



BRCA1, PARP1 and γ H2AX in acute myeloid leukemia: Role as biomarkers of response to the PARP inhibitor olaparib



Isabella Faraoni^{a,b,*}, Mirco Compagnone^b, Serena Lavorgna^{b,c}, Daniela Francesca Angelini^d, Maria Teresa Cencioni^d, Eleonora Piras^d, Paola Panetta^{b,c}, Tiziana Ottone^{b,c}, Susanna Dolci^c, Adriano Venditti^c, Grazia Graziani^{a,1}, Francesco Lo-Coco^{b,c,1}

^a Department of Systems Medicine, University of Rome "Tor Vergata", Rome, Italy

^b Unit of Neuro-Oncohematology, Santa Lucia Foundation-I.R.C.C.S., Rome, Italy

^c Department of Biomedicine and Prevention, University of Rome "Tor Vergata", Rome, Italy

^d Unit of Neuroimmunology, Santa Lucia Foundation-I.R.C.C.S., Rome, Italy

ARTICLE INFO

Article history:

Received 21 July 2014

Received in revised form 24 October 2014

Accepted 1 December 2014

Available online 5 December 2014

Keywords:

Acute myeloid leukemia

PARP1

BRCA1

H2AX

PARP inhibitor

Olaparib

ABSTRACT

Olaparib (AZD-2281, Ku-0059436) is an orally bioavailable and well-tolerated poly(ADP-ribose) polymerase (PARP) inhibitor currently under investigation in patients with solid tumors. To study the clinical potential of olaparib as a single-agent for the treatment of acute myeloid leukemia (AML) patients, we analyzed the *in vitro* sensitivity of AML cell lines and primary blasts. Clinically achievable concentrations of olaparib were able to induce cell death in the majority of primary AML case samples (88%) and tested cell lines. At these concentrations, olaparib preferentially killed leukemic blasts sparing normal lymphocytes derived from the same patient and did not substantially affect the viability of normal bone marrow and CD34-enriched peripheral blood cells obtained from healthy donors. Most primary AML analyzed were characterized by low BRCA1 mRNA level and undetectable protein expression that likely contributed to explain their sensitivity to olaparib. Noteworthy, while PARP1 over-expression was detected in blasts not responsive to olaparib, phosphorylation of the histone H2AFX (γ H2AX) was associated with drug sensitivity. As to genetic features of tested cases the highest sensitivity was shown by a patient carrying a 11q23 deletion. The high sensitivity of AML blasts and the identification of biomarkers potentially able to predict response and/or resistance may foster further investigation of olaparib monotherapy for AML patients unfit to conventional chemotherapy.

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1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease which comprises distinct subtypes defined by specific morphological, cytogenetic, molecular and gene expression characteristics [1]. Modern AML classification is mainly based on chromosome abnormalities (insertions, deletions and translocations) and other recurrent genetic changes in

specific genes such as nucleophosmin (*NPM1*) and *fms*-related tyrosine kinase 3 (*FLT3*). The analysis of these alterations allows the identification at diagnosis of prognostically discrete subtypes of AML which in turn may enable the adoption of risk-adapted therapeutic strategies [2].

Despite important advances in the understanding of the molecular basis of AML, survival outcomes have not significantly improved over the past 20 years, indicating the need of more effective and less toxic

Abbreviations: AML, acute myeloid leukemia; ATR, ATR serine/threonine kinase; ATM, ATM serine/threonine kinase, (ataxia telangiectasia mutated); BM, bone marrow; BRCA1, breast cancer 1, early onset; BRCA2, breast cancer 2, early onset; BSA, bovine serum albumin; CD45, protein tyrosine phosphatase receptor type C; DAPI, 4,6 diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; DSB, DNA double strand breaks; FACS, flow cytometry; FLT3, *fms*-related tyrosine kinase 3; FLT3-ITD, FLT3 internal tandem duplications; FLT3LG, *fms*-related tyrosine kinase 3 ligand; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H2AFX, H2A histone family, member X; γ H2AX, Ser139 phosphorylated H2AFX; HR, homologous recombination; IC₅₀, inhibitory concentration 50; IL-3, interleukin 3; MLH1, mutL homolog 1; MMR, mismatch repair system; MRE11A, MRE11 meiotic recombination 11 homolog A (*S. cerevisiae*); MSH2, mutS homolog 2; MSH3, mutS homolog 3; MSH6, mutS homolog 6; MTS, 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; NAD, nicotinamide adenine dinucleotide; NPM1, nucleophosmin1, (nucleolar phosphoprotein B23, numatrin); NPM1mutA, NPM1 mutation type A; PAR, poly(ADP-ribose); PARP1, poly(ADP-ribose) polymerase 1; PARP2, poly(ADP-ribose) polymerase 2; PARPi, PARP inhibitors; PARylation, poly(ADP-ribosyl)ation; PB, peripheral blood; PI, propidium iodide; qRT-PCR, real-time polymerase chain reaction; RAD51, RAD51 recombinase; SCF, stem cell factor, (KIT ligand); SSB, single strand breaks

* Corresponding author at: Department of Systems Medicine, University of Rome "Tor Vergata", Via Montpellier, 1, 00133 Rome, Italy. Tel.: +39 6 72596329; fax: +39 6 72596323.

E-mail addresses: faraoni@med.uniroma2.it (I. Faraoni), mircocomp@gmail.com (M. Compagnone), serena.lavorgna@hotmail.it (S. Lavorgna), df.angelini@gmail.com (D.F. Angelini), mt.cencioni@hsantalucia.it (M.T. Cencioni), e.piras@hsantalucia.it (E. Piras), paolapanetta@yahoo.it (P. Panetta), tizianaottone@hotmail.com (T. Ottone), dolci@uniroma2.it (S. Dolci), adriano.venditti@uniroma2.it (A. Venditti), graziani@uniroma2.it (G. Graziani), francesco.lo.coco@uniroma2.it (F. Lo-Coco).

¹ GG and FLC contributed equally as last authors to this work.

therapeutic agents. In this context, molecular therapies targeting genetic abnormalities of AML blasts and their altered signaling pathways appear to be promising approaches. In this regard, the inhibitors of poly(ADP-ribose) polymerase (PARP) are attractive candidates for AML treatment.

The PARP family comprises a number of enzymes that transfer ADP-ribose to acceptor proteins using NAD⁺ as substrate resulting in mono- or poly(ADP-ribosyl)ation (PARylation) of proteins [3]. Among the proteins capable of synthesizing ADP-ribose polymers (PAR), PARP1 is responsible for most of the total cellular PARylation activity [4]. Through PARylation, PARP1 controls a wide array of cellular processes, such as DNA repair, gene transcription, cell division and death. The rationale for using PARP inhibitors (PARPi) as single agents, as previously suggested by Ashworth's and Helleday's groups [5,6], relies on their ability to selectively kill BRCA1 or BRCA2 mutated breast or ovarian cancers with a defective repair of DNA double strand breaks (DSB) by homologous recombination (HR) [7]. In the presence of PARPi, single strand breaks (SSB) that are continuously generated by endogenous oxidants or by replication errors, are no longer repaired by the base excision repair (in which PARP1 and PARP2 play a role) and are converted into DSB upon collision with the replication fork. Normal cells will repair DSB by HR and survive, whereas HR-deficient tumor cells will not repair DSB and die or, alternatively, attempt to repair DSB activating the error-prone non-homologous end-joining pathway that causes genomic instability and eventual lethality [8]. Thus, the concomitant inhibition of PARP activity and the lack of function of HR are synthetic lethal conditions.

Besides mutations, low expression of BRCA1/2 and lack of function of other proteins involved in HR pathway or in DSB sensing may cause HR-deficiency and confer tumor cells a "BRCAness" phenotype [9,10]. For instance, cells with defective ataxia telangiectasia mutated (ATM) protein, a kinase quickly activated after the induction of DSB [11], are more sensitive to PARPi than ATM-proficient cells [12–15]. Moreover, aberrations of the MRE11A–RAD50–NBS1 complex, essential in DNA damage detection [12,16–18], silencing or deletion of ATR, RAD51 and Fanconi anemia proteins, which are required for an efficient HR [12, 19,20], reduced expression of mismatch repair components MSH2, MSH6 and MLH1, causing genomic instability [18], all represent conditions that may sensitize cancer cells to PARPi monotherapy.

Recent studies performed in leukemia cell lines and primary myelodysplastic syndromes or AMLs have shown that PARPi might be a suitable therapeutic strategy [21], especially for a subset of myeloid malignancies with microsatellite instability [18]. Among the different PARPi currently under investigation, olaparib (AZD-2281, Ku-0059436) is a well-tolerated and potent PARP1 and PARP2 inhibitor which has shown promising results in clinical trials for solid tumors [22–27]. Presently, olaparib as monotherapy (300 mg twice daily) has reached the phase III development as maintenance treatment for BRCA mutated ovarian cancer after chemotherapy with platinum agents (clinicaltrials.gov).

In the present study we investigated the *in vitro* sensitivity to olaparib of AML cell lines and primary patient blasts and found that exposure to a single clinically relevant concentration of the PARPi exerted anti-proliferative and apoptotic effects in the majority of cases (88%). Moreover, we found that BRCA1 proficiency and PARP1 over-expression designated AML blasts not responsive to olaparib. Finally, the sensitivity of AML cells to the PARPi directly correlated with phosphorylation of the histone H2AFX on Ser139 (γ H2AX), which is regarded as an indicator of DSB [28].

2. Materials and methods

2.1. Primary AML blasts, cell lines and culture conditions

Freshly isolated primary blasts were obtained from bone marrow (BM) aspirates of adult patients with newly diagnosed AML before

starting chemotherapy. The first 14 patients were numbered in order of arrival and selected for a blast infiltration $\geq 60\%$ in the BM at disease presentation. Three patients were selected for blast percentages $\leq 40\%$ to study the influence of olaparib in matched myeloblasts and normal cells; in these latter cases, however, it was not possible to recover enough blasts for immunoblot analyses. Eight additional AML samples (with $\geq 60\%$ blasts) were analyzed only for mRNA expression experiments. All patients provided written informed consent according to institutional guidelines and the study was approved by the IRB at "Policlinico Tor Vergata".

Mononuclear cells from the BM of 25 patients and 10 healthy donors were isolated by Lympholyte-H (Cedarlane) according to the manufacturer's instructions. Two CD34-enriched cells were obtained after G-CSF-mobilization from the peripheral blood of healthy donors and after Lympholyte-H separation.

Since freshly isolated AML cells are typically arrested at G0/G1 phase, cells were exposed to 3 different mitogenic stimuli and cultured for 3 days before starting chemosensitivity assays. Briefly, 10^6 primary AML cells/ml were seeded into a culture flask in complete RPMI medium (Sigma-Aldrich) [with 2 mM L-glutamine (EuroClone) and 1% penicillin/streptomycin (EuroClone)] supplemented with 20% fetal bovine serum (FBS) (PAA Laboratories), 10 ng/ml each of IL-3, SCF and FLT3LG (Peprotech).

The HL-60, U937, NB4 (American Type Culture Collection) and HL-60R (kindly donated by Richard Darley, University of Wales, Cardiff, UK) leukemia cell lines were cultured in complete medium supplemented with 10% FBS, whereas OCI-AML2 and OCI-AML3 (DSMZ-German Collection) leukemia cell lines were cultured in the presence of 20% FBS.

2.2. Drug treatment and survival assay

The stock solution of olaparib (Selleck Chemicals) was prepared by dissolving 10 mg of the drug in 200 μ l of dimethylsulfoxide (DMSO) (Sigma) and then diluted with RPMI to the concentration of 2 mM. The aliquots were stored at -80°C . For each experiment a new aliquot was thawed and used.

For survival assay stimulated primary AML cells were collected, centrifuged, suspended in fresh complete medium (time 0 on the graphics), and seeded at 10^6 cells/ml in duplicate for each point in 24-well culture plates. Olaparib was added at the beginning of each experiment and left in the culture medium during the entire period of incubation. Cells were counted by trypan blue dye exclusion in quadruplicate and the surviving fraction was calculated with respect to the untreated control. Since *in vitro* culture of primary AML blasts could be achieved for 7–14 days only, depending on the AML sample, we performed the assays at up to 7 days after drug exposure. Experiments with normal cells were performed with the same procedure.

For the analysis of survival of AML lines, cells were plated in 96-well plates at 10^4 or 10^3 cells/ml, exposed to graded concentrations of olaparib and cultured for 3 or 7 days, respectively. Cell survival was evaluated by the Kit CellTiter 96[®] AQ_{ueous} One Solution Cell Proliferation Assay (Promega), according to the manufacturer's instructions. At the end of each experiment the absorbance of metabolized MTS was read at 490 nm using a Victor X Plate Reader (PerkinElmer).

The olaparib inhibitory concentration 50 (IC₅₀), defined as the drug concentration capable of inhibiting 50% of cell growth compared to untreated control, was calculated with the GraphPad Prism 5 software by using linear regression. Statistical correlations with IC₅₀ were performed using non-parametric one-tailed Spearman's tests. Results were considered significant for p values ≤ 0.05 .

2.3. Flow cytometry and apoptotic assays

Apoptosis in primary AML samples was assayed using an annexin V apoptosis kit (GFP Certified™ Apoptosis/Necrosis Detection Kit, Enzo Life Sciences), according to the manufacturer's instructions, and

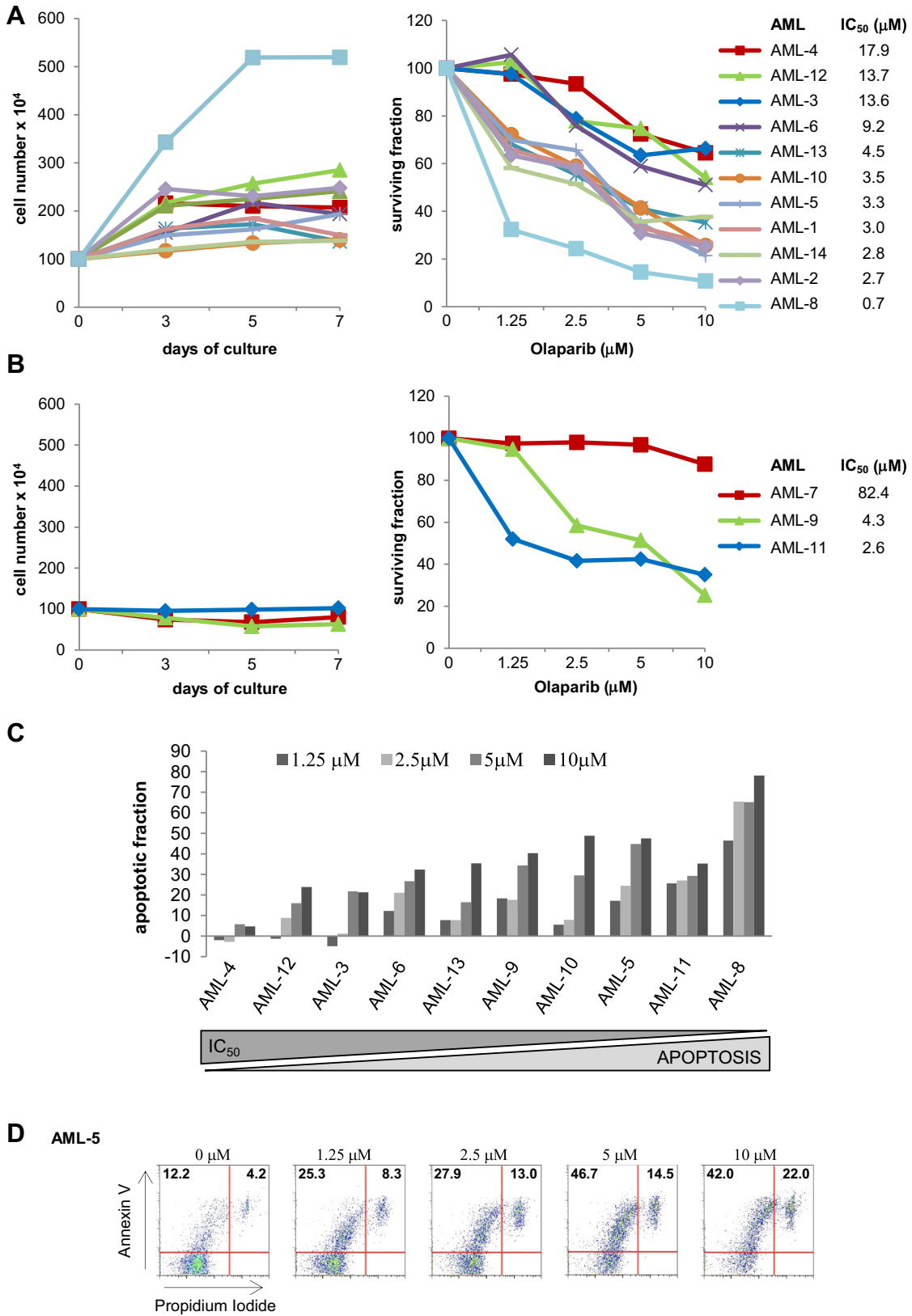


Fig. 1. Primary AML cells are killed *in vitro* by olaparib. Primary AML cells were cultured with IL-3, SCF and FLT3LG for 3 days and then treated (time 0) with increasing concentrations of olaparib. Cell proliferation was evaluated by count of viable cells using trypan blue exclusion at 3, 5 and 7 days. Standard error (SE) of replicate counts was ± 10% and was not indicated in the figure. AML samples were divided in two different groups on the basis of their proliferation rate. The IC₅₀s of olaparib were calculated after 7 days of exposure. (A) Growth curves of 11 proliferating primary AML cells untreated (*left panel*) and the surviving fractions after 7 days of treatment with olaparib (*right panel*). (B) Growth curves of 3 not proliferating primary AML cells untreated (*left panel*) and the surviving fractions after 7 days of treatment with olaparib (*right panel*). (C) Apoptosis induction by olaparib in primary AML cells, following 7 days of exposure to increasing concentrations of olaparib. The percentages of cells treated with olaparib undergoing early and late apoptosis, as detected by Annexin V/PI by FACS analysis, are indicated after subtraction of apoptosis in the untreated controls. In the figure AML samples were arranged from the most resistant to the most sensitive. (D) Representative plot of Annexin V/PI analysis of the AML-5 sample. Necrotic cells (*i.e.*, PI positive and Annexin V negative) were shown in the bottom right quadrant and were <1% in all analyzed AML samples.

analyzed by flow cytometry. To distinguish normal lymphocytes from AML blasts, 5×10^5 cells, untreated or treated with 10 μM olaparib for 7 days, were stained with anti-human CD45 APC-eFluor780 (e-Bioscience), washed with PBS and assayed for apoptosis. Samples were acquired on a CyAN ADP cytometer (Beckman Coulter) and analyzed by FlowJo software (Tree Star Inc.).

Cell death in leukemia cell lines was evaluated after cell fixation in ethanol, treatment with 10 $\mu\text{g}/\text{ml}$ RNase A (Sigma-Aldrich) and staining with 100 $\mu\text{g}/\text{ml}$ propidium iodide (PI) (Sigma-Aldrich).

2.4. mRNA expression and sequencing

Total RNA was isolated using TRIzol reagent (Invitrogen) and checked for quality by gel electrophoresis. One μg of total RNA was reverse-transcribed using random hexamer primers and reagents of Life Technologies.

Quantitative real-time PCR (qRT-PCR) were carried out using Applied Biosystems® TaqMan® Universal PCR Master Mix (Life Technologies). TaqMan primers/probes for PARP1, PARP2, BRCA1, BRCA2 and GAPDH (for internal normalization) were from Integrated DNA Technologies. Analysis was performed in triplicate on ABI-7900HT (Applied Biosystems). The $2^{-\Delta\Delta\text{Ct}}$ relative quantification method was used to calculate relative mRNA expression. A normal BM RNA was used as a calibrator. Statistical analysis was performed using non-parametric one-tailed Mann–Whitney test. Results were considered significant for p values ≤ 0.05 .

NPM1 mutA and *FLT3*-ITD gene status was investigated as previously described [29] with minor changes. Briefly, reverse-transcribed RNA was screened by PCR-capillary electrophoresis methods and the PCR setup was modified for Applied Biosystems 3130 platform.

The following primers were used: for *FLT3* forward primer ITD-F5=5'-tgtcgcagcagctactctaaaca-3', reverse primer ITD-R6=5'-atcctagctaccttcccaaacctc-3'; for *NPM1* forward primer NM-F2=5'-atcaattatgtgaagaattgcttac-3', reverse primer NPM-Rev6=5'-accatttccatgctctgagcacc-3'.

ITD-R6 and NPM-Rev6 primers were labeled with the fluorochromes 6-FAM and the *FLT3* and *NPM1* gene analysis was performed in two different PCR reactions using AmpliTaq Gold DNA-polymerase (Life Technologies). PCR conditions were adapted as follows: pre-incubation at 95 °C for 7 min followed by 30 s at 94 °C, 30 s at 57 °C, and 30 s at 72 °C for 35 cycles, and a final elongation at 72 °C for 10 min. One μl of PCR product was mixed with deionized formamide and ROX500 dye size standard (Life Technologies) according to the manufacturer's instructions, heated to 95 °C for 5 min, and placed on

ice for at least 1 min before electro-kinetic injection to the ABI 3130 capillary electrophoresis instrument (Applied Biosystems).

2.5. Immunoblotting

Total proteins were extracted from AML cells collected before culture and treatment. Cells were lysed 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, 25 mM NaF, 1 mM PMSF and protease inhibitor cocktail (Sigma-Aldrich). Protein aliquots were loaded onto Any KD™ or 7.5% Mini-Protein TGX polyacrylamide gels (Bio-Rad). After transferring to nitrocellulose membrane total proteins were incubated with the following antibodies: anti-PARP1 (M01-3G4, Abnova), anti-PARP2 (ab176330, Abcam), anti-MRE11A (H-300, Santa Cruz), anti-MLH1 (G168-15, BD Pharmingen), anti-MSH2 (Ab-1, Calbiochem), anti-MSH3 (52, BD Pharmingen), anti-BRCA1 (D-9, Santa Cruz), anti-BRCA2 (9012, Cell Signaling Technology), anti-RAD51 (14B4, Thermo), anti- γH2AX (JBW301, Millipore), anti-PAR (4335, Trevigen) and anti- β -actin (4967, Cell Signaling Technology). Horseradish peroxidase-conjugated IgG were used as secondary antibodies. The autoradiograms were subjected to densitometric analysis by ImageJ 1.45s software (Bio-Rad Laboratories). The results were normalized against β -actin and used for statistical correlation analyses.

2.6. Immunofluorescence of γH2AX foci

AML cells were cytocentrifuged ($5\text{--}10 \times 10^4$ cells), fixed with 4% paraformaldehyde, permeabilized in 0.3% triton, blocked in 2% BSA (Sigma-Aldrich) and immunostained with mouse anti- γH2AX . After staining, cells were washed and incubated with goat anti-mouse IgG Alexa Fluor 488 (Invitrogen). Nuclei were then stained with DAPI (4,6 diamidino-2-phenylindole) and mounted in Fluoromount (Sigma-Aldrich). Analysis was performed using a Leica CTR 6000 fluorescence microscope and LAS AF Lite software (Leica). Images were taken at 40 \times magnification.

3. Results

3.1. AML blasts are sensitive to olaparib in vitro

Sensitivity to the PARPi-mediated cytotoxicity was evaluated by trypan blue exclusion cell count (Fig. 1A–B). The requirement of cell progression to S phase for the conversion of PARPi-induced SSB in DSB prompted us to verify whether PARPi sensitivity of AML blasts was influenced by the *in vitro* proliferation rate. Thus, AML samples were

Table 1

Clinical and biological characteristics of AML patients.

IC ₅₀ olaparib (μM)	AML	Age/sex	WBC ($\times 10^6/\text{ml}$)	% blasts (BM)	Karyotype	<i>NPM1</i> mutA	<i>FLT3</i> -ITD	Prognostic risk
82,4	AML-7	56/M	53	80	46,XX,ins(10;11)(p12;q2313)	Neg	Neg	Intermediate
29,9	AML-17	59/M	7	40	NA	Pos	Pos	High
17,9	AML-4	58/M	25	90	NA	Pos	Neg	Low
13,7	AML-12	50/M	57	72	46,XY,t(5;21)(q32;q22)[8]/46,XY[9]	Pos	Pos	High
13,6	AML-3	51/F	6	60	46,XX,t(8;21)	Neg	Neg	Low
9,2	AML-6	35/M	58	90	46,XY,inv(16)	Neg	Neg	Low
4,5	AML-13	34/M	63	73	46,XY	Pos	Pos	High
4,3	AML-9	23/M	102	90	NA [PCR positive for inv(16)]	Neg	Neg	Low
3,5	AML-10	54/F	17	60	46,XX	Pos	Pos	High
3,5	AML-15	58/M	26	40	47,XY,+8[2]/46,XY[28]	Neg	Neg	Intermediate
3,3	AML-5	59/F	105	60	46,XX	Pos	Neg	Low
3,0	AML-1	41/F	28	80	46,XX	Pos	Pos	High
2,8	AML-14	55/M	116	82	46,XY	Pos	Neg	Low
2,7	AML-2	56/F	94	80	46,XX,inv(16)	Neg	Neg	Low
2,6	AML-11	38/M	88	90	47,XY,+6[12]/46,XY[2]	Pos	Pos	High
1,5	AML-16	40/M	5	21	46,XY	Neg	Neg	Intermediate
0,7	AML-8	30/M	16	95	46,XY,del(11)(q23)[7]/46,XY[13]	Neg	Neg	Intermediate

NA = Not Available.

divided in two groups including cycling (panel A) or non-proliferating leukemic blasts (panel B). The doubling times of cycling blasts were extremely variable ranging from 54 to 318 h. The most responsive case (AML-8) ($IC_{50} = 0.7 \mu\text{M}$) was characterized by an extremely high growth rate (Fig. 1A), whereas the olaparib resistant case AML-7 ($IC_{50} = 82.4 \mu\text{M}$) did not proliferate *in vitro* (Fig. 1B). However, 2 out of 3 AMLs that did not proliferate *in vitro* at the assessed time points, showed high responsiveness to olaparib (Fig. 1B). These findings suggest that the antitumor effect of olaparib does not strictly depend on

the *in vitro* proliferative potential of leukemic blasts. Indeed, no statistically significant correlation was observed between the proliferation indexes of tested AMLs and olaparib IC_{50} s. Overall, the results indicated that a single treatment with PARPi induced a dose-dependent decrease of cell survival in 13 out of 14 AML samples, with IC_{50} s of sensitive AML cases ranging from 0.7 to 17.9 μM (Fig. 1A–B). These values are below or within the range of the plasma peak concentrations reached in patients with solid tumors who were treated with 200–600 mg olaparib given twice daily [22,27,30,31].

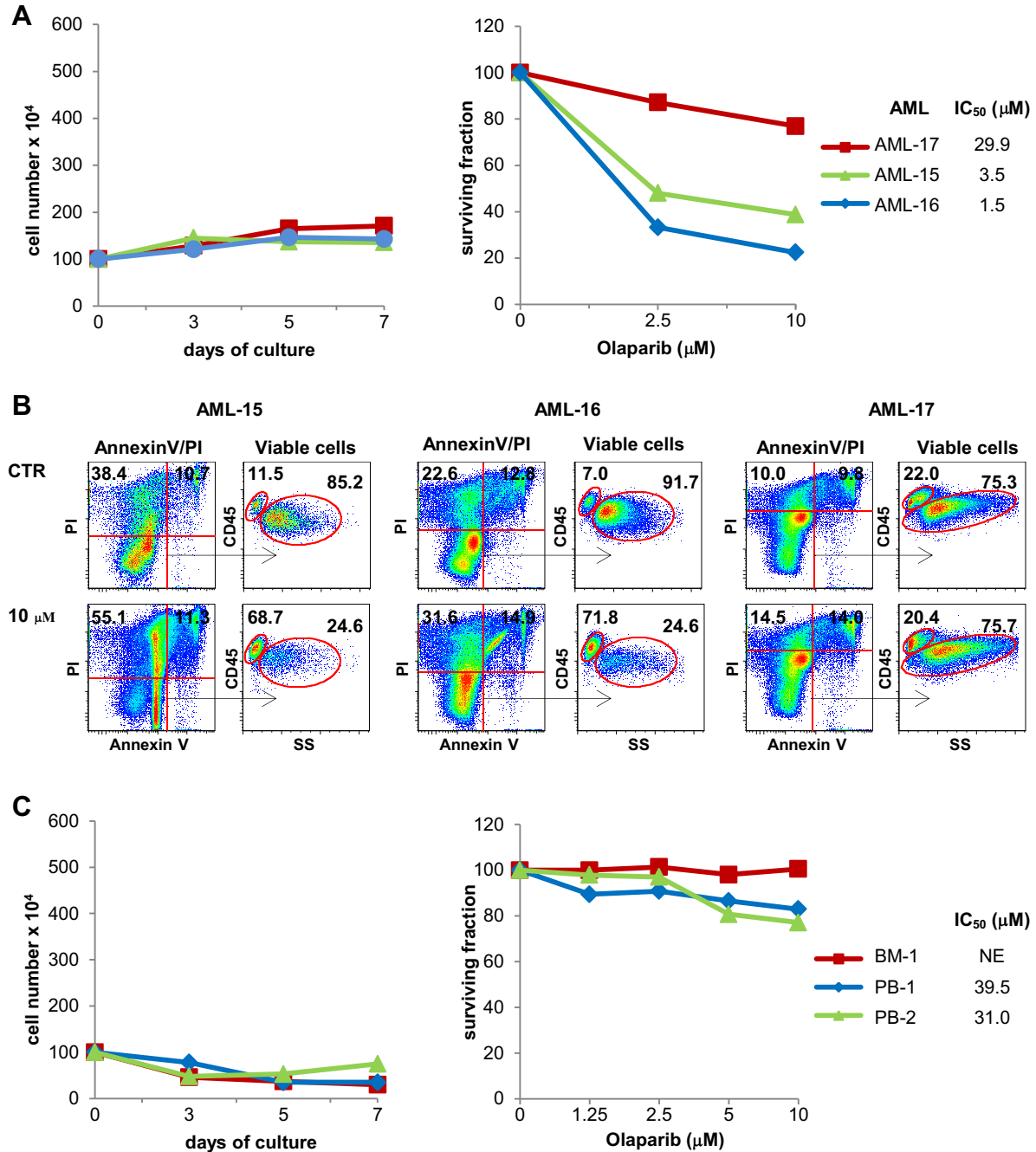


Fig. 2. Lymphocytes from AML patients and normal hematopoietic cells have low sensitivity to olaparib. Three primary proliferating AML samples with percentages of blasts $\leq 40\%$ at diagnosis were treated with increasing concentrations of olaparib. (A) Growth curves of proliferating untreated primary AML cells (left panel) and surviving fractions after 7 days of olaparib treatment (right panel). (B) Apoptosis following 7 days of exposure to 10 μM olaparib detected by FACS analysis after staining with Annexin V/PI. Viable cells were separated in two populations by side scatter and CD45. After separation and culture for 7 days surviving blasts exceeded 75% in all untreated AML samples. In AML-15 and AML-16 sensitive samples olaparib induced a decrease in the percentage of viable leukemic cells (lower cells) which is accompanied by an increase in the percentage of lymphocytes (upper cells). On the contrary, in AML-17 resistant sample the percentage of the two populations remained constant after olaparib treatment. (C) Growth curves of untreated controls (left panel) and surviving fractions after 7 days of olaparib treatment (right panel) of one BM (BM-1) and two CD34-enriched mobilized peripheral blood samples (PB-1 and PB-2) obtained from healthy donors. NE = Not Evaluable.

FACS analysis of AML blasts, stained with annexin V/PI after 7 days of drug exposure, showed that olaparib concentrations ranging from 1.25 to 10 μM were able to induce apoptosis in sensitive AML blasts (Fig. 1C). The PARPi induced apoptosis also in the two responsive leukemia samples that did not proliferate *in vitro* (i.e., AML-9 and AML-11). A representative experiment showing the dose-dependent increase of pre-apoptotic and apoptotic cells mediated by olaparib in AML-5 cells is shown in Fig. 1D.

Next, we analyzed whether a correlation existed between the olaparib IC_{50} values at 7 days and some of the most important clinical prognostic indexes of the AML blasts (i.e., age, white blood cell count, karyotype, *NPM1* mutA and *FLT3*-ITD) (Table 1). However, no statistically significant correlation was found between the sensitivity of leukemic blasts and the above considered prognostic factors. AML-8 cells, which resulted to be the most sensitive to the anti-proliferative and apoptotic effects of olaparib, were characterized by a chromosome 11q23 deletion (Table 1).

3.2. Lymphocytes from AML patients and normal hematopoietic cells have low sensitivity to olaparib

In clinical trials olaparib monotherapy displayed a favorable toxicity profile as compared to most chemotherapeutic agents commonly used for cancer treatment [22–24]. To investigate the effect of olaparib on normal lymphocytes present in the BM of leukemia patients, 3 AML samples, characterized by a low percentage of blasts at the time of

diagnosis ($\leq 40\%$), were treated with olaparib. These AML samples showed comparable growth rates (Fig. 2A, left panel), but different sensitivity to olaparib, being AML-15 and AML-16 more responsive than AML-17 cells (Fig. 2A, right panel). Flow cytometric analysis, performed after 7 days of culture, indicated that in all samples the percentages of blasts, characterized by low expression of CD45, markedly increased due to the cell purification procedures and the *in vitro* culture (Fig. 2B). The higher level of apoptosis induced by olaparib in AML-15 and AML-16 samples is consistent with the results of cell proliferation assay (Fig. 2A). Gate analysis of Annexin V and PI negative cells (viable cells) indicated that in AML-15 and AML-16 sensitive samples the exposure to 10 μM olaparib caused a decrease in the percentage of blasts (CD45 low) and, consequently, an increase in the percentage of normal lymphocytes (CD45 high) (Fig. 2B). These data suggest that olaparib preferentially killed leukemia blasts and spared lymphocytes from the same patient. By contrast, the PARPi minimally affected both cell populations in AML-17 cells that are characterized by an olaparib IC_{50} 8.5-fold and 20-fold higher than that of AML-15 and AML-16 cells, respectively.

The influence of olaparib on normal hematopoietic cells, isolated from one BM and from two CD34-enriched mobilized peripheral blood samples, was also analyzed. Treatment with the PARPi did not affect the survival of BM cells. Mobilized peripheral blood samples showed instead a certain degree of sensitivity to olaparib. However, it should be noted that olaparib IC_{50} values were higher than those obtained with the majority of AML blasts (Fig. 2C vs Figs. 1 and 2A).

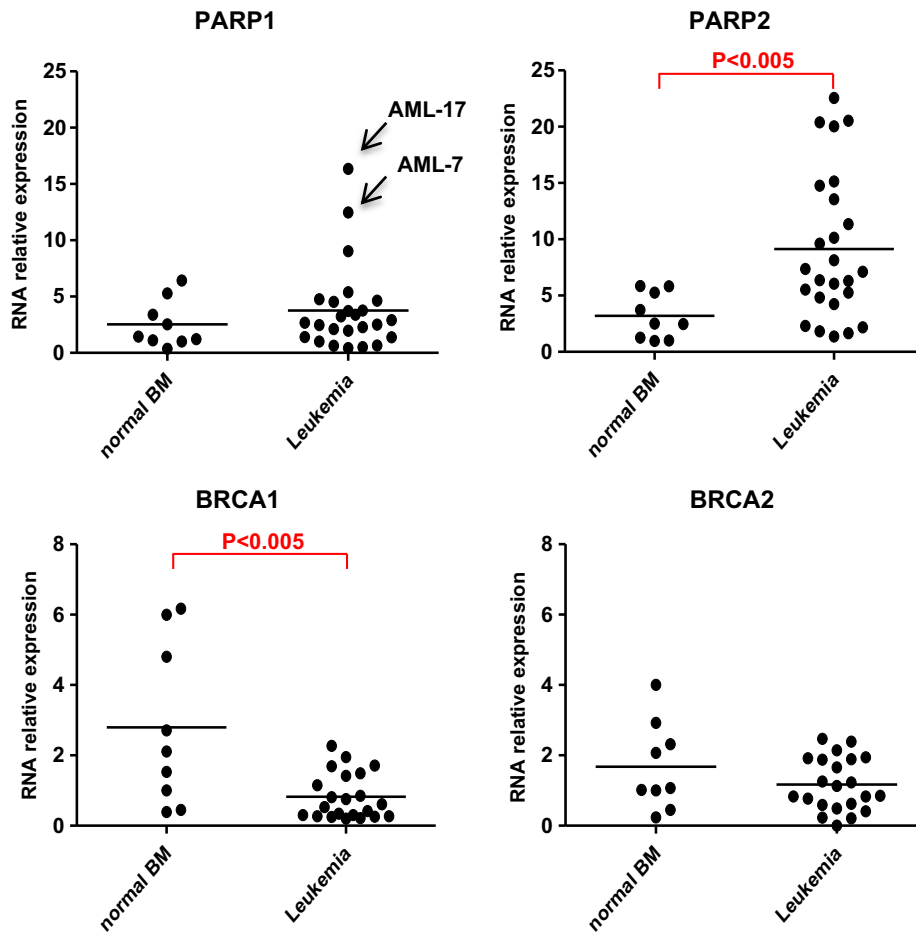


Fig. 3. mRNA expression of PARP1, PARP2, BRCA1 and BRCA2 in normal hematopoietic cells and in *de novo* AML blasts. mRNA expression was measured by qRT-PCR using total RNA extracted from BM of AML patients and healthy donors. Relative expression was obtained normalizing with respect to GAPDH expression and calibrating with respect to a normal BM. Data are presented as the mean of triplicate analysis. Statistical analysis was performed using the non-parametric Mann–Whitney test. Results were considered significant for p values ≤ 0.05 .

3.3. mRNA expression of PARP1/2 and BRCA1/2 is altered in de novo AML cells

It has been recently reported that the expression of PARP1 and PARP2 is required for olaparib cytotoxic effects since PARP1^{-/-} knock-out B-lymphoblast avian cells, which are devoid of PARP2 expression, are unresponsive to the PARPi [32]. In addition, BRCA1/2 homozygous mutations or epigenetic down-regulation of their expression has been widely demonstrated to result in HR-deficiency and responsiveness to the synthetic lethality induced by PARPi monotherapy [8,9]. Based on these observations we speculated that differences in the expression of PARP1, PARP2, BRCA1 and BRCA2 between normal and leukemia cells might account for the resistance of normal hematopoietic cells to olaparib. Thus, we analyzed mRNA expression of these four genes in 9 normal and 25 AML BM samples (including the 17 AML samples tested for chemosensitivity) by qRT-PCR. A higher expression of PARP2 was observed in AML as compared to normal BM samples, whereas PARP1 mRNA levels in leukemic blasts were not significantly different from those present in normal cells (Fig. 3). However, it should be noted that AML-7 and AML-17, regarded as the least sensitive AML, expressed PARP1 transcript largely above the median range.

On the contrary, BRCA1 transcript was down-regulated in AML samples (Fig. 3), supporting a less effective HR repair that might account for the sensitivity to olaparib.

3.4. AML cell lines are sensitive to olaparib: γ H2AX and RAD51 as biomarkers of olaparib sensitivity

In order to verify whether the high rate of response observed in primary AML blasts might be due to an unintended selection of sensitive AML samples we analyzed 6 leukemia cell lines known to be unresponsive to the PARPi Ku0058948 [21]. The olaparib IC₅₀s of leukemia cell lines at 7 days were comprised between 1.0 and 10.7 μ M (Fig. 4). In particular, U937 cells were the least sensitive with an IC₅₀ of about 11-fold higher than that of NB4 cells. Overall, all the AML cell lines tested were responsive to clinically relevant concentrations of olaparib.

In the search of a predictive marker of response to olaparib we evaluated by Western blot analysis the expression of a number of repair proteins that might be involved in sensitivity to PARPi. For this purpose, we initially tested basal PARP1, PARP2 protein expression and PARylated proteins, as a measure of PARP activity, in the AML cell lines (Fig. 5A). Both U937 and NB4 cells, although characterized by different olaparib IC₅₀s (i.e. 10.7 and 1.0 μ M), expressed comparable low PARP1 and PARylated proteins. Indeed, no statistically significant correlation was found between the results of densitometric analysis of PARP1, PARP2 and PAR immunoreactive bands and olaparib IC₅₀s (Fig. 5A). In addition, no correlation was also found between the expression of MRE11A and the mismatch repair proteins MLH1, MSH2, MSH3 and drug sensitivity. In particular, the HL-60R cell line, which overexpresses MSH3 as a result of gene amplification [33], responded to olaparib similarly to the HL-60 wild-type counterpart which expresses 15-fold lower levels of MSH3 (Fig. 5A).

With respect to proteins involved in DSB repair, in our experimental conditions we did not observe detectable levels of BRCA1 and BRCA2 in all the AML cell lines tested. Interestingly, RAD51 directly correlated (Spearman's correlation 0.829; $p = 0.021$) and γ H2AX inversely correlated (Spearman's correlation -1.000 ; $p < 0.0001$) with the olaparib IC₅₀s. In particular, U937 cells showed undetectable basal levels of γ H2AX as compared to the olaparib hypersensitive NB4 cells (Fig. 5A). These data support the role of the repair proteins RAD51 and γ H2AX as potential markers of olaparib sensitivity in AML cell lines.

Cell death and γ H2AX foci formation were then analyzed in U937, HL60 and NB4 cells 24 h after treatment with 1.25 and 5 μ M of olaparib. The results indicated that in the AML cell lines the toxic effects caused by olaparib were evident as early as 24 h after drug exposure, especially in the most sensitive cells (i.e. HL-60 and NB4; Fig. 5B). In the case of

U937 cells, although the percentage of dead cells slightly increased at later time points (i.e., 72 and 120 h), it remained always lower than in HL-60 and NB4 cells (data not shown). Moreover, the PARPi caused a dose-dependent increase in the number γ H2AX positive cells which correlated with sensitivity to olaparib (Fig. 5C). Consistent with the results of immunoblot analysis the number of γ H2AX foci in untreated NB4 and HL-60 cells was higher than in U937 cells.

3.5. In primary AML PARP1 over-expression and BRCA1 proficiency indicate resistance to olaparib, whereas formation of γ H2AX foci indicates sensitivity

Based on the results obtained in the AML cell lines we analyzed by Western blot the expression of PARP1, PARP2, RAD51, γ H2AX, BRCA1, BRCA2 and PARylated proteins in the AML blasts isolated from patients (Fig. 6A). PARP1 protein expression significantly correlated with PAR levels (Spearman's correlation 0.575; $p = 0.02$) and PARP1 mRNA (Spearman's correlation 0.746; $p = 0.002$) (Fig. 6A–B). No statistically significant correlation was found between the results of densitometric analysis of PARP1, PARP2 and PAR immunoreactive bands and olaparib IC₅₀s. Nevertheless, AML-7 cells with the highest olaparib IC₅₀ also showed the highest amount of PARP1 protein and mRNA levels (Fig. 6B and Fig. 3). Differently from the results obtained in AML cell lines, no correlation was observed between olaparib IC₅₀s in primary AML blasts and basal RAD51 or γ H2AX protein expression analyzed before culture.

In keeping with the findings obtained in the AML cell lines, BRCA2 protein was hardly detectable in all AML primary samples, whereas BRCA1 protein was only detected in the olaparib resistant AML-7

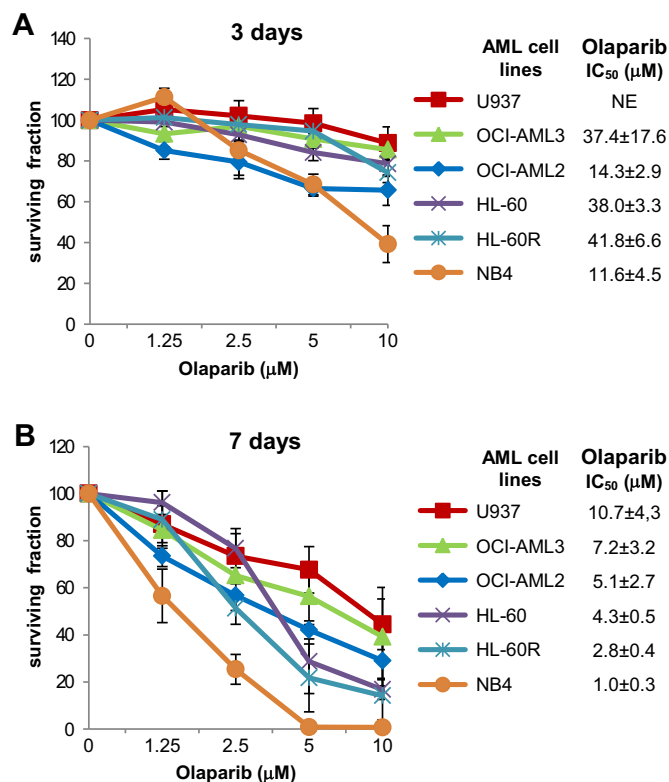


Fig. 4. AML cell lines are sensitive to olaparib. The U937, OCI-AML2, OCI-AML3, HL-60, HL-60R and NB4 cell lines were treated with increasing concentrations of olaparib and analyzed by MTS. The surviving fraction with respect to the untreated control was calculated at 3 days (panel A) and 7 days (panel B) after drug exposure. Values are the mean values \pm SE of at least three independent experiments. The IC₅₀s for each cell line after 3 and 7 days of exposure to olaparib are indicated. NE = Not Evaluable.

sample. Thus, the concomitant condition of high PARP1 expression and the presence of BRCA1 in AML-7 might account for its resistance to olaparib.

AML samples were then examined for their ability to form γ H2AX foci in response to treatment with olaparib. For immunofluorescence analysis we collected 8 AML samples 72 h after drug treatment (Fig. 6C). An inverse correlation was found between the olaparib IC_{50} s

and the percentage of γ H2AX positive cells (i.e. with ≥ 5 γ H2AX foci) in untreated AML blasts (Spearman's correlation -0.633 ; $p = 0.038$) and in those treated with $10 \mu\text{M}$ olaparib (Spearman's correlation -0.817 ; $p = 0.005$). Representative cases of primary AMLs showing γ H2AX after treatment with olaparib are presented in Fig. 6D. Overall, these results outline γ H2AX foci assessment by immunofluorescence as a useful method to monitor response of primary AML cells to olaparib.

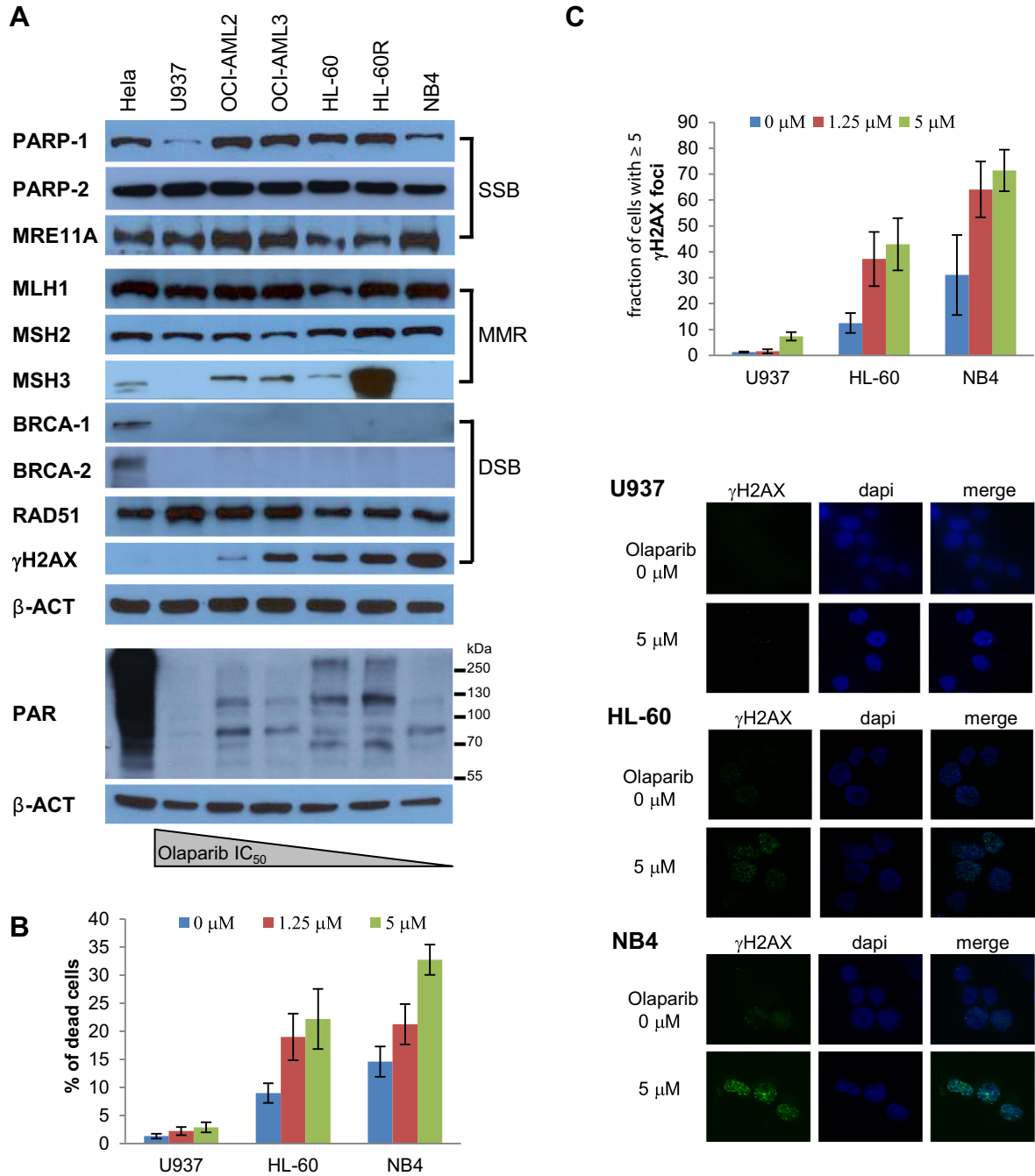


Fig. 5. Analysis of repair proteins, γ H2AX foci formation and cell death in leukemia cell lines with different sensitivity to olaparib. (A) Western blot analysis of proteins (30 μg) extracted from HeLa, U937, OCI-AML2, OCI-AML3, HL-60, HL-60R and NB4 cell lines. HeLa cells were used as a positive control. Cell lines were arranged on the gel from the most resistant to the most sensitive to olaparib. Basal expression of PARP1, PARP2, MRE11A (repair of SSB), MLH1, MSH2, MSH3 (components of the mismatch repair system, MMR), BRCA1, BRCA2, RAD51 and γ H2AX (repair of DSB), PAR and β -actin, was analyzed by immunoblots. One representative blot of β -actin is shown. (B) Cell death induced by graded concentrations of olaparib (0, 1.25, 5 μM) evaluated after 24 h in U937, HL-60 and NB4 leukemia cell lines by flow cytometry after PI staining. The percentage of PI positive cells in the untreated cells is shown. Values are the mean \pm SE of three independent experiments. (C) Frequency of cells displaying γ H2AX foci (%) as detected by immunofluorescence after 24 h exposure to 0, 1.25 and 5 μM olaparib. At least 300 nuclei were counted per experiment. Values are the mean \pm SE of three independent experiments. (D) Representative nuclei of U937, HL-60 and NB4 cells probed with γ H2AX (green stain) and DAPI (blue) in untreated cells or cells treated with $5 \mu\text{M}$ olaparib.

4. Discussion

In the present study we show that the PARPi olaparib as single agent exerts antiproliferative and apoptotic effects in AML blasts at clinically achievable concentrations. By contrast, normal lymphocytes or hematopoietic cells from healthy donors showed low sensitivity to olaparib regardless of the sensitivity/resistance profile of matched leukemic blasts. The exposure to a single dose of olaparib was able to exert cell killing in a high percentage (88%) of primary AML samples. Only in 2 out of 17 cases the olaparib IC_{50} was $\geq 25 \mu\text{M}$, i.e. higher than the maximum concentration achievable in patients with solid tumors who were treated with 200–600 mg olaparib twice daily.

At present, the efficacy of PARPi as monotherapy has been mostly confined to patients with *BRCA1/2* mutations and related to synthetic lethal interaction between defective *BRCA1/2* function and inhibition of

PARP1 activity. However, mutations in *BRCA1/2* genes are not common in acute leukemia [34]. Nevertheless, a decreased expression of *BRCA1/2* proteins causing impairment of HR may also derive from mechanisms different from gene mutations [9,35,36]. In addition, olaparib has been shown to induce objective responses in patients with ovarian cancer also in the absence of *BRCA* mutations [25].

Our results show that AML blasts express lower levels of *BRCA1/2* transcripts as compared to BM samples derived from healthy donors. Indeed, a reduced *BRCA1* expression due to promoter hypermethylation was reported in therapy-related AML cells [37]. Moreover, although *BRCA* transcripts were measurable in all samples, the corresponding proteins were barely detectable or not present in the AML cell lines and patient blasts. Thus, alterations in post-transcriptional regulatory mechanisms might influence the expression of *BRCA* proteins in AML. Moskwa et al. [38] reported that over-expression of miR-182 in the

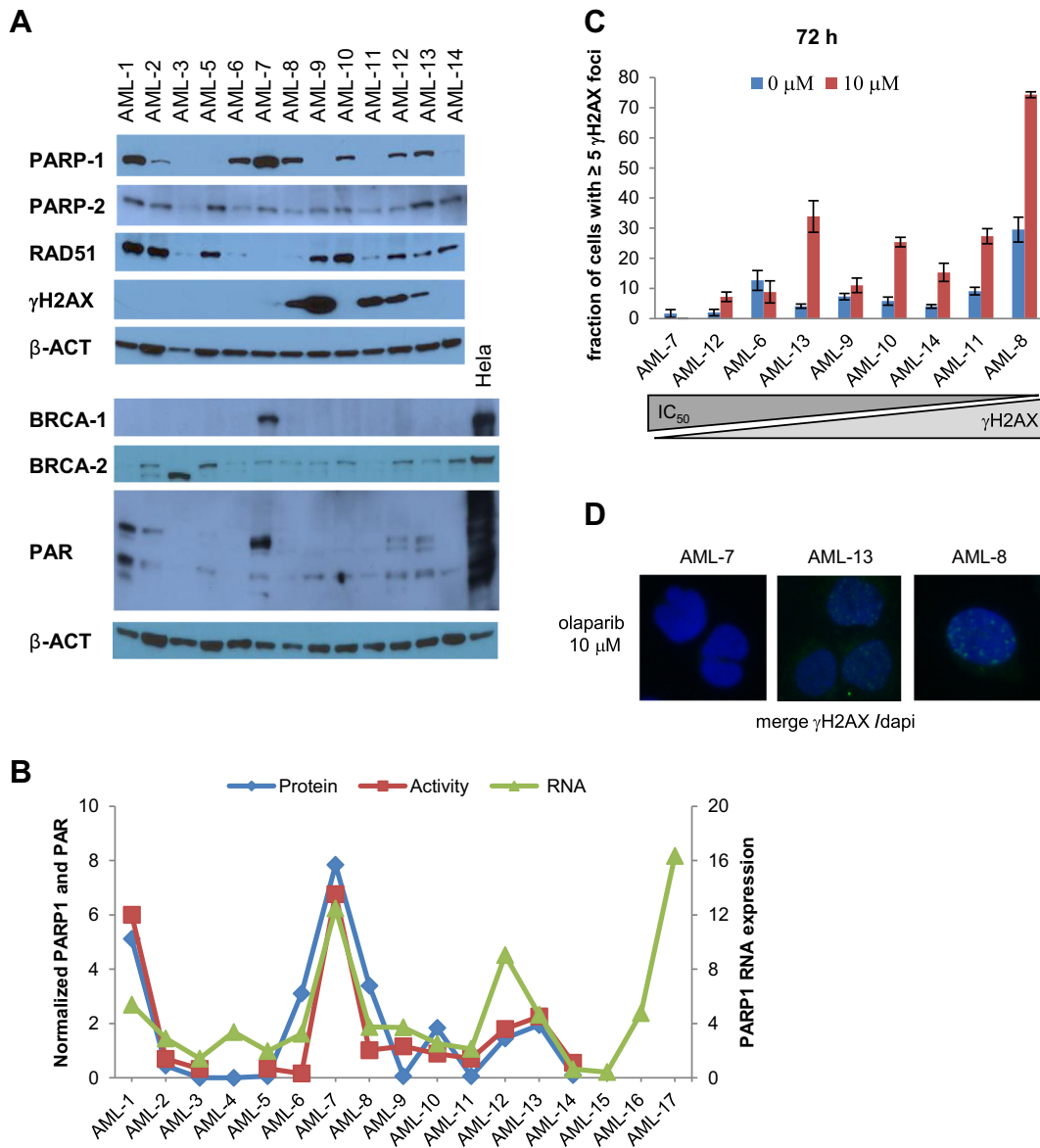


Fig. 6. Analysis of repair proteins and γH2AX foci formation in primary AML blasts. (A) Western blot analysis of proteins (30 μg in all cases, except for BRCA protein analysis in which 60 μg was loaded) from primary AML cells at the time of diagnosis. Immunoblots of PARP1, PARP2, RAD51, γH2AX , BRCA1, BRCA2 and PARylated proteins (PAR), were shown. β -actin was used to normalize samples. (B) Expression of PARP1 protein, PARylated proteins and mRNA in AML blasts. Optical density values of PARP1 and PAR were normalized against β -actin. The mRNA values refer to the data shown in Fig. 3. (C) Analysis of γH2AX foci by immunofluorescence after 72 h of exposure to 0 and 10 μM olaparib. At least 200 nuclei were counted. Values are the mean percentage \pm SE of the cells with ≥ 5 γH2AX foci in four quadrants with at least 50 cells each. (D) Representative nuclei of AML blasts after 72 h of exposure to 10 μM olaparib. Cells were probed with γH2AX (green stain) and DAPI (blue).

MDA-MB-231 breast cancer cell line hampers mRNA translation of BRCA1 and confers sensitivity to two different PARPi. Since the only resistant AML sample (*i.e.*, AML-7) that we could analyze by Western blot showed the highest expression of BRCA1 and all the remaining samples presented undetectable or low levels of BRCA1 and BRCA2, we can reasonably assume that the low expression of these proteins contributes to the sensitivity of AML to olaparib.

It should be observed that the drug concentrations used in our study are higher than those required by olaparib to inhibit the catalytic activity of purified PARP1 and PARP2 proteins (*i.e.* IC₅₀: PARP1 = 0.005 μM, PARP2 = 0.001 μM). In previous works, *in vitro* sensitivity of certain tumors to nanomolar concentrations of olaparib was linked to inhibition of PARP catalytic activity [15,17]. However, higher concentrations of the PARPi may cause cell death by additional mechanisms. For instance, in the work by Hegan et al. [39] several PARPi at μM concentrations down-regulated BRCA1 and RAD51 expression increasing the occupancy of their promoters by the repressive E2F4/p130 complexes. Recently, Nowsheen et al. [40] observed that concentrations of veliparib and olaparib ranging from 2.5 to 10 μM inhibited the HER2/NF-κB signaling rendering HER2-positive breast cancers sensitive to the PARPi. Moreover, Murai et al. [32,41] reported a cytotoxic mechanism based on trapping of PARP1/2 on DNA complexes which varied in extent among the different PARPi and was independent from their inhibitory effect on PARP catalytic activity. Additional mechanisms of olaparib that have been recently reported involve up-regulation of death receptors such as FAS and DR5 leading to sensitization of tumor cells to death receptor ligands [42]. Therefore, it is conceivable that the cytotoxicity exerted by olaparib in AML cells also depends on inhibition of other PARP functions in addition to the proven block of PARylation and synthetic lethality.

Since PARP1 and PARP2 are the primary targets of PARPi we also investigated whether a relationship existed between their levels and the response to olaparib. No statistically significant correlation was found between olaparib IC₅₀s and PARP1, PARP2 mRNA or protein expression. These data are in agreement with our recent findings on glioblastoma stem cells using a different PARPi [43]. However, the two highly resistant cases AML-17 and AML-7 showed the highest levels of PARP1 expression. In our experimental conditions, a threshold of PARP1 mRNA relative expression set at 10 (the 2^{-ΔΔCt} value) may be considered to identify patients who may not benefit from treatment with olaparib. To the best of our knowledge, the up-regulation of PARP1 was reported in some solid tumors [44,45] but, no information is still available from clinical trials on the relationship between basal PARP1 expression in primary tumors and resistance to PARPi.

Primary blasts from AML-8 showing high sensitivity to olaparib (IC₅₀ = 0.7 μM) had a deletion at chromosome 11q23. The region 11q comprises several genes known to be involved in the response to DSB, including *MRE11A*, *ATM* and *H2AFX* alleles. Our findings are in agreement with a recent report showing that lymphoid tumors with 11q deletion are highly responsive to olaparib [15]. Moreover, Gaymes et al. [18] reported mutations in *ATM*, as well as in *MRE11A*, in cell lines characterized by microsatellite instability that were responsive to PARPi, including the hypersensitive NB4 cells.

Cancer treatment might be improved if information on the patient individual response to a drug is available at diagnosis or early in the course of treatment. The use of γH2AX as a pharmacodynamic response to a DNA cross-linking agent has been recently adopted in a clinical trial setting [46,47]. In our study we observed a strong correlation between olaparib IC₅₀s and basal γH2AX protein expression in AML cell lines analyzed by immunoblot, but not in primary AMLs when tested at baseline before culture. On the other hand, a significant correlation was found between the sensitivity to olaparib and the number of basal γH2AX foci in cultured AML blasts (Figs. 5C and 6C). It can be hypothesized that the pattern of γH2AX expression in cycling blasts more likely reflects the situation of leukemia cells in patients, since just after their isolation from BM specimens blasts are typically arrested at G1/G0 phase. An abnormal ability to repair the DNA damage caused by endogenous

insults might cause an increase of basal γH2AX foci in proliferating AML blasts enhancing the sensitivity to PARPi. Indeed, elevated basal γH2AX levels, which may result in dysregulation of DSB response, have been recently reported in some therapy-related AML [48]. Moreover, we found an increased number of γH2AX foci in sensitive AML blasts after a single treatment with olaparib, as a result of the DNA damage caused by the drug.

In conclusion, we show here that a single treatment with olaparib caused cell killing in the majority of primary AML blasts tested at concentrations achievable in cancer patients, likely due to low expression of BRCA proteins. Moreover, PARP1 mRNA over-expression and γH2AX foci appear to be promising candidate biomarkers to predict resistance or response. The favorable side effects profile and the good oral bioavailability of olaparib render this drug an attractive therapeutic option as monotherapy for AML patients not suitable for conventional chemotherapy.

Disclosure of conflicts of interest

The authors declare no competing financial interests.

Authorship contributions

IF designed the work, performed the experiments, interpreted the data and wrote the paper; MC, DFA and EP performed the experiments; PP performed karyotype of AML blasts; SL and TO provided leukemic samples and performed the experiments; MTC contributed to experimental design and performed the experiments; SD contributed to experimental design and writing; AV provided clinical data on AML sample; GG and FLC planned the research strategy and wrote the paper. All authors reviewed and approved the manuscript.

Acknowledgments

We would like to thank Michela Cittadini, Serena Zaza and Mariadomenica Divona for providing essential reagents and thoughtful comments. We are grateful to Gaspare Adorno, Gianpaolo Del Proposto, and Francesca Agostini for supplying the control samples from healthy donors and to Carla Rossi for statistical support.

This work was supported by grants from “Associazione Italiana per la Ricerca sul Cancro” (AIRC) to F.L.C. (IG 11586) and in part to G.G. (IG 14042).

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