nature portfolio

Peer Review File

TOPORS E3 ligase Mediates Resistance to Hypomethylating Agent Cytotoxicity in Acute Myeloid Leukemia Cells

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, Truong et al. perform a CRISPR screen to identify genes which deficiency cause sensitivity to HMAs in MDS. They identify TOPORS as a factor which deficiency cause sensitivity to these agents. Next, the authors perform a variety of genome/proteome wide studies where the observe HMAs-dependent efeccts in the TOPORS affected cells. Finally, the authors explore the efficacy of combined therepy with HMA and SUMO inhibitors against MDS, they conclude that this combinatorial treatment kills cancer cells but do not affect healthy cells.

Overall, the experiments are well executed and the amount of -omics data is cummulous, no extra experiments should be required in this regard. However, the authors cannot exclude the fact that when differences between control and TOPORS-deficient arise,for example in the splicing experiments, these are due to an increased amount of DNA damage due to the lack of TOPORS and presence of AZA-caused DNA-protein crosslinks. Mechanistical aspects are lacking in the manuscript.

While the efficacy of of double treatment of AZA+TAK943 in hematopietical malignancies is not a novel observation, the authors prove that these in combination do not affect healthy cells. Thus, enhancing its potential as a therapeutical approach.

THis reviewer thinks that the conclusions proposed by the authors from the omics data are sufficiently supoported in a mechanistic manner and could all be due to indirect effect of the differential induction of DNA damage that cannot be repaired in the absence of TOPORS. THe novelty of the other data is not sufficient for publication in Nature Communications.

Reviewer #2 (Remarks to the Author):

Truong et al. identified TOPORS as a critical driver of HMA/VEN resistance through a high-throughput screen. The implications of these findings are highly significant to the field, as resistance to HMA/VEN leads to dismal outcomes. The study is rigorous and well-described. I have several questions that need clarification:
• In Figure 1, the deletion of TOPORS, UBE2K, and UBXN7 only leads to partial suppression of AZA-treated cells. However, in subsequent figures, it appears that AZA treatment leads to complete suppression of TOPORS-edited cells. Please address.
• The authors need to specify why the cells are pre-treated with AZA. This was not clear in the description of the results.
• What is the expression of TOPORS in normal hematopoietic cells compared to MDS/AML?
• In Figure 4E, TOPORS-edited MDSL cells showed greater sensitivity to AZA. An additional cell line should be validated to increase rigor.
• The role of E2F1 in AZA-treated TOPORS-edited MDSL cells is overstated (Figure 5/S4). I suggest the authors provide alternative interpretations of these findings. For example, E2F1 activity may be enhanced in these cells as a compensatory mechanism but not one that drives the phenotype. The role of SRSF1 is also overstated (Figure 4/S4C).
• The immunoblots in Figure 6 are not convincing. For example, the molecular weight of DNMT1 is 250 kDa, but the bands are well below 198 kDa.

Reviewer #3 (Remarks to the Author):

This manuscript by Pimanda and colleagues presents an investigation into overcoming resistance to hypomethylating agents (HMAs) in Myelodysplastic Syndromes (MDS) and Acute Myeloid Leukemia (AML) through targeting TOPORS, a dual E3 ubiquitin and SUMO ligase, identified via a genome-wide CRISPR-Cas9 screen. The study demonstrates that depletion of TOPORS or inhibition of SUMOylation sensitizes leukemic cells to HMAs, enhancing treatment efficacy without affecting healthy hematopoiesis. TAK-981 emerges as a promising pharmacological surrogate for TOPORS targeting, offering a new therapeutic strategy for high-risk MDS and AML patients. This approach, leveraging the impaired DNA damage response in TOPORS-depleted cells, presents a rational framework for developing combinatorial treatments to enhance HMA effectiveness. The study is very extensive, using multiple models and state-of-the-art techniques.

Major Issues:
- Selection of Cell Models: The rationale behind using MOLM13 cells (TP53 wild type) for further experiments contradicts the initial aim to identify synergistic drugs for TP53 mutant refractory MDS. The authors should clearly explain their choice of model and how it relates to their research goal.

- Marginal Improvement in Drug Response: The difference in azacitidine (AZA) drug response curves for TOPORS knockout in TF-1 and KASUMI-1 cells appears minimal, questioning the general clinical significance to all types of AML. Similarly, the median survival extension by ~4 days in MOLM13-transplanted mice with TOPORS knockout may not be biologically significant. These limitations should be clearly stated and discussed.

- The assertion that TOPORS knockout minimally affects normal hematopoiesis, based on peripheral blood and bone marrow analysis in MISTRG mice, lacks direct comparison to primary AML blasts (or even cell lines), potentially oversimplifying the impact on normal hematopoiesis.

- The omission of survival analysis in patient-derived xenograft (PDX) models treated with AZA and TAK-981 shows limitation on the full therapeutic potential and synergy of the treatment, which should be clearly stated in the manuscript.

- The claim that defective SRSF6 binding underlies the crucial mechanism of TOPORS deficiency lacks direct experimental validation, such as whether SRSF6 knockdown would mimic TOPORS knockout effects.

- The manuscript's alternating focus between decitabine (DAC) and AZA without clear rationale could confuse the readership regarding therapeutic context and implications for treatment strategy.

Minor points:
- Clarity and Size of Figures: Figures 2F, 5B, and 5E are too small and confusing, impairing interpretability.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, Truong et al. perform a CRISPR screen to identify genes which deficiency cause sensitivity to HMAs in MDS. They identify TOPORS as a factor which deficiency cause sensitivity to these agents. Next, the authors perform a variety of genome/proteome wide studies where the observe HMAs-dependent efeccts in the TOPORS affected cells. Finally, the authors explore the efficacy of combined therepy with HMA and SUMO inhibitors against MDS, they conclude that this combinatorial treatment kills cancer cells but do not affect healthy cells.

Overall, the experiments are well executed and the amount of -omics data is cummulous, no extra experiments should be required in this regard. However, the authors cannot exclude the fact that when differences between control and TOPORS-deficient arise, for example in the splicing experiments, these are due to an increased amount of DNA damage due to the lack of TOPORS and presence of AZA-caused DNA-protein crosslinks. Mechanistical aspects are lacking in the manuscript.

We too were concerned that differential effects such as RNA splicing, may be a result of increased DNA damage rather than a direct consequence of TOPORS-deficiency. We have now included analysis of mis-splicing induced by *TOPORS*-editing in vehicle-treated cells (Fig. 5C–D), which demonstrates enrichment in *TOPORS*-edited cells for mis-splicing in DDR genes even in the absence of AZA-treatment. Nonetheless, we acknowledge that we cannot “exclude the likelihood that some form of DNA damage is necessary for elevated mis-splicing to occur in *TOPORS*-edited cells.” (page 13, lines 20-22) We were already careful to make comparisons in the presence and absence of AZA in our proteomics analyses to ensure that a distinction was made between differentials induced by TOPORS-deficiency alone versus TOPORS-deficiency in the presence of AZA.

While the efficacy of of double treatment of AZA+TAK943 in hematopietical malignancies is not a novel observation, the authors prove that these in combination do not affect healthy cells. Thus, enhancing its potential as a therapeutical approach. THis reviewer thinks that the conclusions proposed by the authors from the omics data are sufficiently supoported in a mechanistic manner and could all be due to indirect effect of the differential induction of DNA damage that cannot be repaired in the

absence of TOPORS. THe novelty of the other data is not sufficient for publication in Nature Communications.

If the novelty that the reviewer comments on here refers to reference #58 (the publication by Gabellier et al https://doi.org/10.3324/haematol.2023.282704), please note that although the authors in that paper treated cell line (THP1) engrafted mice for 3 cycles of AZA/TAK-981 and calculated probability of survival, their PDX mice were treated for a single cycle and culled at day 9 for assessment of hCD45 blasts in mouse bone marrows. We consider this inadequate for a pre-clinical assessment of drug tolerance and efficacy. In addition to experiments with healthy CD34+ engrafted MISTRG mice to assess impact of these drugs alone and in combination on renewal/differentiation of normal blood stem/progenitor cells, we now include PDX data from two independent AML patients (AML-5 and AML-16) with 2 or 3 cycles of treatment (AZA, TAK-981 and AZA+TAK-981) and weekly follow up assessment of peripheral blood chimerism and proper estimation of event/survival. Please note that our ethics approval for AML PDX required survival endpoint to be defined by % engraftment, not illness. Taken together, from the viewpoint of preclinical assessment of HMA+ TAK-981- these data are both novel and impactful.

If the comment was in reference to #62 and #63, where the authors also identified TOPORS as a resistance gene to hypomethylating agents, it is remarkable that several research groups working independently of each other identified the same gene despite variations in methodology. As editorialised by Nature Communications (*Nat Commun* **11**, 4466 (2020). https://doi.org/10.1038/s41467-020-17817-x), there is strength in numbers, and although we believe that our oral presentation at the American Society of Hematology meeting (2022; *Blood* (2022) 140 (Supplement 1): 220–221.https://doi.org/10.1182/blood-2022-165713) and target identification preceded all others (International (PCT) Patent Application No. PCT/AU2023/050210) - we have taken care to progress outcomes of our screen towards human trials by rigorous *in vivo* pre-clinical testing of AZA+TAK981 as a promising combination for AML.

Reviewer #2 (Remarks to the Author):

Truong et al. identified TOPORS as a critical driver of HMA/VEN resistance through a high-throughput screen. The implications of these findings are highly significant to the field, as resistance to HMA/VEN leads to dismal outcomes. The study is rigorous and well-described. I have several questions that need clarification:

• In Figure 1, the deletion of TOPORS, UBE2K, and UBXN7 only leads to partial

suppression of AZA-treated cells. However, in subsequent figures, it appears that AZA treatment leads to complete suppression of TOPORS-edited cells. Please address.

Figure 1 shows increasing depletion of TOPORS-edited cells over time (up to 16 days) using treatment with a single dose (0.3μM) of AZA. Figures 2 and 7 show EC50 curves produced after culturing cells for 5 days with serial diluted doses of AZA or other drugs. The highest drug concentration in the EC50 curves was selected to be almost 100% lethal over the 5 day time frame of EC50 experiments.

• The authors need to specify why the cells are pre-treated with AZA. This was not clear in the description of the results.

Cells were pre-treated with AZA only in specific contexts. In Fig 1A, AZA-treatment prior to sgRNA sequencing was the means by which drop-out hits were selected from the genome wide sgRNA library. In Fig. 3B, the purpose was to test in vitro how exposure of healthy *TOPORS*-edited HSPC to AZA affected their differentiation capacity; CRISPR-engineered cord blood CD34+ cells were cultured with HSPC-supporting cytokines and treated with AZA before CD34+ cells were re-sorted and plated into methylcellulose medium to undergo differentiation in vitro. The text on page. 9, lines 16-17 has been edited to clarify this. In Fig. 3C, mice engrafted with CRISPR-engineered cord blood CD34+ cells were treated with AZA or vehicle several weeks before subsequent treatment of the same mice with DAC or vehicle to test if healthy *TOPORS*-ko CD34+ cells continued to produce mature blood cells following exposure to either drug. Mature human blood cells were quantified after each drug treatment as indicated in Fig. 3C.

• What is the expression of TOPORS in normal hematopoietic cells compared to MDS/AML?

We have included an extra panel (now panel A) in Fig. S3 preceding experiments that we performed to evaluate the consequences of TOPORS depletion on healthy hematopoiesis, to show *TOPORS* mRNA levels in various HSPC and AML cell types, and added the following text to p.9, lines 9-11: “In AML cells, TOPORS mRNA levels are highest in LSC, but they are even higher in healthy HSC, MPP and MEP (Fig. S3A). This raised the possibility that targeting TOPORS would be toxic to healthy haematopoiesis.”

• In Figure 4E, TOPORS-edited MDSL cells showed greater sensitivity to AZA. An additional cell line should be validated to increase rigor.

In Fig. 2 and Fig. S2 we showed that four different MDS/AML lines in (MDS-L, Kasumi-1, MOLM-13 and TF-1 cell lines) were sensitized to AZA or DAC once depleted of TOPORS, demonstrating with rigor that sensitization to HMA by TOPORS depletion was reproducible in AML. Furthermore, combinatorial treatment with TAK-981 and AZA

(Fig. 9A and Fig. S7A) or TAK-981 and DAC (Fig. 9B and Fig. S7B) was also performed in all four cell lines.

• The role of E2F1 in AZA-treated TOPORS-edited MDSL cells is overstated (Figure 5/S4). I suggest the authors provide alternative interpretations of these findings. For example, E2F1 activity may be enhanced in these cells as a compensatory mechanism but not one that drives the phenotype. The role of SRSF1 is also overstated (Figure 4/S4C).

We agree with the reviewer and do not claim that E2F1 is driving a DNA damage response specifically when TOPORS is depleted; we only point out that much of the DDR transcription evident in AZA-treated *TOPORS*-edited cells correlates with the presence of E2F binding sites in the DDR gene promoters, as does the DDR transcription in DDR responses described by others (references 43-44). We have further clarified this in the revised text (page 12, line 17-18).

We also agree that our data suggesting a role for SRSF1 in the RNA mis-splicing we characterized in AZA-treated *TOPORS*-edited cells may have been over-interpreted and have removed the supplemental motif maps and modified the text accordingly (pages 13-14).

• The immunoblots in Figure 6 are not convincing. For example, the molecular weight of DNMT1 is 250 kDa, but the bands are well below 198 kDa.

We believe the immunoblots are convincing for the following reasons. The molecular weight of the common human DNMT1 isoform is 183 kDa (the minor DNMT1b variant is 16 residues (1%) larger). 183 kDa is consistent with the mobility of the major anti-DNMT1 signal detected in Fig 6C. The immunoblot was sequentially probed with anti-DNMT1, then anti-actin, then anti-SUMO2/3, with stripping between probes. The DNMT1 signal is obviously lower in the DAC-treated triplicates than in the vehicle-treated triplicates, and the SUMO signal is obviously highest in the DAC-treated sgTOPORS triplicates. Furthermore, the immunoblot data are consistent with the independent LC-MS/MS data presented alongside the immunoblot data. We have no doubt therefore that (1) DNMT1 is depleted in both sgTOPORS and sgCONTROL cells following days exposure to AZA, and (2) that protein SUMO2/3ylation is markedly elevated in sgTOPORS cells exposed to AZA.

Reviewer #3 (Remarks to the Author):

This manuscript by Pimanda and colleagues presents an investigation into overcoming resistance to hypomethylating agents (HMAs) in Myelodysplastic Syndromes (MDS)

and Acute Myeloid Leukemia (AML) through targeting TOPORS, a dual E3 ubiquitin and SUMO ligase, identified via a genome-wide CRISPR-Cas9 screen. The study demonstrates that depletion of TOPORS or inhibition of SUMOylation sensitizes leukemic cells to HMAs, enhancing treatment efficacy without affecting healthy hematopoiesis. TAK-981 emerges as a promising pharmacological surrogate for TOPORS targeting, offering a new therapeutic strategy for high-risk MDS and AML patients. This approach, leveraging the impaired DNA damage response in TOPORS-depleted cells, presents a rational framework for developing combinatorial treatments to enhance HMA effectiveness. The study is very extensive, using multiple models and state-of-the-art techniques.

Major Issues:

- Selection of Cell Models: The rationale behind using MOLM13 cells (TP53 wild type) for further experiments contradicts the initial aim to identify synergistic drugs for TP53 mutant refractory MDS. The authors should clearly explain their choice of model and how it relates to their research goal.

Our study was initially designed around MDS-L because it is uniquely an MDS cell line and pertinent to our goal of overcoming HMA resistance where such monotherapy is used. The study was not designed to target *TP53*-mutant AML specifically, nor did it become focussed on *TP53*-mutant cells. Nonetheless, we came to notice differences in synergy between TAK-981 and HMA that correlated with *TP53* status in our cohort of cell lines. Our attention switched to AML cells for later biochemical and *in vivo* studies because the study became focussed on cytotoxic cell killing, which is more pertinent to AML than MDS (see reference 70 in the manuscript), rather than differentiation or differentiation-induced killing. The choice of MOLM13 for the SUMO pull down assays and *in vivo* drug testing rather than MDS-L, was dictated more by the higher proliferation rate of MOLM-13. We have made a text change to the introduction to clarify our intent (page 4, lines 14-16).

- Marginal Improvement in Drug Response: The difference in azacitidine (AZA) drug response curves for TOPORS knockout in TF-1 and KASUMI-1 cells appears minimal, questioning the general clinical significance to all types of AML. Similarly, the median survival extension by ~4 days in MOLM13-transplanted mice with TOPORS knockout may not be biologically significant. These limitations should be clearly stated and discussed.

The impact of *TOPORS*-ko on AZA sensitivity was indeed lower in TF1 and Kasumi than MDS-L and MOLM-13. However, EC50 responses are measured after 4 days frug exposure. The impact of small changes in sensitivity amplifies over longer time frames as demonstrated by Fig 1E. We have clarified this in the revised text on page 8, lines 18-21.

The impact of *TOPORS*-ko on *in vivo* AZA sensitivity of MOLM-13 was reduced by the fact that only one cycle of AZA was used. Nonetheless, as our updated text on page 9, line 4-5 points out, that one cycle of treatment "increased median post-treatment survival time by 50% relative to controls".

- The assertion that TOPORS knockout minimally affects normal hematopoiesis, based on peripheral blood and bone marrow analysis in MISTRG mice, lacks direct comparison to primary AML blasts (or even cell lines), potentially oversimplifying the impact on normal hematopoiesis.

Given that HMAs are already used to treat MDS and AML in patients, our foremost objective was to evaluate whether the capacity of TOPORS knockout human CD34+ cells to proliferate and differentiate *in vivo* in the presence of either AZA or DAC (versus vehicle control) was comparable to that of control human CD34+ (Fig.3C-G). *TOPORS*-edited xenografts were in competition with non-edited cord blood cells plus mouse blood cells at all times. This was an experimental tour de force that allowed us to establish that editing TOPORS did not confer a selective disadvantage to engraftment, proliferation, or differentiation in the presence of HMAs or vehicle control.

Direct comparison using AML/cord blood BM chimaeras were not practicable for the following reasons: (1) it would have been difficult to reliably distinguish healthy human myeloid cells in circulation from AML cells in mixed BM chimaeras of the complexity proposed by the reviewer; (2) over-engraftment of MISTRG mice with human haematopoietic cells kills the host mice *via* anaemia, because human RBC cannot substitute in circulation for mouse RBC (see PMID 30696625) – an experience that we are all too familiar with; and (3) co-engrafted AML cells had potential to kill directly host mice before treatment of cord blood cells was complete.

Nonetheless, our data show that novel drug targeting of TOPORS activity is unlikely to enhance HMA toxicity in healthy hematopoiesis with clinical use alongside HMAs. We have clarified these points in the revised text (page 9, lines 22-24 & page 10, lines 19-20).

- The omission of survival analysis in patient-derived xenograft (PDX) models treated with AZA and TAK-981 shows limitation on the full therapeutic potential and synergy of the treatment, which should be clearly stated in the manuscript.

This omission has been rectified in the revised manuscript now that two cohorts of independent AML PDX have completed survival analysis. Fig. 9 and text on page 19, lines 4-12 have been updated accordingly.

- The claim that defective SRSF6 binding underlies the crucial mechanism of TOPORS

deficiency lacks direct experimental validation, such as whether SRSF6 knockdown would mimic TOPORS knockout effects.

We did not intend to convey the impression that defective SRSF6 binding underlies the crucial mechanism of TOPORS mediated HMA resistance. We acknowledge and now emphasise in the revised manuscript that TOPORS' potential functions in splicing and regulation of SRSF6 are secondary to TOPORS' function in clearing DNA-protein adducts. We have added an rMAPS analysis for SRSF6 motifs in vehicle-treated *TOPORS*-edited versus control cells (Fig. 5G) showing that SRSF6 motifs are enriched in differentially spliced mRNAs from *TOPORS*-edited cells even in the absence of AZA, and downplayed the functional importance of splicing in mediating AZA-resistance on pp. 13 (lines 12-22), 14 (lines 3-12), 17 (line 21) and 22 (lines 11-12).

SRSF6 sgRNAs produced beta values close to zero in both arms of our original CRISPR screen (see Table. S2B), so we do not expect SRSF6 knockdown can mimic TOPORS-deficiency. We have made that apparent on p. 22 lines 11-12.

- The manuscript's alternating focus between decitabine (DAC) and AZA without clear rationale could confuse the readership regarding therapeutic context and implications for treatment strategy.

We used DAC in some experiments (1) to make it clear that TOPORS-mediated AZA-resistance extends to the other common HMA, DAC, (2) that TOPORS-deficient healthy cord blood cells could survive treatment with either AZA or DAC, or to be certain that observed phenomena were due to incorporation of HMA into DNA and not RNA. We have removed DAC from Fig. 4 (where it was superfluous) and made minor alterations to text on pages 8 (lines 18-21), 10 (lines 6, 19-20), 14 (lines 3-12), 15 (lines 24- 25) to clarify the purpose of DAC usage.

Minor points:

- Clarity and Size of Figures: Figures 2F, 5B, and 5E are too small and confusing, impairing interpretability.

Panel sizes and layout have been adjusted in these Figures.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

I am convinced by the explanations given by the authors. The novelty issue was not only based on references #58, #60 & #61 but also on (Kroonen et al. 2023 Leukemia). Mechanisitcal aspects are still missing. However, given thre recent publications of #60 & #61, these aspects should be overseen for publicaion.

Reviewer #2 (Remarks to the Author):

The authors have addressed my major concerns. I have no additional comments.

Reviewer #3 (Remarks to the Author):

The authors have sufficently addressed the reviewers' concerns to further improve their manuscript.

RESPONSE TO REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

I am convinced by the explanations given by the authors. The novelty issue was not only based on references #58, #60 & #61 but also on (Kroonen et al. 2023 Leukemia). Mechanisitcal aspects are still missing. However, given the recent publications of #60 & #61, these aspects should be overseen for publicaion.

We thank the reviewer for their comments and have included Kroonen et al as an additional reference.

Reviewer #2 (Remarks to the Author):

The authors have addressed my major concerns. I have no additional comments.
We thank the reviewer for their comments.

Reviewer #3 (Remarks to the Author):

The authors have sufficently addressed the reviewers' concerns to further improve their manuscript.
We thank the reviewer for their comments.