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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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ABSTRACT

Arsenic trioxide (ATO) is an anticancer agent used for the treatment ofacute promyelocytic leukemia (APL). However, 5%–10% of patients fail to respond or experience disease relapse. Based on poly(ADP-ribose) polymerase (PARP) 1 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 C_{max} reported in patients, likely as a result of 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 nonoverlapping 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.

SIGNIFICANCE STATEMENT

This study found that poly(ADP-ribose) inhibitors (PARPi) show activity as single agents against human acute promyelocytic leukemia cells resistant to arsenic trioxide at clinically 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 as a result 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 that is characterized by the reciprocal balanced translocation t(15;17), involving the promyelocytic leukemia (*PML*) and retinoic acid receptor α (*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, leading to impaired stress response, decreased DNA damage repair, and reduced cell propensity to undergo senescence and apoptosis (Gurnari et al., 2019).

For 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 treatment guidelines for APL have also included ATO in the frontline therapy of high-risk APL patients without cardiac dysfunction, 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 the chemotherapeutic agents used for first-line therapy (https://www.nccn.org/professionals/physician_gls/pdf/aml.pdf).

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-nuclear bodies 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, enrollment 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), which 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 also be 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; Iaccarino et al., 2019; Noguera 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 (Faraoni et al., 2015, 2018; Esposito et al., 2015; 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 recently been 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 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.

Materials and Methods

Generation of NB4 Clones and Cell Culture Conditions. The promyelocytic leukemia cell line NB4 (American Type Culture Collection, Manassas, VA) was cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) 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

ABBREVIATIONS: AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; ATO, arsenic trioxide; ATRA, all-*trans* retinoic acid; BER, base excision repair; CI, combination index; DCF, 2,7-dichlorodihydrofluorescein; DHA, dehydroascorbate; DHE, dihydroethidium; Fa, fraction affected; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; 5hmC, 5-hydroxymethylcytosine; MDS, myelodysplastic syndrome; PARP, poly(ADP-ribose) polymerase; PARPi, PARP inhibitors; PI, propidium iodide; PML, promyelocytic leukemia; RARA, retinoic acid receptor α; ROS, reactive oxygen species; TET, ten-eleven translocation.

initial stock purchased from American Type Culture Collection (Fig. 1). To generate ATO-resistant cells, cell clones were exposed to increasing concentrations of ATO (0.1–1 μM) for about 1 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).

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 (2 mM olaparib, talazoparib, and veliparib; 20 mM niraparib and rucaparib; Selleck Chemicals, Houston, TX) were prepared by dissolving the powder of each compound in DMSO (Sigma-Aldrich), followed by dilution in RPMI 1640 medium. Decitabine (Cayman Chemical, Ann Arbor, MI) 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).

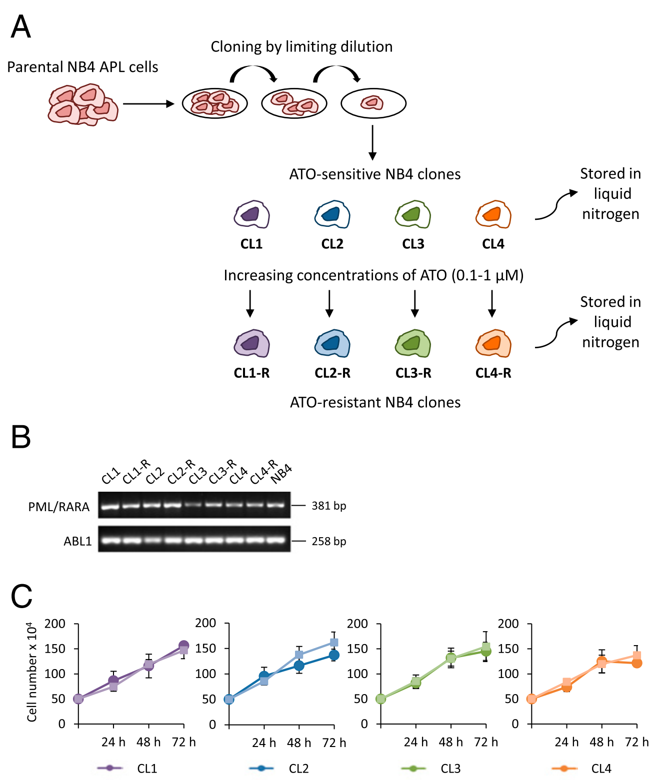

Fig. 1. ATO-resistant clones maintain APL phenotype. (A) Flowchart of the generation of ATO-sensitive and -resistant NB4 clones. Four different clones were isolated by limiting dilution from the bulk NB4 cell line (CL1, CL2, CL3, CL4). Each clone was independently exposed to increasing concentrations 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 *ABL proto-oncogene 1, non-receptor tyrosine kinase* (ABL1) was used as a housekeeping gene. (C) Proliferation rate of ATO-sensitive and -resistant clones analyzed by cell count and trypan blue exclusion assay (triplicate counts) at 24, 48, and 72 hours. Each plot shows cell growth of the ATO-sensitive clone and its ATO-resistant counterpart at the indicated times. Values are means ± S.D. of three independent experiments.

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 (C_{max}) values reached in patients with cancer.

For survival assays, cells were analyzed by the MTS viability test (Promega, Madison, WI), 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 with the untreated control was extrapolated from the dose-response curves by using linear regression (GraphPad Prism 5 software; GraphPad Inc., San Diego, CA). The dose-effect curves were analyzed by the median-effect method of Chou and Talalay with the CompuSyn software (ComboSyn Inc., Paramus, NJ). 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 with 100 μg/ml propidium iodide (PI) (Sigma-Aldrich) for 20 minutes at 37°C in the dark. Samples (5×10^4 cells) were acquired on a BD FACSCalibur flow cytometer and evaluated using CellQuest Software (BD Biosciences, San Jose, CA).

Molecular Analysis of PML/RARA. Total RNA was isolated by Trizol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA). In total, 1 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). 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 polymerase chain reaction 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), anti–caspase 8 (1:500, 12F5; Enzo Life Sciences, NY), anti–cleaved caspase 8 (1:400, Asp374; Cell Signaling Technology, Danvers, MA), anti–caspase 3 (1:1000, D3R6Y; Cell Signaling Technology), anti–cleaved caspase 3 (1:1000, D175; Cell Signaling Technology), anti-γH2AX (1:1000, JBW301; Millipore, Burlington, MA), anti–glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1000, 14C10; Cell Signaling Technology), and 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). The autoradiograms were subjected to densitometric analysis using the ImageJ 1.45s software (Schneider et al., 2012), and results were normalized against GAPDH or β-actin.

Determination of Intracellular ROS. Total intracellular ROS were evaluated by the 2,7-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; Invitrogen, Thermo Fisher Scientific) reagent that is deacetylated by nonspecific 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, which becomes fluorescent after intercalation into DNA. For ROS analysis, cells were harvested after 4 hours of

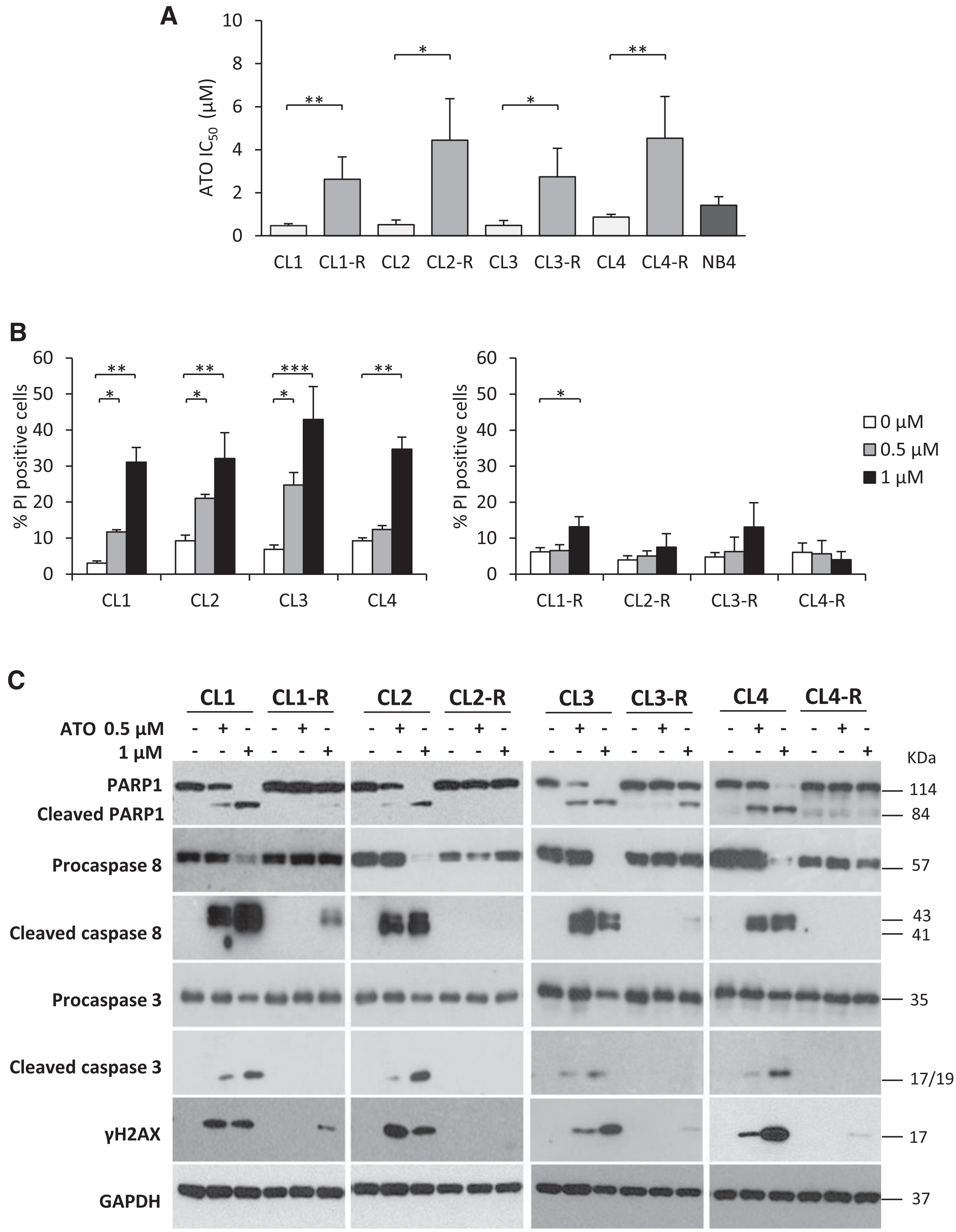

Fig. 2. Differential sensitivity of ATO-sensitive and -resistant APL clones to the antiproliferative, apoptotic, and DNA-damaging effects of ATO. (A) Analysis of APL clones' susceptibility to the antiproliferative effects of ATO. All NB4-derived cell clones were treated with ATO (0–2 μM), and after 3 days, proliferation was evaluated by the MTS assay. The mean IC_{50} values ± S.D., 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 (gray bars) and 1 μM (black bars) ATO at 48 hours after drug exposure. The results of three independent experiments are expressed as mean percentages ± S.D. of PI-positive cells in ATO-sensitive (left panel) and -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 a housekeeping gene.

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. Cells were treated with ascorbate or PARPi (i.e., olaparib, niraparib, or talazoparib), as single agents or in combination, for 24 hours, and then genomic DNA was extracted with DNeasy Blood and 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 minutes and neutralized with an equal volume of cold 4 M ammonium acetate (pH 7.0). Starting from 2 μg of denatured DNA, 2-fold dilutions of each sample were spotted on the nylon membrane Hybond-N+ (GE Healthcare) in an assembled Bio-Dot apparatus (Bio-Rad Laboratories, Hercules, CA). Each well was washed with 0.4 N NaOH, 10 mM EDTA, and then 2× saline-sodium citrate buffer. After baking at 80°C for 15 minutes, air-dried membranes were blocked in 5% skimmed milk in Tris-buffered saline/Tween 20and incubated with anti-5hmC antibody (39769; Active Motif, Carlsbad, CA) and anti-rabbit horseradish peroxidase–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 means ± S.D. 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 least significant difference (LSD) test was used. All statistical tests were two-sided. Differences were considered statistically significant when $P < 0.05$.

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 (French-American-British classification system M3) (Lanotte et al., 1991). From this cell line, we generated four clones by limiting dilution (CL1, CL2, CL3, and CL4), and these were subsequently exposed to increasing concentrations of ATO (0.1–1 μM) within a time frame of 1 year to generate the corresponding ATO-resistant clones (CL1-R, CL2-R, CL3-R, and CL4-R) (flowchart in Fig. 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 (Fig. 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 (Fig. 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 3 days of treatment with graded drug concentrations. The ATO IC_{50} in the resistant clones (2.6–4.5 μM range) was 5- to 9-fold higher than in the corresponding sensitive clones (0.5–0.9 μM range) (Fig. 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, whereas no or marginal apoptotic effects (at the higher concentration tested) were observed in ATO-resistant cells (Fig. 2B). Consistent 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) (Fig. 2C).

ATO-Resistant Clones Are Sensitive to PARPi. Recent preclinical studies have shown that PARPi exert cytotoxic effects against myeloid malignancies (Faraoni, et al., 2019a and b; Fritz et al., 2021). 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 6 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, 2018, 2019a). The drug concentrations tested in the experiments always included the plasma $C_{\max}$ reported in patients with cancer during phase 1 clinical trials ($C_{\max}$ ranges were represented by dotted lines in Fig. 3) (Fong et al., 2009; Kummar et al., 2009; Sandhu et al., 2013; Mateo et al., 2016; de Bono et al., 2017; Kristeleit et al., 2017; Nishikawa et al., 2017; Shapiro et al., 2019). All clones and the NB4 cell line were sensitive to olaparib with IC_{50} values lower (2.9–12.1 μM) than the reference $C_{\max}$ values (range 16–22 μM). Moreover, the ATO-resistant CL1-R and CL4-R clones showed significantly lower susceptibility to olaparib compared with their ATO-sensitive counterparts (Fig. 3A). All clone pairs presented comparable susceptibility to niraparib with IC_{50} values (0.7–2.0 μM) below or within the $C_{\max}$ range (1.2–4.4 μM) (Fig. 3B). In the case of talazoparib, the IC_{50} values of most clones (five of eight clones; 16.0–56.7 nM) were below or within the $C_{\max}$ (30–60 nM), and no significant differences were observed between ATO-sensitive and -resistant clones, except for the CL3/CL3-R couple, with CL3-R being significantly more sensitive to talazoparib than CL3 (Fig. 3C). Regarding rucaparib and veliparib, in almost all cases (seven of eight clones for rucaparib and eight of eight clones for veliparib), the obtained IC_{50} values were above the $C_{\max}$ values (0.6–9.5 μM and 2.6–13.5 μM, respectively) (Fig. 3, D and E). Similar to what was observed with talazoparib, CL3-R was also more susceptible to these PARPi compared with 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., 2019a), 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 Fig. 4, A and B, in all cell clones, azacitidine and decitabine

IC_{50} values, calculated 3 days after drug exposure, were within the range of clinically relevant concentrations previously reported for these drugs (Cashen et al., 2008; Keating, 2012). Interestingly, the growth of CL1-R and CL4-R clones was significantly more inhibited compared with their 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, which showed higher sensitivity to decitabine compared with its ATO-resistant counterpart (Fig. 4, A 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 because no significant differences were observed in the susceptibility to the PARPi tested between the ATO-sensitive and -resistant cells (Fig. 3). Analysis of the inhibitory effects on cell proliferation exerted by the drug combination was performed after 3 days of treatment because of the more rapid antiproliferative effects of the cytidine analogs compared with PARPi. Results indicated that the drug combination induced a greater inhibition of cell growth compared with the single hypomethylating agents in both ATO-sensitive and ATO-resistant clones (Fig. 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 (Fig. 4C), with CI values largely below 1 (dotted line of the Fa-CI plots) (Fig. 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., 2019b) and primary cultures of MDS samples (Faraoni et al., 2019a).

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; Cimmino et al., 2017; Noguera 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 3 days of culture (Fig. 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} values ranged from 0.6 to 1.8 mM (Fig. 5B), values comprising the range of plasma concentrations detected in cancer patients treated with high doses of this agent (Hoffer et al., 2008; Ngo

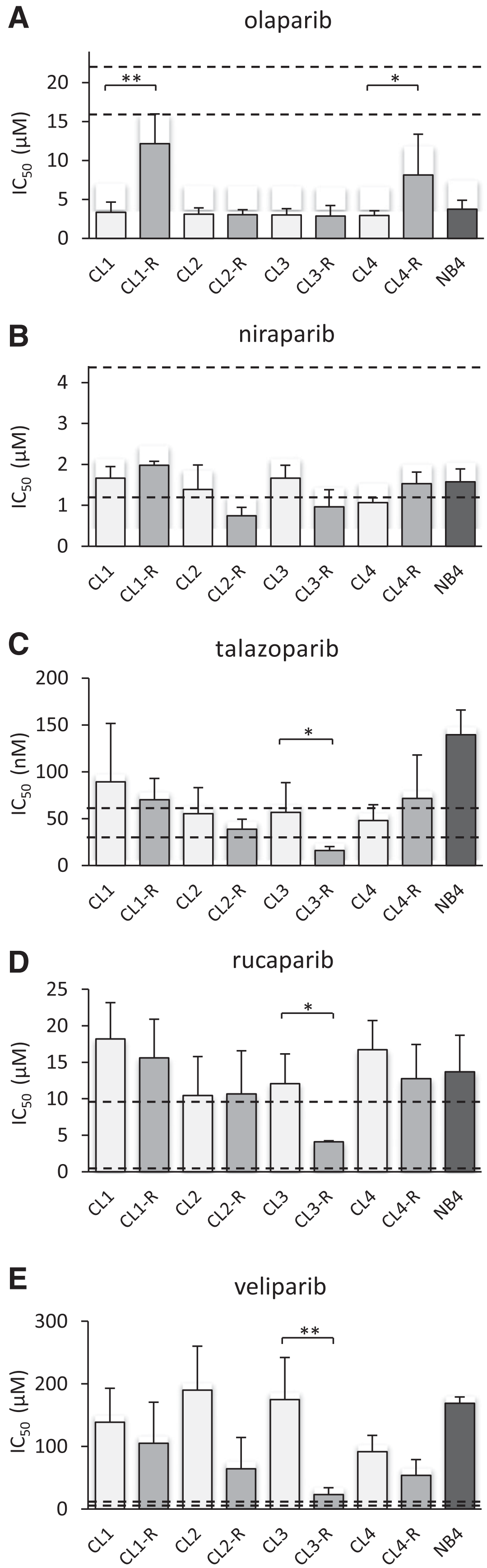

Fig. 3. Susceptibility of ATO-sensitive and -resistant APL clones to the antiproliferative effects of different PARPi. NB4 cell clones were treated with increasing concentrations of the indicated PARPi, as described in *Materials and Methods*. After 6 days, proliferation was assessed by MTS assay, and the mean IC_{50} values were calculated. A) olaparib; B) niraparib; C) talazoparib; D) rucaparib; E) veliparib. The dotted line in each histogram represents the C_{max} range for each PARPi reported in clinical studies. Values are means ± S.D. of three independent experiments. Statistical analysis was calculated by unpaired t test (*$P < 0.05$; **$P < 0.01$).

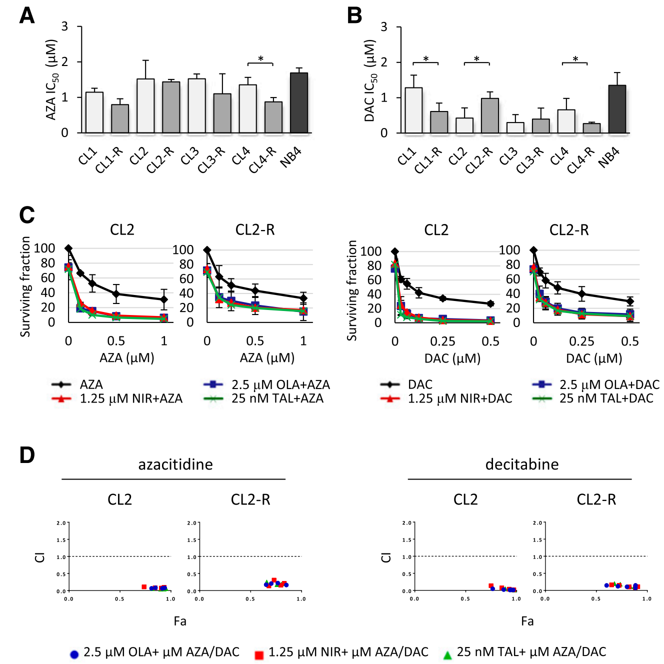

Fig. 4. Antiproliferative effects of hypomethylating agents as single agents or combined with PARPi in ATO-sensitive and -resistant APL cells. Susceptibility of NB4 clones to (A) azacitidine (AZA) or (B) decitabine (DAC) as single agents. NB4 cell clones were treated with azacitidine (0.125–2 μM) and decitabine (0.125–1 μM), followed by MTS assay. Drug IC_{50} values were evaluated after 3 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 concentrations of azacitidine (AZA, 0.125–1 μM) or decitabine (DAC, 31.25–500 nM). After 3 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 means ± S.D. of three independent experiments. (D) PARPi/azacitidine (left panel) or PARPi/decitabine (right panel) combined effects were 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.

et al., 2019). Interestingly, no significant differences were observed between ATO-sensitive and -resistant cells, indicating that there is no crossresistance 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 (Pastor et al., 2013; Cimmino et al., 2017), 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 3 days of treatment by cell count, and results were reported as proliferation curves in Fig. 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), whereas its combination with niraparib resulted in additive effects in CL1 and CL1-R clones and synergistic effects in all the other clones. Notably, the ascorbate/talazoparib combination was highly effective in all ATO-sensitive and -resistant clones, with extremely low CI values (Fig. 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 cotreatment 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, in which the combination was synergistic with all 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 (Fig. 6A). Conversely, the ascorbate/PARPi combination significantly increased γH2AX expression compared with 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 three PARPi, whereas in CL3-R cells, it was observed mainly with talazoparib (Fig. 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 (Fig. 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 cofactor of TET enzymes, which catalyze the oxidation of 5-methylcytosine 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 5-methylcytosine 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 with untreated or PARPi-treated cells (Fig. 6C). More interestingly, the addition of 50 nM talazoparib significantly increased 5hmC accumulation compared with ascorbate alone (Fig. 6C). Similar results were also obtained when ascorbate was combined with olaparib or niraparib and in another ATO-resistant clone (i.e., CL3-R), in which 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 with ascorbate alone (Fig. 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 (Fig. 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.

Discussion

In the present study, we demonstrated for the first time that PARPi increase the in vitro antiproliferative activity of

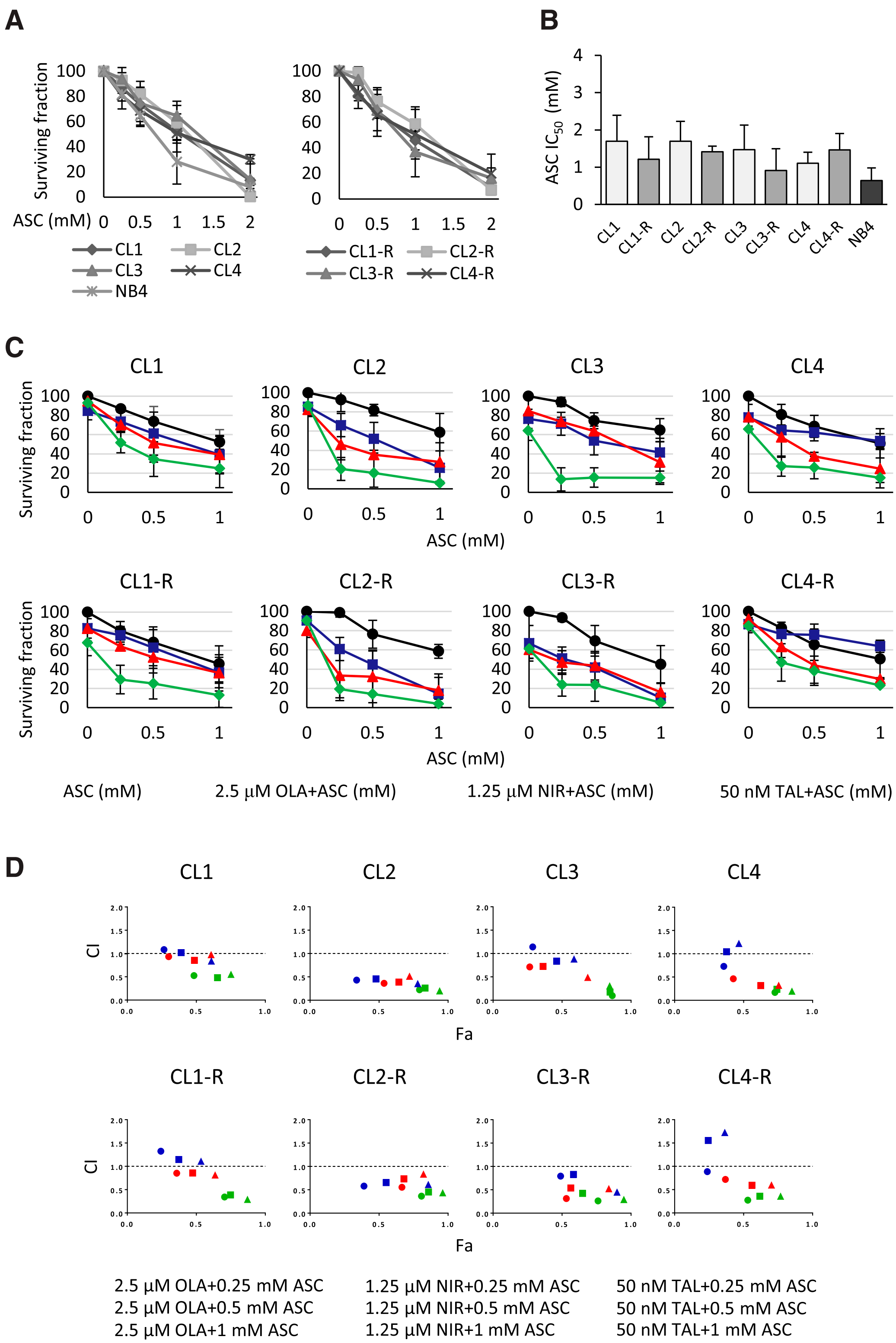

Fig. 5. Antiproliferative effects of ascorbate as a single agent or combined with PARPi in ATO-sensitive and -resistant APL cells. (A and B) Susceptibility of ATO-sensitive and -resistant clones to ascorbate as a single agent. NB4 clones were treated with increasing concentrations of ascorbate (0.25–2 mM), and after 3 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 ± S.D. 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 3 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 ± S.D. of three independent experiments are represented. (D) Combined treatment effects were analyzed by CompuSyn software as indicated in the legend of Fig. 4.

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 *BRCA1/2* 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 with 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 in both ATO-sensitive and -resistant APL cells at clinically relevant concentrations. Moreover, our data suggest the lack of crossresistance between ATO and PARPi. Indeed, crossresistance to ATO and conventional chemotherapeutic agents is uncommon, since ATO is not a substrate of the p-glycoprotein encoded by *multidrug resistance protein 1* gene or other members of the ATP-binding cassette family of transporters, such as multidrug resistance associated protein1 or breakpoint cluster region pseudogene 1 (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 (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 *multidrug resistance protein 1*(Lawlor et al., 2014). However, in our cellular model, the pattern of response of APL clones did not suggest the occurrence of common resistance 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 vs. PARP1 trapping at DNA breaks). Indeed, although their inhibitory effects on PARP1 catalytic activity are not largely different (with IC_{50} values in the nanomolar range), PARPi markedly differ in the trapping ability, with talazoparib and veliparib being the most and least potent, respectively (Murai et al., 2012, 2014). Moreover, PARPi trapping potency correlated with cytotoxicity in tumor cells (Murai et al., 2012; Murai and Pommier, 2015). Consistent 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 patients with AML who are 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 patients with AML who achieve first complete remission but are not able to complete intensive induction chemotherapy. Both drugs are cytidine analogs 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, 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, and solid tumors (e.g., ovarian cancer and non–small-cell lung cancer) (Muvarak et al., 2016; Zhao and So, 2017; Pulliam et al., 2018; Faraoni et al., 2019a; 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 (Muvarak et al., 2016; Zhao and So, 2017; Faraoni et al., 2019a; Abbotts et al., 2019). The mechanisms underlying the observed synergistic activity include altered processing by BER and PARP1 of the aberrantly incorporated cytidine analog and trapped DNA (cytosine-5)-methyltransferase 1 (Orta et al., 2014), induction of a BRCAness (i.e., similar to hereditary BRCA-mutated tumors) phenotype by downregulating 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), and 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 millimolar concentrations of ascorbate. At physiologic concentrations, ascorbate acts as an antioxidant and cofactor of metabolic enzymes; conversely, when pharmacological doses are administered intravenously (resulting in plasma concentrations in the millimolar range), ascorbate behaves as a 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 patients with AML resulted in clinical benefit, especially in the presence of loss-of-function mutations of *TET2* (Zhao et al., 2018; Das et al., 2019), which 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

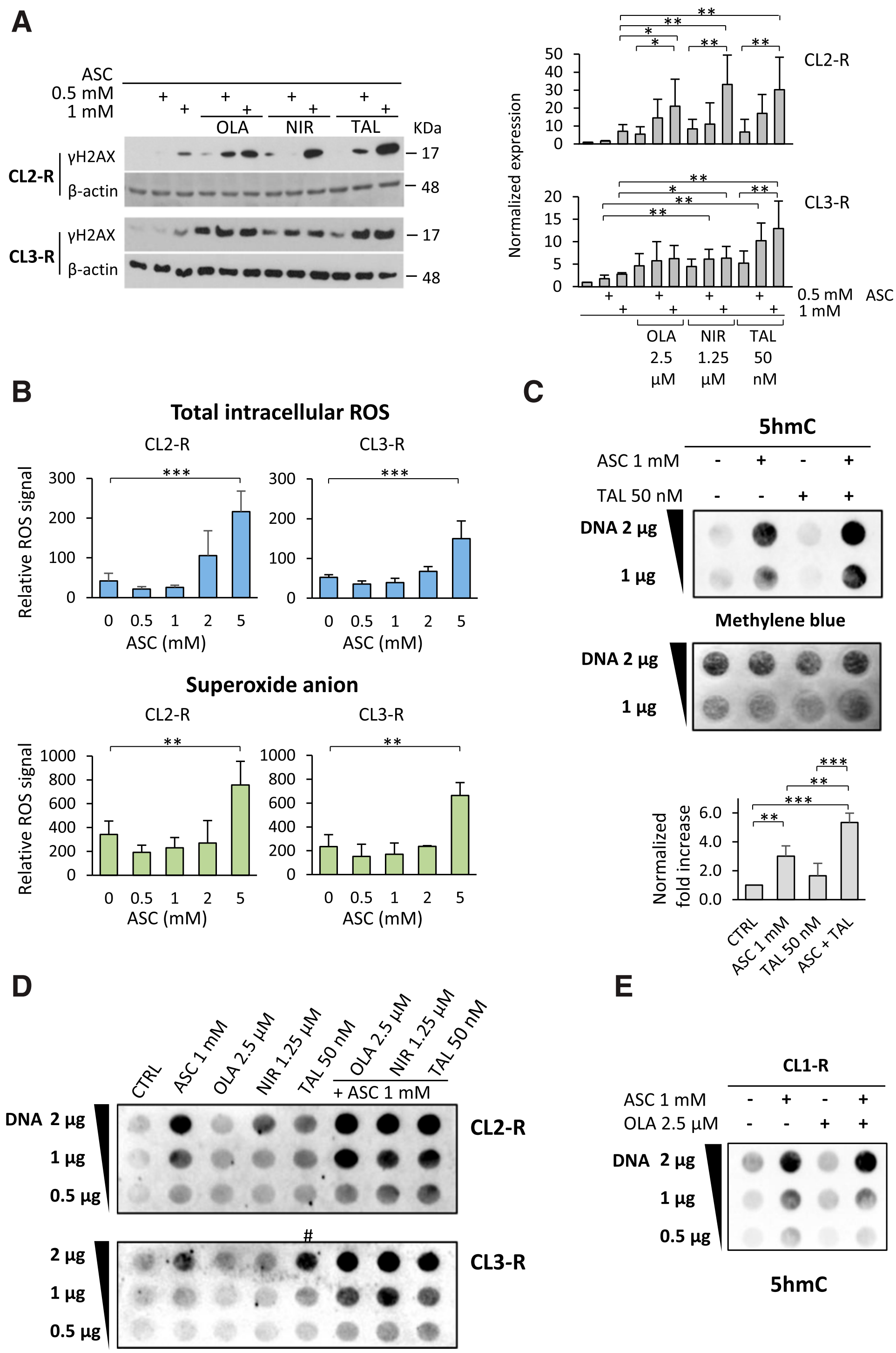

Fig. 6. Combined treatment with PARPi/ascorbate increases DNA damage and 5hmC levels. Two ATO-resistant clones (CL2-R, CL3-R), in which the PARPi/ascorbate combination was synergistic in terms of antiproliferative effects, were treated with ascorbate (ASC, 0.5 and 1 mM) in combination with olaparib (OLA, 2.5 μM), niraparib (NIR, 1.25 μM), or talazoparib (TAL 50 nM). After 24 hours, 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 means ± S.D. of three independent experiments. Only the statistical significance of cotreatments compared with 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 after treatment with increasing concentrations of ascorbate. (C) DNA dot blots for 5hmC in CL2-R cells untreated (CTRL) or 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 untreated (CTRL) or 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, treated with 1 mM ascorbate, 2.5 μM olaparib, and the drug combination. The mean values ± S.D. of each histogram were obtained from three different experiments. Statistical analysis was evaluated by unpaired one-way ANOVA (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

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 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 central nervous system. 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).

The antileukemic activity of PARPi may also be increased by their combination with agents used for APL treatment, such as anthracyclines and gemtuzumab 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.

Authorship Contributions

Participated in research design: Prete, Ciccarone, Voso, Graziani, Faraoni.

Conducted experiments: Giansanti, De Gabrieli, Prete, Ottone, Divona, Karimi, Ciccarone.

Performed data analysis: Giansanti, Ottone, Karimi.

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

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