JPET Fast Forward. Published on April 5, 2021 as DOI: 10.1124/jpet.121.000537
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Poly (ADP-ribose) polymerase inhibitors for arsenic trioxide-resistant acute promyelocytic leukemia: synergistic *in vitro* antitumor effects with hypomethylating agents or high-dose vitamin C

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Running Title:

PARPi with decitabine, azacitidine or vitamin C for APL (56)

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Manuscript data:

Number of text pages: **23** (excluding Abstract, References and Figure legends)

Number of tables: **0**

Number of figures: **6**

Number of references: **82**

Number of words in the Abstract: **250**

Number of words in the Introduction: **780** (excluding reference citations)

Number of words in the Discussion: **1388** (excluding reference citations)

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Abbreviations:

AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; ATO, arsenic trioxide; ATRA, all-trans retinoic acid; BER, base excision repair; CI, combination index; Cmax, plasma maximum concentration; DCF, 2,7-dichlorodihydrofluorescein; DHE, dihydroethidium; DNMT1, DNA (cytosine-5)-methyltransferase 1; Fa, fraction affected; FAB, French-American-British classification system; MDS, myelodysplastic syndrome; PARPi, PARP inhibitors.

Section

Cellular and Molecular

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ABSTRACT

Arsenic trioxide (ATO) is an anticancer agent used for the treatment of acute promyelocytic leukemia (APL). However, 5-10% of patients fail to respond or experience disease relapse. Based on poly(ADP-ribose) polymerase 1 (PARP1) involvement in the processing of DNA demethylation, here we have tested the *in vitro* susceptibility of ATO-resistant clones, derived from the human APL cell line NB4, to PARP inhibitors (PARPi) in combination with hypomethylating agents (azacitidine and decitabine) or high-dose vitamin C (ascorbate), which induces 5-hydroxymethylcytosine (5hmC)-mediated DNA demethylation. ATO-sensitive and -resistant APL cell clones were generated and initially analyzed for their susceptibility to five clinically used PARPi (olaparib, niraparib, rucaparib, veliparib and talazoparib). The obtained PARPi IC_{50} values were far below (olaparib and niraparib), within the range (talazoparib) or above (rucaparib and veliparib) the Cmax reported in patients, likely due to differences in the mechanisms of their cytotoxic activity. ATO-resistant APL cells were also susceptible to clinically relevant concentrations of azacitidine and decitabine and to high-dose ascorbate. Interestingly, the combination of these agents with olaparib, niraparib or talazoparib resulted in synergistic antitumor activity. In combination with ascorbate, PARPi increased the ascorbate-mediated induction of 5hmC, which likely resulted in stalled DNA repair and cytotoxicity. Talazoparib was the most effective PARPi in synergizing with ascorbate, in accordance with its marked ability to trap PARP1 at damaged DNA. These findings suggest that ATO and PARPi have non-overlapping resistance mechanisms and support further investigation on PARPi combination with hypomethylating agents or high-dose ascorbate for relapsed/ATO-refractory APL especially in frail patients.

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Significance Statement:

In this study we found that poly(ADP-ribose) inhibitors (PARPi) show activity as single agents against human acute promyelocytic leukemia cells resistant to arsenic trioxide at clinical relevant concentrations. Furthermore, PARPi enhance the *in vitro* efficacy of azacitidine, decitabine and high-dose vitamin C, all agents that alter DNA methylation. In combination with vitamin C, PARPi increase the levels of 5-hydroxymethylcytosine, likely because of altered processing of the oxidized intermediates associated with DNA demethylation.

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INTRODUCTION

Acute promyelocytic leukemia (APL) is a subtype of acute myeloid leukemia (AML) with aggressive clinical presentation, characterized by the reciprocal balanced translocation t(15;17) involving the promyelocytic leukemia (*PML*) and retinoic acid receptor alpha (*RARA*) genes. The PML/RARA fusion protein blocks myeloid differentiation at the promyelocyte stage and induces aberrant self-renewal of APL cells with disruption of normal hematopoiesis. PML-RARA acts as a transcriptional repressor of RARA target genes, deregulating the homeostatic control of development, expansion and maturation/differentiation of hematopoietic stem cells. Moreover, PML-RARA interferes with the normal formation of PML-Nuclear Bodies (PML-NBs) leading to impaired stress response, decreased DNA damage repair and reduced cell propensity to undergo senescence and apoptosis (Gurnari *et al.*, 2020).

Since several years, treatment of newly diagnosed APL has been centered on the use of all-*trans* retinoic acid (ATRA) in combination with an anthracycline resulting in long-term remission rates above 80% (Sanz *et al.*, 2009). For low risk APL, ATRA plus arsenic trioxide (ATO) is the standard of care in the frontline setting (Sanz *et al.*, 2019), with long-term response rates exceeding 90% (Lo-Coco *et al.*, 2013)(Cicconi *et al.*, 2020). The current National Comprehensive Cancer Network (NCCN) treatment guidelines for APL have included ATO in the frontline also for high risk APL patients without cardiac issues, in combination with ATRA and an anthracycline or ATRA and the antibody-drug conjugate gemtuzumab ozogamicin (i.e., an anti-CD33 monoclonal antibody conjugated with the DNA damaging agent calicheamycin). Moreover, ATO is used for relapsed/refractory APL with or without ATRA, gemtuzumab ozogamicin or an anthracycline, depending on remission duration and

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the chemotherapeutic agents used for first-line therapy (NCCN guidelines version 3.2021).

In APL blasts, ATO binds to the PML portion of the hybrid oncoprotein and stimulates its sumoylation, polyubiquitination and proteasomal degradation. This process is followed by the restoration of PML-NBs and induction of apoptosis in APL cells (Noguera *et al.*, 2019). ATO also possesses additional mechanisms, including generation of reactive oxygen species (ROS) (Miller *et al.*, 2002). Despite the excellent results obtained with ATRA/ATO therapy, 5-10% of patients develop relapsed/refractory disease (Sanz *et al.*, 2019) and, in patients not eligible for allogeneic hematopoietic cell transplantation or who fail to respond to second-line agents, enrolment in a clinical trial is encouraged.

The best characterized molecular mechanism involved in ATO resistance is represented by missense somatic mutations within the B2 ATO-binding domain of *PML* gene (~40% of ATO-resistant APL cases) that prevent ATO binding and impede degradation of PML/RARA oncoprotein (Goto *et al.*, 2011)(Zhu *et al.*, 2014)(Madan *et al.*, 2016). The most common PML-A216V/T amino acidic mutation can be also found in the unrearranged *PML* allele (Iaccarino *et al.*, 2016). Other *PML*-unrelated mechanisms may contribute to ATO resistance such as cellular metabolic adaptation, dysregulation of redox signaling, presence of the X-RARA oncoprotein instead of PML-RARA, and mutations in other genes (Alex *et al.*, 2014)(Balasundaram *et al.*, 2016) (Noguera *et al.*, 2019)(Iaccarino *et al.*, 2019).

In the search of potential therapeutic approaches for APL relapsed/refractory to ATO, we have generated an *in vitro* model of APL human sublines with acquired resistance to ATO and focused our attention on poly (ADP-ribose) polymerase inhibitors (PARPi), based on preclinical evidence of their activity against myeloid malignancies

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(Faraoni *et al.*, 2015)(Esposito *et al.*, 2015)(Faraoni *et al.*, 2018)(Nieborowska-Skorska *et al.*, 2017)(Zhao and So, 2017)(Kohl *et al.*, 2019). These agents belong to a new class of orally administered anticancer drugs that mainly act by dampening the activity of PARP1, a nuclear enzyme required for sensing and repairing DNA damage. Five PARPi have been recently approved for advanced/recurrent ovarian, breast, pancreatic or prostate cancers with defective homologous recombination due to mutated *BRCA1/2* genes or other genetic/epigenetic alterations leading to reduced repair of DNA double-strand breaks (Faraoni and Graziani, 2018). Moreover, these and other PARPi are currently under clinical investigation as monotherapy and in combination with targeted agents or chemotherapy for several types of cancers, including hematological malignancies (www.clinicaltrials.gov).

Our previous studies revealed that PARPi exerted cytotoxic effects in primary cultures of AML blasts and leukemia cell lines. Among the different AML cell lines tested, the promyelocytic cell line NB4 was the most sensitive to the PARPi olaparib (Faraoni *et al.*, 2015). In addition, studies in murine and human AML grafts, revealed that PML/RARA translocation-driven leukemia was extremely sensitive to olaparib and veliparib (Esposito *et al.*, 2015).

In the present study, we have compared the antitumor activity of different PARPi (olaparib, niraparib, rucaparib, talazoparib and veliparib) in APL cells rendered resistant to ATO as monotherapy and combined with agents endowed with antileukemic activity and whose mechanism of action involves a DNA damage response with PARP1 intervention. In particular, PARPi have been tested in combination with the DNA hypomethylating agents azacitidine and decitabine or with high-dose vitamin C (hereafter referred to as ascorbate), which has been shown to promote 5-hydroxymethylcytosine (5hmC)-mediated DNA demethylation by

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enhancing the activity of Ten-Eleven Translocation (TET) enzymes (Minor *et al.*, 2013). Results indicated that olaparib, niraparib and talazoparib in combination with the aforementioned DNA demethylating agents exerted synergistic antiproliferative effects against APL cells, including those resistant to ATO. The increased DNA damage observed in APL cells exposed to PARPi plus ascorbate was associated with a significant increase in the levels of 5hmC, likely as a consequence of altered processing of the oxidized intermediates associated with DNA demethylation.

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MATERIALS AND METHODS

Generation of NB4 clones and cell culture conditions

The promyelocitic leukemia cell line NB4 (American Type Culture Collection, ATCC, Manassas, VA, USA) was cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2 mM L-glutamine (EuroClone, Pero, Milan, Italy), 1% penicillin/streptomycin (EuroClone) and 20% fetal bovine serum at 37°C in a humidified CO_2 incubator. Four different clones (CL1, CL2, CL3 and CL4) were produced by limiting dilution from the NB4 cell line at early passages from the initial stock purchased from ATCC (Figure 1). In order to generate ATO-resistant cells, cell clones were exposed to increasing concentrations of ATO (0.1-1 μM) for about one year and the corresponding ATO-resistant clones were named CL1-R, CL2-R, CL3-R and CL4-R. At this time, to preserve the resistant phenotype, cells were frozen in several aliquots. ATO-resistant clones were maintained in culture with 1 μM ATO; experiments were performed after at least two passages from the last ATO-treatment. The parental NB4 cell line and its ATO-sensitive and -resistant clones were authenticated by testing the expression of the fusion PML/RARA gene (see below).

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Drug treatment and survival assay

The ATO (As_2O_3, Sigma-Aldrich) stock solution was prepared by dissolving the drug in 1 N NaOH and diluting it in PBS (EuroClone) at a final concentration of 2 mM. The stock solutions of PARPi (Selleck Chemicals, Houston, TX, USA; 2 mM olaparib, talazoparib and veliparib; 20 mM niraparib and rucaparib) were prepared by dissolving the powder of each compound in dimethyl sulfoxide (DMSO; Sigma-Aldrich) followed by dilution in RPMI-1640 medium. Decitabine (Cayman Chemical,

Ann Arbor, MI, USA) and azacitidine (Sigma-Aldrich) were dissolved in PBS (2 mM) and DMSO (20 mM), respectively. Ascorbate (L-ascorbate, Sigma-Aldrich) was diluted in RPMI-1640 medium at 250 mM concentration. Drug aliquots were stored at −80 °C and for each experiment a new aliquot was thawed and used. In all experiments, the DMSO final concentration in the culture medium was always <0.01% (v/v).

For cell treatment, drugs were added at the beginning of each experiment and left in culture medium until cell harvesting. NB4 parental cell line and its clones were treated with the PARPi olaparib (1.25-20 μM), niraparib (1.25-10 μM), talazoparib (12.5-100 nM), rucaparib (1.25-10 μM) and veliparib (5-20 μM), as single agents or in combination with azacitidine (1.25-1 μM), decitabine (25-500 nM) or ascorbate (0.25-1 mM). Drug concentrations tested always included the plasma peak concentration (Cmax) values reached in cancer patients.

For survival assays, cells were analyzed by the MTS viability test (Promega, Madison, WI, USA), according to the manufacturer's instructions, or by trypan blue dye exclusion count. The drug concentration capable of inhibiting 50% of cell growth (IC_{50}), compared to the untreated control, was extrapolated from the dose-response curves by using linear regression (GraphPad Prism 5 software; GraphPad Inc., San Diego, CA, USA). The dose-effect curves were analyzed by the median-effect method of Chou and Talalay with the CompuSyn software (ComboSyn Inc., Paramus, NJ, USA). The combination index (CI) indicates a quantitative measure of the drug combination effects in terms of synergistic (CI <1), additive (CI =1) or antagonistic effect (CI >1).

Apoptosis was evaluated by flow cytometry analysis of the sub-G1 fraction after cell fixation in ethanol, treatment with 10 μg/ml RNase A (Sigma-Aldrich) and staining

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with 100 µg/ml propidium iodide (PI) (Sigma-Aldrich) for 20 minutes at 37°C in the dark. Samples (5 x 10^4 cells) were acquired on a BD FACSCalibur flow cytometer and evaluated using CellQuest Software (BD Biosciences, San Jose, CA, USA).

Molecular analysis of PML/RARA

Total RNA was isolated by Trizol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). One µg of RNA was reverse-transcribed with random hexamer primers and amplified (reagents from Life Technologies, Thermo Fisher Scientific) with the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). RT-PCR for PML/RARA detection was carried out using standard protocols (van Dongen *et al.*, 1999).

For sequencing the region of PML gene corresponding to the B2 ATO-binding domain, the PML/RARA fusion transcript was amplified by PCR and analyzed by Sanger sequencing as reported elsewhere (Iaccarino *et al.*, 2016).

Immunoblot analysis of apoptosis and DNA damage markers

Total cellular proteins were extracted using a buffer containing 50 mM Tris–HCl (pH 7.5), 5 mM EDTA, 5 mM EGTA, 150 mM NaCl, 1% Nonidet P-40, 1 mM Na orthovanadate, 20 mM β-glycerophosphate, 1 mM AEBSF (Sigma-Aldrich) and protease inhibitor cocktail (Thermo Fisher Scientific). Protein aliquots were loaded onto SDS polyacrylamide gel electrophoresis and blotted to nitrocellulose membranes. Filters were incubated with the following antibodies: anti-PARP1 (1:1000; C2-10, Trevigen, Gaithersburg, MD, USA), anti-caspase 8 (1:500; 12F5, Enzo Life Sciences, NY, USA), anti-cleaved caspase 8 (1:400; Asp374, Cell Signaling Technology, Danvers, MA, USA), anti-caspase 3 (1:1000; D3R6Y, Cell

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Signaling Technology), anti-cleaved caspase 3 (1:1000; D175, Cell Signaling Technology), anti-γH2AX (1:1000; JBW301, Millipore, Burlington, MA, USA), anti-glyceraldehyde 3-phosphate dehydrogenase (1:1000; GAPDH, 14C10, Cell Signaling Technology), anti-β-actin (1:2000; A2066, Sigma-Aldrich). Horseradish peroxidase-conjugated IgGs were used as secondary antibodies (1:5000; anti-mouse A4416, Sigma-Aldrich; anti-rabbit NA934, GE Healthcare, Chicago, IL, USA). The autoradiograms were subjected to densitometric analysis using the ImageJ 1.45s software (Bio-Rad Laboratories, Hercules, CA, USA) and results normalized against GAPDH or β-actin.

Determination of intracellular ROS

Total intracellular ROS were evaluated by 2,7-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, Invitrogen, Thermo Fisher Scientific) reagent that is deacetylated by non-specific esterases and oxidized in 2,7-dichlorodihydrofluorescein (DCF) fluorescent compound by hydroxyl and peroxyl radicals or other intracellular ROS in the cells. Cytosolic superoxide anion production was detected by dihydroethidium (DHE, Invitrogen, Thermo Fisher Scientific) reagent. DHE is oxidized by superoxide anion in 2-hydroxyethidium that becomes fluorescent after intercalation into DNA. For ROS analysis, cells were harvested after 4 h treatment with graded concentrations of ascorbate. Cells (5×10^5) were incubated with 5 μM of CM-H_2DCFDA or DHE at 37°C for 30 minutes in 5% fetal bovine serum in PBS and analyzed by the BD FACSCalibur flow cytometer.

Analysis of 5hmC by dot blot assay

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Cells were treated with ascorbate or PARPi (i.e., olaparib, niraparib or talazoparib), as single agents or in combination, for 24 h and then genomic DNA was extracted with DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Dot blots were performed as previously published (Ciccarone *et al.*, 2018). Briefly, DNA was denatured in 0.4 N NaOH, 10 mM EDTA at 95°C for 10 min and neutralized with an equal volume of cold 4 M ammonium acetate (pH 7.0). Starting from 2 μg of denatured DNA, two-fold dilutions of each sample were spotted on the nylon membrane Hybond-N+ (GE Healthcare) in an assembled Bio-Dot apparatus (Bio-Rad Laboratories). Each well was washed with 0.4 N NaOH, 10 mM EDTA and then 2x SSC buffer. After baking at 80°C for 15 min, air-dried membranes were blocked in 5% skimmed milk in TBS-T and incubated with anti-5hmC antibody (39769, Active Motif, Carlsbad, CA) and anti-rabbit HRP-conjugated secondary antibody. Dot-blot signals were revealed by chemiluminescence. Equal spotting of total DNA onto the membrane was checked by staining the same blotted filter with 0.02% methylene blue in 0.3 M sodium acetate (pH 5.2).

Statistical Analysis

Statistical analysis was performed by using the GraphPad Prism 5 software and data were reported as mean ±SD. Statistical analysis of the differences in IC_{50} values between two groups was performed by unpaired Student's *t*-test. For multiple comparisons, unpaired one-way ANOVA analysis, followed by LSD test was used. All statistical tests were two-sided. Differences were considered statistically significant when $p<0.05$.

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RESULTS

Generation of ATO-resistant clones

The NB4 cell line was originally derived from the bone marrow of a 23-year-old woman with APL (FAB M3) (Lanotte *et al.*, 1991). From this cell line, we generated four clones by limiting dilution (CL1, CL2, CL3 and CL4) that were subsequently exposed to increasing concentrations of ATO (0.1-1 μM) within a time-frame of one year to generate the corresponding ATO-resistant clones (CL1-R, CL2-R, CL3-R and CL4-R) (flow chart in Figure 1A). Sensitive and resistant clones were characterized for PML/RARA expression by RT-PCR. All NB4 clones, including the ATO-resistant ones, maintained the expression of the fusion PML/RARA gene (Figure 1B). Sequencing of the PML allelic region corresponding to the B2 ATO-binding domain indicated that all ATO-resistant clones lacked the PML-A216V/T mutation or other mutations in the PML B2 domain (data not shown).

Since the proliferation rate might affect tumor cell response to ATO, we analyzed the growth pattern of the APL clones by cell count and found no significant differences between the ATO-sensitive clones and their corresponding ATO-resistant counterparts (Figure 1C).

The *in vitro* susceptibility to ATO antiproliferative effects of the parental and ATO-selected clones or of the NB4 bulk cell line was analyzed by MTS assay after three days of treatment with graded drug concentrations. The ATO IC_{50} in the resistant clones (2.6-4.5 μM range) was 5-9 fold higher than in the corresponding sensitive clones (0.5-0.9 μM range) (Figure 2A). Analysis of apoptosis after treatment with 0.5 and 1 μM ATO revealed the induction of cell death in ATO-sensitive clones in a concentration-dependent manner, while no or marginal apoptotic effects (at the higher concentration tested) were observed in ATO-resistant cells (Figure 2B).

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Consistently with the results of flow cytometry showing apoptotic induction in ATO-sensitive cells, immunoblot analysis demonstrated that this effect was associated with cleavage of PARP1, caspase 3 and caspases 8, the latter indicating activation of the apoptotic extrinsic pathway. Moreover, ATO induced DNA damage only in ATO sensitive clones, as evidenced by the high expression levels of histone 2AX phosphorylation at serine 139 (γH2AX) (Figure 2C).

ATO-resistant clones are sensitive to PARPi

Recent preclinical studies have shown that PARPi exert cytotoxic effects against myeloid malignancies (Faraoni, *et al.*, 2019)(Fritz *et al.*, 2020). To investigate the potential activity of different PARPi in APL cells, ATO-sensitive and -resistant NB4 clones as well as the bulk cell line were exposed to increasing concentrations of olaparib, niraparib, talazoparib, rucaparib and the investigational PARPi veliparib. Cell growth was analyzed by MTS assay on day six after a single exposure to the PARPi. In fact, based on our previous studies, the antiproliferative activity of PARPi in myeloid tumor cells requires prolonged drug exposure (Faraoni *et al.,* 2015)(Faraoni *et al.,* 2018)(Faraoni *et. al,* 2019). The drug concentrations tested in the experiments always included the plasma Cmax reported in cancer patients during phase 1 clinical trials (Cmax ranges were represented by dotted lines in figure 3) (Fong *et al.*, 2009)(Mateo *et al.*, 2016)(Sandhu *et al.*, 2013)(Kristeleit *et al.*, 2017)(Shapiro *et al.*, 2019)(de Bono *et al.*, 2017)(Kummar *et al.*, 2009)(Nishikawa *et al.*, 2017). All clones and the NB4 cell line were sensitive to olaparib with IC_{50} values lower (2.9-12.1 μM) than the reference Cmax values (range 16-22 μM). Moreover, the ATO-resistant CL1-R and CL4-R clones showed significantly lower susceptibility to olaparib compared to their ATO-sensitive counterparts (Figure 3A). All clone pairs

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presented comparable susceptibility to niraparib with IC_{50} values (0.7-2.0 μM) below or within the Cmax range (1.2-4.4 μM) (Figure 3B). In the case of talazoparib, the IC_{50}s of most clones (5/8 clones; 16.0-56.7 nM) were below or within the Cmax (30-60 nM) and no significant differences were observed between ATO-sensitive and -resistant clones, except for the CL3/CL3R couple, being CL3-R significantly more sensitive to talazoparib than CL3 (Figure 3C). Regarding rucaparib and veliparib, in almost all cases (7/8 clones for rucaparib and 8/8 clones for veliparib) the obtained IC_{50}s were above the Cmax values (0.6-9.5 μM and 2.6-13.5 μM, respectively) (Figures 3D and 3E). Similarly to what was observed with talazoparib, CL3-R was more susceptible also to these PARPi compared to its parental CL3 clone. Overall, these results indicated that ATO-resistant NB4 clones were still responsive to clinically achievable concentrations of the PARPi olaparib, niraparib and, in most cases, talazoparib.

PARPi in combination with hypomethylating agents induce synergistic growth inhibitory effects in ATO-sensitive and -resistant APL clones

Based on our previous report on the synergistic cytotoxic effects induced by the PARPi olaparib in AML and MDS cells when tested in combination with hypomethylating agents (Faraoni, *et al.*, 2019), we have investigated the activity of azacitidine and decitabine against ATO-sensitive and –resistant APL cells, as single agents or in association with the PARPi. As shown in Figure 4A and B, in all cell clones azacitidine and decitabine IC_{50} values, calculated three days after drug exposure, were within the range of clinically relevant concentrations previously reported for these drugs (Keating, 2012)(Cashen *et al.*, 2008). Interestingly, the growth of CL1-R and CL4-R clones was significantly more inhibited compared to their

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corresponding ATO-sensitive counterparts. Conversely, in other cases no differences in azacitidine or decitabine IC_{50} values were observed between ATO-sensitive and -resistant clones, except for the CL2 clone that showed higher sensitivity to decitabine compared to its ATO-resistant counterpart (Figure 4A and B). These findings suggested that there was no direct correlation between the susceptibility profile to ATO of APL clones and the response to both hypomethylating agents.

To assess the activity of hypomethylating agents in combination with PARPi, CL2 and CL2-R cells were exposed to increasing concentrations of azacitidine (0.125-1 μM) or decitabine (0.031-0.5 μM) together with a fixed concentration of the PARPi that, based on our analysis, were the most active when tested as single agents (i.e., olaparib, niraparib and talazoparib). The clone 2/2R couple was chosen since no significant differences were observed in the susceptibility to the PARPi tested between the ATO-sensitive and -resistant cells (Figure 3). Analysis of the inhibitory effects on cell proliferation exerted by the drug combination was performed after three days of treatment due to the more rapid antiproliferative effects of the cytidine analogues compared to PARPi. Results indicated that the drug combination induced a greater inhibition of cell growth compared to the single hypomethylating agents in both ATO-sensitive and ATO-resistant clones (Figure 4C). As assessed by the CompuSyn method (Chou, 2010), synergistic effects were observed regardless of the type of PARPi associated with azacitidine or decitabine, both in ATO-sensitive and -resistant clones (Figure 4C), with combination index (CI) values largely below 1 (dotted line of the Fa-CI plots) (Figure 4D). The strong synergism observed with the combination of these drugs in APL cells is in agreement with previous data obtained with AML cell lines (Orta *et al.*, 2014)(Muvarak *et al.*, 2016)(Faraoni *et al.*, 2019) and primary cultures of MDS samples (Faraoni *et al.*, 2019).

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Ascorbate induces synergistic antiproliferative effects in combination with the PARPi niraparib and talazoparib.

Recent *in vitro* and *in vivo* preclinical evidence indicates that high-dose ascorbate (i.e., mM concentrations) has cytotoxic activity against AML and APL cells (Mastrangelo *et al.*, 2015)(Noguera *et al.*, 2017)(Cimmino *et al.*, 2017). In this study, NB4 clones were treated with graded concentrations of ascorbate (0.125-2 mM) and cell proliferation was assessed by cell count after three days of culture (Figure 5A), a time point commonly used for evaluating ascorbate antiproliferative activity in other cellular models (Cimmino *et al.,* 2017)(Noguera *et al.,* 2017). The obtained ascorbate IC_{50}s ranged from 0.6 to 1.8 mM (Figure 5B), values comprised with the range of plasma concentrations detected in cancer patients treated with high doses of this agent (Hoffer *et al.*, 2008)(Ngo *et al.*, 2019). Interestingly, no significant differences were observed between ATO-sensitive and -resistant cells, indicating that there is no cross-resistance between ascorbate and ATO.

Since ascorbate has been reported to induce a TET2-mediated increase of 5hmC levels whose processing requires base excision repair (BER) and PARP1 intervention (Cimmino *et al.*, 2017)(Pastor *et al.*, 2013), we investigated the effect of its combination with PARPi on APL cells. Fixed PARPi concentrations were combined with graded concentrations of ascorbate. The antiproliferative effects were assessed after three days of treatment by cell count and results were reported as proliferation curves in Figures 5C (top panels, ATO-sensitive clones; bottom panels, ATO-resistant clones). The ascorbate combination with olaparib exerted synergistic effects only in three out of the eight clones (i.e., CL2, CL2-R, CL3-R), while its combination with niraparib resulted in additive effects in CL1 and CL1-R clones and

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synergistic effects in all the other clones. Noteworthy, the ascorbate/talazoparib combination was highly effective in all ATO-sensitive and -resistant clones, with extremely low CI values (Figure 5D), indicating this PARPi as the best candidate to be combined with ascorbate.

PARPi in combination with ascorbate increase DNA damage and 5-hydroxymethylcytosine levels

To investigate whether co-treatment with PARPi and ascorbate might enhance DNA damage, we first evaluated γH2AX expression by immunoblot analysis in the two ATO-resistant CL2-R and CL3-R clones, where the combination was synergistic with all the three PARPi tested (i.e., olaparib, niraparib and talazoparib). As single agents, ascorbate (1 mM) and the PARPi induced low or moderate levels of γH2AX (Figure 6A). Conversely, the ascorbate/PARPi combination significantly increased γH2AX expression compared to both single agents, indicating a significantly higher level of unrepaired DNA damage. This effect was more evident with 1 mM ascorbate: in CL2-R cells, it was observed with all the three PARPi, whereas in CL3-R cells mainly with talazoparib (Figure 6A).

High-dose ascorbate was previously reported to generate ROS (Chen *et al.*, 2007) that contribute to DNA damage (Kim *et al.*, 2018). However, in our cellular model, ascorbate concentrations below 2 mM did not increase either DCF or DHE fluorescence, tested as indicators of total intracellular ROS and cytosolic superoxide anion, respectively (Figure 6B). Having excluded induction of DNA damage by oxidative stress, we evaluated whether the increase of γH2AX expression observed after APL cell exposure to ascorbate in combination with PARPi could be related to altered processing of 5hmC. In fact, ascorbate is a co-factor of TET enzymes, which

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catalyze the oxidation of 5-methylcytosine (5mC) to 5hmC and other intermediates that are processed by BER during active DNA demethylation. Thus, when cells are treated with ascorbate, the rate of initial oxidation of 5mC increases with consequent augmented formation of 5hmC (Gillberg *et al.*, 2018). Since inhibition of PARP1 was suggested to block the BER-mediated processing of the oxidized intermediates associated with DNA demethylation (Cimmino *et al.*, 2017), we verified whether the increase of DNA damage detected when PARPi were added to ascorbate was due to ineffective BER-mediated processing of 5hmC with a consequent rise of its levels. Indeed, in CL2-R cells 1 mM ascorbate induced higher 5hmC levels compared to untreated or PARPi treated cells (Figure 6C). More interestingly, the addition of 50 nM talazoparib significantly increased 5hmC accumulation compared to ascorbate alone (Figure 6C). Similar results were obtained also when ascorbate was combined with olaparib or niraparib and in another ATO-resistant clone (i.e., CL3-R), where the drug combination resulted in synergistic antiproliferative effects. Conversely, combined treatment with ascorbate and olaparib of clone CL1-R did not further increase 5hmC levels compared to ascorbate alone (Figure 6D). For this clone, the olaparib concentration tested (i.e., 2.5 μM) was markedly below the IC_{50} and did not result in synergistic antiproliferative effects with ascorbate (Figure 5C). These results suggest that the synergistic cytotoxic effects observed when ascorbate was associated with PARPi are at least in part due to the blockade of BER activity during the demethylation process with consequent accumulation of 5mhC.

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DISCUSSION

In the present study, we demonstrated for the first time that PARPi increase the *in vitro* antiproliferative activity of hypomethylating agents and ascorbate against APL cells, including those resistant to ATO.

PARPi efficacy was first demonstrated in patients with ovarian cancer harboring germline or somatic deleterious mutations of *BRCA1/2* genes, but a significant clinical benefit was also reported in the absence of *BRCA*1/2 mutations (Ledermann *et al.*, 2014)(Friedlander *et al.*, 2018). In fact, genetic alterations affecting other genes involved in the repair of DNA double-strand breaks may render cancer cells more sensitive to PARPi compared to normal cells (Faraoni and Graziani, 2018). In APL cells, the presence of PML/RARA has been reported to alter the repair of DNA single- and double-strand breaks (Alcalay *et al.*, 2003), sensitizing them to PARPi (Esposito *et al.*, 2015). On this basis, in an attempt to identify alternative therapies for patients with APL refractory to ATO, we have generated an APL cellular model represented by clones sensitive or resistant to this arsenic derivative and tested their susceptibility to PARPi.

Among the different PARPi tested, olaparib, niraparib and talazoparib, but not rucaparib and veliparib, induced antiproliferative and cytotoxic effects both in ATO-sensitive and -resistant APL cells at clinically relevant concentrations. Moreover, our data suggest the lack of cross-resistance between ATO and PARPi. Indeed, cross-resistance to ATO and conventional chemotherapeutic agents is uncommon, since ATO is not a substrate of the p-glycoprotein encoded by *MDR-1* gene or other members of the ATP-binding cassette family of transporters, such as MRP-1 or BCRP (Takeshita *et al.*, 2003)(Sertel *et al.*, 2012). However, repeated exposure of APL cells to ATO has been reported to induce expression of the p-glycoprotein

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(Takeshita *et al.*, 2003). Regarding PARPi, although the most frequent resistance mechanism is the emergence of secondary mutations restoring BRCA1/2 function, for olaparib or other inhibitors (e.g., rucaparib, talazoparib), which are substrates of the p-glycoprotein or other efflux pumps, low intratumoral drug concentrations might also contribute to treatment failure in tumors overexpressing *MDR-1* (Lawlor *et al.*, 2014). However, in our cellular model, the pattern of response of APL clones did not suggest the occurrence of common resistant mechanisms between the different PARPi tested.

The distinct susceptibility profile of APL clones to each PARPi may be attributed to the different mechanisms involved in the cytotoxic activity of the individual agents (i.e., catalytic inhibition versus PARP1 trapping at DNA breaks). Indeed, while their inhibitory effects on PARP1 catalytic activity are not largely different (with IC_{50}s in the nanomolar range), PARPi markedly differ in the trapping ability, being talazoparib and veliparib the most and least potent, respectively (Murai *et al.*, 2012)(Murai *et al.*, 2014). Moreover, PARPi trapping potency correlated with cytotoxicity in tumor cells (Murai *et al.*, 2012)(Murai and Pommier, 2015). Consistently with their trapping efficiency, talazoparib, niraparib and olaparib were, in this order, the most effective PARPi as a single agent in inhibiting cell proliferation of the APL clones.

ATO-sensitive and -resistant APL cells were also susceptible to clinically relevant concentrations of the hypomethylating agents azacitidine and decitabine, showing a chemosensitivity profile that did not parallel that of ATO. These agents are inhibitors of DNA methyltransferase and are used in clinical practice particularly in elderly AML patients ineligible for intensive chemotherapy and in intermediate/high-risk MDS. More recently, oral formulations of both drugs have been FDA-approved as maintenance therapy of AML patients who achieve first complete remission but are

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not able to complete intensive induction chemotherapy. Both drugs are cytidine analogues that cause DNA damage as a consequence of their random incorporation into DNA (azacitidine also in RNA), covalent complex formation with DNA (cytosine-5)-methyltransferase 1 (DNMT1) leading to its trapping onto DNA (Patel *et al.*, 2010)(Maes *et al.*, 2014) and loss of methylated cytosines with a widespread change in gene expression (Santi *et al.*, 1984). The synergistic effects observed in APL cells treated with hypomethylating agents plus PARPi (i.e., talazoparib, niraparib and olaparib) are consistent with those previously reported in other experimental models, including AML, MDS, as well as solid tumors (e.g., ovarian cancer and non-small cell lung cancer) (Zhao and So, 2017)(Faraoni *et al.*, 2019)(Muvarak *et al.*, 2016)(Pulliam *et al.*, 2018)(Abbotts *et al.*, 2019). These effects are likely the result of increased DNA damage, as we and others have previously reported in several tumor models (Zhao and So, 2017)(Faraoni et al., 2019)(Muvarak et al., 2016)(Abbotts et al., 2019). The mechanisms underlying the observed synergistic activity include: altered processing by BER and PARP1 of the aberrantly incorporated cytidine analogue and trapped DNMT1 (Orta *et al.*, 2014); induction of a BRACness phenotype by down-regulating the expression of DNA repair enzymes (Abbotts *et al.*, 2019); accumulation of ROS with consequent DNA damage that triggers PARP1 activation and becomes deleterious in the presence of PARPi (Pulliam *et al.*, 2018); increased drug retention at the DNA damage sites (Muvarak *et al.*, 2016). It is reasonable to hypothesize that similar PARPi and hypomethylating agent interactions may also occur in APL cells.

ATO-sensitive and -resistant APL cells showed comparable susceptibility to mM concentrations of ascorbate. At physiological concentrations, ascorbate acts as antioxidant and cofactor of metabolic enzymes; conversely, when pharmacological

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doses are administered intravenously (resulting in plasma concentrations in the millimolar range), ascorbate behaves as pro-oxidant favoring the formation of large amounts of ROS (Mastrangelo *et al.*, 2015)(Chen *et al.*, 2007)(Kim *et al.*, 2018)(Gillberg *et al.*, 2018). Treatment with ascorbate of AML patients resulted in clinical benefit, especially in the presence of loss-of-function mutations of *TET2* (Zhao *et al.*, 2018)(Das *et al.*, 2019) that are frequently detected in AML and result in altered DNA demethylation (Abdel-Wahab *et al.*, 2009)(Delhommeau *et al.*, 2009). Moreover, several clinical studies are testing high-dose ascorbate, as single agents or in combination with chemotherapeutic agents, in a variety of tumors, including AML and APL (www.clinicaltrials.gov). In our APL experimental model, ascorbate inhibited cell proliferation at concentrations devoid of pro-oxidant effects but capable of inducing DNA damage, as indicated by H2AX phosphorylation, which was likely the result of increased 5hmC formation (Cimmino *et al.*, 2017)(Agathocleous *et al.*, 2017)(Wu and Zhang, 2017).

In APL cells, the combination of ascorbate with PARPi resulted in a significant increase of cytotoxicity, DNA damage and 5hmC levels, which is likely due to ineffective BER-mediated processing of the oxidized intermediates associated with DNA demethylation. Indeed, 5hmC is detected as DNA damage and triggers the intervention of BER and PARP1. Thus, in the presence of PARPi, the ascorbate-mediated activation of TET2 and increased generation of 5hmC in DNA may result in stalled repair and greater cytotoxicity (Kharat *et al.*, 2020). In this context, talazoparib more potently synergized with ascorbate, as compared with olaparib and niraparib, in accordance with its higher ability to trap PARP1 on DNA.

A potential drawback associated with ascorbate treatment relies on its complex pharmacokinetics and potential heterogeneous distribution in tumor and

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normal tissues (Giansanti *et al.,* 2021). Although ascorbate millimolar concentrations can be achieved in plasma after intravenous injection, these high concentrations might not be easily reached at the tumor site, especially in the case of APL involving the CNS. In fact, only administration of its oxidized form dehydroascorbate (DHA) may generate pharmacological levels of vitamin C in the brain, since DHA more readily crosses the blood-brain barrier via the glucose transporter GLUT1 (Spoelstra-de Man *et al.,* 2018). However, high-dose DHA cannot always be considered a valid alternative to ascorbate, since DHA antitumor activity depends on its conversion to ascorbate by glutathione and glutathione transferases and tumor cells might have different reducing ability and not always efficiently accumulate ascorbate (Ferrada *et al*., 2019). Moreover, modulation of TET activity, likely required for the observed synergism with PARPi, is mediated by ascorbate and not by vitamin C oxidized forms (Minor *et al*., 2013)(Dickson *et al*., 2013) (Yin *et al*., 2013)(Guan *et al*., 2020).

A limitation of our study is represented by the use of different clones deriving from one cell line only (i.e., NB4). However, it should be noted that few human APL cell lines are presently available for in vitro studies. Furthermore, bone marrow samples collected from patients with APL resistant to ATO for establishing primary cultures are not readily available. Thus, preclinical in vivo studies in murine APL models might further validate our data. Moreover, our cellular model of ATO-resistant cells, lacking mutations in the PML B2 domain, did not allow evaluating the activity of the pharmacological treatment against APL cells harboring PML-A216V/T mutations. Thus, additional studies are required to evaluate drug treatment in specific genetic contexts or more complex resistance phenotypes (e.g., double resistance to both ATRA and ATO).

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The anti-leukemic activity of PARPi may be also increased by their combination with agents used for APL treatment such as anthracyclines and gentuzumab ozogamicin. In fact, both agents are able to induce DNA damage, the repair of which can be hampered by inhibiting PARP1 activity (Yamauchi *et al.*, 2014)(Portwood *et al.,* 2019). Previous reports have also suggested a potential role of PARPi in reducing the risk of cardiotoxicity associated with the use of anthracyclines, based on the involvement of PARP1 overactivation in cardiomyocyte damage induced by these chemotherapeutic agents (Pacher *et al.*, 2002)(Ali *et al.*, 2011). However, the protective effect of PARPi on anthracycline-induced cardiotoxicity is still debated (Damiani *et al.*, 2018).

The favorable safety profile of PARPi, decitabine, azacitidine and ascorbate encourages further investigation on their therapeutic potential as components of combination regimens for relapsed/ATO-refractory APL especially in the case of frail patients who cannot tolerate the proarrhythmic effects of ATO or the adverse effects of more aggressive therapies.

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Wrote or contributed to the writing of the manuscript: Faraoni, Graziani and Voso

DISCLOSURE OF CONFLICTS OF INTEREST

The authors declare no financial conflict of interest.

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Footnotes

This work was supported by the "Associazione Italiana per la Ricerca sul Cancro" (AIRC) [IG 2017-ID. 20353 project-PI Grazia Graziani; AIRC 5x1000 call "Metastatic disease: the key unmet need in oncology to MYNERVA project; #21267 (MYeloid NEoplasms Research Venture AIRC) and PRIN grant N. 2017WXR7ZT to Maria Teresa Voso].

Congress abstracts where the work was previously presented:

1) M. Giansanti, I. Faraoni, S.P. Prete, T. Karimi, M. Divona, T. Ottone, M. I.Consalvo, M.T. Voso and G. Graziani. XVI Congresso Nazionale SIES (Società Italiana di Ematologia Sperimentale). Postponed until a later date. Giansanti M, Faraoni I, Prete SP, Karimi T, Divona M, Ottone T, Consalvo MI, Voso MT, Graziani G (2020) Poly(ADP-ribose) polymerase inhibitors synergize with ascorbate and hypomethylating agents in arsenic trioxide-resistant promyelocytic leukaemia cells. Abstract Book, Proceedings of the XVI Congress of the Italian Society of Experimental Hematology (SIES), Napoli, Italy, 15-17 October 2020. *Haematologica.* 2020, 105(s2), p. S65, abstract n. C079. Available online: https://haematologica.org/article/view/haematol.2020.s2

2) M. Giansanti, I. Faraoni, S.P. Prete, T. Karimi, M. Divona, T. Ottone, F. Ciccarone, M.T. Voso and G. Graziani. XIII SIF Seminar on Pharmacology for PhD Students, Fellows, Post Doc and Specialist Trainees", "The 40th Congress of the Italian Pharmacological Society (SIF), Digital Edition", 8-13 March, 2021. Giansanti M, Faraoni I, Prete SP, Karimi T, Divona M, Ottone T, Ciccarone F, Voso MT, Graziani G. Role of poly(ADP-ribose) polymerase inhibitors in combination with ascorbate and hypomethylating agents for acute promyelocytic leukaemia resistant to arsenic

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FIGURE LEGENDS

Figure 1 ATO-resistant clones maintain APL phenotype.

(A) Flow chart of the generation of ATO-sensitive and -resistant NB4 clones. Four different clones were isolated by limiting dilution from the bulk NB4 cell line (CL1, CL2, CL3, CL4). Each clone was independently exposed to increasing concentrations of ATO (0.1-1 μM) to generate the corresponding four ATO-resistant clones (CL1-R, CL2-R, CL3-R, CL4-R). **(B)** Expression of the PML/RARA transcript evaluated by RT-PCR analysis in ATO-sensitive and -resistant clones and the NB4 cell line. The *ABL1* was used as housekeeping gene. **(C)** Proliferation rate of ATO-sensitive and -resistant clones analyzed by trypan blue cell count exclusion (triplicate counts) at 24, 48 e 72 h. Each plot shows cell growth of the ATO-sensitive clone and its -resistant counterpart at the indicated times. Values are the mean ±SD of three independent experiments.

Figure 2. Differential sensitivity of ATO-sensitive and -resistant APL clones to the antiproliferative, apoptotic and DNA damaging effects of ATO.

(A) Analysis of APL clones susceptibility to the antiproliferative effects of ATO. All NB4-derived cell clones were treated with ATO (0-2 μM) and after three days, proliferation was evaluated by the MTS assay. The mean IC_{50} values ±SD, calculated from at least three independent experiments, are reported. Statistical analysis was assessed by unpaired *t*-test: *, $p<0.05$; **, $p<0.01$. **(B)** Apoptosis analysis. Induction of apoptosis was evaluated by PI staining and flow cytometry of untreated cells (white bars) or cells treated with 0.5 μM (grey bars) and 1 μM (black bars) ATO at 48 h after drug exposure. The results of three independent experiments are expressed as mean percentage ±SD of PI-positive cells in ATO-sensitive (left panel) and -

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resistant (right panel) clones. Statistical analysis was performed by unpaired One-Way ANOVA (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). **(C)** Western blot analysis of proteins associated with the apoptotic pathway (PARP1, caspase 8 and 3) and DNA damage (γH2AX). GAPDH was used as housekeeping gene.

Figure 3. Susceptibility of ATO-sensitive and -resistant APL clones to the antiproliferative effects of different PARPi.

NB4 cell clones were treated with increasing concentrations of the indicated PARPi as described in Material and Methods. After six days, proliferation was assessed by MTS assay and the mean IC_{50} values were calculated. The dotted line in each histogram represents the Cmax range for each PARPi reported in clinical studies. Values are the mean ±SD of three independent experiments. Statistical analysis was calculated by unpaired *t*-test (*, $p<0.05$; **, $p<0.01$).

Figure 4. Antiproliferative effects of hypomethylating agents as single agents or combined with PARPi in ATO-sensitive and -resistant APL cells.

Susceptibility of NB4 clones to **(A)** azacitidine (AZA) or **(B)** decitabine (DAC) as single agents. NB4 cell clones were treated with azacitidine (0.125–2 μM) and decitabine (0.125–1 μM), followed by MTS assay. Drug IC_{50} values were evaluated after three days of treatment. Statistical analysis of the results from three independent experiments was performed by unpaired *t*-test (*, $p<0.05$). **(C)** Antiproliferative effects of azacitidine or decitabine with a fixed concentration of the PARPi olaparib, niraparib or talazoparib. ATO-sensitive (CL2) and -resistant (CL2-R) clones were treated with the indicated concentrations of olaparib (OLA, 2.5 μM), niraparib (NIR, 1.25 μM) or talazoparib (TAL, 25 nM) in combination with increasing

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concentrations of azacitidine (AZA, 0.125-1 μM) or decitabine (DAC, 31.25-500 nM). After three days, proliferation was evaluated by cell count in triplicate. Data are represented as surviving fraction of PARPi/azacitidine (left panel) or PARPi/decitabine (right panel) combined treatments in ATO-sensitive and -resistant CL2 clones. Values are the mean ±SD of three independent experiments, **(D)** PARPi/azacitidine (left panel) or PARPi/decitabine (right panel) combined effects analyzed by CompuSyn software. Each Fa-CI plot (or Chou-Talalay plot) indicates the CI as a function of the Fraction affected (Fa). CI <1, synergistic (values below the dotted line); CI =1, additive; CI >1, antagonist.

Figure 5. Antiproliferative effects of ascorbate as single agent or combined with PARPi in ATO-sensitive and -resistant APL cells.

(A, B) Susceptibility of ATO-sensitive and -resistant clones to ascorbate as a single agent. NB4 clones were treated with increasing concentrations of ascorbate (0.25-2 mM) and, after three days, proliferation was evaluated by cell count. The surviving fraction **(A)** and IC_{50} values **(B)** were calculated for the parental NB4 cell line and ATO-sensitive clones (left panel), and ATO-resistant clones (right panel) (A). Results are the mean values ±SD of three independent experiments. **(C)** Antiproliferative effects of ascorbate in combination with a fixed concentration of the PARPi olaparib, niraparib and talazoparib. ATO-sensitive and -resistant clones were treated with olaparib (OLA, 2.5 μM), niraparib (NIR, 1.25 μM) or talazoparib (TAL, 50 nM) in combination with ascorbate (ASC, 0.25-1 mM) and, after three days, cells viability was evaluated by cell count. Data are represented as surviving fraction of ATO-sensitive (upper panel) and ATO-resistant (lower panel) clones after PARPi/ascorbate combined treatment. Mean values ±SD of three independent

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experiments are represented. **(D)** Combined treatment effects were analyzed by CompuSyn software as indicated in the legend to Figure 4.

Figure 6. Combined treatment with PARPi/ascorbate increases DNA damage and 5hmC levels.

Two ATO-resistant clones (CL2-R, CL3-R), in which the PARPi/ascorbate combination was synergistic in terms of antiproliferative effects, were treated with ascorbate (ASC, 0.5 and 1 mM) in combination with olaparib (OLA, 2.5 μM), niraparib (NIR, 1.25 μM) or talazoparib (TAL 50 nM). After 24 h, cells were collected and analyzed for γH2AX as a marker of DNA damage, ROS production and 5hmC levels. **(A)** γH2AX immunoblot analysis. Histograms represent the results of densitometric analysis of γH2AX normalized with β-actin and are the mean ± SD of three independent experiments. Only the statistical significance of co-treatments compared to single treatments is reported. **(B)** Total Intracellular ROS and cytosolic superoxide anion were quantified by DCF and DHE fluorescence, respectively, in CL2-R and CL3-R cells following treatment with increasing concentrations of ascorbate. **(C)** DNA dot blots for 5hmC in CL2-R cells treated with 1 mM ascorbate, 50 nM talazoparib and a combination of the two drugs. The same dot blot was stained with methylene blue as loading control. The graph shows the densitometric analysis of 5hmC normalized for methylene blue. **(D)** DNA dot blots for 5hmC in CL2-R and CL2-R clones treated with 1 mM ascorbate, 2.5 μM olaparib, 1.25 μM niraparib, 50 nM talazoparib and PARPi/ascorbate combination. #This DNA sample was slightly overloaded. **(E)** DNA dot blots for 5hmC in the CL1-R clone, in which the PARPi/ascorbate combination was not synergistic in terms of antiproliferative effects,

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JPET Fast Forward. Published on April 5, 2021 as DOI: 10.1124/jpet.121.000537
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treated with 1 mM ascorbate, 2.5 μM olaparib, and the drug combination. The mean values ±SD of each histogram were obtained from three different experiments. Statistical analysis was evaluated by unpaired One-Way ANOVA (*p, <0.05; **, p<0.01; ***, p<0.001).

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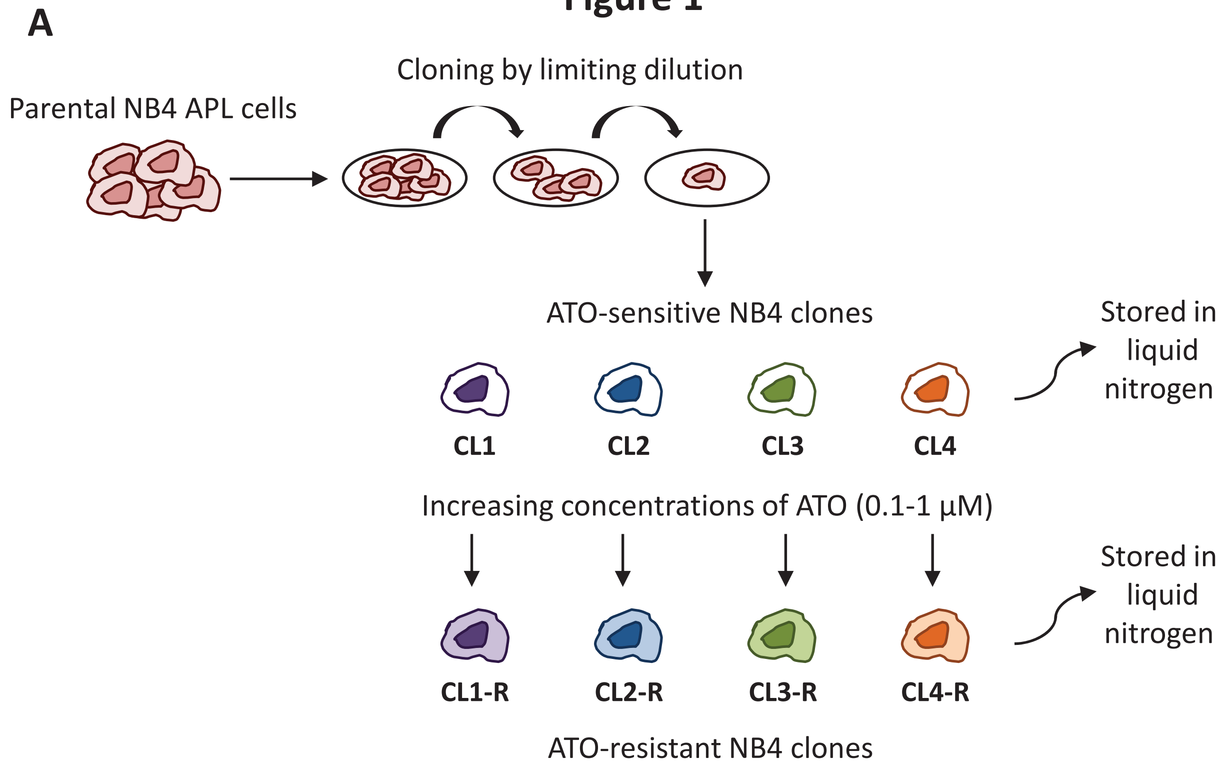
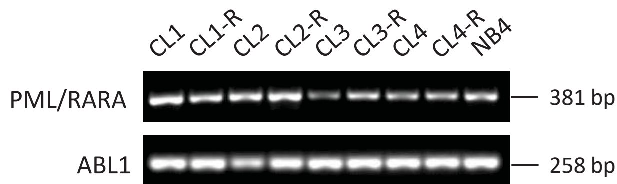
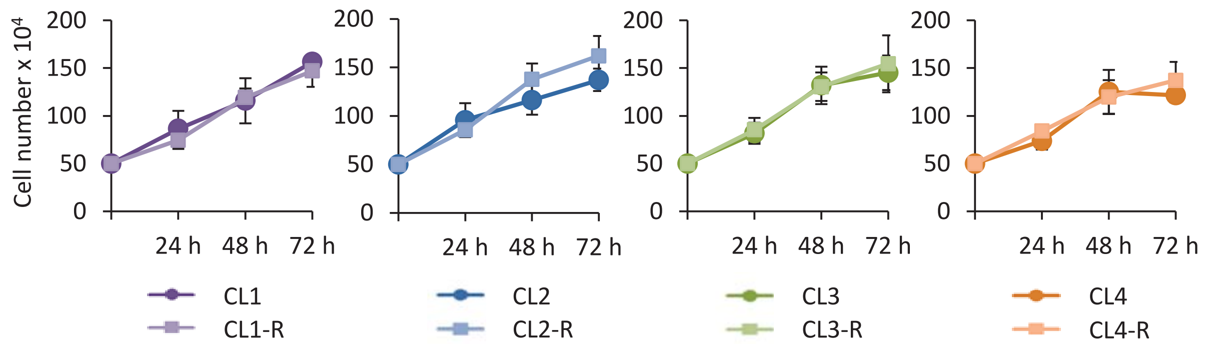
Figure 1







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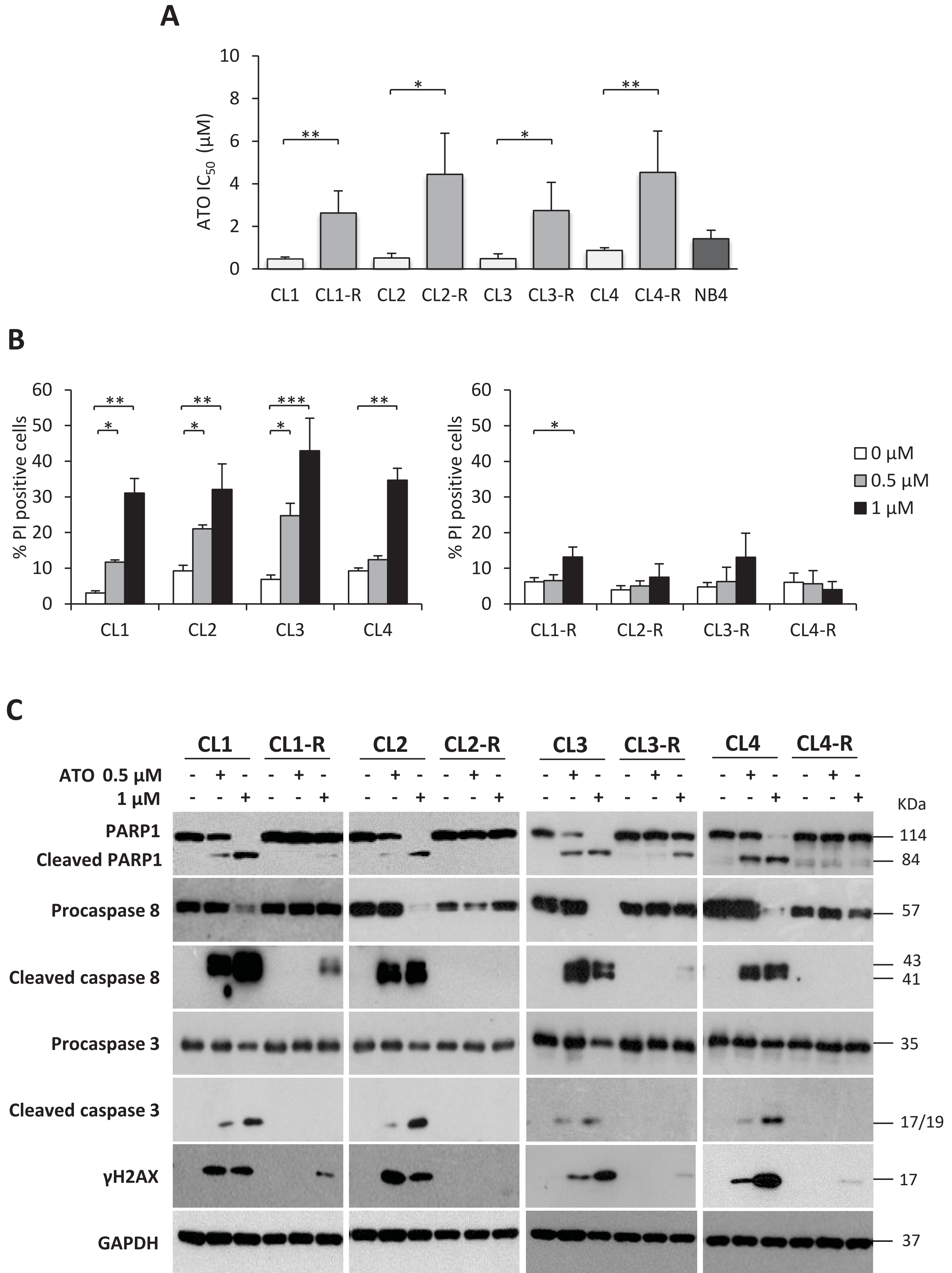

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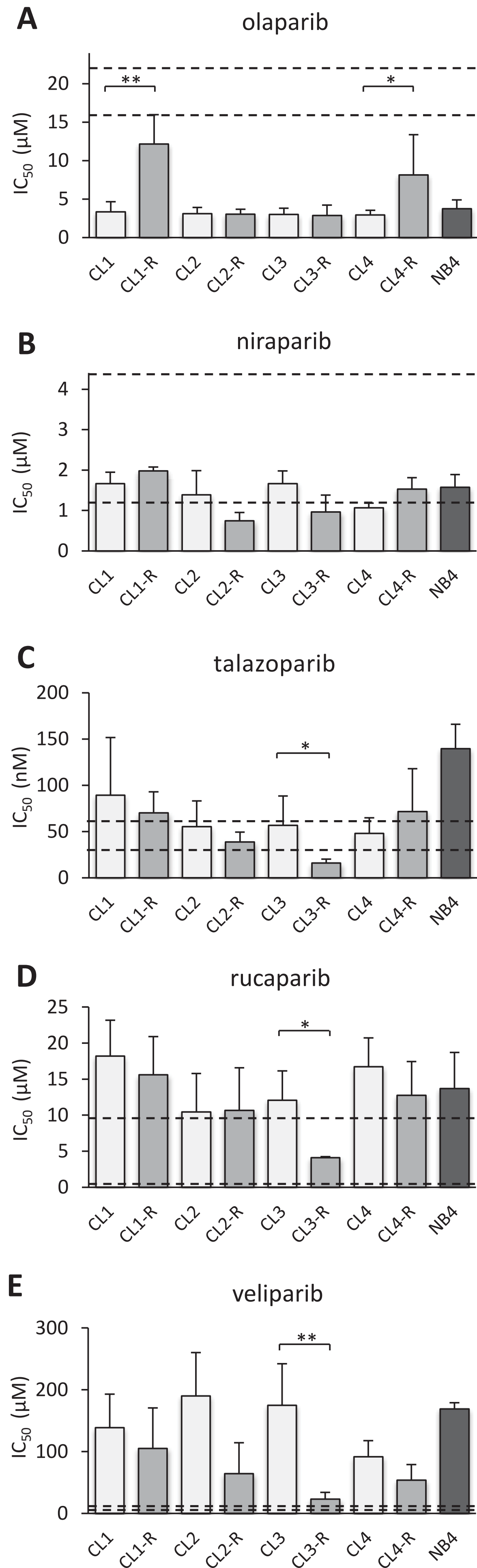

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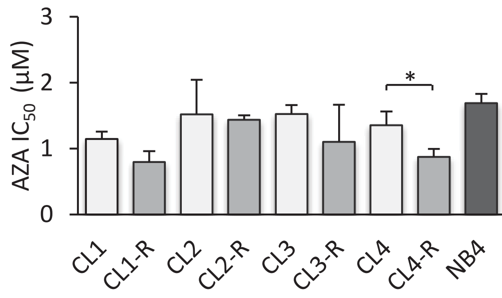

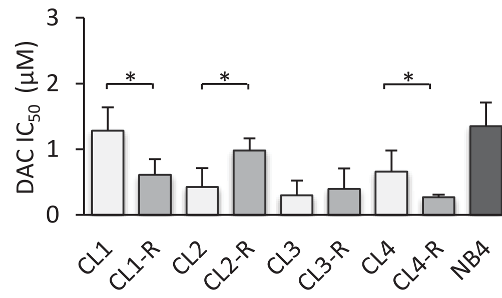

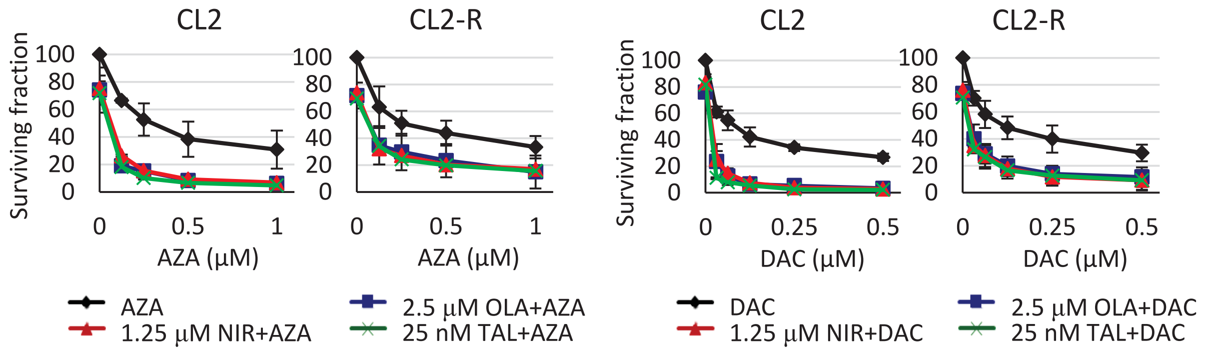

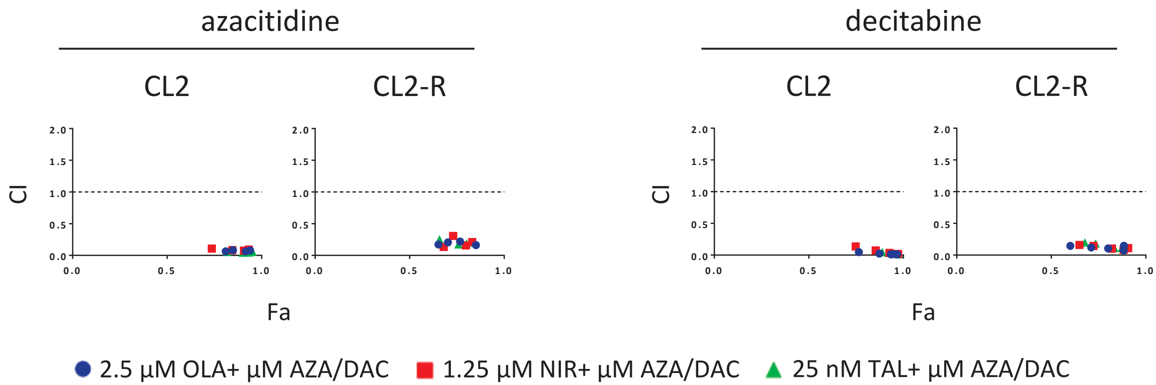

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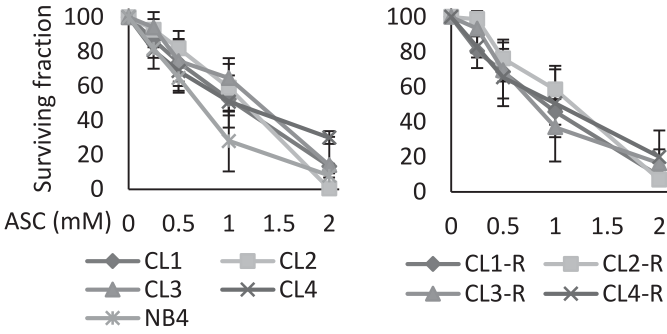
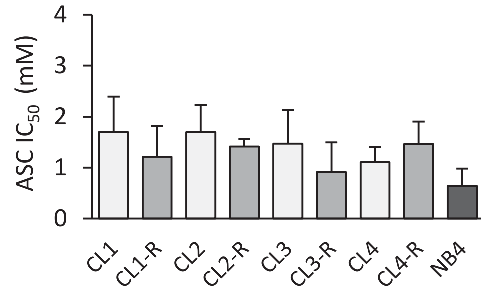
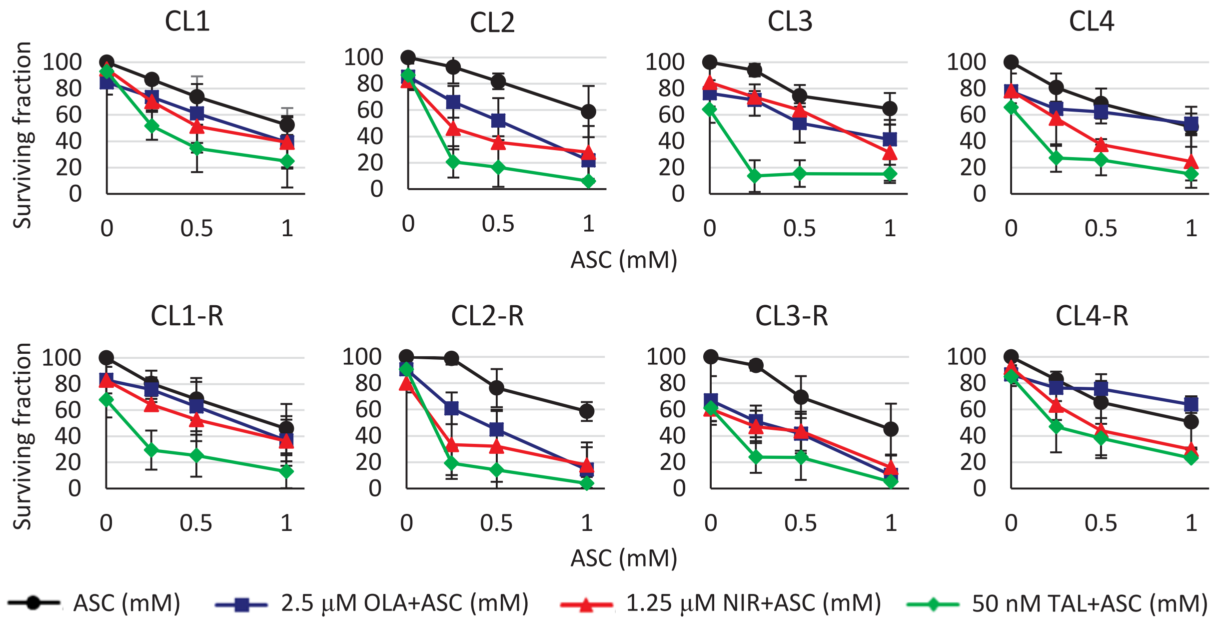
Figure 5







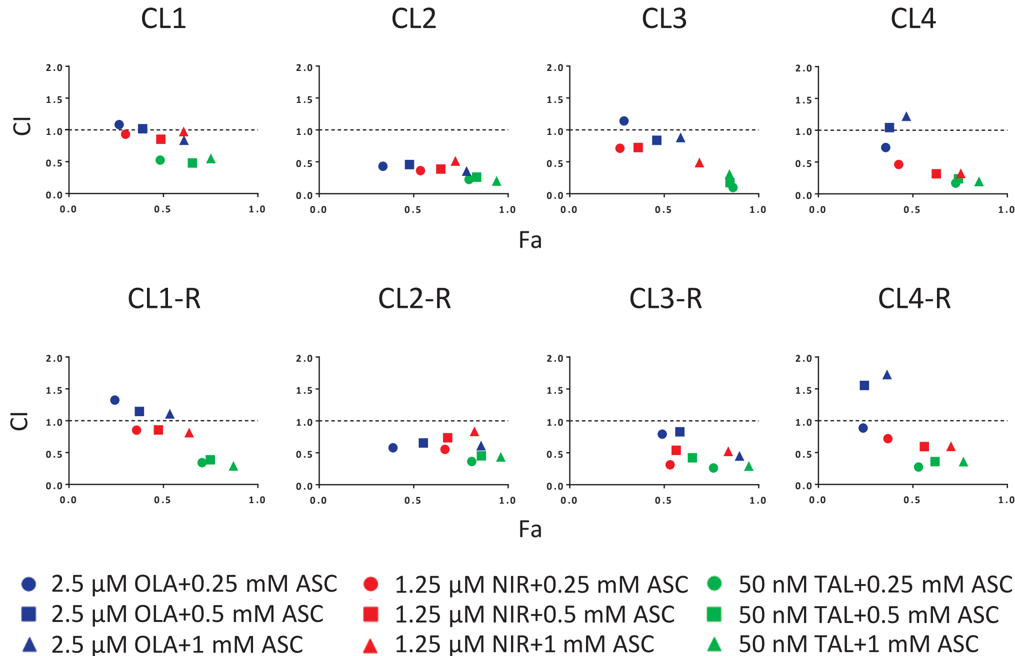


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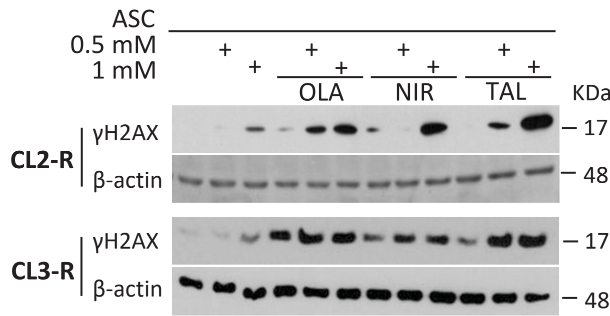
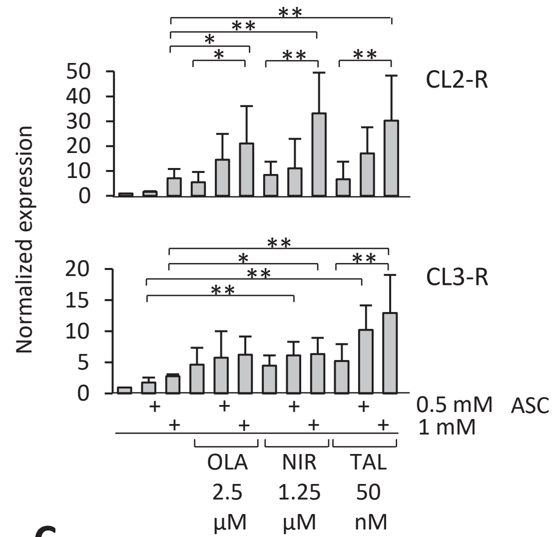
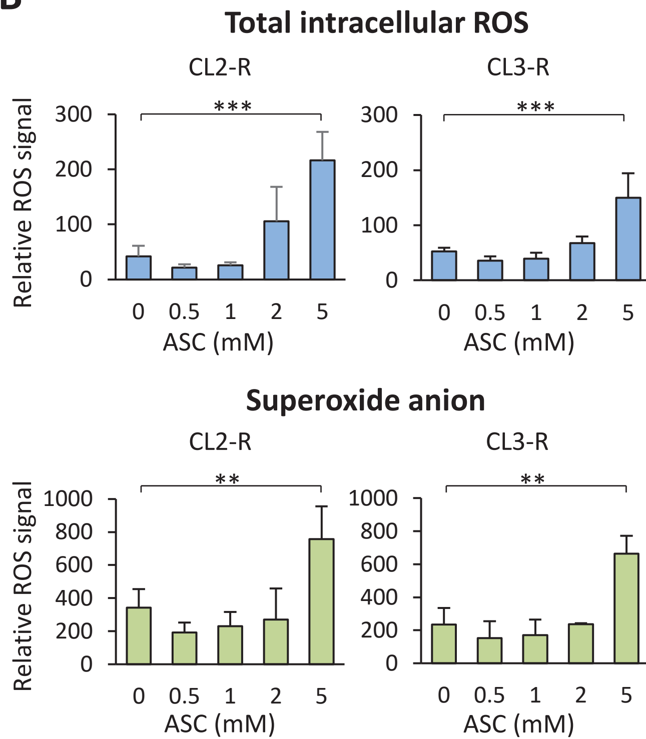
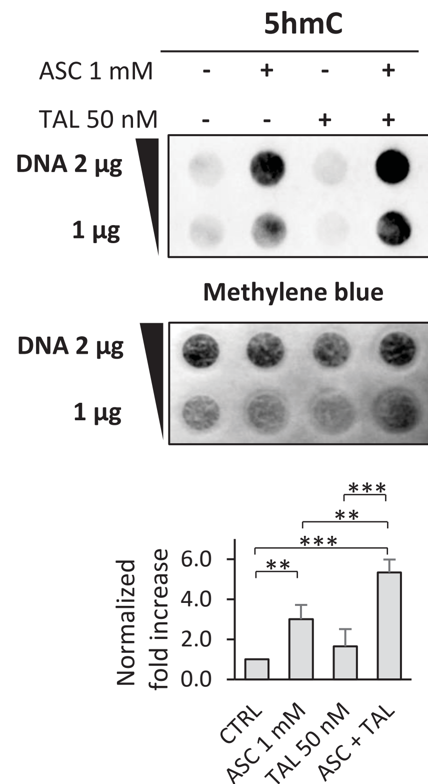
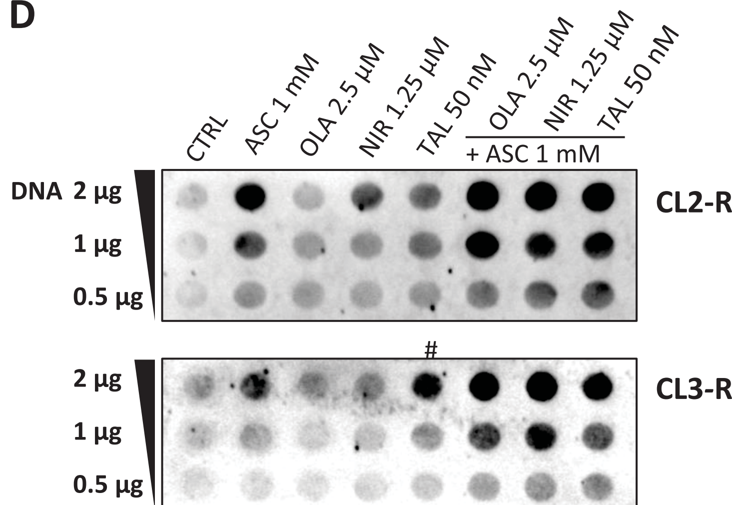
Figure 6

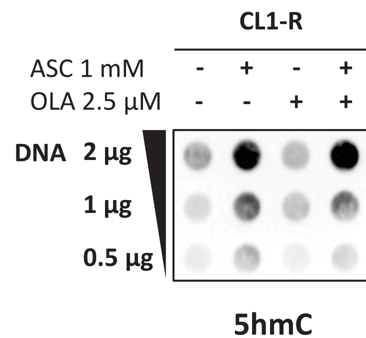












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