However, other ligands than MICA were only moderately affected by APTO235. The expression of *ULBP2*, *5*, and *6* was not significantly up-regulated in OVCAR8 or OVCAR4 cells, and only a moderate induced expression of *MICB* was detectable in OVCAR8 and for all ligands in PANC1 cells (Figure 3) excluding a general regulation of NKG2D-L by APTO235. (page 4, line 84-90).

Legend to new Figure 3:

Figure 3: The effect of APTO253 on the expression of *MICB*, *ULBP2*, *-5*, and *-6* in different tumor cell lines. Ovarian and pancreatic cell lines were treated with 300 nM or 600 nM APTO253 for 24 hours. Then, cells were subjected to RT-qPCR analysis to evaluate mRNA expression of **A.** *ULBP2*, **B.** *ULBP5,* **C.** *ULBP6,* and **D.** *MICB*. Data are presented as mean ± SEM of three to five independent experiments, performed in three technical replicates. Statistical significance was calculated using one-way ANOVA followed by Tukey's multiple comparison test. $p^* \leq 0.05$, $p^{**} \leq 0.01$, $p^{***} \leq 0.001$.

2) Comment 2: MICA is highly polymorphic with numerous alleles, which can have different biological properties (e.g. MICA*008 is a highly prevalent truncated allele that can have low expression). Do you know if OVCAR4, OVCAR8 and PANC1 share

the same allele? This may explain the different responses seen.
Reply: The *MICA* genotype for OVCAR4, OVCAR8, and PANC1 was not determined. Given that *MICA* is highly polymorphic (2) it is possible that polymorphism, e.g. in the promoter region, affects the transcriptional expression. This was added to the main text.
Changes in the text: The high degree of *MICA* polymorphisms (2) may further contribute to the differences of cellular responses. (page 4, line 80-81).

3) Comment 3: The data so far is only transcript, but MICA and other NKG2D ligands can exhibit significantly different profiles at the protein level. For example, shedding of NKG2D ligands is very common is some tumor types. Is the MICA expression you see at the transcript level reflected at the protein level?
Reply: We measured the surface expression of MICA/B using flow cytometry and added a new figure (Fig.2). APTO253 treatment resulted in an increase of MICA/B surface expression in OVCAR8, but not in OVCAR4 or PANC1 cells, which is in line with the transcripts.
Changes in the text: 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). (page 4, line 81-83)
Legend to new Figure 2:
Figure 2: The effect of APTO253 on the protein expression of MICA/B in different tumor cell lines. Cells were treated with 300 nM 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 +/- SEM of four to five independent experiments. Statistical significance was calculated using one-way ANOVA followed by Turkey's multiple comparison test*$p < .05$; ** $p < .01$; *** $p < .001$; **** $p < .0001$

4) You note that "The molecular mechanisms responsible for the induction of NKG2D-L expression on malignant cells are still not fully understood" (lines 48-49), however this has been extensively researched and reviewed (e.g. see reviews by Baugh et al., 2020 (10.3390/cancers12123827) and Alves et al., 2021 (doi:10.3389/fimmu.2021.712722)). I would suggest clarifying that you are referring to mechanisms driving the induction of NKG2D-Ls in response to therapeutics. NKG2D-L regulation at baseline and in wildtype tumor/virally-infected cells has been extensively characterised.
Reply: Thanks for this comment. We followed this suggestion and changed the text accordingly.
Changes in the text: Several mechanisms regulating NKG2D-L expression are described(3, 4), however, the mechanisms driving the induction of NKG2D-Ls in

response to therapeutics are not well studied. (page 2, line 49-51).

5) Please overlay data points on the graph.
Reply: done

Reviewer B

This is a very brief report demonstrating that the small molecule APTO253 has divergent effects on two transcription factors (KLF4, Myc) and the NKG2DL, MICA when added to three solid tumor cell lines. However, I do not find the story convincing and the data do not really lead to a convincing conclusion.

Major comments

1. In the introduction, authors should comment on the double-edged role of the NKG2D/L axis in some cancers eg Curio et al Cancers (Basel). 2023 Mar 16;15(6):1792; Curio et al Discov Immunol. 2022 May 10;1(1):kyac002 and Sheppard et al Nat Commun. 2017 Jan 27;8:13930.
Reply: Thanks for this comment, see changes in the text below.
Changes in the text: Of note, recent studies report a pro-tumorigenic role of NKG2D reflecting the paradoxical role of NKG2D/NKG2D-L in cancer immunity(5-7). (page 2, line 47-48).

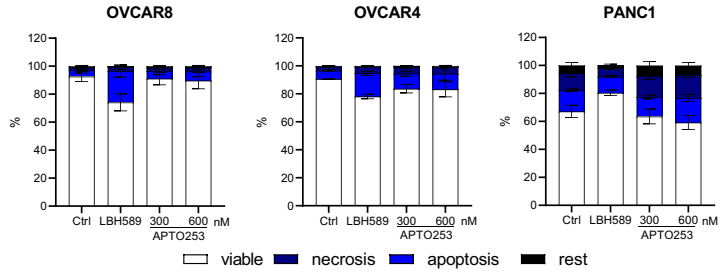
2. Have the authors attempted mechanistic experiments to support the assertion that both induction of KLF4 and down-regulation of c-MYC are important for the induction of MICA expression. This could have been addressed using RNA knockdown for example. Do the OVCAR8 data not refute this hypothesis since both Myc and KLF4 are significantly upregulated in addition to MICA upon treatment with APTO253.
Reply: The molecular mechanism of APTO253-induced expression via KLF4/c-MYC is of course interesting and part of our ongoing research activities, however beyond the scope of this Brief Report.

3. Have authors looked at MICA induction/ expression at the protein level.
Reply: Yes, we did and added a new Figure.
Changes in the text: 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). (page 4, line 81-83)
Legend to new Figure 2:

Figure 2: The effect of APTO253 on the protein expression of MICA/B in different tumor cell lines. Cells were treated with 300 nM 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 +/- SEM of four to five independent experiments. Statistical significance was calculated using one-way ANOVA followed by Turkey's multiple comparison test*$p < .05$; ** $p < .01$; *** $p < .001$; **** $p < .0001$

4. Unpaired t-test is not a suitable analysis where there are more than 2 comparators.
Reply: This was changed to one-way ANOVA with Tukey's multiple comparison test as indicated in the figure legends.

5. How were inhibitor concentrations selected.
Reply: We used sublethal APTO325 concentrations. The viability of the cells was not affected by the APTO253 concentrations used as depicted in the figure below.



Cellular toxicity of LBH589 and APTO253. Ovarian and pancreatic cell lines were treated with 100 nM LBH589 or 300 nM & 600 nM APTO253 for 24 hours. Then, cells and supernatant were collected and cell death was measured using AnnexinV / PI staining. Data are presented as mean ± SEM of three to four independent experiments.
Changes in the text: ….APTO253 at sublethal, non-toxic concentrations..(page 3, line 73).

1. Zingoni A, Molfetta R, Fionda C, Soriani A, Paolini R, Cippitelli M, et al. NKG2D and Its Ligands: "One for All, All for One". Front Immunol. 2018;9:476.
2. Klussmeier A, Massalski C, Putke K, Schafer G, Sauter J, Schefzyk D, et al. High-Throughput MICA/B Genotyping of Over Two Million Samples: Workflow and Allele Frequencies. Front Immunol. 2020;11:314.
3. Baugh R, Khalique H, Seymour LW. Convergent Evolution by Cancer and Viruses in Evading the NKG2D Immune Response. Cancers (Basel). 2020;12(12).
4. Alves E, McLeish E, Blancafort P, Coudert JD, Gaudieri S. Manipulating the

NKG2D Receptor-Ligand Axis Using CRISPR: Novel Technologies for Improved Host Immunity. Front Immunol. 2021;12:712722.
5. Curio S, Edwards SC, Suzuki T, McGovern J, Triulzi C, Yoshida N, et al. NKG2D signaling regulates IL-17A-producing gammadeltaT cells in mice to promote cancer progression. Discov Immunol. 2022;1(1):kyac002.
6. Curio S, Lin W, Bromley C, McGovern J, Triulzi C, Jonsson G, et al. NKG2D Fine-Tunes the Local Inflammatory Response in Colorectal Cancer. Cancers (Basel). 2023;15(6).
7. Sheppard S, Guedes J, Mroz A, Zavitsanou AM, Kudo H, Rothery SM, et al. The immunoreceptor NKG2D promotes tumour growth in a model of hepatocellular carcinoma. Nat Commun. 2017;8:13930.