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Original Articles

The poly(ADP-ribose) polymerase inhibitor olaparib induces up-regulation of death receptors in primary acute myeloid leukemia blasts by NF-κB activation

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ABSTRACT

Olaparib is a potent orally bioavailable poly(ADP-ribose) polymerase inhibitor (PARPi), approved for BRCA-mutated ovarian and breast cancers. We recently showed that olaparib at clinically achievable concentrations exerts anti-proliferative and pro-apoptotic effects *in vitro* as monotherapy against primary acute myeloid leukemia (AML) blasts, while sparing normal bone marrow (BM) hematopoietic cells. Since AML expresses low levels of death receptors that may contribute to apoptosis resistance, in this study we investigated whether the anti-leukemia activity of olaparib involves modulation of FAS and TRAIL receptors DR5 and DR4. Our data show that the primary AML samples tested express FAS and DR5 transcripts at levels lower than normal BM. In this context, apoptosis triggered by olaparib is associated with a dose-dependent up-regulation of death receptors expression and caspase 8 activation. Olaparib-mediated FAS up-regulation requires NF-κB activation, as indicated by the increase of p65 phosphorylation and decrease of IκBα. Moreover, FAS up-regulation is abrogated by pretreatment of AML cells with two different NF-κB inhibitors. These results indicate that NF-κB activation and consequent induction of death receptor expression contribute to the anti-leukemia effect of olaparib in AML.

Introduction

Acute myeloid leukemia (AML) is a genetically heterogeneous and rapidly progressing disease characterized by clonal expansion of abnormal myeloid progenitors arrested in their ability to differentiate into mature blood cells [1]. In physically fit patients, current treatment includes intensive induction chemotherapy followed by consolidation chemotherapy or allogeneic hematopoietic stem cell transplantation. However, the disease persists or recurs in most cases and the outcome of patients with relapsed or refractory AML is poor. In the last four decades, only few new drugs have been approved for this disease and, in the absence of effective conventional care regimens, patients with relapsed/refractory AML are often recruited in clinical trials aimed at testing the efficacy and safety of new investigational therapies [1]. In 2017, some agents have been approved in the front-line setting, such as midostaurin for *FLT3*-mutated AML (by FDA and EMA), the liposomal co-formulation of cytarabine and daunorubicin (CPX-351) for

Non standard abbreviations: BRCA1, DNA repair associated; BRCA2, DNA repair associated; DR4, TNF receptor superfamily member 10a (TNFRSF10A); DR5, TNF receptor superfamily member 10b (TNFRSF10B); DSBs, DNA double strand breaks; FAS, Fas cell surface death receptor; FASL, FAS Ligand; FLT3, fms-related tyrosine kinase; FLT3LG, FLT3 ligand; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HR, homologous recombination; IκBα, NFKB inhibitor alpha (NFKBIA); NF-κB, nuclear factor kappa B subunit 1; NPM1, nucleophosmin1, (nucleolar phosphoprotein B23, numatrin); PAR, poly(ADP-ribose); PARP1, poly(ADP-ribose) polymerase 1; PARP2, poly(ADP-ribose) polymerase 2; PARPi, PARP inhibitors; PARylation, poly(ADP-ribosyl)ation; SCF, stem cell factor, (KIT ligand); SSBs, single strand breaks; TRAIL, TNF-related apoptosis-inducing ligand.

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therapy-related AML or AML with myelodysplasia-related changes (by FDA), and gemtuzumab ozogamicin for CD33-positive AML (by FDA; also for subsequent lines of treatment). Furthermore, FDA has recently approved enasidenib for IDH-mutated relapsed/refractory AML. However, treatment of resistant or recurrent AML is still an unmet medical need [2].

We recently demonstrated that the poly(ADP-ribose) polymerase inhibitor (PARPi) olaparib (Lynparza, AstraZeneca) induces apoptosis *in vitro*, at clinically achievable concentrations, in primary AML blasts characterized by a *BRACness* phenotype due to low BRCA1/2 expression [3]. Interestingly, olaparib preferentially kills leukemic blasts and spares normal lymphocytes, and has little influence on the viability of bone marrow (BM) or $CD34^+$ enriched peripheral blood cells obtained from healthy donors [3]. Olaparib is an orally active agent, approved as monotherapy by FDA and EMA for the treatment of recurrent BRCA-mutated ovarian cancer, in 2014, and by FDA for BRCA-mutated/HER2-negative metastatic breast cancer, in 2018. As single agent, it has an acceptable safety profile, being fatigue, gastro-intestinal disorders and anemia the most frequent adverse events -mostly of grade 2 or less -reported in patients pretreated with at least 3 previous lines of chemotherapy [4]. In addition, olaparib is being actively investigated, either as monotherapy or in combination with other regimens (chemotherapy, radiotherapy or targeted agents), for the treatment of a variety of cancer types, mainly represented by solid tumors also lacking BRCA1/2 mutations [5–7]. Similar to other PARPi, olaparib interacts with the binding site of β-NAD^+ in the catalytic domain of PARP1 and PARP2, which are enzymes able to synthesize negatively charged, branched poly(ADP-ribose) (PAR) chains on target proteins (PARylation), such as histones and PARP1 itself, using β-NAD^+ as substrate. The negative charges added to PARP1 cause its later release from repaired DNA.

Of the 17 members of the PARP family, only PARP1, PARP2 and PARP3 are involved in the repair of damaged DNA, with PARP1 having a prevalent role as DNA damage sensor and signal transducer [reviewed in 8]. Although initially investigated as chemo- and radio-sensitizers, the main clinical interest in PARPi is related to their ability, as single agents, to induce synthetic lethality in tumors unable to repair DNA double strand breaks (DSBs) due to homologous recombination (HR) deficits (e.g., BRCA1/2-mutated ovarian or breast cancer) [9,10]. The mechanism underlying synthetic lethality induced by PARPi relies on accumulation of single strand breaks (SSBs) (generated by endogenous oxidant or replication stress), which are no longer repaired by base excision repair that requires intact PARP1 and PARP2. Unrepaired SSBs lead to stalling of replication forks and subsequent formation of highly cytotoxic DSBs, which cause cell death if not corrected by HR, as seen in tumors with mutated or epigenetically silenced BRCA or other HR-related genes [11]. An additional mechanism involved in the PARPi-induced lethality relates to the ability of certain PARPi, including olaparib, to trap PARP1 at the damaged DNA site due to inhibition of auto-PARylation [12]. Moreover, PARP1 and PARP2 have multiple important roles beyond DNA repair, including for instance transcription, inflammation, apoptosis; thus, the anti-tumor activity of PARPi may derive also from inhibition of additional PARP functions [13].

The cell surface death receptors FAS (CD95, APO1), TRAIL-R2/DR5 (TNFRSF10B) and TRAIL-R1/DR4 (TNFRSF10A) receptors of the tumor necrosis factor (TNF) receptor superfamily participate in the extrinsic apoptotic system. Their activation represents an attractive therapeutic strategy to induce cell death by apoptosis in cancer cells [14]. In the present study, we found that olaparib cytotoxicity in primary AML blasts, involves not only DNA damage but also modulation of death receptors expression, which in turn requires NF-κB activation.

Materials and methods

AML samples

Freshly isolated primary blasts were obtained from BM aspirates of 21 adult patients with newly diagnosed AML. Cases were numbered in order of consecutive arrival without any kind of selection. All patients provided written informed consent according to institutional guidelines and the study was approved by the institutional review board at “Policlinico Tor Vergata”. Routine morphological, immunophenotypic, and genetic analyses were carried out at presentation. Conventional karyotyping was performed on BM diagnostic aspirates after short-term culture and analyzed after G-banding. For molecular analysis, total RNA was extracted from Lympholyte-H (Cedarlane, Burlington, Canada) isolated BM mononuclear cells. *FLT3* and *NPM1* mutational status was investigated by a multiplex PCR strategy, as previously described [3]. Mononuclear cells were also collected from 9 normal BM samples.

Cell culture and drug treatment

Mononuclear cells from AML patients were cultured at 37 °C in a humidified atmosphere of 5% CO_2 for 1–2 days before starting chemosensitivity assays. Briefly, 2×10^6 primary AML cells/ml were seeded into culture flasks in RPMI medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2 mM L-glutamine (EuroClone, Pero, Milan, Italy), 1% penicillin/streptomycin (Euroclone), 20% fetal bovine serum (FBS) (Gibco, ThermoFisher Scientific, Waltham, MA, USA) and 10 ng/ml each of IL-3, SCF and FLT3LG (PeproTech, Rocky Hill, NJ, USA).

For survival assay, stimulated primary AML cells were collected and seeded in 48 or 24-well culture plates (10^6 cells/ml, in duplicate) and treated with olaparib. 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 maintained for 7–14 days only, depending on the AML sample, we performed the assays up to 7 days after drug exposure.

The stock solution of olaparib (kindly provided by AstraZeneca) was prepared by dissolving 10 mg of the drug in 200 µl of dimethylsulfoxide (DMSO) (Sigma-Aldrich) and then diluted with RPMI to the concentration of 2 mM. The aliquots were stored at −80 °C. For each experiment, a new drug aliquot was thawed and used. The olaparib inhibitory concentration 50 (IC_{50}), 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.

The NF-κB inhibitor dehydroxylmethyllepoxyquinomicin (DHMEQ, kindly provided by Dr. Kazuo Umezawa, Department of Molecular Target Medicine, Aichi Medical University School of Medicine, Nagakute, Japan) was dissolved in DMSO (10 mg/ml), stored at −20 °C and diluted in complete medium just before use. The NF-κB SN50 cell permeable inhibitory peptide and the SN50 M cell-permeable inactive control peptide (Enzo Life Sciences, Farmingdale, NY, USA) were diluted in PBS (10 mg/ml), stored in aliquots at −20 °C and diluted at 50 µg/ml for the experiments.

mRNA expression

Total RNA was isolated by Trizol reagent (Invitrogen, Carlsbad, CA, USA) and checked for quality by gel electrophoresis. One µg of total RNA was reverse-transcribed using random hexamer primers and reagents of Life Technologies (Carlsbad, CA, USA). Quantitative real-time PCR (qRT-PCR) was carried out using the Applied

Biosystems® TaqMan® Universal PCR Master Mix (Life Technologies). TaqMan primers/probes for PARP1, FAS, DR5, DR4 and GAPDH (for internal normalization) were purchased from Integrated DNA Technologies (Coralville, IA, USA). Analysis was performed in triplicate on an ABI-7900HT instrument (Applied Biosystems, Foster City, CA, USA). The $2^{-\Delta\Delta Ct}$ relative quantification method was used to calculate relative mRNA expression. Normal BM RNA was used as a calibrator. To analyze the expression of target genes following olaparib treatment, RNA was extracted from cultured cells at the times indicated in the text. In this case, RNA from untreated cells was used as calibrator.

Quantification of FASL and TRAIL in AML by ELISA

FAS ligand (FASL) and TNF-related apoptosis-inducing ligand (TRAIL) production was analyzed in supernatants from primary AML cells or in cell lysates. Supernatants were concentrated at least 10-fold in Centriplus concentrators (Amicon, Beverly, MA, USA). Quantification of the amount of FASL and TRAIL was performed using R&D Systems (Minneapolis, MN, USA) Elisa kits. Absorbance was read at 450 nm in a 3550-UV Microplate reader (Bio-Rad, Hercules, CA, USA) with a reference wavelength of 570 nm, according to the manufacturer's instructions. Death receptor ligand values were expressed as pg/ml and normalized by the total number of cells or μg/protein in the case of supernatants or cell lysates, respectively.

Immunoblotting

Total proteins were extracted from AML cells using a lysis 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 a protease inhibitor cocktail (Sigma-Aldrich). Protein aliquots were loaded onto polyacrylamide gels, transferred to nitrocellulose membrane, and incubated with the following antibodies: anti-PARP1 (M01-3G4, Abnova, Taipei, Taiwan), anti-PARP2 (ab77270, Abcam, Cambridge, UK), anti-NF-κB p65 (6956, Cell Signaling Technology, Danvers, MA, USA), anti-phospho-NF-κB, Ser536 (pNF-κB) (3033, Cell Signaling Technology), anti-IκBα (sc-371, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-caspase 8 (ALX-804-242, Enzo Life Sciences) and anti-β-actin (4967, Cell Signaling Technology). Horseradish peroxidase-conjugated IgGs were used as secondary antibodies. The autoradiograms were analyzed by densitometric analysis by ImageJ 1.45s software. The results were normalized against β-actin and used for statistical analysis.

Flow cytometry and apoptosis 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 analyzed by flow cytometry.

For analysis of cell surface FAS and DR5 expression in AML cells by flow cytometry, 2×10^5 cells were incubated with anti-human CD95 APC-conjugated (BD Biosciences, Buccinasco, Milan, Italy) and TRAIL/R2 PE-conjugated (R&D Systems) antibodies for 20 min.

Samples were acquired on a BD FACSCanto II flow cytometer and evaluated using DIVA and FlowJo softwares for apoptosis and cell surface receptor expression analyses, respectively (BD Biosciences).

Immunofluorescence of γH2AX foci

AML cells were cytocentrifuged (10×10^4 cells), fixed with 4% paraformaldehyde, permeabilized in 0.3% triton and blocked in 2% bovine serum albumin (Sigma-Aldrich). Slides were incubated with mouse anti-γH2AX (JBW301, Millipore, Burlington, MA, USA) and, after washing, 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, Wetzlar, Germany). Images were taken at 40 × magnification.

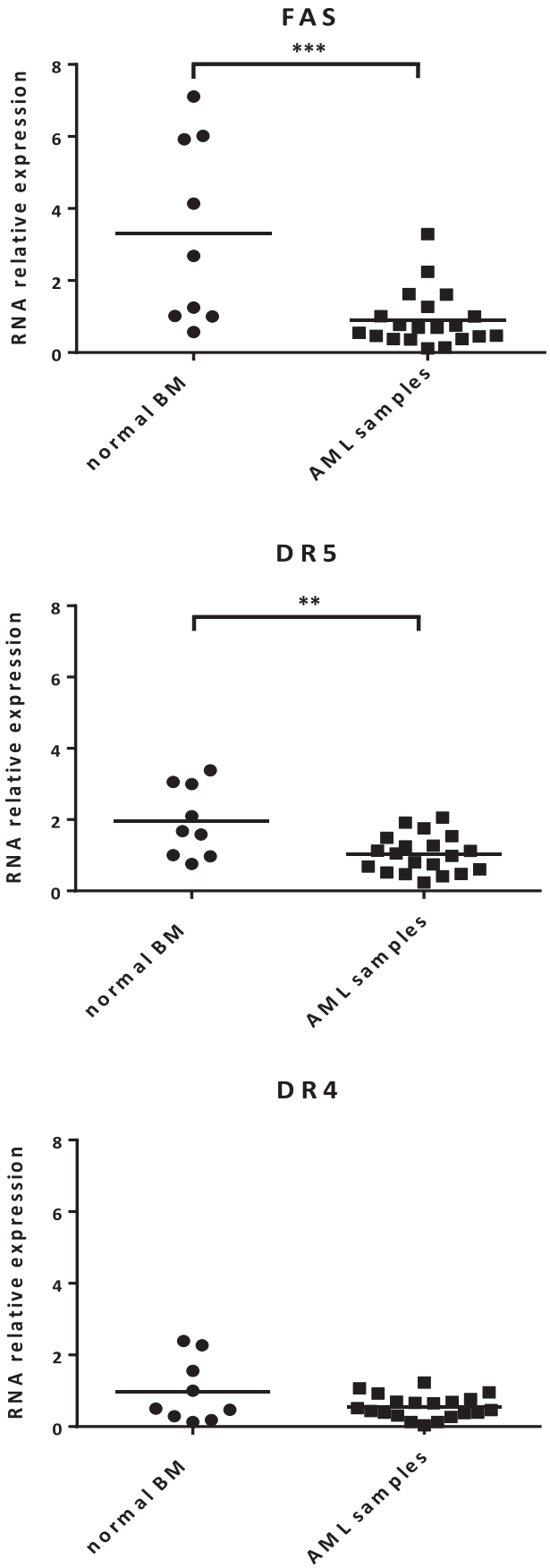
Statistical analyses

Relationship between protein expression levels and olaparib IC_{50}s was performed by the non-parametric two-tailed Spearman's

Table 1
Clinical and biological characteristics of AML patients.

IC_{50} olaparib (μM)	Patient number	Age/ Sex	% Blasts (BM)	WBC (x10^6/ ml)	Karyotype	*NPM1* mutA*	*FLT3*-ITD**	Prognostic Risk
20.3	upn-12	21/F	48	4	46,XX [1]/46,XX,t(8;21) [8]/46,XX,t(8;21),add(7)(q32) [18]	neg	neg	low
7.1	upn-11	53/M	78	22	46,XY	pos	neg	low
7.0	upn-15	59/M	68	13	46,XY	pos	pos	high
5.9	upn-9	53/M	30	3	45X,-Y	neg	pos	high
5.9	upn-13	38/M	80	167	46,XY	neg	pos	high
5.7	upn-21	66/M	30	10	NA [PCR neg for inv(16) and t(8:21)]	neg	neg	intermediate
5.5	upn-14	43/M	80	40	46,XY,add(7q)	neg	neg	high
5.5	upn-7	49/F	53	28	49,XX,+8,+mar1,+mar2 [7]/46,XX [13]	neg	neg	high
5.2	upn-17	46/F	NA	8	46,XX	pos	neg	low
5.0	upn-2	51/F	98	87	NA [PCR neg for inv(16) and t(8:21)]	pos	pos	high
4.7	upn-16	21/M	92	14	46,XY	neg	neg	intermediate
4.3	upn-1	51/F	100	121	46,XX	pos	neg	low
4.1	upn-8	37/F	25	22	46,XX	NA	NA	NA
4.0	upn-4	60/F	65	18	NA [PCR neg for inv(16) and t(8:21)]	pos	neg	low
3.5	upn-19	34/M	70	20	46,XY,t(8;21)	neg	neg	low
3.5	upn-5	53/M	46	3	46,XY	pos	neg	low
3.3	upn-6	48/M	NA	4	46,XY	neg	neg	intermediate
2.6	upn-10	49/F	98	185	46,XX	pos	pos	high
2.6	upn-18	56/M	80	12	46,XY,t(1,11),del(7q),t(8,21) [15]/46 XY, t(1,11), del(7q) [5]	neg	neg	high
2.5	upn-3	60/M	90	16	46,XY	neg	pos	high
1.7	upn-20	51/M	84	45	46,XY,+11	neg	pos	high

IC_{50}, inhibitory concentration 50; BM, bone marrow; WBC, white blood cell count; NA, not available; **NPM1*mutA, *NPM1* mutation type A; ***FLT3-ITD*, fms-related tyrosine kinase internal tandem duplications.



correlation coefficients. Statistical analysis of the differences between two groups was performed by two-tailed Student's *t*-test. For multiple comparisons ANOVA analysis, followed by LSD test was used. Differences were considered statistically significant when $p < 0.05$.

Results

Treatment with the PARPi olaparib increases death receptor expression in AML blasts

We initially analyzed the sensitivity to olaparib of 21 AML primary blast cultures by trypan blue exclusion cell count, 7 days after a single exposure to graded concentrations of the PARPi. BM blast infiltration at disease presentation was ≥50% in about two third of AML samples tested (14/21) and most of patients (13/21) had an intermediate or high prognostic risk (Table 1). All samples, except one (UPN-12), were highly responsive to olaparib. In fact, olaparib IC_{50} values were largely below the plasma peak concentrations (i.e., 16–25 μM) detected in cancer patients after treatment with the PARPi, administered twice daily [15–17]. There was no correlation between the olaparib IC_{50} values calculated on day 7 after treatment and some of the most important clinical prognostic indexes indicated in Table 1 (i.e., age, white blood cell count, karyotype, NPM1mutA and FLT3-ITD).

It has been previously reported that low expression of FAS and DR5 death receptors in AML correlates with poor prognosis and resistance to apoptosis [18–22]. Thus, we tested the expression of FAS, DR5 and DR4 by qRT-PCR in BM samples obtained from nine healthy donors and twenty AML patients. FAS and DR5 mRNA expression was significantly lower in AML cells than in normal BM, whereas no statistically significant differences were observed in DR4 transcript levels (Fig. 1). To investigate whether the anti-leukemic activity of olaparib involves up-regulation of death receptors, blasts from five AML patients were exposed to increasing concentrations of olaparib (1.25–10 μM), and analyzed for FAS, DR5 and DR4 expression by qRT-PCR after 7 days of treatment. The PARPi induced a statistically significant up-regulation of FAS transcript at concentrations from 2.5 to 10 μM (Fig. 2A). Interestingly, the increase of FAS transcript was detected in all AML tested and a dose-dependent up-regulation of DR5 and DR4 transcripts was observed in 3 out of 5 samples (Supplementary Fig. S1). The increase in DR5 and DR4 expression was statistically significant only for blasts treated with 10 μM olaparib (Fig. 2A). The olaparib-induced increase of FAS and DR5 transcripts was associated by a dose-dependent increase of the corresponding protein, evaluated by FACS analysis. This effect was detected at 72 h and after one week of culture (Fig. 2B). Since death receptor-induced apoptosis is mediated by the interaction with their ligands, AML cultures were analyzed for the production of FAS and of DR-5/DR-4 ligands (i.e., FASL and TRAIL, respectively). The results indicated that AML culture supernatants as well as AML cell extracts contained detectable levels of death receptor ligands (Fig. 2C).

Since we previously reported that the antitumor activity of olaparib in AML does not depend on the *in vitro* proliferative rate of leukemic blasts [3], we investigated whether the up-regulation of death receptors in AML cells could be influenced by the presence of growth factors (i.e., IL-3, SCF and FLT3LG). Our results indicated that

Fig. 1. RNA expression of FAS, DR5 and DR4 in AML blasts. Analysis of FAS, DR4 and DR5 expression was performed by qRT-PCR using total RNA extracted from mononuclear cells isolated from BM of nine healthy donors and twenty AML patients. Relative RNA expression was obtained following normalization with GAPDH expression, and calibration with a normal BM value. Data are the mean of triplicate analysis. Statistical analysis by unpaired Student's *t*-test: $^{**}P < 0.01$, $^{***}P < 0.001$.

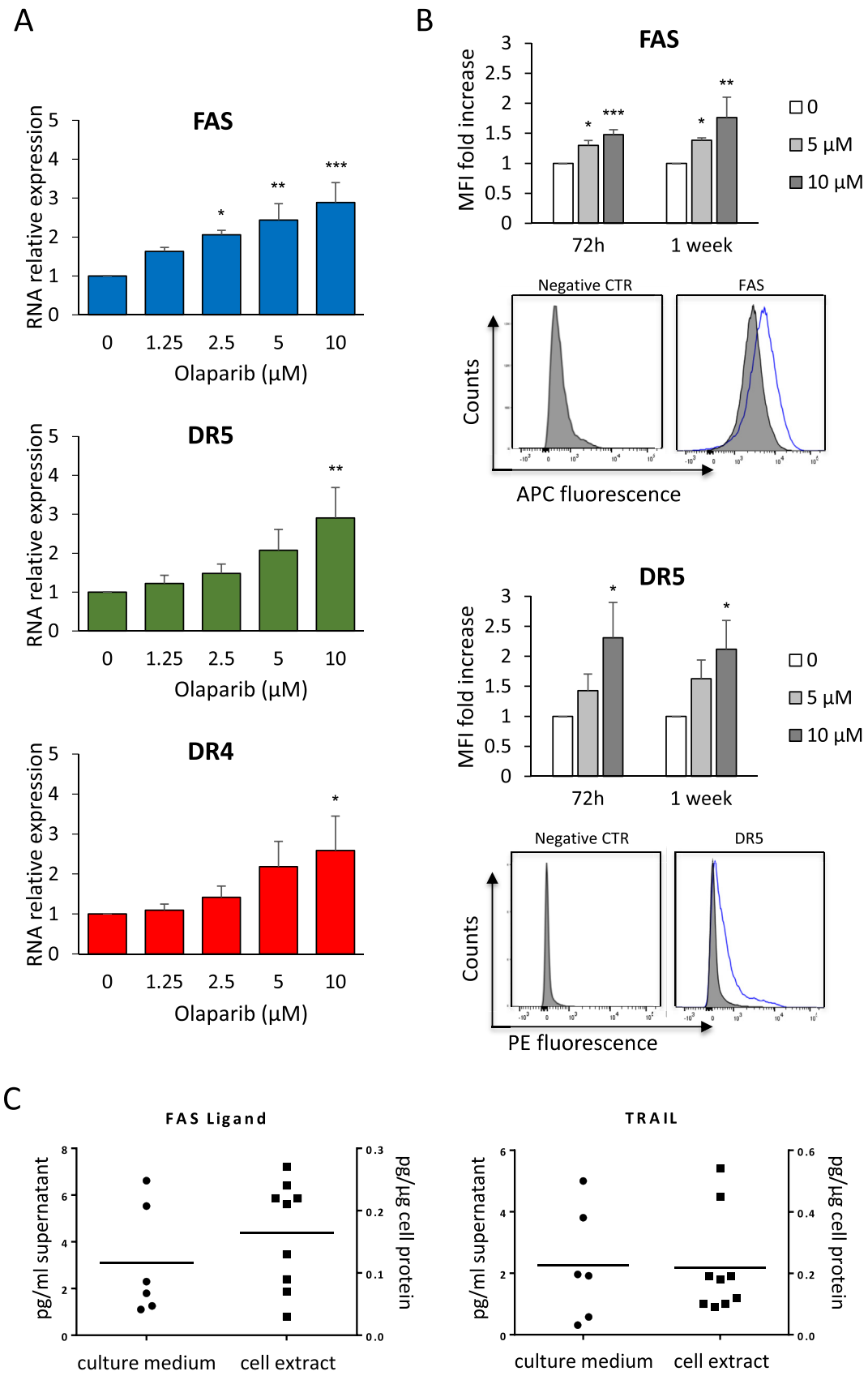


Fig. 2. Modulation of FAS, DR5 and DR4 RNA and protein expression by olaparib in AML blasts. A) FAS, DR5 and DR4 expression was evaluated by qRT-PCR after treatment of AML samples with the indicated concentrations of olaparib for 7 days. Mean gene expression levels (±SE) of five AML samples are shown (UPN-1, UPN-10, UPN-13, UPN-18 and UPN-20). **B)** FAS and DR5 protein expression was evaluated by flow cytometry in four AML samples (UPN-10, UPN-13, UPN-18 and UPN-21) treated with 5 and 10 μM olaparib at the indicated times. Representative plots are shown. The Mean fluorescence intensity (MFI) was calculated by subtracting the MFI values of unstained cells. Histograms represent the ratio of MFI values of treated to control cells. Data are the mean (±SE) of four independent determinations. **C)** FASL and TRAIL were quantified by ELISA in AML culture supernatants ($n = 6$) or in cell extracts ($n = 9$) and normalized by the total number of cells or μg of protein, respectively. Statistical analysis by one-way ANOVA, followed by LDS test: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

olaparib induced a dose-dependent decrease in the number of viable cells also in an AML sample incubated in the absence of growth factors (Fig. 3A). Although non-proliferating cells were less sensitive to olaparib than their proliferating counterparts (IC_{50} 14.9 *vs* 2.6 μM), both cell types underwent similar up-regulation of death receptor expression when exposed to the PARPi (Fig. 3B).

Olaparib triggers NF-κB phosphorylation and decreases IκBα, PARP1 and PARP2 protein expression in AML blasts

NF-κB is known to be constitutively activated in the majority of AML cells, contributing to apoptosis resistance to anticancer agents [24]. Beside its demonstrated role in cell survival and protection from apoptosis, NF-κB, and in particular the RelA (p65) subunit, has been shown to adopt a pro-apoptotic function stimulating the expression of death receptors [25]. On this basis, we analyzed in AML blasts the basal levels of total p65 protein and its phosphorylated form. In fact, degradation of IκBα and nuclear NF-κB translocation are not sufficient to promote a maximal NF-κB transcriptional response, which requires NF-κB phosphorylation [26]. The results of Western blot analysis in freshly isolated AML blasts indicated that ~50% of samples (7/15) constitutively expressed high p65 levels, which correlated with protein phosphorylation at serine 536 (Fig. 4A and B). Conversely, sensitivity of AML blasts to olaparib did not correlate with the phosphorylated NF-κB to total NF-κB ratio, since no significant differences were observed in olaparib IC_{50}s of AML blasts with high or low/undetectable total or phosphorylated NF-κB levels (4.3 ±1.1 *vs* 5.7±1.5, p = 0.19, Fig. 4C).

We then investigated the influence of olaparib on p65

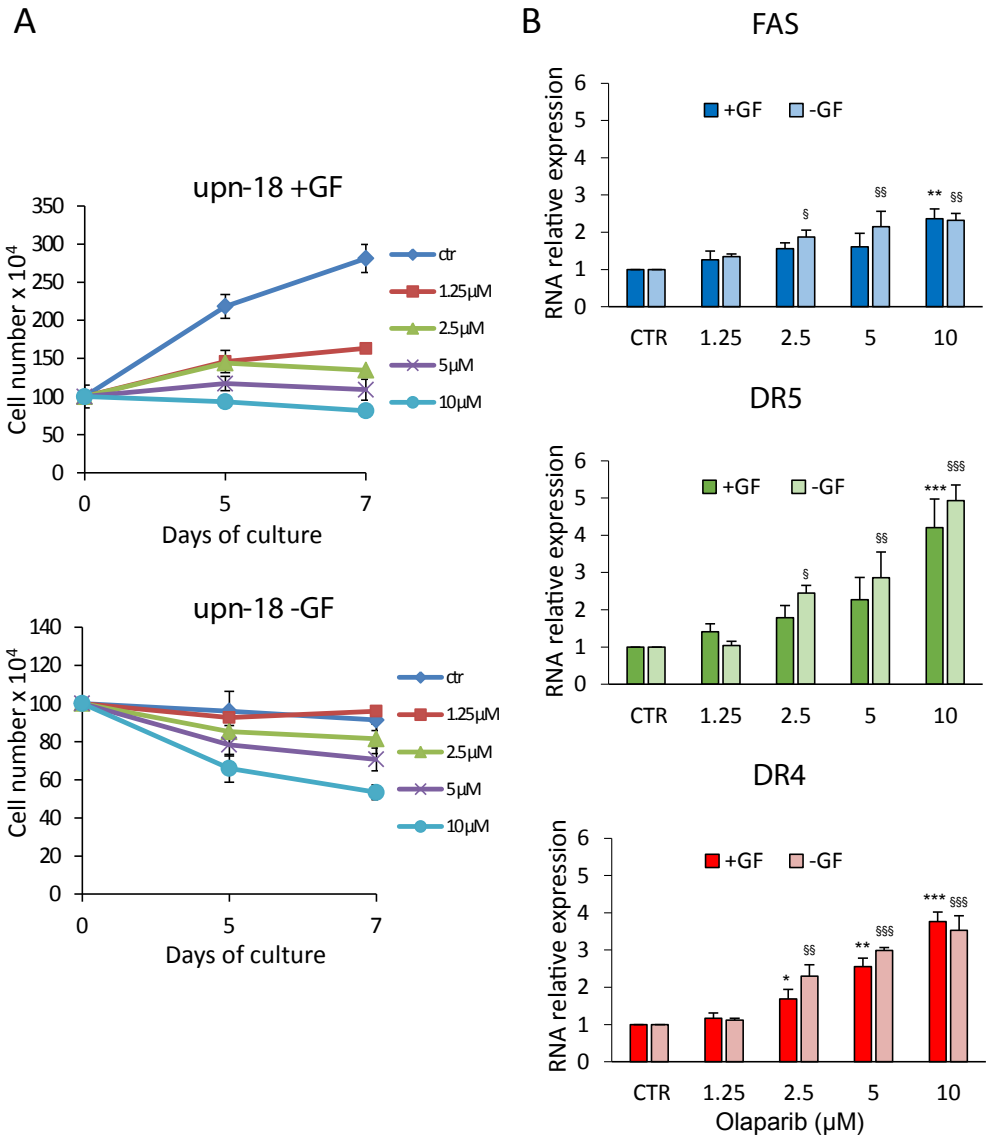


Fig. 3. Olaparib-induced modulation of FAS, DR5 and DR4 RNA expression in proliferating and non-proliferating AML blasts. AML cells from UPN-18 were grown in complete medium supplemented with 20% FBS, IL-3, SCF and FLT3LG at 10 ng/ml each, or with 20% FBS only, and exposed to the indicated concentrations of olaparib. **A)** Cell proliferation was evaluated by counting viable cells using trypan blue dye exclusion, at 5 and 7 days. Data are the mean (±SD) of triplicate counts. **B)** The expression level of FAS, DR5 and DR4 transcripts in proliferating and non-proliferating cells form UPN-18 was evaluated by qRT-PCR. Results are the mean (±SE) of three qRT-PCR analyses, each performed in triplicate. Symbols indicate statistically significance difference, evaluated by one-way ANOVA, between drug treated proliferating (*) or non-proliferating cells (§) in comparison with the corresponding untreated control group. No statistically significant differences were observed between proliferating (cultured with GF) and non-proliferating (cultured without GF) cells at all of the concentrations, tested by unpaired Student's *t*-test. (For interpretation of the references to colour in this and other figure legends, the reader is referred to the Web version of this article.)

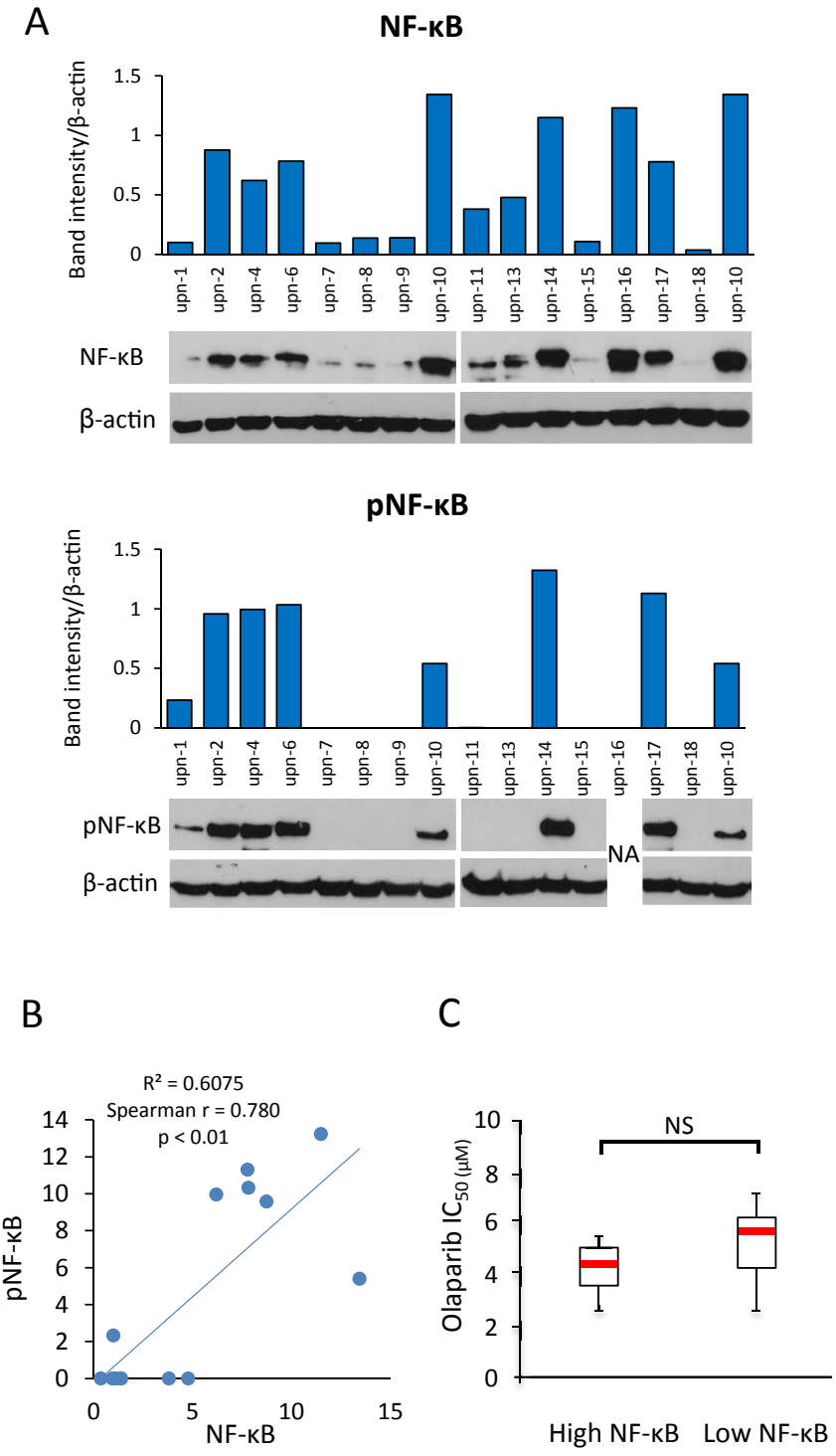


Fig. 4. Basal and phosphorylated NF-κB protein levels in AML blasts and their correlation with olaparib IC_{50}s. A) Western blot analysis of p65 (NF-κB) and phosphorylated p65 (pNF-κB). Bar graphs represent the densitometric analysis of p65 or pNF-κB proteins (normalized to β-actin). NA, protein sample not available. **B)** Relationship between total NF-κB and pNF-κB in AML blasts. The non-parametric Spearman's correlation coefficients and their significance levels are indicated. **C)** Olaparib IC_{50} values in AML cells expressing high and low/undetectable NF-κB or pNF-κB levels are presented as box and whiskers plots. Unpaired Student's *t*-test: p = 0.19.

expression in four AML samples exposed to graded concentrations of the PARPi for 7 days. Olaparib increased NF-κB phosphorylation in a dose-dependent manner, without significant effects on p65 expression levels (Fig. 5A). Conversely, AML exposure to the PARPi resulted in a dose-dependent decrease of PARP1 and PARP2 proteins (Fig. 5A). Immunoblots showed the up-regulation of phosphorylated p65 and the down-regulation of PARP proteins induced by olaparib in UPN-6 (Fig. 5B). After 7 days of culture, phosphorylation of p65 was not detectable; however, exposure to as low as 1.25 μM olaparib restored NF-κB activation, that reached the maximum level with 10 μM PARPi (Fig. 5B).

We then investigated whether reduced expression of IκBα might

also contribute to olaparib-induced increase of NF-κB. In fact, IκBα keeps NF-κB proteins sequestered in an inactive state in the cytoplasm, by masking their nuclear localization signals. Results of Western blot analysis in four AML samples revealed that exposure to 5 and 10 μM olaparib induced a marked and statistically significant decrease of IκBα (Fig. 6A). The immunoblot shows the dose-dependent decrease of IκBα expression in blasts from UPN-8 treated with the PARPi (Fig. 6B).

Olaparib induces early increase of death receptor expression that requires NF-κB activation

In order to investigate whether the olaparib-induced up-regulation of NF-κB phosphorylation and death receptor expression were correlated events, we initially analyzed their induction at earlier time points. Time-course qRT-PCR analysis of FAS and DR5 RNA expression using five different AML samples indicated that the increase FAS and DR5 expression started to appear at 24 h and reached statistical significance at 48 h and 72 h after treatments

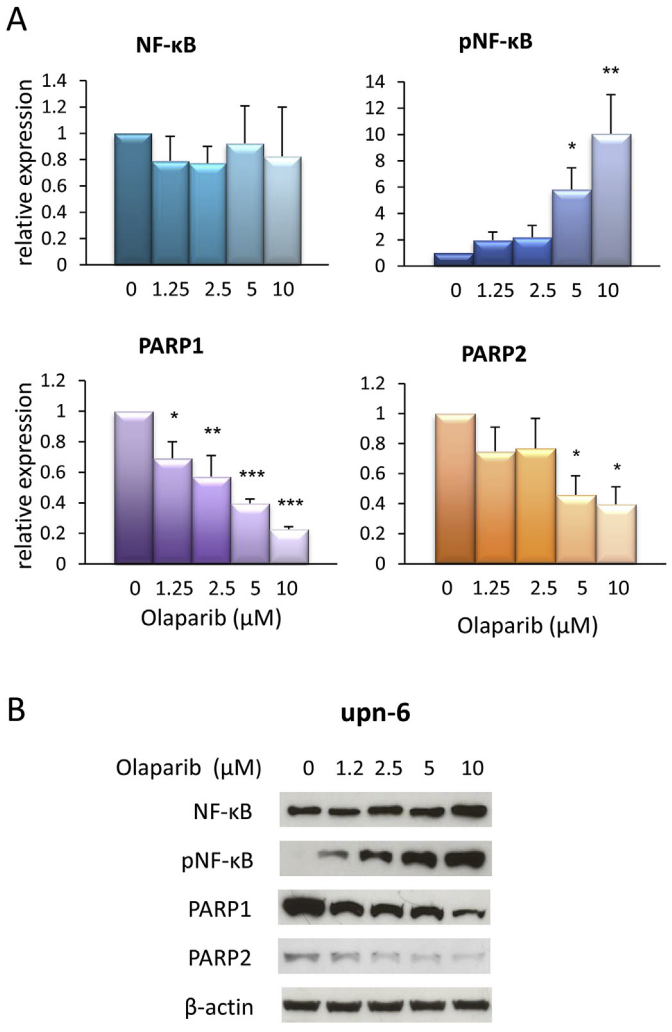


Fig. 5. Modulation of phosphorylated NF-κB, PARP1 and PARP2 protein expression levels induced by olaparib in AML blasts. A) AML samples (UPN-1, UPN-6, UPN-8 and UPN-10) were cultured in the presence of the indicated concentrations of olaparib for 7 days and then analyzed for p65 (NF-κB), phosphorylated p65 (pNF-κB), PARP1 and PARP2 expression by Western blot. Bar graphs represent the mean (±SE) expression of each protein(normalized to β-actin) relative to the untreated control in four AML samples. One-way ANOVA, followed by LDS test: *P < 0.05, **P < 0.01, ***P < 0.001. **B)** Representative immunoblot showing data from one AML sample (i.e., UPN-6).

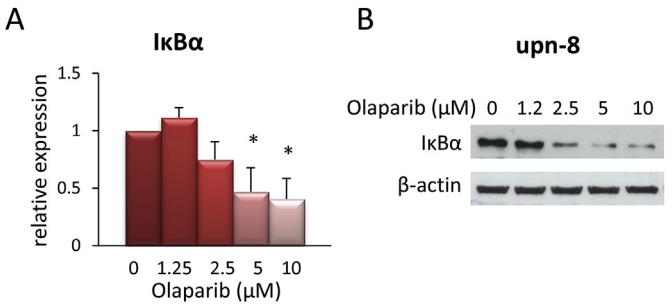


Fig. 6. Inhibition of IκBα expression in AML blasts treated with olaparib. A) AML samples (UPN-1, UPN-6, and UPN-8) were cultured in the presence of the indicated concentrations of olaparib for 7 days and then analyzed for IκBα expression by Western blot. Bar graphs represent the mean (±SE) expression of IκBα (normalized to β-actin) relative to the untreated control in three AML samples. One-way ANOVA, followed by LDS test: *P < 0.05. **B)** Representative immunoblot showing data from one AML sample (i.e., UPN-8).

(Fig. 7A). Modulation of DR4 expression was less pronounced and statistically significant only at 72 h (Fig. 7A). In UPN-10 sample, where olaparib induced a marked increase of FAS transcript as early as 24 h after treatment (Fig. 7B), we also analyzed NF-κB phosphorylation that resulted to be up-regulated at the same time point (Fig. 7C).

We then investigated whether AML exposure to NF-κB inhibitors prevented olaparib-induced up-regulation of FAS expression. To this purpose, UPN-10 cells were treated with olaparib in the presence of DHMEQ, a specific inhibitor of NF-κB nuclear translocation and DNA binding [27,28], and FAS expression was analyzed using qRT-PCR. Olaparib induced a marked increase of FAS transcript and this effect was abrogated by pre-treatment of the cells with DHMEQ, which also decreased constitutive FAS mRNA levels in AML cells (Fig. 8A). Treatment with DHMEQ also counteracted olaparib-induced up-regulation of FAS protein at the cell membrane level as indicated by the results of flow cytometry (Fig. 8A).

Due to the ability of DHMEQ itself to decrease basal FAS mRNA levels, it could be hypothesized that DHMEQ and olaparib might exert opposite and independent effects on FAS expression. To exclude this possibility, the NF-κB involvement in FAS up-regulation induced by olaparib was tested using the NF-κB inhibitor SN-50 (50 μg/ml), a cell membrane permeable peptide that inhibits nuclear translocation [29]. Indeed, leukemia cell pretreatment with SN50 did not affect basal FAS expression levels in another AML sample (UPN-21), but it prevented the increase of FAS mRNA and protein (Fig. 8B) triggered by olaparib. This effect was not observed in cells treated with the inactive control SN50 M before exposure to olaparib.

Overall, these findings support a temporal and functional correlation between the transcription factor and death receptor expression.

Olaparib induced cell death is associated with activation of the extrinsic apoptotic pathway

We previously demonstrated that olaparib, used as single agent, is able to cause DNA damage in primary cultures of AML blasts (3). In order to investigate whether also activation of death receptors might contribute to the cytotoxic activity of the PARPi in AML cells, we evaluated the induction of apoptosis by olaparib and expression of the cleaved active form of caspase 8, which plays a pivotal role in the extrinsic apoptotic signaling [23]. Analysis of apoptosis by flow cytometry in five AML samples indicated that olaparib induced a statistically significant increase in the percentage of dead cells at

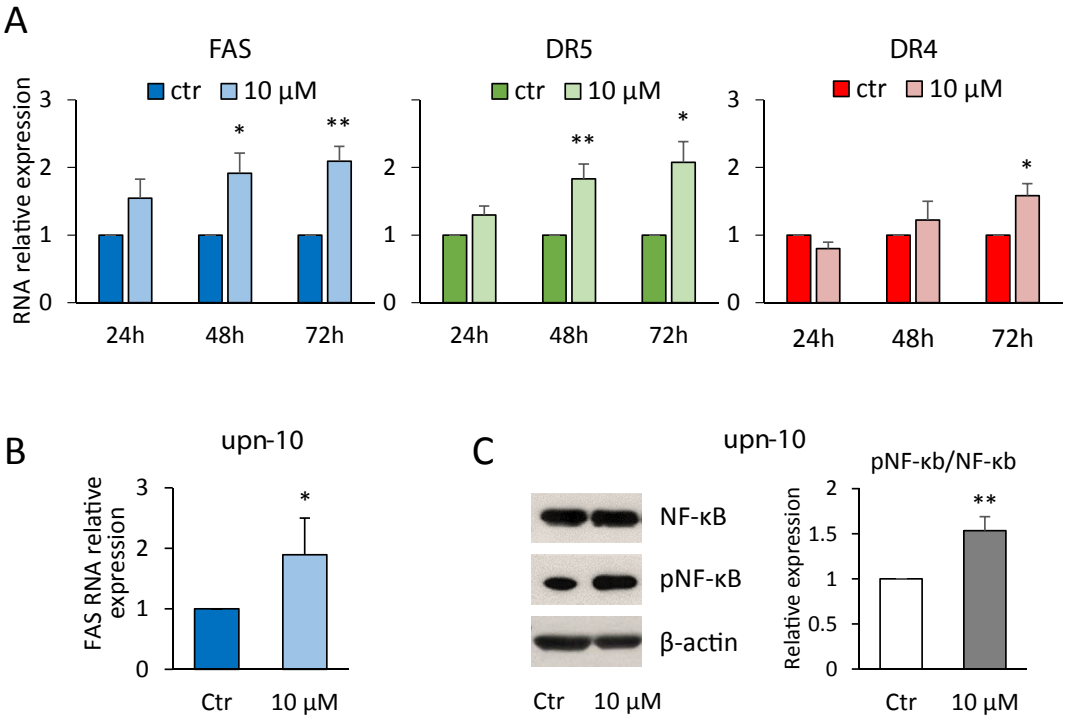


Fig. 7. Olaparib-induced early up-regulation of death receptor expression is associated with NF-κB phosphorylation. A) FAS, DR5 and DR4 expression was evaluated by qRT-PCR at 24, 48 and 72 h after treatment with 10 μM olaparib. Mean gene expression levels (±SE) of five AML samples are shown (UPN-1, UPN-6, UPN-10, UPN-20 and UPN-21). **B)** FAS expression was evaluated in UPN-10 blasts by qRT-PCR after 24 h treatment with the indicated concentration of olaparib. Means of triplicate experiments ±SD are shown. **C)** Western blot analysis of total p65 NF-κB and pNF-κB in untreated or olaparib treated UPN-10 blasts at 24 h. Bar graphs represent the mean (±SD) expression of pNF-κB/NF-κB (normalized to β-actin) relative to the untreated control in three independent determinations. Statistical analysis by unpaired Student's *t*-test: *P < 0.05, **P < 0.01.

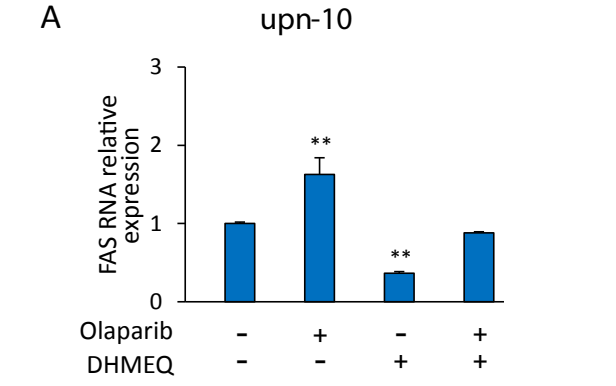
48 h (Fig. 9A). Western blot analysis in three AML blast samples demonstrated that treatment with olaparib resulted in a significant caspase 8 activation at 48 h after treatment (Fig. 9B). Nevertheless, in UPN-1 sample, which presented a significant increase of FAS expression and apoptosis induction at 24 h after drug exposure, caspase 8 activation was also observed at the same time point (Supplementary Fig. S2, panels A–C). Analysis of histone H2AX phosphorylated on serine 139 (γH2AX), as a hallmark of DNA damage response, revealed that olaparib caused a parallel increase in the percentage of γH2AX positive cells in this sample (Fig. S2, panel D). Overall, these data suggested that both death receptor activation and DNA damage might contribute to the anti-leukemia activity of the PARPi.

Discussion

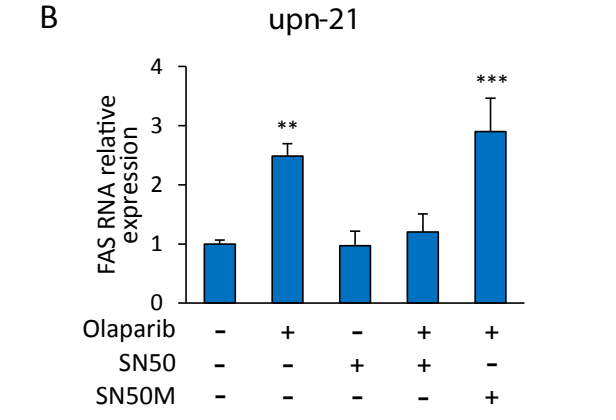
Olaparib is the first-in-class PARPi approved as monotherapy for cancer treatment. In the present study, we demonstrate that olaparib as single agent, at clinically relevant concentrations, induces anti-proliferative and cytotoxic effects in primary cultures of human AML blasts through a mechanism involving up-regulation of death receptors, which requires activation of the transcription factor NF-κB.

The therapeutic potential of PARPi in AML has been recently suggested by our and other groups [3,30–32], indicating that different PARP molecular functions may be critical for AML cell survival [33]. Indeed, we previously demonstrated that exposure of primary AML blasts to a single clinically achievable concentration of the PARPi preferentially kills leukemic blasts, compared to normal BM and CD34-enriched peripheral blood cells [3]. While low expression of BRCA1/2 in AML blasts indicates synthetic lethality involvement in the anti-leukemic activity of olaparib against proliferating cells, additional mechanisms may be involved in the cytotoxicity of the PARPi on quiescent leukemia cells [3,34]. In fact, according to the synthetic lethality model, proliferation is needed to convert PARPi induced-SSBs in DSBs upon stalling and collapse of replication forks. Indeed, our data in primary AML blasts, obtained at disease presentation from patients with an intermediate or high prognostic risk, demonstrate that apoptosis induced by olaparib is associated with up-regulation of FAS, DR5 and DR4 expression and caspase 8 activation. The increase of death receptor transcripts was detected not only in actively proliferating cells, but also in resting leukemia cells, cultured in the absence of growth factors, suggesting that cytotoxicity of olaparib is not strictly dependent on cell growth rate. Actually, up-regulation of receptors that promote extrinsic apoptosis with caspase 8 activation becomes particularly relevant when referred to quiescent leukemia stem cells, which are generally resistant to chemotherapy. Nevertheless, this mechanism is alternative but not mutually exclusive with DNA damage and synthetic lethality induced by olaparib in AML blasts, which is accompanied by a dose-dependent increase of γH2AX foci in treated cells. Synthetic lethality likely prevails in proliferating AML cells since leukemia blasts cultured in presence of grow factors are more sensitive to olaparib than quiescent cells (Fig. 3 and references 3, 30–32). Moreover, the AML samples tested in the present study were characterized by lower basal levels of FAS and DR5 transcripts, as compared to normal BM stem cells, and this property has been commonly associated with resistance to apoptosis [18–22].

Our data carried out on primary AML blasts are in agreement with recent findings in immortalized solid tumor and leukemia cell lines, showing that PARP inhibition may increase FAS and DR5 transcript and protein levels [35]. In addition, Meng et al. reported that exposure of AML blasts to olaparib at concentrations likely



FAS cell surface expression				
Corrected MFI	1812	2129	1374	1608
Fold increase	1	1.2	0.8	0.9
Olaparib	-	+	-	+
DHMEQ	-	-	+	+



FAS cell surface expression					
Corrected MFI	1790	2591	2013	2205	2772
Fold increase	1	1.4	1.1	1.2	1.5
Olaparib	-	+	-	+	+
SN50	-	-	+	+	-
SN50M	-	-	-	-	+

Fig. 8. NF-κB inhibitors counteract FAS up-regulation induced by olaparib. A) AML cells (UPN-10) treated or not with 10 μM olaparib were cultured for 3 days, and exposed to 5 μg/ml DHMEQ or to an equivalent volume of DMSO during the last 18 h before cell harvesting. FAS RNA expression was evaluated by qRT-PCR and data are the mean of triplicate real time analyses ±SD. In one experiment cells were also analyzed for the expression of FAS protein by flow cytometry. **B)** AML cells (UPN-21) treated or not with 10 μM olaparib were cultured for 2 days and exposed to 50 μg/ml SN-50 or control SN-50 M peptides during the last 18 h before cell harvesting, and then analyzed for FAS transcript by qRT-PCR and. Data referring to qRT-PCR are the mean of triplicate real time analyses ±SD. In one experiment cells were also analyzed for FAS protein expression by flow cytometry.
Statistical analysis by one-way ANOVA, followed by LDS test: in UPN-10, olaparib *vs* all the other groups and DHMEQ *vs* all the other groups, **P < 0.01; in UPN-21, olaparib *vs* all the other groups except SN50 M, **P < 0.01; SN50 M *vs* all the other groups except olaparib, ***P < 0.001.
Numbers in the tables indicate the corrected MFI values, calculated by subtracting the MFI values of unstained cells, and the fold increase of fluorescence intensity, evaluated as ratio between drug treated samples and vehicle treated control.

devoid of cytotoxic effects results in sensitization to the anti-proliferative effects of recombinant TRAIL [35]. Herein, we show that olaparib as single agent directly inhibits AML cell proliferation and induces cell death. Interestingly, we found that the primary cultures of leukemia BM samples produce FASL and TRAIL that may directly interact with their respective receptors. In our model, represented by a mixed cell population obtained from the processing of patient BM specimens, FASL and TRAIL may derive either from leukemia blasts or from normal cells present in the cultures, thus hampering AML blasts viability through autocrine or paracrine mechanisms, respectively. These findings are in line with previous reports showing that both leukemia and normal hematopoietic cells can produce death receptor ligands [36–39]. Interestingly, up-regulation of FAS induced by olaparib is associated with an increase of NF-κB activation at early time points after treatment and this effect is abrogated by pre-treatment with two different NF-κB inhibitors.

The transcription factor NF-κB in the form of homo or hetero-dimeric complex, most commonly formed by p65-p50 proteins, regulates a number of cellular pathways. Activation of NF-κB results in up-regulation of both anti-apoptotic and pro-apoptotic genes. Indeed, in various cellular models, NF-κB promotes apoptosis in a cell type- and stimulus-dependent manner, through direct induction of FAS, DR5 and DR4 death receptors [25,40–44]. In AML blasts, olaparib induced a dose-dependent decrease of IκBα and an increase of NF-κB p65 phosphorylation at serine 536. In fact, phosphorylation at this site is critical for the expression of NF-κB-dependent genes [45,46]. It is known that NF-κB is frequently constitutively activated in AML, likely as a result of autocrine/paracrine cytokine signaling and this feature seems to be required

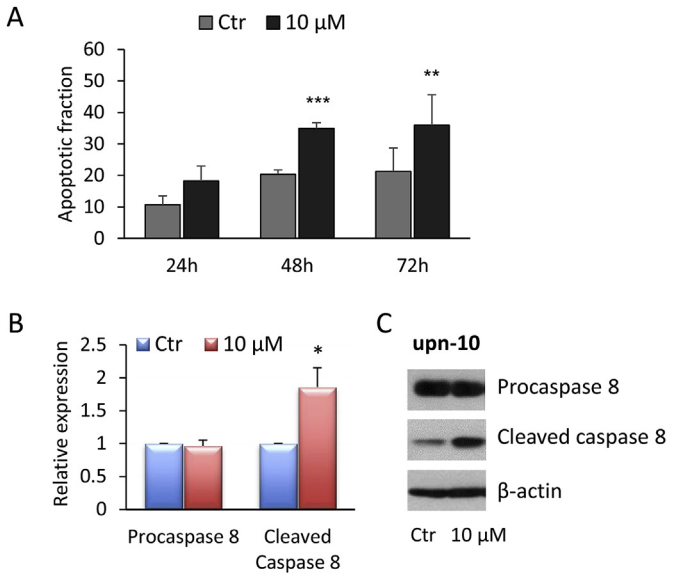


Fig. 9. Olaparib induces apoptosis and activation of caspase-8 in AML blasts. A) Induction of apoptosis was studied by flow cytometry analysis of Annexin-V/propidium iodide (PI) stained blasts at the indicated time points after exposure to 10 μM olaparib. Data are the mean percentages ±SE of apoptotic cells from five AML blast samples. **B)** Western blot analysis of caspase 8 at 48 h after treatment. Bar graphs represent the mean (±SE) expression of procaspase 8 and active caspase 8 (normalized to β-actin) relative to the untreated control in three AML samples (UPN-1, UPN-10 and UPN-6). **C)** Representative immunoblot showing procaspase 8 and active caspase 8 in one AML sample (i.e., UPN-10). Statistical analysis by unpaired Student's *t*-test: *P < 0.05, **P < 0.01, ***P < 0.001.

for the maintenance of AML cells [24,47,48]. Indeed, we found that ~50% of primary AML samples expressed high basal levels of p65, which is also constitutively phosphorylated at tyrosine 536.

In the present study, we found no correlations between basal NF-κB expression/phosphorylation levels in AML blasts and sensitivity to olaparib, being the mean IC_{50} values very similar in blasts expressing high or low/undetectable NF-κB levels. These data indicate that, constitutive NF-κB expression does not contribute to resistance to olaparib. By contrast, high basal NF-κB protein levels predicted responsiveness to olaparib in head and neck cancer cell lines, and the PARPi caused down-regulation of this transcription factor [49], which instead remained unchanged in AML blasts. On the other hand, treatment of AML blasts with olaparib resulted in a dose-dependent decrease of its target proteins PARP1 and PARP2, probably as a result of protein degradation.

The mechanism by which olaparib stimulates NF-κB activation may be related to the role of PAR in the regulation of protein functions. Actually, the requirement of PARylation for NF-κB transcriptional activity is controversial, and seems to be dependent on cell-type and stimulus. While PARP1 may promote NF-κB activity through direct interaction with histone acetyl-transferases p300 and CREB-binding proteins [50], enzymatic activation of PARP1 may differentially affects NF-κB function, depending on the type of PAR acceptor protein [51]. Moreover, the influence of PARylation on NF-κB itself is not clear, with several reports showing decreased, increased, or unaffected DNA-binding activity [52–56].

In conclusion, we identified a novel molecular mechanism, requiring NF-κB activation that may contribute to olaparib activity against primary AML blasts. Taking into account its bioavailability through oral administration and the acceptable tolerability profile also in heavily pretreated cancer patients, olaparib monotherapy might represent an attractive treatment option for relapsed/refractory AML.

Author contributions

I.F. designed and performed the experiments, interpreted the data and revised the manuscript; F.A., A.D.G., M.I.C., S.L. performed the experiments and analyzed the data; M.T.V. and F.L.C. contributed to plan the research strategy and revised the manuscript; G.G. planned the research strategy and wrote the paper.

Disclosure of conflicts of interest

The authors declare no conflict of interest. The funding sponsor had no role in the design of the study, in the collection, analyses or interpretation of data, and in the writing of the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.canlet.2018.03.008.

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