Exploring the potential of saruparib in overcoming venetoclax resistance in AML

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Abstract

Acute myeloid leukaemia (AML) is a highly aggressive type of blood cancer. In particular, elderly and medically unfit patients face a poor prognosis. Treatment with venetoclax and azacitidine improved the outcome for these patients, yet drug resistance remains a significant issue. Combining the poly (ADP-ribose) polymerase (PARP)1/2 inhibitor talazoparib with venetoclax and azacitidine was suggested as a potential treatment option. However, PARP2 inhibition is associated with haematological toxicities. This study explored the therapeutic potential of the selective PARP1 inhibitor saruparib compared to talazoparib in overcoming venetoclax resistance when administered combined with venetoclax and/or azacitidine. Cytotoxic effects in venetoclax resistant AML cell lines were studied in drug sensitivity assays and time to progression assays mimicking the clinical administration of the drugs. Induction of DNA damage and apoptotic signalling were measured to assess molecular mechanisms upon treatment. The triple combination of saruparib, venetoclax and azacitidine effectively induced DNA damage and apoptotic signalling leading to cell death. Talazoparib combined with venetoclax and/or azacitidine induced slightly higher levels of DNA damage and apoptotic signalling, resulting in lower cell viability. In time to progression assays, the triple combination with saruparib, venetoclax and azacitidine effectively inhibited cell expansion but only for the duration of azacitidine administration, suggesting azacitidine as an essential cytotoxic factor. Conversely, the talazoparib triple combination effectively eradicated AML cells with intrinsic venetoclax resistance, possibly due to the additional effect of PARP2 and/or PARP16 inhibition. This indicates that PARP1 inhibition in combination with venetoclax and azacitidine might not be sufficient to overcome venetoclax resistance.

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Introduction

Drug resistance is one of the major challenges in cancer treatment. Many patients fail to respond initially to treatment or will eventually relapse and develop resistance. Therefore, new approaches are needed to overcome treatment resistance and improve survival rates (1).

Acute myeloid leukaemia

Acute myeloid leukaemia (AML) is a highly aggressive type of blood cancer that mainly affects the elderly population, with a median age at diagnosis of 68 years. The prognosis is poor with a 5-year overall survival rate of 30%. However, for patients above 60 years the survival rate decreases to less than 10% (2).

AML is a heterogeneous disease that is characterised by multiple mutations in genes involved in haematopoiesis (3). In normal haematopoiesis, haematopoietic stem cells differentiate into multipotent progenitor cells, which can further differentiate into all lineages of blood cells. In AML, haematopoietic stem or progenitor cells acquire recurrent mutations that can lead to increased survival and proliferation, turning them into leukemic stem cells. These give rise to leukemic blasts, which are immature cells that lack the ability to differentiate into functioning blood cells (4). The increased proliferation of leukemic cells causes an accumulation of myeloid blasts in the bone marrow, which can eventually infiltrate the blood and other tissues. This causes life-threatening symptoms, such as immunodeficiency and cytopenia (5).

For diagnosis, AML is generally defined as having at least 10-20% myeloid blasts in the peripheral blood or bone marrow. However, more emphasis is now being put on including genetic markers as diagnostic factors. In the 2022 guidelines from the World Health Organization, patients with genetic abnormalities such as *RUNX1::RUNX1T1* fusion or *NPM1* mutation can be diagnosed with AML irrespective of the blast cell count. Genetic markers are also being used to determine the prognosis of the disease, where mutations in genes such as *TP53* and gene fusions such as *BCR::ABL1* are associated with a poor prognosis (2). Another classification of AML is as de novo (dn-AML), secondary (s-AML) or therapy-related AML (t-AML). Most cases arise de novo, without pre-existing medical history of haematological diseases or cancers, as compared to s-AML where the leukaemia arises from another haematological disease. T-AML is caused by previous treatment with cytotoxic agents. Both s-AML and t-AML are associated with poorer outcomes compared to dn-AML (6).

Current treatments

The standard of care in AML is an intensive chemotherapy regimen with 3 days of cytarabine and 7 days of anthracycline. In patients that respond and achieve complete remission (CR), this is followed by consolidation treatment to achieve long-term remission. Patients with low-risk disease receive additional cycles of chemotherapy whereas patients with intermediate or high-risk disease are considered for allogeneic haematopoietic stem cell transplantation. However, elderly patients are often ineligible for intensive treatments due to comorbidities and medical unfitness. Instead, the new standard of care for these individuals is treatment with venetoclax in combination with a hypomethylating agent, such as azacitidine (2). Azacitidine is given for the first seven days whereas venetoclax is given once daily throughout the 28-day treatment cycle (7).

In haematological malignancies, the DNA is extensively methylated within promoter regions, resulting in the silencing of tumour suppressor genes. As these genes are involved in apoptosis

and cell cycle regulation, silencing can allow the cancer cells to proliferate uncontrollably and avoid apoptosis (8). Azacitidine reverses methylation by incorporating into the DNA and irreversibly binding to DNA methyltransferase (DNMT)1, inhibiting its methylating function during replication, and causing its degradation. This induces DNA hypomethylation leading to reactivation of aberrantly silenced genes. In addition, the azacitidine-DNMT1 complex also induces DNA damage repair pathways, which triggers cell cycle arrest and apoptosis (9). In the clinics, combining azacitidine with venetoclax increases the overall survival rates compared to administering azacitidine as a monotherapy (10).

Venetoclax is an inhibitor that induces apoptosis by selectively blocking the anti-apoptotic protein B-cell lymphoma-2 (BCL-2), which is part of the BCL-2 family (11). BCL-2 family proteins are the key regulators of the intrinsic apoptosis pathway and consists of both pro-apoptotic protein such as BCL-2-associated X protein (BAX) and BCL-2 homologous antagonist/killer (BAK) as well as anti-apoptotic proteins, such as BCL-2, myeloid cell leukemia-1 (MCL-1) and B-cell lymphoma-extra large (BCL-XL). In the absence of apoptosis-inducing signals, the pro-apoptotic proteins are bound to the anti-apoptotic proteins and are thereby inactivated. Upon intrinsic cellular stress, the pro-apoptotic proteins are released which leads to BAX and BAK forming pores through the outer mitochondrial membrane, a process known as mitochondrial outer membrane permeabilization (MOMP). This causes release of cytochrome c into the cytoplasm, which triggers the formation of the apoptosome and activation of the effector caspases-3 and -7 (11, 12). These cleave poly (ADP-ribose) polymerase (PARP) and other substrates which leads to DNA degradation, cell disintegration and eventually cell death (13). In AML cells, the anti-apoptotic protein BCL-2 is often overexpressed, making it an interesting therapeutic target. Overexpression of BCL-2 disrupts the balance between pro- and anti-apoptotic proteins, which causes increased survival of the cancer cells as they can evade apoptosis (11). Figure 1 shows the function of venetoclax and how the BCL-2 family proteins are involved in the intrinsic apoptosis pathway.

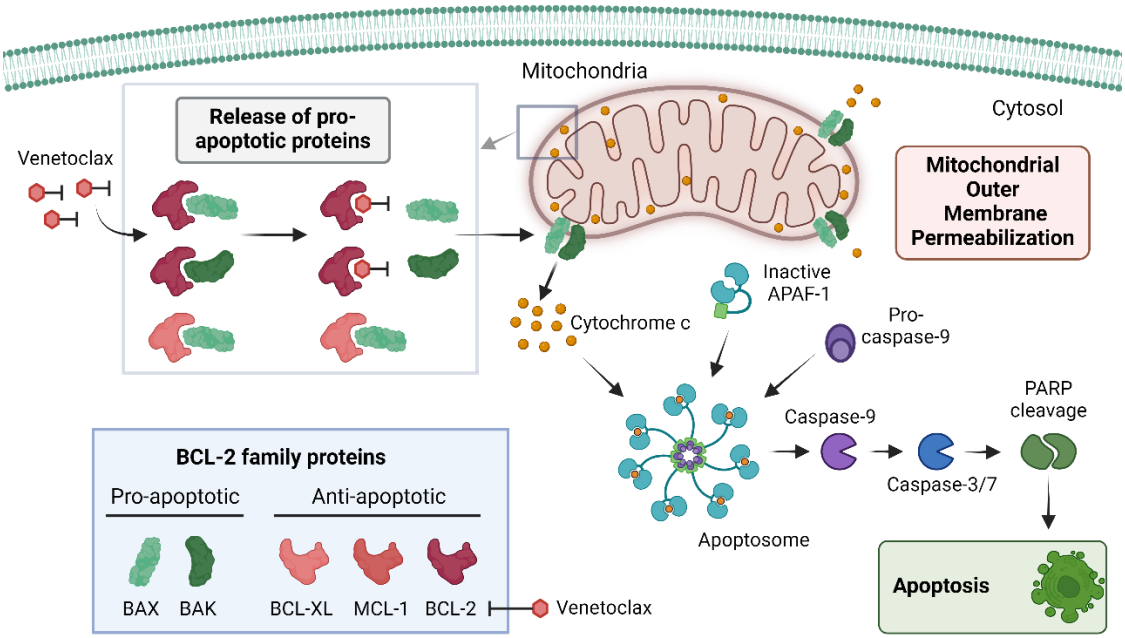

Figure 1. The role of venetoclax and the BCL-2 family in the intrinsic apoptosis pathway. Following exposure to venetoclax, pro-apoptotic proteins are released from BCL-2, causing mitochondrial outer membrane permeabilization. This leads to formation of the apoptosome, which activates caspases that induce apoptosis. Created with BioRender.com.

Although treatment with venetoclax and azacitidine has proven effective for many patients ineligible for chemotherapy, around 30-40% of patients are clinically resistant to venetoclax. Furthermore, most patients that respond initially eventually relapse because of acquired resistance to the treatment. This shows that while the azacitidine-venetoclax treatment provides a significant clinical benefit and extended survival for the elderly/unfit AML patient group, it is not a curative treatment and there is therefore a need for novel treatment options (11).

Potential treatment options

Previous investigation in the Wennerberg group indicated that venetoclax induces DNA damage and apoptotic signalling in *in vitro* generated venetoclax resistant (venR) AML cell lines. While the cell growth and survival remained unaffected, an increase in cytochrome c in the cytoplasm and cleavage of caspase-3 were observed. This indicates the activation of the intrinsic apoptotic pathway but not to the extent of causing cell death (14). These effects could be due to minority MOMP, which is when only a minority of mitochondria undergo membrane permeabilization, resulting in limited cytochrome c release and caspase activation. Repeated triggering of minority MOMP leads to DNA damage and genomic instability (15). The increased DNA damage following exposure to venetoclax might make cells more dependent on the DNA damage repair machinery. Consequently, inhibition of DNA damage repair could provide a targetable vulnerability to overcome venetoclax resistance (14).

One of the first enzymes recruited to DNA lesions following DNA damage is PARP1, which is part of the PARP family. PARP1 and PARP2 can repair multiple kinds of DNA lesions in a process called PARylation, where PARP binds to the damaged DNA and adds chains of poly (ADP-ribose) to itself and surrounding proteins. This leads to recruitment of other proteins involved in DNA damage repair (16). PARP1 accounts for 80-95% of the total PARylation and PARP2 for the remaining 5-20% (17). The importance of PARP enzymes in the DNA damage response makes them interesting therapeutic targets (18).

PARP inhibitors (PARPis) are currently approved for treatment of various cancers with a deficiency in DNA damage repair. PARPis compete for the active site, and thus block the catalytic function of PARP (19). Additionally, in a mechanism called PARP-trapping, they stabilise the DNA-PARP1 or -PARP2 complex, which creates a physical block for the replisome, causing replication fork collapse and DNA double-strand breaks (17). Inhibition of PARP1 therefore creates accumulated DNA damage, genomic instability and eventually cell death (18). Figure 2 shows the function of PARPis.

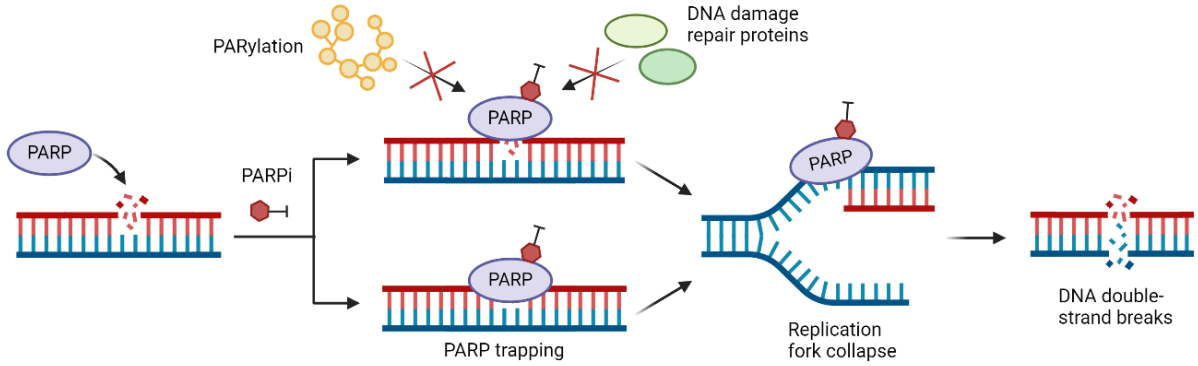

Figure 2. The functions of PARP inhibitors. Binding of PARP inhibitor (PARPi) blocks the catalytic function of PARP and traps PARP on the DNA. This causes replication fork collapse and leads to DNA double-strand breaks. Created with BioRender.com.

Talazoparib is a very potent PARP1/2 inhibitor that is approved for treatment of multiple cancers (20). In AML cells, combination treatment of talazoparib with azacitidine demonstrated synergistic effects by increased PARP-trapping compared to the monotherapies *in vitro*. Moreover, the combinational treatment reduced the tumour burden *in vivo* and increased the survival of AML xenograft mice (21). Similarly, treatment of AML cells with a combination of venetoclax and talazoparib increased DNA damage and apoptosis *in vitro* as compared to the single agents (22). However, in clinical settings talazoparib treatment is associated with toxicities similar to those observed with chemotherapeutics, including anaemia, neutropenia and thrombocytopenia (20). Toxicities are mainly thought to be associated with PARP2 inhibition, which led to development of the selective PARP1 inhibitor saruparib. Compared to PARP1/2 inhibitors, saruparib demonstrated greater efficacy in decreasing the tumour burden with minimal haematologic toxicity in animal models (17). Currently, saruparib is tested in a phase I/IIa clinical trial (NCT04644068) as a monotherapy and in combination with other anti-cancer agents in patients with advanced solid malignancies (23). Saruparib could therefore present a new therapeutic opportunity.

The aim of this project was to explore if the selective PARP1 inhibitor saruparib can be used to overcome venetoclax resistance in AML. This was tested in short-term drug sensitivity assays to assess the efficacy of saruparib compared to talazoparib and if there is an additional cytotoxic effect when combined with venetoclax and/or azacitidine. The drugs were then tested in time to progression assays mimicking clinical administration of the drugs to study long-term effects. Functional assays were performed measuring DNA damage and apoptotic signalling to understand the mechanism of action and underlying causes of cell death.

Materials and methods

Cell lines

Human AML cell lines (DSMZ) with either intrinsic or acquired resistance towards venetoclax were cultured at 37°C with 5% CO_2. Cell lines with acquired venetoclax resistance were previously generated by Mahesh Tambe (Wennerberg group) by exposing parental cells to increasing doses of venetoclax. Cells were grown either in RPMI medium (Gibco) supplemented with 10% or 20% FBS (Sigma-Aldrich) and a mix of 100 IU/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher), or in IMDM medium (Gibco) supplemented with 20% FBS (Sigma-Aldrich) and a mix of 100 IU/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher). The growth medium of cell lines with acquired venetoclax resistance was supplemented with 500 nM venetoclax once a week. Additional information about the cell lines is displayed in table I.

Table I. Cell line information (24, 25). Pen/strep denotes the mix of 100 IU/ml penicillin and 100 µg/ml streptomycin.

Cell line	Resistance type	Growth medium	Mutations	Gene fusions
NB-4	Intrinsic	RPMI + 10% FBS + pen/strep	*TP53, KRAS*	*PML::RARA*
KG-1	Intrinsic	IMDM + 20% FBS + pen/strep	*TP53, NRAS*	*FGFR1OP2::FGFR1*
MOLM-13	Acquired	RPMI + 10% FBS + pen/strep	*FLT3-ITD*	*KMT2A::MLLT3*
MV4-11	Acquired	IMDM + 20% FBS + pen/strep	*FLT3-ITD*	*KMT2A::AFF1*

Pharmaceutical compounds

All pharmaceutical compounds were dissolved in DMSO (Sigma-Aldrich). Information about the compounds is presented in Table II.

Table II. Information about the pharmaceutical compounds used in the experiments.

Pharmaceutical compound	Function	Supplier	Supplier ref.	Stock conc. (mM)
Venetoclax	BCL-2 inhibitor	ChemieTek	CT-A199	80
Azacitidine	Hypomethylating agent	ChemieTek	CT-5AZA	80
Saruparib	PARP1 inhibitor	Medchem Express	HY-1321	10
Talazoparib	PARP1/2 inhibitor	Medchem Express	HY-16106	50
Benzethonium Chloride	Apoptosis inducer	Sigma-Aldrich	B8879	100
Etoposide	Topoisomerase II inhibitor	Medchem Express	HY-13629	40
Staurosporine	Protein kinase inhibitor	Medchem Express	HY-15141	1

Cell culture

Prior to setting up experiments, cells were stained with Trypan Blue (Thermo Fisher) and counted using the automated cell counter Countess II (Thermo Fisher). The cells were then centrifuged at 350×g for 5 min, the supernatant discarded, and the cell pellet resuspended in medium to the desired concentration.

Drug sensitivity assay

Cells were plated in a 384-well v-bottom plate (Corning) with a cell density of 150 000 cells/ml in a volume of 30 µl per well (4500 cells/well). The cells were treated with venetoclax (1-1 000 nM), azacitidine (1-3 000 nM), saruparib (0.1-1 000 nM) and talazoparib (0.1-1 000 nM) as single agents and in combination with 100 nM venetoclax and/or 300 nM azacitidine. Figure 3 shows the drug combinations and concentrations used. The cells were incubated for 4 days and azacitidine was added every day. Benzethonium chloride (100 µM) was used as a positive control. Compounds were added to the plate prior to cell plating using the acoustic dispenser ECHO550 (LabCyte).

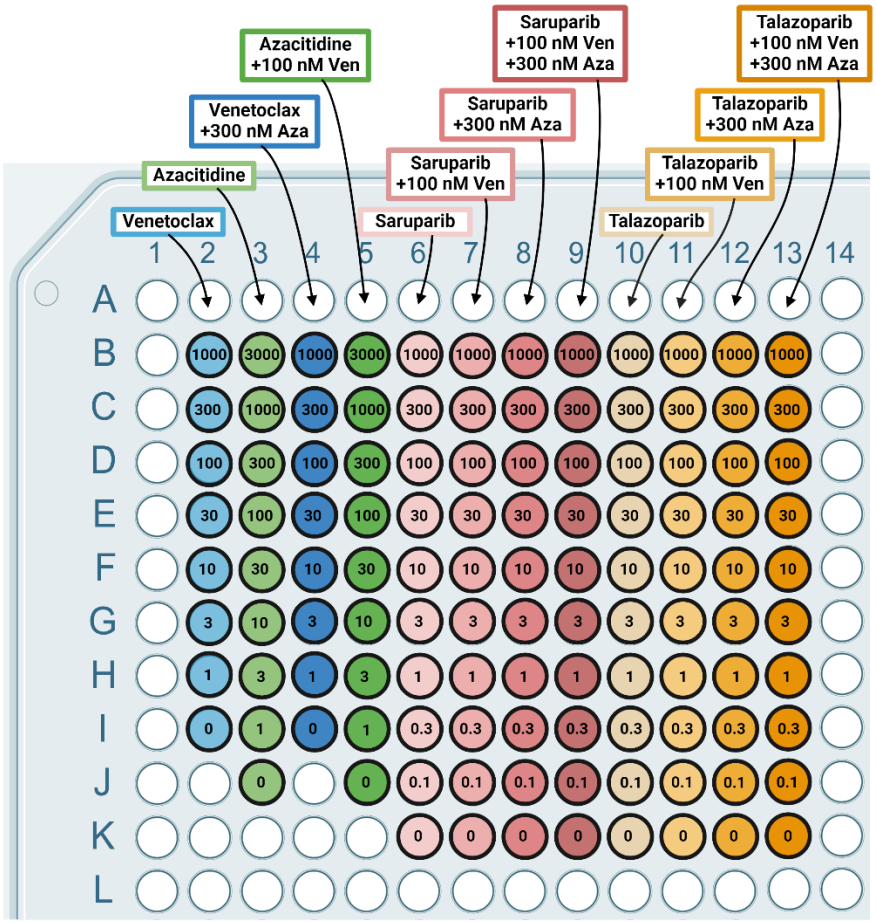

Figure 3. The drug combinations and concentrations used in the drug sensitivity assay. A representative layout of part of the 384-well plate used for the drug sensitivity assay showing the drug concentrations in nM and drug combinations the cells were treated with. For combinations with 100 nM venetoclax (ven) and/or 300 nM azacitidine (aza), the concentrations of ven and aza are the same in the whole column. Created with BioRender.com.

Time to progression assay

Cells were plated in a 24-well plate (Corning) with a cell density of 500 000 cells/ml in a volume of 1 ml per well. The cells were treated for 28 days with venetoclax (100 nM), azacitidine (300 nM), saruparib (30 nM) and talazoparib (10 nM) as single agents and in combinations. Azacitidine was added daily for the first 5 days, while venetoclax, saruparib and talazoparib were added 3 times per week throughout the experiment. Cells were counted via flow cytometry and split back to 500 000 cells/ml in 1 ml fresh medium 3 times per week. Treatment conditions that reached a 4-fold cell expansion were terminated after at least 10 days of treatment.

Functional assays
On day 5 of the time to progression assay, cells were assessed for induction of DNA damage and apoptotic signalling via flow cytometry. Thus, on day 3, a volume of 100 µl with a cell density of 500 000 cells/ml (50 000 cells/well) was transferred to 96-well v-bottom plates (Thermo Fisher). The cells were treated with the same drug concentrations as in the time to progression assay. Staurosporine (1 µM) and etoposide (40 µM) were added the day before readout as positive controls for apoptosis and DNA damage respectively.

Flow cytometry

Flow cytometry was used to assess cell viability, apoptosis and markers for DNA damage and apoptotic signalling. The antibodies used for staining the cells prior to flow cytometric analysis are displayed in Table III. Data were acquired using an iQue Screener PLUS flow cytometer (Sartorius) and analysed with the ForeCyte software (Sartorius). The gating strategy for all experiments can be found in Supplemental Figure 1.

Table III. Information about the antibodies and stains used in the experiments.

Target	Fluorophore	Isotype	Detector on iQue	Clone	Supplier	Cat no.	Stock conc. (mg/ml)	Dilution
Viability								
Annexin V	PE	N/A	BL2	N/A	BD Biosciences	556421	0.003	100
Annexin V	PE-Cy7	N/A	BL5	N/A	Biolegend	640950	0.009	50
Draq7	N/A	N/A	RL2	N/A	Biolegend	424001	0.3	800
eFluor™ 780	eF780	N/A	RL2	N/A	Thermo Fisher	65-0865-18	N/A	1000
Intracellular								
γH2Ax	AF488	IgG1, k	BL1	N1-431	BD Biosciences	560445	0.025	100
cPARP	PE-CF594	IgG1, k	BL3	F21-852	BD Biosciences	564130	0.05	40
Caspase-3/7	FAM	N/A	BL1	N/A	Biorad	Ict094	N/A	300

Drug sensitivity assay
In the drug sensitivity assay, cell viability and apoptosis were measured by adding Draq7 and Annexin V-PE, followed by a 10-minute incubation at room temperature in the dark.

Time to progression assay
In the time to progression assay, the cell number and viability were measured 3 times per week by adding Draq7, followed by a 10-minute incubation at room temperature in the dark.

Functional assays
In the functional assays, DNA damage was assessed by measuring the levels of phosphorylated H2Ax (γH2Ax), and apoptotic signalling was assessed by measuring the levels of cleaved PARP (cPARP) and activity of caspase-3/7.

<u>γH2Ax and cPARP</u>

Prior to flow cytometric analysis, the cells were stained using the BD Pharmingen™ Transcription Factor Buffer Set according to the supplier's instruction. In short, the cells were stained with the viability dye eFluor 780 for 30 minutes at 4°C, followed by fixation and permeabilization for 45 minutes at 4°C and intracellular staining for 45 minutes.

Caspase-3/7 activation

Apoptosis was also measured by caspase-3 activation, using the Bio-Rad FAM FLICA™ Caspase-3/7 Kit. Prior to flow cytometric analysis, the cells were stained according to the manufacturer's instruction. In short, the caspase-3/7 inhibitor FAM FLICA was added, and the cells were incubated for 60 minutes at 37°C, followed by washing with apoptosis buffer and staining with Annexin V PE-Cy7 and Draq7 for 10 minutes.

Data analysis and normalisation

Statistical analysis was performed using GraphPad Prism version 10.2.0 to calculate mean, SEM, SD and absolute IC_{50} values. For the drug sensitivity assay, the raw data was normalised in two different ways. The data was normalised to the average of the DMSO control to be able to compare results between experiments since the cell lines have different growth rates. This shows the overall growth inhibition of the drug combinations since DMSO does not affect cell growth. To show synergistic effects of the drug combinations, the data was normalised to the average of the corresponding controls (DMSO, 100 nM venetoclax, 300 nM azacitidine or 100 nM venetoclax combined with 300 nM azacitidine). This removes the effects of the background and only shows the added effect caused by synergies between the combined drugs. In the time to progression assay, cumulative cell numbers were calculated based on the fold changes from previous cell counts. For the functional assays, the ratio between positive (upper right in the gating) and negative cells (upper left in the gating) was normalised to the average of the background, see Supplemental Figure 1 for gating.

Results

Aiming to find new treatments to overcome venetoclax resistance in AML, PARPis were tested alone and in combination with venetoclax and/or azacitidine. Human AML cell lines with intrinsic or acquired venetoclax resistance were used, representing patients that either show upfront resistance or relapse and develop resistance, respectively. To ensure that the cell lines used were relevant for this study, their response to venetoclax was tested. All cell lines showed resistance to venetoclax, as seen in Supplemental Figure 2, and can therefore be used as venetoclax resistant models.

The triple combination of PARPi, venetoclax and azacitidine re-sensitises venR cells to venetoclax

To investigate whether PARPis can be used to overcome venetoclax resistance, drug sensitivity assays were performed. Cell lines with intrinsic or acquired venetoclax resistance were treated for 4 days with either saruparib or talazoparib, alone and in combination with venetoclax and/or azacitidine. Data showing the response of the cell lines to venetoclax and/or azacitidine, which were used as controls, can be found in Supplemental Figure 3.

First, the response was tested in cell lines with intrinsic venetoclax resistance. In NB-4 cells, saruparib alone or in combination with venetoclax did not induce significant cell death. However, saruparib together with azacitidine or in combination with venetoclax and azacitidine resulted in reduced cell viability, where the triple combination exerted the strongest effect (Fig. 4). The combination of saruparib and azacitidine exhibited a stronger synergistic effect than saruparib combined with venetoclax (Fig. 4A). The stronger effect of saruparib and azacitidine can also be seen by the lower IC_{50} value in Table IV. The triple combination with saruparib, venetoclax and azacitidine decreased the IC_{50} more than 6-fold compared to saruparib and azacitidine. At a concentration of 30 nM, the triple combination reached its strongest effect where the cell viability was decreased to almost 0% (Fig. 4B). Talazoparib displayed a higher potency in reducing cell viability than saruparib, both when administered as a single agent and in combination with venetoclax and/or azacitidine. Combining talazoparib with venetoclax and/or azacitidine significantly enhanced the cytotoxic effect (Fig. 4, Table IV).

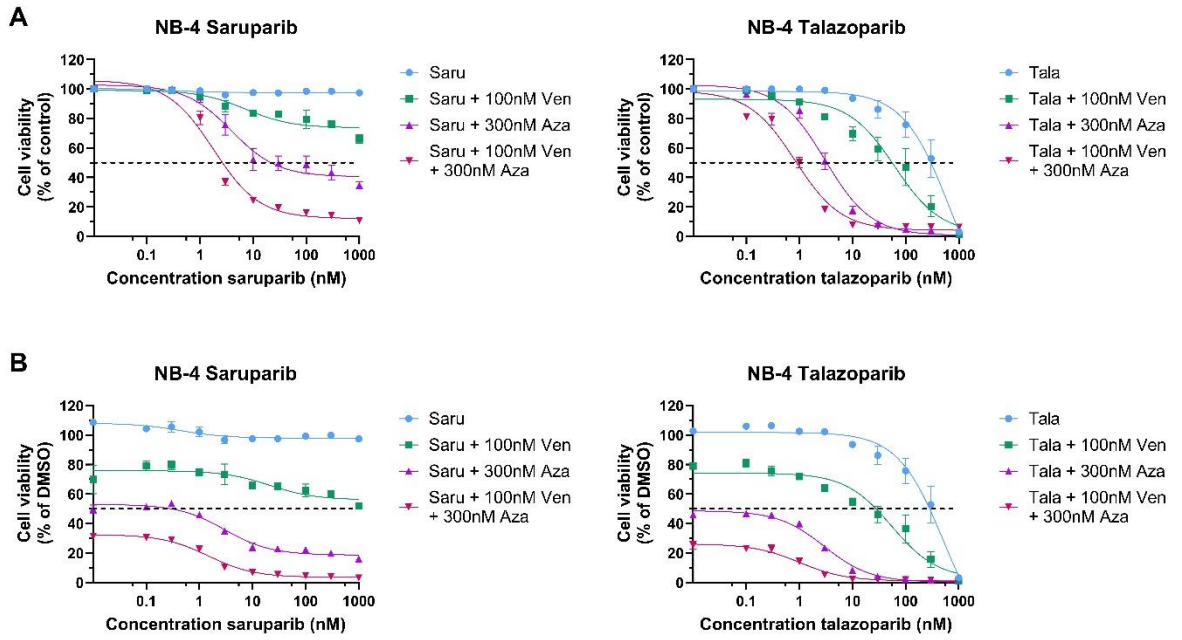

Figure 4. Venetoclax and azacitidine enhance the cytotoxic effect of PARPis in NB-4 cells. NB-4 cells were treated with DMSO, saruparib (saru) (0.1-1000 nM) or talazoparib (tala) (0.1-1000 nM) alone and in combination with 100 nM venetoclax (ven) and/or 300 nM azacitidine (aza) for 4 days. Cell viability was measured via flow cytometry (Annexin V-/Draq7- population). The dose response curves present the cell viability representing the cell counts as percentages either (**A**) normalised to their respective controls (for double combinations either the response to 100 nM venetoclax or 300 nM azacitidine alone, for the triple combination to the response to 100 nM venetoclax and 300 nM azacitidine in combination) or (**B**) normalised to the response to DMSO. Data are shown as mean ± SEM (n = 4 from four independent experiments).

In KG-1 cells, saruparib alone did not decrease cell viability significantly and combining saruparib with venetoclax and/or azacitidine only slightly enhanced the cytotoxic effect (Fig. 5A). The triple combination exerted the strongest effect but none of the saruparib conditions killed the cells completely (Fig. 5B). Talazoparib exerted a stronger effect than saruparib, both as a monotherapy and in combination with venetoclax and/or azacitidine. The addition of venetoclax and/or azacitidine only slightly enhanced the effect of talazoparib, where the IC_{50} value was decreased about 3-fold for the triple combination compared to the monotherapy (Table IV).

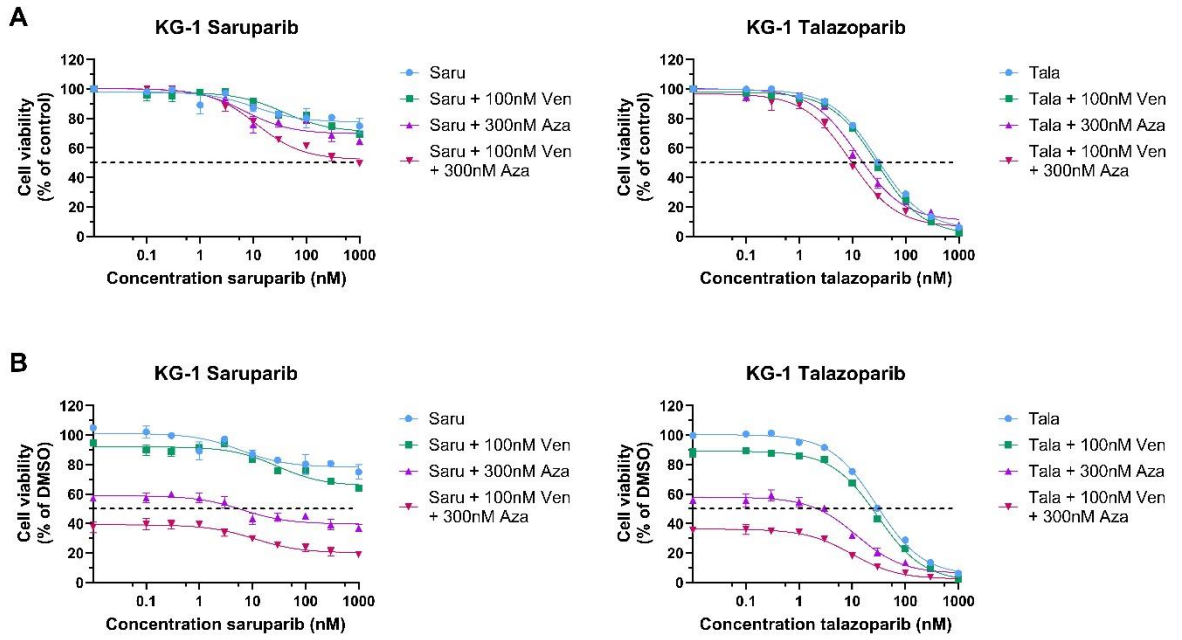

Figure 5. **Venetoclax and azacitidine slightly enhance the effect of PARPis in KG-1 cells, where talazoparib was more effective than saruparib.** KG-1 cells were treated with DMSO, saruparib (saru) (0.1-1000 nM) or talazoparib (tala) (0.1-1000 nM) alone and in combination with 100 nM venetoclax (ven) and/or 300 nM azacitidine (aza) for 4 days. Cell viability was measured via flow cytometry (Annexin V-/Draq7- population). The dose response curves present the cell viability representing the cell counts as percentages either (**A**) normalised to their respective controls (for double combinations either the response to 100 nM venetoclax or 300 nM azacitidine alone, for the triple combination to the response to 100 nM venetoclax and 300 nM azacitidine in combination) or (**B**) normalised to the response to DMSO. Data are shown as mean ± SEM (n = 3 from three independent experiments).

Table IV. Absolute IC_{50} values for the different cell lines and treatments. The IC_{50} values (in nM) are based on the graphs shown in Figures 4A, 5A, 6A and 7A. The colour gradient is based on the logarithm of the IC_{50} values where red colours represent the lowest IC_{50} values and blue the highest.

Treatment \ Cell line	NB-4	KG-1	MOLM-13	MV4-11
Saruparib	>1000	>1000	>1000	>1000
Saruparib + Ven	>1000	>1000	>1000	223
Saruparib + Aza	13.7	>1000	>1000	3.5
Saruparib + Ven + Aza	2.2	>1000	2.8	2.5
Talazoparib	325	32.5	29.6	10.2
Talazoparib + Ven	59.9	28.1	10.1	6.9
Talazoparib + Aza	3.1	15.2	4.8	2.0
Talazoparib + Ven + Aza	0.9	9.5	1.6	0.7

The response to the treatments was also tested in cell lines with acquired venetoclax resistance. MOLM-13 venR cells responded slightly to the treatment with saruparib. Adding venetoclax or azacitidine enhanced the cytotoxic effect, where saruparib and azacitidine exhibited a stronger synergistic effect than saruparib and venetoclax (Fig. 6A). The triple combination exhibited the highest potency in decreasing cell viability (Fig. 6, Table IV). Still, the cell viability did not decrease below 20% even at the highest saruparib concentration (Fig. 6B). Talazoparib exerted a stronger effect than saruparib and combining talazoparib with venetoclax and/or azacitidine increased the cytotoxic effect.

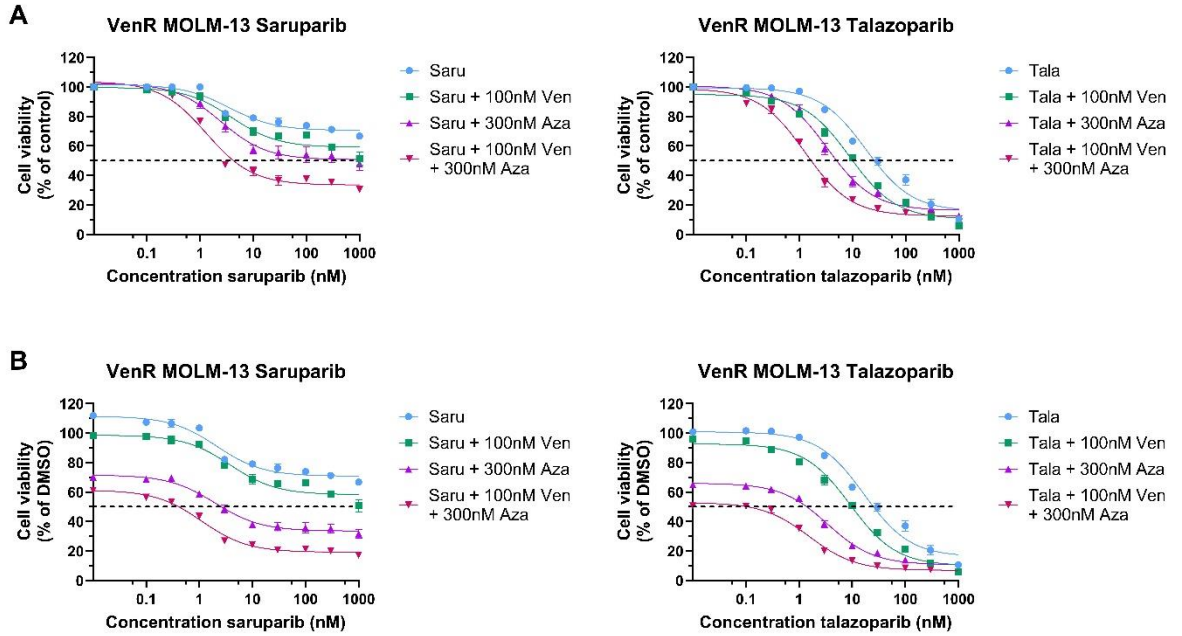

Figure 6. Talazoparib decreases cell viability more in venR MOLM-13 cells than saruparib. VenR MOLM-13 cells were treated with DMSO, saruparib (saru) (0.1-1000 nM) or talazoparib (tala) (0.1-1000 nM) alone and in combination with 100 nM venetoclax (ven) and/or 300 nM azacitidine (aza) for 4 days. Cell viability was measured via flow cytometry (Annexin V-/Draq7- population). The dose response curves present the cell viability representing the cell counts as percentages either (**A**) normalised to their respective controls (for double combinations either the response to 100 nM venetoclax or 300 nM azacitidine alone, for the triple combination to the response to 100 nM venetoclax and 300 nM azacitidine in combination) or (**B**) normalised to the response to DMSO. Data are shown as mean ± SEM (n = 3 from three independent experiments).

Cell viability was significantly decreased in venR MV4-11 cells following treatment with saruparib and azacitidine. A strong synergistic effect between saruparib and azacitidine was observed, whereas venetoclax did not increase the effect of saruparib or the combination of saruparib and azacitidine (Fig. 7A). Cells treated with saruparib and azacitidine, or with saruparib, venetoclax and azacitidine were effectively killed at saruparib concentrations above 10 nM (Fig. 7B). Talazoparib was more effective than saruparib alone and combined with venetoclax. Combinations with talazoparib and azacitidine exhibited only slightly stronger cytotoxic effects compared to combinations with saruparib and azacitidine (Fig. 7B, Table IV).

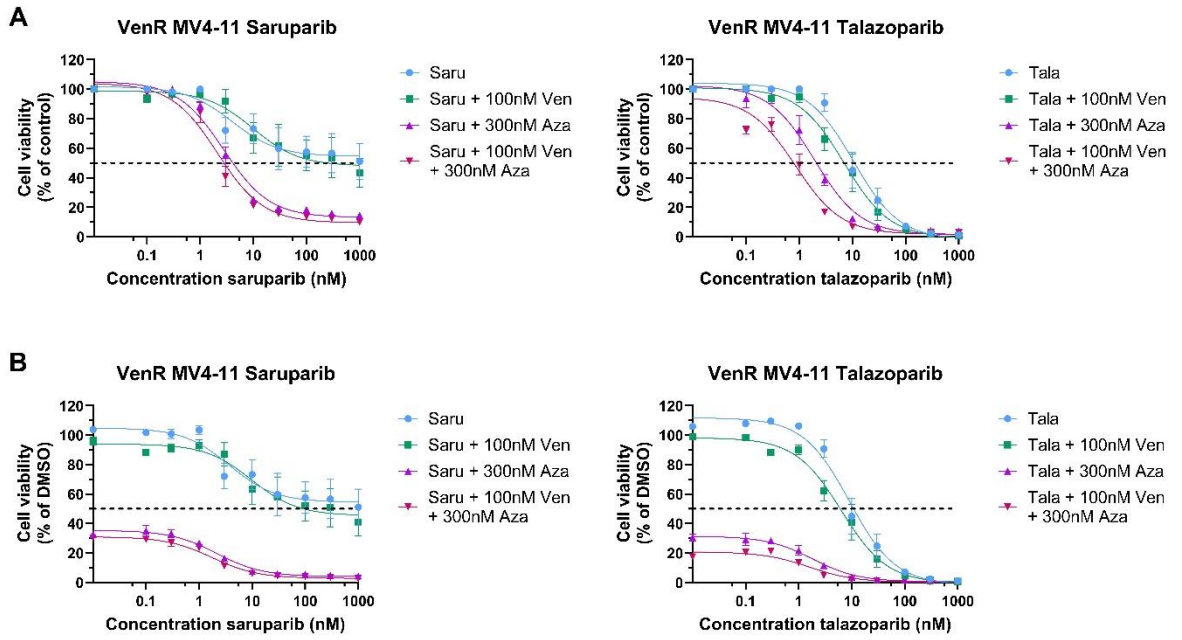

Figure 7. Azacitidine strongly enhances the effect of both PARPis by effectively decreasing cell viability in venR MV4-11 cells. VenR MV4-11 cells were treated with DMSO, saruparib (saru) (0.1-1000 nM) or talazoparib (tala) (0.1-1000 nM) alone and in combination with 100 nM venetoclax (ven) and/or 300 nM azacitidine (aza) for 4 days. Cell viability was measured via flow cytometry (Annexin V-/Draq7- population). The dose response curves present the cell viability representing the cell counts as percentages either (**A**) normalised to their respective controls (for double combinations either the response to 100 nM venetoclax or 300 nM azacitidine alone, for the triple combination to the response to 100 nM venetoclax and 300 nM azacitidine in combination) or (**B**) normalised to the response to DMSO. Data are shown as mean ± SEM (n = 3 from three independent experiments).

In summary, saruparib alone was generally not effective in reducing the cell viability significantly, but combining saruparib with venetoclax and azacitidine increased the cytotoxicity in all cell lines tested. At saruparib concentrations of 30 nM and above, the triple combination of saruparib, venetoclax and azacitidine decreased the cell viability to 20% or lower. Talazoparib was more effective than saruparib, both as a monotherapy and combined with venetoclax and/or azacitidine.

The triple combination of saruparib, venetoclax and azacitidine induces cell death only in the presence of azacitidine

To test if the triple combination of saruparib, venetoclax and azacitidine would be effective to overcome venetoclax resistance during longer treatment periods, a time to progression assay was performed over 28 days. Azacitidine was added daily for the first 5 days whereas the other drugs were added three times per week throughout the treatment cycle to mimic clinical administration of the drugs. The drug concentrations used were based on the results from the drug sensitivity assays, where saruparib reached its strongest effect around 30 nM and talazoparib around 10 nM. The time to progression assay was performed for one cell line with intrinsic and one cell line with acquired venetoclax resistance to represent different resistance types seen in patients. NB-4 cells with intrinsic venetoclax resistance and MV4-11 cells with acquired venetoclax resistance were used since they showed sensitivity to the saruparib combination treatments in the drug sensitivity assays.

For NB-4 cells, the combinations with saruparib, venetoclax and azacitidine, and saruparib and azacitidine decreased the cell numbers for the duration of the azacitidine administration (Fig. 8). The triple combination exhibited a deeper response than the combination with saruparib and azacitidine. However, the cells started expanding once the administration of azacitidine stopped. Cell growth was inhibited slightly longer for the triple combination, as seen by the low cell number at day 10. Combinations with talazoparib and azacitidine or talazoparib, azacitidine and venetoclax exhibited deeper responses than the same combinations with saruparib. The effect lasted as the talazoparib combinations did not regrow after azacitidine treatment was stopped. Treatment with venetoclax alone or combined with azacitidine, saruparib or talazoparib resulted in slight growth inhibition, but the cells quickly expanded after day 5. All other treatment conditions resulted in cell expansion from the start.

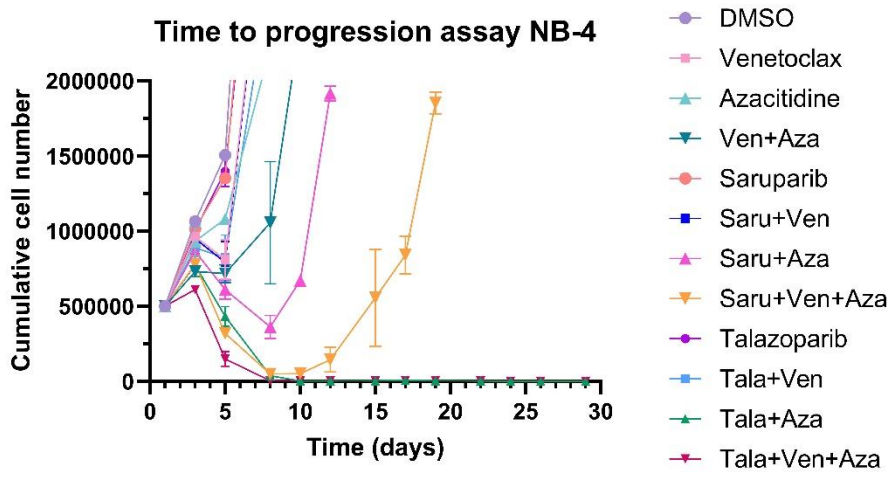

Figure 8. Combinations with saruparib and azacitidine inhibit cell growth only in the presence of azacitidine. NB-4 cells were treated with DMSO, 100 nM venetoclax (ven), 300 nM azacitidine (aza), 30 nM saruparib (saru) or 10 nM talazoparib (tala) as monotherapies and in combination with 100 nM ven and/or 300 nM aza. Azacitidine was added for the first five days whereas the other drugs were added three times per week throughout the 28-day treatment cycle. At the indicated timepoints, the number of live cells was measured via flow cytometry (Draq7- population), the cells were split back to 500 000 cells/ml per well and the growth medium was changed. The curves show the cumulative cell number, which is the product of the starting cell number and fold changes from previous counts. Data are shown as mean ± SD (n = 2 from a single experiment).

In venR MV4-11 cells, treatment with saruparib and azacitidine, or saruparib, azacitidine and venetoclax inhibited cell growth for the duration of the azacitidine administration (Fig. 9). The response was slightly deeper following treatment with the triple combination and the cells also regrew slightly slower. Combinations with talazoparib and azacitidine decreased the cell numbers slightly more than the same combinations with saruparib. The addition of venetoclax to the combination with talazoparib and azacitidine had no effect. Combinations with talazoparib and azacitidine resulted in extended growth inhibition and no clear cell expansion was observed until day 17, after which the cells started to expand again. Treatment with azacitidine alone or combined with venetoclax resulted in slight growth inhibition, but the cells quickly regrew after day 5. All other treatment conditions led to cell expansion.

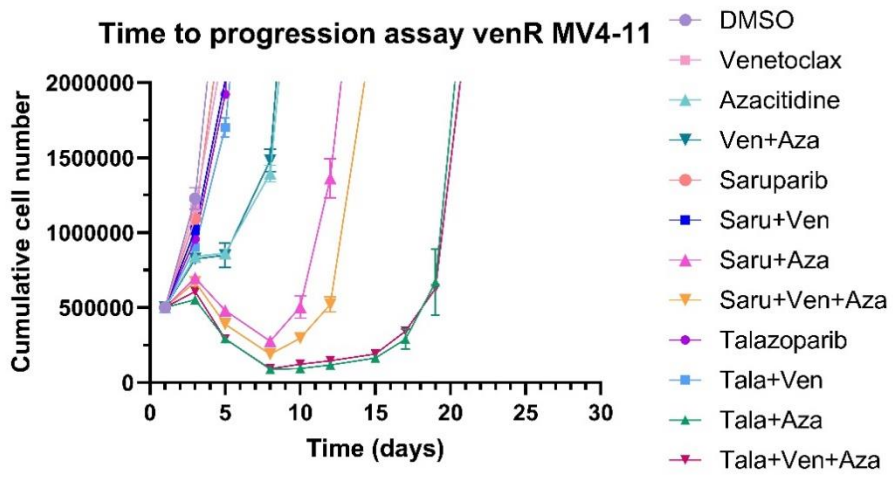

Figure 9. **Combination treatments with PARPi and azacitidine inhibit cell growth and delay exponential cell expansion in venR MV4-11 cells**. VenR MV4-11 cells were treated with DMSO, 100 nM venetoclax (ven), 300 nM azacitidine (aza), 30 nM saruparib (saru) or 10 nM talazoparib (tala) as monotherapies and in combination with 100 nM ven and/or 300 nM aza. Azacitidine was added for the first five days whereas the other drugs were added three times per week throughout the 28-day treatment cycle. At the indicated timepoints, the number of live cells was measured via flow cytometry (Draq7- population), the cells were split back to 500 000 cells/ml per well and the growth medium was changed. The curves show the cumulative cell number, which is the product of the starting cell number and fold changes from previous counts. Data are shown as mean ± SD (*n* = 2 from a single experiment).

In conclusion, the triple combination of saruparib, venetoclax and azacitidine effectively inhibited cell growth for the duration of azacitidine treatment. Once administration of azacitidine was stopped, the cells quickly regrew. The triple combination with talazoparib had a slightly stronger effect and delayed the regrowth. In NB-4 cells, talazoparib in combination with azacitidine and with venetoclax and azacitidine resulted in an apparent complete cell eradication without regrowth throughout the treatment cycle.

Combination treatments increase induction of DNA damage and apoptotic signalling

To elucidate whether PARPi combinations induced a higher level of DNA damage which eventually could lead to apoptosis, functional assays were set up over 4 days. A marker for DNA damage is phosphorylation of H2Ax into γH2Ax. Apoptosis induction can be detected by measuring the levels of cPARP and the activity of caspases-3 and -7.

First, venR MOLM-13 cells were treated with saruparib as a single agent and in combination with azacitidine and/or venetoclax to assess DNA damage and cPARP. Increased DNA damage corresponded with increased apoptosis for all conditions. Saruparib alone neither induced DNA damage nor apoptosis but combining saruparib with venetoclax and/or azacitidine resulted in elevated DNA damage and apoptosis (Fig. 10). The highest levels were observed when treating the cells with the saruparib triple combination. Surprisingly, the venetoclax and saruparib combination as well as the venetoclax and azacitidine combination exhibited slightly higher levels of cPARP and DNA damage than the combination of saruparib and azacitidine. This was interesting since the saruparib and azacitidine combination induced higher levels of cell death in the drug sensitivity assays (Fig. 6). Moreover, the saruparib and azacitidine combination slowed down cell expansion more in the time to progression assay on

day 5 of the treatment (Supplemental Fig. 4). The positive controls for the functional assays are exhibited in Supplemental Figure 5.

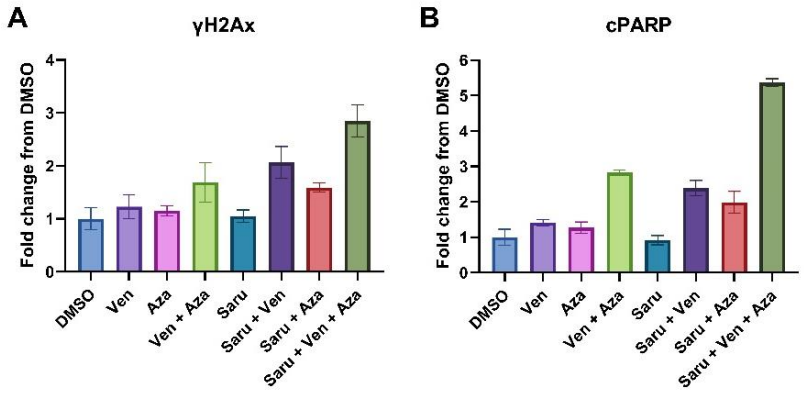

Figure 10. **The triple combination with saruparib, venetoclax and azacitidine induces DNA damage and cPARP in venR MOLM-13 cells**. VenR MOLM-13 cells were taken from the time to progression assay on day 3 and treated with DMSO, 100 nM venetoclax (ven), 300 nM azacitidine (aza) or 30 nM saruparib (saru) as single agents and in combination with 100 nM ven and/or 300 nM aza. Levels of (**A**) DNA damage, as measured by phosphorylated H2Ax (γH2Ax), and (**B**) apoptosis, as measured by cleaved PARP (cPARP), were assessed on day 5 of the functional assays via flow cytometry. (**A, B**) Data are displayed as ratio between γH2Ax or cPARP positive and negative cells, normalised to the response to DMSO. Data are shown as mean ± SD (n = 3 from a single experiment).

For venR MV4-11 cells, a slight increase in DNA damage was observed for all conditions, but more pronounced for the saruparib triple combination and combinations with talazoparib and azacitidine (Fig. 11A). However, there was no clear increase in DNA damage for the positive control in the γH2Ax experiment so the results should therefore be treated with extreme caution. The positive controls are shown in Supplemental Figure 6. A clear increase in apoptotic signalling was detected for all treatments that contained azacitidine (Fig. 11B, 11C). The levels of cPARP were similar for all treatments with azacitidine (Fig. 11B). The highest levels of active caspases-3 and -7 were observed for talazoparib and azacitidine as well as for the talazoparib triple combination. The triple combination of saruparib, venetoclax and azacitidine also resulted in elevated caspase activity and was slightly higher compared to the combination with saruparib and azacitidine (Fig. 11C). Since venR MV4-11 cells exhibited high sensitivity to azacitidine in the drug sensitivity assay (Fig. 7), it is not surprising that combinations with azacitidine induced the highest levels of apoptotic signalling. It could also be seen in the time to progression assay that all azacitidine conditions resulted in a cytostatic or cytotoxic effect on day 5 (Fig. 9).

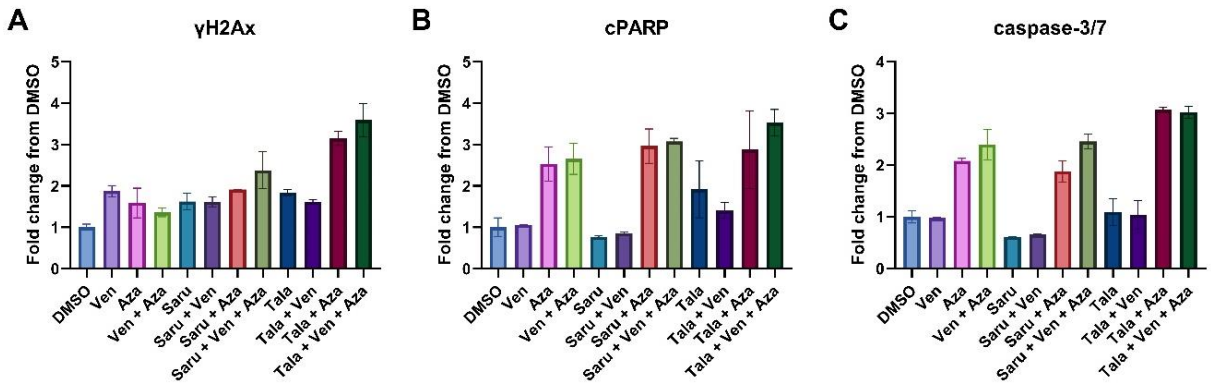

Figure 11. **The triple combination with saruparib and combinations with talazoparib and azacitidine induce high levels of DNA damage and apoptotic signalling in venR MV4-11 cells**. VenR MV4-11 cells were taken from the time to progression assay on day 3 and treated with DMSO, 100 nM venetoclax (ven), 300 nM azacitidine (aza), 30 nM saruparib (saru) or 10 nM talazoparib (tala) as single agents and in combination with 100 nM ven and/or 300 nM aza. Levels of (**A**) DNA damage measured by phosphorylated H2Ax (γH2Ax), (**B, C**) apoptosis measured by (**B**) cleaved PARP (cPARP), and (**C**) caspase-3/7 activity, were assessed on day 5 of the functional assays via flow cytometry. (**A, B, C**) Data are displayed as ratio between γH2Ax, cPARP or caspase-3/7 positive and negative cells, normalised to the response to DMSO. In (**A**), there was no clear increase in DNA damage for the positive control, so the results should be treated with caution. Data are shown as mean ± SD (n = 2 from a single experiment).

In conclusion, the triple combinations of both saruparib and talazoparib with venetoclax and azacitidine resulted in increased induction of DNA damage and apoptotic signalling.

Discussion

The lack of treatment options and development of drug resistance are major challenges in treating AML. Talazoparib has previously shown potential to overcome venetoclax resistance (14) but may be too toxic for elderly and medically unfit patients. Since toxicities are mainly associated with PARP2 inhibition (17), a selective PARP1 inhibitor might be better tolerated. Here, the efficacy of the selective PARP1 inhibitor saruparib was tested and compared to talazoparib. While saruparib was less cytotoxic than talazoparib, combination with venetoclax and azacitidine led to decreased cell viability and growth inhibition, where azacitidine in particular enhanced the effect of saruparib. Accumulated DNA damage could explain the cytotoxic effect since increased DNA damage corresponded to increased induction of apoptosis.

Saruparib exerted a cytotoxic effect on venR AML cells, but talazoparib was more effective as it induced slightly higher levels of apoptotic signalling and cell death. Talazoparib is an unselective PARP1/2 inhibitor that also targets PARP16 as an off-target event (26). In contrast, saruparib is a selective PARP1 inhibitor that has a 500-fold selectivity for PARP1 compared to PARP2 (17). Since talazoparib exhibited a stronger cytotoxic effect than saruparib, this indicates that targeting PARP2 and/or PARP16 has an additional effect on venR AML cell lines compared to only targeting PARP1. Both PARP1 and PARP2 are involved in DNA damage repair, where PARP1 accounts for the vast majority of PARylation in cells, whereas PARP2 only for 5-20% (17). Saruparib combinations did not increase DNA damage and apoptosis as much as talazoparib combinations, suggesting that the PARylation activity of PARP2 was enough to keep the DNA damage at a tolerable level in cells treated with saruparib. The enhanced effect of talazoparib compared to saruparib could also be due to inhibition of PARP16, as it has previously been reported that silencing PARP16 increased the effect of other PARPis (26). Irrespective of whether PARP2 or PARP16 inhibition caused the additional effect of talazoparib compared to saruparib, this shows that selective PARP1 inhibition was not as effective in inducing cell death in venR AML cells.

Muvarak *et al*. recently reported a synergistic effect between a hypomethylating agent and PARPi. The DNMT inhibitor (DNMTi) decitabine, which works similarly to azacitidine, enhanced the cytotoxic effect of PARPi in AML. DNMTi recruits PARP1 to sites of DNA damage, where it is trapped by a PARPi and becomes tightly bound to the DNA. This blocks the catalytic activity of PARP1 and causes an increase of DNA double-stranded breaks (21). These results are in line with our findings where azacitidine enhanced the cytotoxic effect of saruparib and talazoparib. In the functional assays, combining PARPi with azacitidine or azacitidine and venetoclax increased the DNA damage and apoptotic signalling. For saruparib, only a slight increase in DNA damage was measured when combined with azacitidine, whereas a stronger increase in DNA damage was measured for talazoparib combined with azacitidine. This mostly correlated with increases observed in apoptotic signalling, suggesting that increased DNA damage and PARP trapping could explain the increased cytotoxic effect of combinations with PARPi and azacitidine. In the drug sensitivity assays, azacitidine enhanced the effects of the PARPis the most. In the time to progression assays, only combinations with PARPi and azacitidine inhibited cell growth. The cells treated with saruparib and azacitidine started expanding once azacitidine treatment was stopped, suggesting that azacitidine is crucial to sensitise the cells to PARPi treatment.

Venetoclax contributed to increased cytotoxic stress in venR cell lines when given in combination with PARPi alone or with azacitidine. As seen in the drug sensitivity assays, there was an additional effect of venetoclax. This was seen both as a synergistic effect, where venetoclax enhanced the effect of PARPi alone or in combination with azacitidine, as well as an increased overall response. Venetoclax in combination with azacitidine and/or saruparib also increased DNA damage and apoptosis markers, as seen in the functional assay with MOLM-13 venR cells (Fig. 10). This indicates that even though the cell lines are resistant to venetoclax, treatment with venetoclax increased cellular stress and genomic instability. In other studies aimed at overcoming venetoclax resistance in haematological malignancies, synergistic effects have been observed with venetoclax combination treatment in venR cells (27). This indicates that the synergistic effects of combination treatments could sensitise venR cells to venetoclax-based combination treatments. Synergy between venetoclax and talazoparib has previously been observed by Giorgi *et al* in venetoclax sensitive cells (22). This suggests that the increased effect of venetoclax and PARPi compared to PARPi alone could be due to synergistic effects and shows that venetoclax increases the effect of combination treatments in venR cells.

In the functional assays, increased induction of DNA damage and apoptotic signalling was measured for combination treatments where the cells were expanding. As observed in the time to progression assay for MOLM-13, all conditions were expanding on day 5 when the functional assays were performed (Supplemental Fig. 4). Still, higher levels of DNA damage and apoptotic signalling was observed for saruparib combined with venetoclax and/or azacitidine as well as for the combination with venetoclax and azacitidine (Fig. 10). For MV4-11, increased apoptosis was observed for all treatments with azacitidine (Fig. 11). This relates to the results seen in the time to progression assay, where azacitidine treatments showed cytostatic or cytotoxic effects (Fig. 9). However, the increase in apoptotic markers was similar regardless of if the treatment exhibited a cytostatic or cytotoxic effect. Most treatments also resulted in increased DNA damage, although these results were not completely reliable. It has previously been reported by Ichim *et al* that increased cellular stress leads to minority MOMP, which causes an increase in apoptotic markers and genomic instability when the apoptotic signals are not strong enough to induce apoptosis (15). This could explain the results from the functional assays and time to progression assays where the cells expanded despite exhibiting increased levels of DNA damage and apoptotic markers. This suggests that even when saruparib combinations do not provide a cytotoxic effect, they increase the cellular stress and lead to genomic instability.

Variations in drug response between cell lines were consistently observed across all experiments. The clearest differences were seen in the drug sensitivity assays, as it included the most cell lines. There were differences both in the overall response to the various treatments, as well as in the additional effects of venetoclax and/or azacitidine. Cell lines with acquired resistance were slightly more responsive to PARPis than intrinsically resistant cell lines. The strongest response was seen in MV4-11 cells. NB-4 cells were least responsive to PARPis alone, where saruparib did not decrease the cell viability irrespective of concentration. However, the synergistic effect of PARPi in combination with venetoclax and/or azacitidine was most pronounced for these cells. For KG-1 cells, the addition of venetoclax and/or azacitidine only minimally enhanced the effect of PARPis. In the time to progression assays, durable growth inhibition was achieved by combinations with talazoparib and azacitidine in NB-4 cells but not in MV4-11 cells. The saruparib triple combination also

provided a deeper response in NB-4 cells compared to MV4-11 cells. In the functional assays, the addition of venetoclax to saruparib treatment increased apoptosis and DNA damage more in MOLM-13 cells, as compared to MV4-11 cells where azacitidine had a stronger impact on apoptosis. AML is a very heterogenous disease, which is also represented in the drug response. However, even though differences were observed, the same drug combinations were the most effective in all experiments. This indicates that while variability in drug response should be expected, combining PARPi with venetoclax and azacitidine will provide a better response than the monotherapies. As some cell lines had similar genetic profiles but still showed differences in drug response, even larger differences should be expected for patients as the genetic variation is greater.

The time to progression assays made it obvious that azacitidine was needed in combination with PARPis to provide a lasting cytotoxic effect. Since cells treated with combinations with saruparib and azacitidine started expanding once azacitidine administration was stopped, a potential option for this treatment to be viable is to extend the duration of azacitidine treatment. Azacitidine is quickly degraded with a plasma half-life of around 40 minutes. The hypomethylating effect of azacitidine is transient and disappears 1-2 weeks after azacitidine treatment is stopped (28). In the time to progression assay, it was seen that the cells treated with azacitidine and saruparib started regrowing about 5 days after azacitidine treatment was stopped. Increasing the duration of azacitidine treatment could therefore extend the response and possibly deepen the effect of the combination treatments. However, this may not be clinically possible. The triple combination of saruparib, venetoclax and azacitidine led to strong growth inhibition and almost as deep response as combinations with talazoparib and azacitidine. Compared to venetoclax and azacitidine, which is the current standard of care, the addition of saruparib provided a stronger cytotoxic effect with prolonged growth inhibition. This indicates that the triple combination of saruparib, venetoclax and azacitidine could prove to be a viable treatment option for venR patients if azacitidine administration can be extended.

Further experiments are needed to evaluate whether prolonged azacitidine treatment will exert an extended cytotoxic effect and provide a durable response, or if the cells will gain resistance. This could indicate whether combination treatment with saruparib and azacitidine could be an effective treatment option for venR patients. Toxicity studies are also needed to assess the additional toxic effect of prolonged azacitidine treatment, as well as the toxic effects of saruparib at effective concentrations. As only cell lines were tested, further experiments need to be conducted with patient samples to see if the results found here will remain true. Due to the heterogeneity of the disease, patient samples are also needed to show whether the treatments could be effective for most patients or only for a specific subgroup. To better understand the differences in drug response observed between cell lines, it would be of interest to test the PARP levels in the cells. It would be interesting to test if there is a shift in PARP1 and PARP2 levels after treatment with saruparib, as well as if the increased effect of talazoparib is due to inhibition of PARP2, PARP16 or both. Further, the staining for DNA damage should be redone. Either the staining did not work properly, or there were issues with the positive control where maybe there were too few live cells to see an effect. Therefore, the staining would need to be done again using a lower concentration of etoposide or treating the cells for a shorter amount of time to see if the positive control was the issue. Otherwise, DNA damage could be measured using a different kind of assay, such as a comet assay.

In conclusion, the cytotoxic effect of saruparib was not as strong as talazoparib in venR cell lines, indicating that selective PARP1 inhibition was not as effective. However, the additional effect of venetoclax and azacitidine impacted cytotoxicity more and the triple combination with saruparib, venetoclax and azacitidine killed cells almost as effectively as the talazoparib triple combination. The stronger cytotoxic effect seemed to be due to increased DNA damage. Azacitidine contributed the most to the enhanced effect and extending azacitidine treatment could lead to the saruparib triple combination being a viable treatment option.

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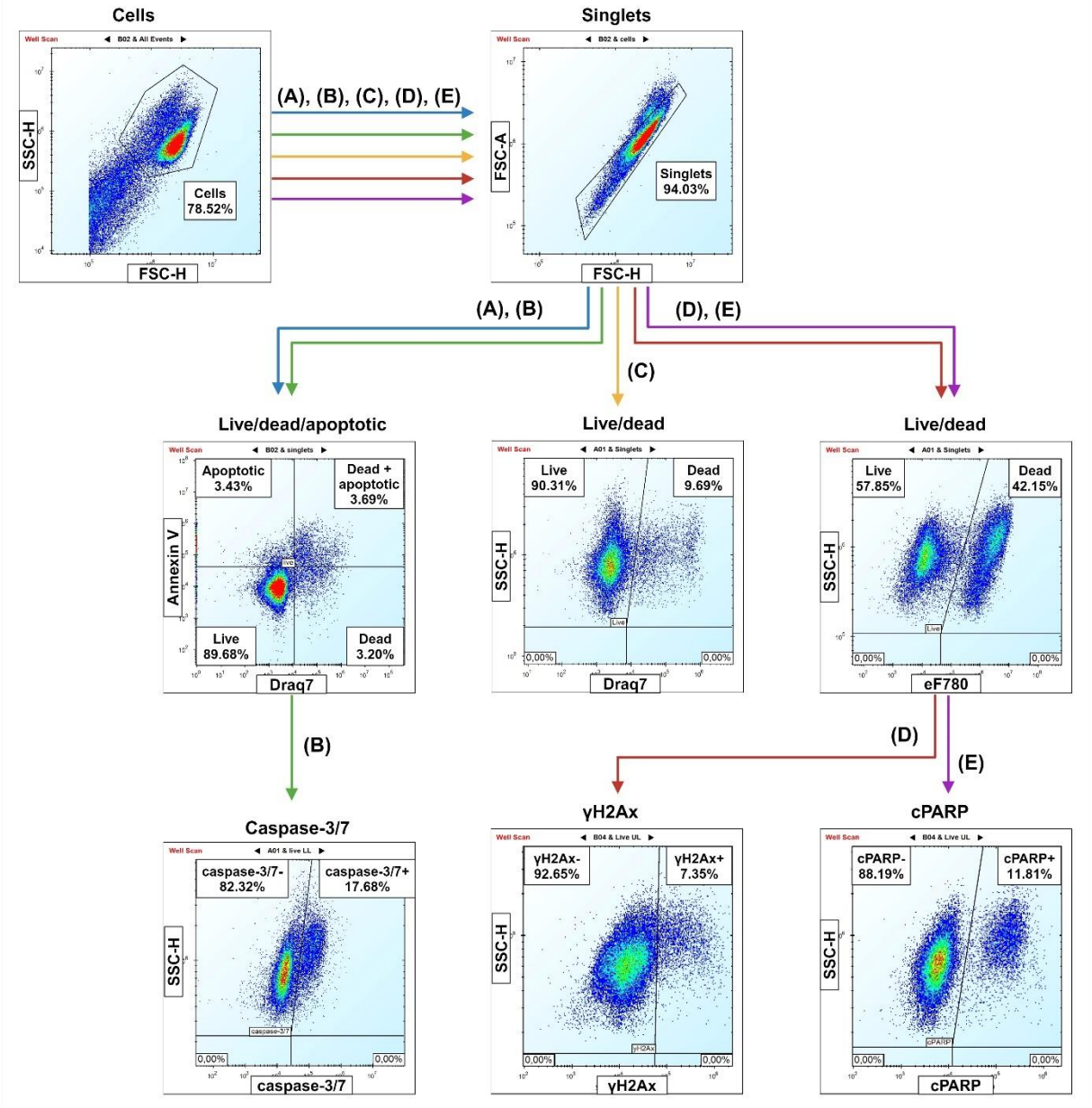
References

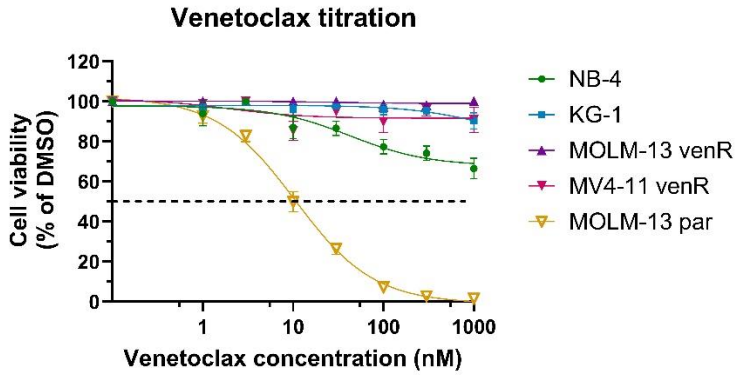
1. Zhang J, Gu Y, Chen B. 2019. Mechanisms of drug resistance in acute myeloid leukemia. *OncoTargets and therapy* 12: 1937-45
2. Shimony S, Stahl M, Stone RM. 2023. Acute myeloid leukemia: 2023 update on diagnosis, risk-stratification, and management. *American Journal of Hematology* 98: 502-26
3. Vakiti A, Mewawalla P. 2023. Acute Myeloid Leukemia. In *StatPearls [Internet]*. Treasure Island (FL): StatPearls Publishing
4. Chopra M, Bohlander SK. 2019. The cell of origin and the leukemia stem cell in acute myeloid leukemia. *Genes, Chromosomes and Cancer* 58: 850-8
5. Behrmann L, Wellbrock J, Fiedler W. 2018. Acute Myeloid Leukemia and the Bone Marrow Niche—Take a Closer Look. *Frontiers in Oncology* 8
6. Goel H, Rahul E, Gupta I, Chopra A, Ranjan A, Gupta AK, Meena JP, Viswanathan GK, Bakhshi S, Misra A, Hussain S, Kumar R, Singh A, Rath GK, Sharma A, Mittan S, Tanwar P. 2021. Molecular and genomic landscapes in secondary & therapy related acute myeloid leukemia. *American journal of blood research* 11: 472-97
7. Garciaz S, Hospital MA, Alary AS, Saillard C, Hicheri Y, Mohty B, Rey J, D'Incan E, Charbonnier A, Villetard F, Maisano V, Lombardi L, Ittel A, Mozziconacci MJ, Gelsi-Boyer V, Vey N. 2022. Azacitidine Plus Venetoclax for the Treatment of Relapsed and Newly Diagnosed Acute Myeloid Leukemia Patients. *Cancers* 14: 2025
8. Stomper J, Rotondo JC, Greve G, Lübbert M. 2021. Hypomethylating agents (HMA) for the treatment of acute myeloid leukemia and myelodysplastic syndromes: mechanisms of resistance and novel HMA-based therapies. *Leukemia* 35: 1873-89
9. Diesch J, Zwick A, Garz A-K, Palau A, Buschbeck M, Götze KS. 2016. A clinical-molecular update on azanucleoside-based therapy for the treatment of hematologic cancers. *Clinical epigenetics* 8: 71
10. Pratz KW, Jonas BA, Pullarkat V, Thirman MJ, Garcia JS, Döhner H, Récher C, Fiedler W, Yamamoto K, Wang J, Yoon S-S, Wolach O, Yeh S-P, Leber B, Esteve J, Mayer J, Porkka K, Illés Á, Lemoli RM, Turgut M, Ku G, Miller C, Zhou Y, Zhang M, Chyla B, Potluri J, DiNardo CD. 2024. Long-term follow-up of VIALE-A: Venetoclax and azacitidine in chemotherapy-ineligible untreated acute myeloid leukemia. *American Journal of Hematology*
11. Dhakal P, Bates M, Tomasson MH, Sutamtewagul G, Dupuy A, Bhatt VR. 2023. Acute myeloid leukemia resistant to venetoclax-based therapy: What does the future hold? *Blood Reviews* 59: 101036
12. Singh R, Letai A, Sarosiek K. 2019. Regulation of apoptosis in health and disease: the balancing act of BCL-2 family proteins. *Nature reviews. Molecular cell biology* 20: 175-93
13. Elmore S. 2007. Apoptosis: A Review of Programmed Cell Death. *Toxicologic Pathology* 35: 495-516
14. Heckman C, Tambe M, Kriegbaum M, Vänttinen I, Olgac E, Kontro M, Wennerberg K. 2023. Venetoclax triggers sub-lethal apoptotic signaling in venetoclax-resistant acute myeloid leukemia cells and induces vulnerability to PARP inhibition and azacitidine. PREPRINT.
15. Ichim G, Lopez J, Ahmed SU, Muthalagu N, Giampazolias E, Delgado ME, Haller M, Riley JS, Mason SM, Athineos D, Parsons MJ, Kooij Bvd, Bouchier-Hayes L, Chalmers AJ, Rooswinkel RW, Oberst A, Blyth K, Rehm M, Murphy DJ, Tait SWG. 2015. Limited Mitochondrial Permeabilization Causes DNA Damage and Genomic Instability in the Absence of Cell Death. *Molecular cell* 57: 860-72

16. Chaudhuri AR, Nussenzweig A. 2017. The multifaceted roles of PARP1 in DNA repair and chromatin remodelling. *Nature Reviews. Molecular Cell Biology* 18: 610-21
17. Illuzzi G, Staniszewska AD, Gill SJ, Pike A, McWilliams L, Critchlow SE, Cronin A, Fawell S, Hawthorne G, Jamal K, Johannes J, Leonard E, Macdonald R, Maglennon G, Nikkilä J, O'Connor MJ, Smith A, Southgate H, Wilson J, Yates J, Cosulich S, Leo E. 2022. Preclinical Characterization of AZD5305, A Next-Generation, Highly Selective PARP1 Inhibitor and Trapper. *Clinical cancer research* 28: 4724-36
18. Morales J, Li L, Fattah FJ, Dong Y, Bey EA, Patel M, Gao J, Boothman DA. 2014. Review of Poly (ADP-ribose) Polymerase (PARP) Mechanisms of Action and Rationale for Targeting in Cancer and Other Diseases. *Critical Reviews in Eukaryotic Gene Expression* 24: 15-28
19. Kontandreopoulou C-N, Diamantopoulos PT, Tiblalexi D, Giannakopoulou N, Viniou N-A. 2021. PARP1 as a therapeutic target in acute myeloid leukemia and myelodysplastic syndrome. *Blood advances* 5: 4794-805
20. Boussios S, Abson C, Moschetta M, Rassy E, Karathanasi A, Bhat T, Ghumman F, Sheriff M, Pavlidis N. 2020. Poly (ADP-Ribose) Polymerase Inhibitors: Talazoparib in Ovarian Cancer and Beyond. *Drugs in R&D* 20: 55-73
21. Muvarak NE, Chowdhury K, Xia L, Robert C, Choi EY, Cai Y, Bellani M, Zou Y, Singh ZN, Duong VH, Rutherford T, Nagaria P, Bentzen SM, Seidman MM, Baer MR, Lapidus RG, Baylin SB, Rassool FV. 2016. Enhancing the Cytotoxic Effects of PARP Inhibitors with DNA Demethylating Agents – A Potential Therapy for Cancer. *Cancer cell* 30: 637-50
22. Giorgi M, Portwood SM, Boncek M, Wang ES. 2021. PARP Inhibition with Talazoparib Enhances DNA Damage and Anti-Leukemic Activity of Venetoclax in Preclinical Human Acute Myeloid Leukemia (AML) Models. *Blood* 138: 1176-
23. AstraZeneca. 2024. Study of AZD5305 as Monotherapy and in Combination With Anti-cancer Agents in Patients With Advanced Solid Malignancies (PETRA). ClinicalTrials.gov
24. Cellosaurus.
25. DSMZ.
26. Palve V, Knezevic CE, Bejan DS, Luo Y, Li X, Novakova S, Welsh EA, Fang B, Kinose F, Haura EB, Monteiro AN, Koomen JM, Cohen MS, Lawrence HR, Rix U. 2022. The non-canonical target PARP16 contributes to polypharmacology of the PARP inhibitor talazoparib and its synergy with WEE1 inhibitors. *Cell Chemical Biology* 29: 202-14
27. Yue X, Chen Q, He J. 2020. Combination strategies to overcome resistance to the BCL2 inhibitor venetoclax in hematologic malignancies. *Cancer Cell International* 20: 524
28. Garcia-Manero G, Döhner H, Wei AH, Torre IL, Skikne B, Beach CL, Santini V. 2022. Oral Azacitidine (CC-486) for the Treatment of Myeloid Malignancies. *Clinical Lymphoma Myeloma and Leukemia* 22: 236-50

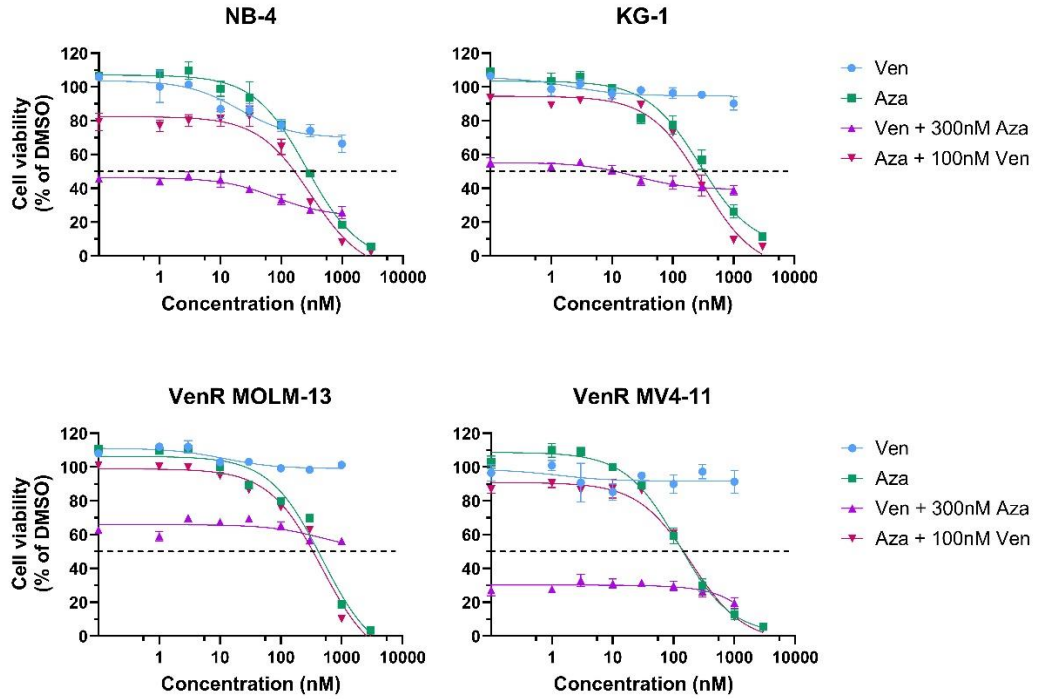
Supplementary data



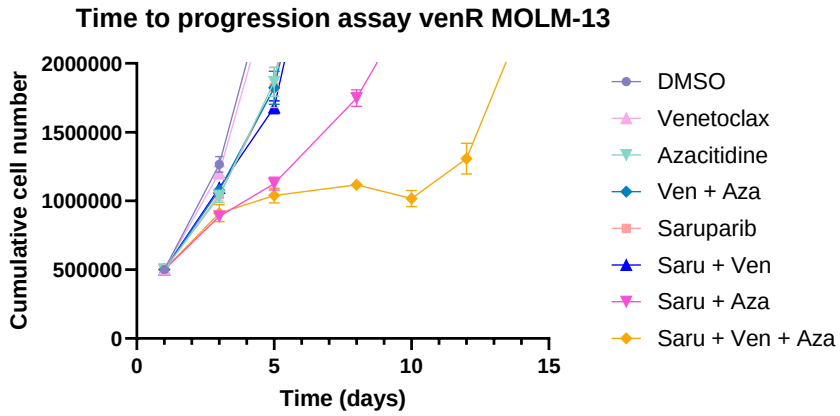
Supplemental Figure 1. Gating strategy for flow cytometric analysis. The gating for flow cytometric analysis was performed using the Forecyte software on the iQue for all experiments. The gating strategy for (**A**) drug sensitivity assays, (**B**) caspase-3/7 functional assay, (**C**) time to progression assay, (**D**) γH2Ax functional assay and (**E**) cPARP functional assay. Created with BioRender.com.



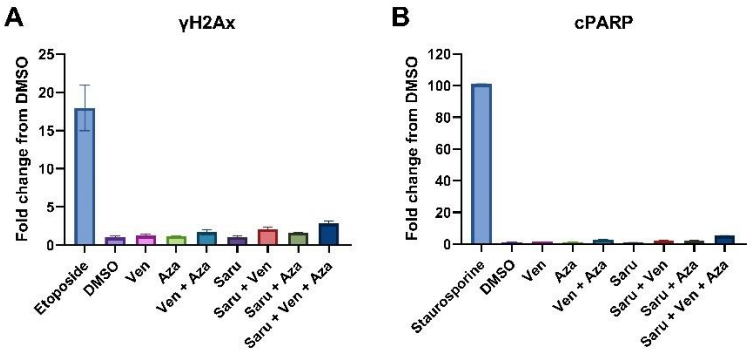
Supplemental Figure 2. Venetoclax induces cell death in parental but not in venetoclax resistant cells. The cells were treated with DMSO or venetoclax (1-1000 nM) for 4 days. Cell viability was measured via flow cytometry (Annexin V-/Draq7- population). The dose response curves present the cell viability representing the cell counts as percentages normalised to the response to DMSO. Parental (par) MOLM-13 cells are included to show the response to venetoclax of a venetoclax sensitive cell line. Data are shown as mean ± SEM (*n* = 3 from three independent experiments).



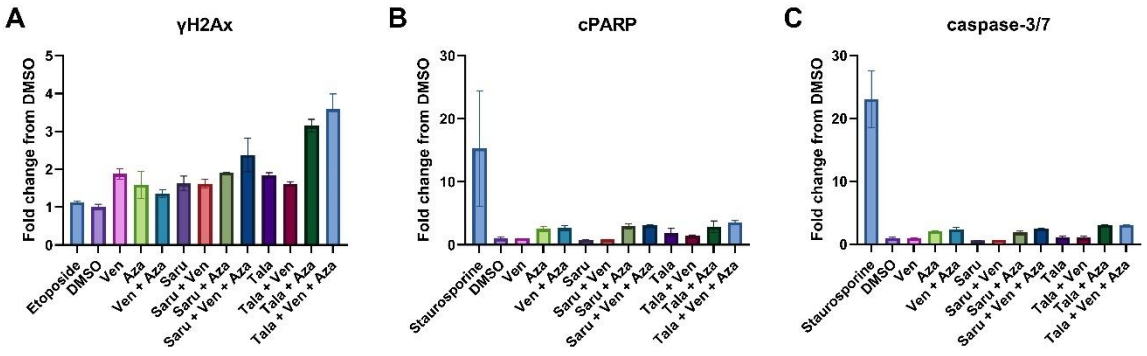
Supplemental Figure 3. **The response to venetoclax and azacitidine for the cell lines tested**. The cells were treated with DMSO, venetoclax (ven) (1-1000 nM) or azacitidine (aza) (1-3000 nM) alone and in combination with 100 nM ven or 300 nM aza for 4 days. Cell viability was measured via flow cytometry (Annexin V-/Draq7- population). The dose response curves present the cell viability representing the cell counts as percentages normalised to the response to DMSO. Data are shown as mean ± SEM (*n* = 3 from three independent experiments).



Supplemental Figure 4. The saruparib triple combination slows down cell growth in venR MOLM-13 cells. VenR MOLM-13 cells were treated with DMSO, 100 nM venetoclax (ven), 300 nM azacitidine (aza) or 30 nM saruparib (saru) as monotherapies and in combination with 100 nM ven and/or 300 nM aza. Azacitidine was added for the first five days whereas the other drugs were added three times per week throughout the treatment cycle. At the indicated timepoints, the number of live cells was measured via flow cytometry (Draq7- population), the cells were split back to 500 000 cells/ml per well and the growth medium was changed. The curves show the cumulative cell number, which is the product of the starting cell number and fold changes from previous counts. Data are shown as mean ± SD (n = 3 from a single experiment).



Supplemental Figure 5. **The results from the functional assays for venR MOLM-13 cells including positive controls.** VenR MOLM-13 cells were taken from the time to progression assay on day 3 and treated with DMSO, 100 nM venetoclax (ven), 300 nM azacitidine (aza) or 30 nM saruparib (saru) as single agents and in combination with 100 nM ven and/or 300 nM aza. Etoposide (40 µM) was used as a positive control for DNA damage and staurosporine (1 µM) for apoptosis. Levels of (**A**) DNA damage, as measured by phosphorylated H2Ax (γH2Ax), and (**B**) apoptosis, as measured by cleaved PARP (cPARP), were assessed on day 5 of the functional assays via flow cytometry. (**A, B**) Data are displayed as ratio between γH2Ax or cPARP positive and negative cells, normalised to the response to DMSO. Data are shown as mean ± SD (n = 3 from a single experiment).



Supplemental Figure 6. **The results from the functional assays for venR MV4-11 cells including positive controls.** VenR MV4-11 cells were taken from the time to progression assay on day 3 and treated with DMSO, 100 nM venetoclax (ven), 300 nM azacitidine (aza), 30 nM saruparib (saru) or 10 nM talazoparib (tala) as single agents and in combination with 100 nM ven and/or 300 nM aza. Etoposide (40 µM) was used as a positive control for DNA damage and staurosporine (1 µM) for apoptosis. Levels of (**A**) DNA damage measured by phosphorylated H2Ax (γH2Ax), (**B, C**) apoptosis measured by (**B**) cleaved PARP (cPARP), and (**C**) caspase-3/7 activity, were assessed on day 5 of the functional assays via flow cytometry. (**A, B, C**) Data are displayed as ratio between γH2Ax, cPARP or caspase-3/7 positive and negative cells, normalised to the response to DMSO. In (**A**), there was no clear increase in DNA damage for the cells treated with etoposide, so the results should be treated with caution. Data are shown as mean ± SD (n = 2 from a single experiment).