The Combination of the PARP Inhibitor Rucaparib and 5FU Is an Effective Strategy for Treating Acute Leukemias

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Abstract

The existing treatments to cure acute leukemias seem to be nonspecific and suboptimal for most patients, drawing attention to the need of new therapeutic strategies. In the last decade the anticancer potential of poly ADP-ribose polymerase (PARP) inhibitors became apparent and now several PARP inhibitors are being developed to treat various malignancies. So far, the usage of PARP inhibitors has been mainly focused on the treatment of solid tumors and not too much about their efficacy on leukemias is known. In this study we test, for the first time on leukemic cells, a combined therapy that associates the conventional chemotherapy agent fluorouracil (5FU), used as a source of DNA damage, and a PARP inhibitor, rucaparib. We demonstrate the efficacy and the specificity of this combined therapy in killing both acute myeloid leukemia and acute lymphoid leukemia cells in vitro and in vivo. We clearly show that the inhibition of DNA repair induced by rucaparib is synthetic lethal with the DNA damage caused by 5FU in leukemic cells. Therefore, we propose a new therapeutic strategy able to enhance the cytotoxic effect of DNA-damaging agents in leukemia cells via inhibiting the repair of damaged DNA.

Introduction

Based on their cell of origin, the most common types of acute leukemias in adults are subdivided in acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL). Both pathologies are biologically heterogeneous and display different clinical behaviors according to the morphologic and cytogenetic subtypes they belong to. For AMLs, the standard-of-care treatment has remained relatively unchanged over the past four decades. The outcomes vary considerably according to the karyotype of the patients, and even within groups of patients with the same karyotype and the prognosis is poor (1, 2). For ALLs, the situation is even more complex, especially in adult patients. Whereas children have a better outcome with an overall survival of about 80%, adults have a less optimistic perspective with an overall survival of only approximately 45% (3, 4). Clinical data clearly indicate how the available treatments for AMLs and ALLs are nonspecific and inadequate for most patients, underlining the need for new therapeutic approaches. Although several new agents, such as tyrosine kinase inhibitors and epigenetic modulators, have shown a promise in the treatment of leukemia, it is expected that they will be more effective when used in combination with other new agents, or with conventional therapies rather than as a monotherapy. The targeted therapies of new generation aim to weaken redundant pathways or augment deficiencies to kill the tumor.

Tumor cells and particularly tumor stem cells tolerate better DNA damage, present alterations in cellular checkpoints and in DNA repair pathways. Previous studies from our laboratory suggest a general mechanism of tumorigenesis in which initiating oncogenes, due to their ability to induce DNA damage, might trigger tumorigenesis by inducing genomic instability in the long-living hematopoietic stem cells (5, 6). Inhibition of DNA repair might be synthetic lethal with oncogene expression. Therefore, a good therapeutic strategy could be enhancing the cytotoxic effect of DNA-damaging agents in tumor cells via inhibiting the DNA damage repair (DDR).

There are extensive evidences of the success of poly ADP-ribose polymerase inhibitors (PARPi) in treatment of cancer. In several preclinical models both in vitro and in vivo, PARP inhibition results in enhanced killing of tumor cells of different origins. PARP-1 plays a fundamental role in DDR and is central to the base excision repair (BER) pathway. It is, indeed, able to sense and bind DNA breaks, favor opening of chromatin and recruit at the site of damage other BER factors (7, 8). The successful therapeutic strategies that take advantage of PARP inhibition are based on

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two rationales: (i) the use of PARPi as single agent, exploiting the synthetic lethality mediated by mutations in genes involved in other DNA repair pathways (i.e., BRCA1, BRCA2, and PTEN) with loss of PARP activity, (ii) the use of PARPi in combination with other DNA-damaging chemotherapeutic agents to override the cell ability to repair damaged DNA (9). A proof of the synthetic lethality of DNA damage with PARP inhibition in DNA-repair deficient cancers comes from both preclinical studies and clinical trials on patients affected by breast or ovarian cancers harboring BRCA1/2 mutations (10–12). Several studies on different tumor types have shown that PARPi enhances the cytotoxic effects of various DNA-damaging agents via inhibition of BER (9, 13). A number of clinical trials are in progress assessing the safety and efficacy of PARPi in various solid tumors (14). Nonetheless, not much is known about the usefulness of PARPi in the treatment of hematopoietic tumors, in fact only few studies suggest a role of PARPi in killing both lymphoid and myeloid tumor cells in vivo (15–19).

In the present study, we explored the possibility of boosting the DNA damage of AML and ALL blasts using the conventional chemotherapeutic agent fluorouracil (5FU), an antimetabolite that induces BER (20) in combination with a PARPi. We observed a significant synergism between PARPi (Clovis Oncology, rucaparib; previously AG14447) and 5FU in an in vivo mouse model of ALL and in xenotransplants of human AML and ALL. Moreover, we describe the molecular mechanism of such new drug-therapy showing that rucaparib and 5FU cooperate to induce DNA damage and apoptosis of leukemic cells via targeting S-phase.

We propose a combination therapy that may have the advantage of killing specifically leukemic cells by an overload of DNA damage without increasing the cytotoxicity of 5FU to normal hematopoietic cells. Therefore we offer a novel therapeutic approach for curing both ALL and AML.

Materials and Methods

**IC50 measurements by cell viability assays**

We used two human cell lines: OCI-AML2 (AML) and RPMI-8402 (ALL). Originally obtained from ATCC, our cell lines have been validated in April 2012 performing a short tandem repeat (STR) analysis using the StemElite ID system (Promega) and comparing them to cell banks databases or published profiles.

Cell viability was measured 48 hours after treatment with increasing concentrations of drugs or vehicle controls. Forty-eight hours after treatment, OCI-AML2 cells were incubated for 4 hours in a MTT reagent solution (0.5 mg/mL, final concentration) and resuspended in DMSO to be read on the Glomax Multidetection System (Promega). The concentration-response curve was calculated by the ratio of absorbance 560 nm/750 nm. For RPMI-8402, we used the WST-1 assay, according to the manufacturer’s instructions (Roche Applied Science).

**In vitro pharmacologic treatment**

OCI-AML2 cells were treated with rucaparib (3 μmol/L) every 24 hours for 3 consecutive days. The first day of treatment, cells were also treated with a 3 μmol/L single dose of 5FU (Teva). RPMI-8402 cells were treated with a single dose of rucaparib (25 μmol/L), a single dose of 5FU (50 μmol/L), or a combination of rucaparib and 5FU at the same time. Cell viability was measured 48 hours after treatment as described above.

Mice and antileukemic treatments in vivo

Experiments involving mice were performed according to the Italian guidelines and after approval of the Institutional Review Board, in agreement with the guidelines reported in (21). All mice used in the study were purchased from Charles River Laboratories: C57BL6/Ly5.2 (strain C57BL/6Ncrl), C57BL6/Ly5.1 (strain B6.SJL-PtprcaPepcb/BayCrl) and NOD SCID IL2RG null (strain NOD.Cg-Prkcsd1 Il2rgm1Wjl/SzJ, NSG). We used 8- to 12-week-old males and females (average weight, 20–25 g).

The xenograft of the human AML sample (hAML) was generated by transplantation in NSG mice of blasts derived from a patient affected by an M4 AML, harboring the translocation t(9;11). Our transplantation protocol consists of intravenous injection of 1 × 10^6 CD3-depleted mononuclear bone marrow (BM) cells in 6- to 8-week-old mice. The xenograft of the human T-ALL (hALL) was generated by transplantation of leukemia cells obtained at diagnosis from a patient with a pre-T ALL onto NOD/SCID mice as described previously (22). Human engraftment is determined by FACS analysis following staining of peripheral blood with the anti-human CD45-APC antibody (Becton Dickinson). For our experiments, human blasts collected from spleen of second or third passages of xenotransplantation were injected intravenously (1 × 10^6 and 4 × 10^5 cells/mouse, for hAML and hALL, respectively) into nonirradiated NSG recipients.

All leukemia mice were generated by a single intraperitoneal (i.p.) administration of 50 mg/kg N-ethyl-N-nitrosourea (ENU; Sigma) to C57BL6/Ly5.2 WT mice. At onset of the disease, the mice were euthanized and blasts collected from spleen and BM (mALL). By immunophenotypic analysis, the blasts were positive for CD4, CD8, and CD3, confirming the development of ALL in the ENU-treated animals. Splenocytes from ALL leukemia C57BL6/Ly5.2 mice were injected intravenously (2 × 10^4 cells/mouse) into nonirradiated C57BL6/Ly5.1 recipients.

For all models, drugs were administered i.p. 3 days after transplantation of blasts (time necessary for engraftment in the host BM). Mice received five consecutive daily doses of rucaparib (1 mg/kg and 1.3 mg/kg each dose for murine and for human blasts, respectively) and a single dose (administered at day 2) of 150 mg/kg 5FU for mALL and hAML and of 75 mg/kg 5FU for hALL.

Western blotting

C57BL6/Ly5.1 healthy mice were treated as follows: i.p. of PBS every day for two days (control group); an i.p. of PBS the first day and an i.p. of 5FU (150 mg/kg) the day after (5FU group); an i.p. of rucaparib (1 mg/kg) every day for two days (rucaparib group); an i.p. of rucaparib (1 mg/kg) the first day and an i.p. of 5FU (150 mg/kg) the day after (rucaparib + 5FU group). Zero, 5, and 24 hours after the second i.p., BM cells were collected and lysed by Laemlli Buffer.

OCI-AML2, RPMI-8402, and blasts from the hAML xenograft were treated in vitro as described above. Cells were collected at the indicated time points and lysed (buffer, 50 mMol/L Heps, pH7.4; 150 mMol/L NaCl; 1.5 mMol/L EDTA; 10% glycerol; 1% NP-40). Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Detection was carried out using chemiluminescent substrate (Pierce Biotechnology) after incubation with the following antibodies: anti-H2AX-Phosphorylated (Ser139; BioLegend); anti-cleaved caspase-3,
anti-Chk1, anti-Phospho-Chk1 (Ser317), anti-Chk2, and anti-Phospho-Chk2 (Thr68, all from Cell Signaling Technology). Vinculin was used as loading control.

**Immunofluorescence**

OCI-AML2 were seeded onto poly-D lysine-coated slides, fixed with 4% paraformaldehyde, and incubated with the following antibodies: anti-Lamin B (Santa Cruz Biotechnology), anti-Phospho-Histone H3 serine-10 (Cell Signaling Technology), and anti-H2AX. Phosphorylated Alexa 647-conjugated (BioLegend). Detection was performed using anti-goat Cy3-conjugated and anti-rabbit Alexa 488-conjugated antibodies (Invitrogen). Following nuclear staining with DAPI (4,6 diamidino-2-phenylin- dolde; Sigma-Aldrich) and mounting with Mowiol (Calbiochem), images were acquired with the Olympus BX61 microscope driven by the Metamorph software. Analysis was performed using the Image software (W. Rasband, NIH).

Ethynyl-deoxy-Uridine staining of proliferating cells

Cells were treated with 10 μmol/L Ethynyl-deoxy-Uridine (EdU; Invitrogen) for 30 minutes, fixed in 2% formaldehyde, and seeded in CultureWell Chambered Coverglasses (Invitrogen) for staining with the Click.iT EdU Immunofluorescence Assay Kit (Invitrogen), according to the manufacturer’s instructions. Cells were then incubated with anti-γH2AX (BioLegend) and stained with Alexa 488-conjugated anti-mouse (Molecular Probes; Invitrogen).

**Image cytometry**

Images were collected using a BX61 upright fluorescence microscope (Olympus) controlled by Scan R 2.6.1 software equipped with an ORCA ER Hamamatsu CCD camera (Hamamatsu, Italy). Evaluation of cell-cycle progression and DNA damage was performed using a dedicated package developed in our laboratory (23). Bivariate DNA versus EdU content distribution was measured to distinguish between G1, G2–M, and active DNA replicating cells at each time point. Evaluation of drug-induced DNA damage was based on the integration of fluorescence intensity associated to detected γH2AX foci in every cell-cycle phase.

**Flow cytometry analysis**

FACS analysis (BD FACS) was performed on BM cells of leukemic mice postpharmacologic treatment. Cells were fixed, permeabilized, and stained using the following antibodies: anti-H2AX-Phosphorylated Alexa 647-conjugated (BioLegend) for murine blasts or anti-H2AX-Phosphorylated (BioLegend) and secondary antibody anti-mouse Alexa488-conjugated (Molecular Probes, Invitrogen) for human blasts; anti mouse CD45.2 FITC-conjugated (eBioscience) or anti-human CD45 APC-conjugated (Becton Dickinson); rabbit anti-cleaved caspase-3 (Asp175; Cell Signaling Technology) and secondary antibody anti-rabbit PE-conjugated (Jackson Immunoresearch).

**Statistical analysis**

Data were analyzed for statistical significance with a two-sided t test. The Kaplan–Meyer rank test was used to compare survival rate among groups of treated mice. Data are presented as mean ± SD.

**Results**

**PARP inhibition prevents the DNA damage repair caused by 5FU**

To optimize a chemotherapeutic strategy that takes advantage of impaired DDR activation, we tested the efficacy of the combination of 5FU, a conventional chemotherapeutic source of DNA damage, and a PARP inhibitor, rucaparib. The histone variant H2AX is rapidly phosphorylated on Ser139 (γH2AX) in response to different types of DNA damage (24). We evaluated the effect of PARP inhibition in vivo on the DNA damage induced by 5FU in normal bone marrow mononuclear cells (BM-MNC). C57BL/6 Ly5.1 nonleukemic mice were pretreated for 24 hours with rucaparib before cotreatment with rucaparib and 5FU (one dose). BM-MNCs were collected 0, 5, and 24 hours posttreatment and analyzed for expression of γH2AX. Normal BM-MNCs have low physiological levels of DNA damage and inhibition of PARP by rucaparib does not induce DNA damage per se. The administration of 5FU, in contrast, causes DNA damage at 5 hours that is repaired during time. However, in presence of rucaparib the DNA damage induced by 5FU is retained up to 24 hours (Fig. 1). Our data suggest that rucaparib is able to block the DDR in normal BM-MNCs without toxic side effects. The lack of changes in γH2AX expression in animals treated with PARPi alone may be because rucaparib inhibits PARP-mediated DDR in cells where the DNA damage is already present.

**PARP inhibition kills AML and ALL cells in vitro, sensitizing them to chemotherapy**

We tested the effect of rucaparib on AML and ALL in vitro, using OCI-AML2 and RPMI-8402 cell lines, respectively. Cell survival was evaluated by colorimetric assays. Both cell lines are sensitive to increasing concentrations of rucaparib; however, OCI-AML2 seems to be more responsive: with an EC50 of 3 μmol/L versus 75 μmol/L for RPMI-8402 (Fig. 2A). Similarly, OCI-AML2 appears more sensitive to 5FU treatment (EC50 = 3 μmol/L versus 75 μmol/L for RPMI-8402; Fig. 2B). Moreover, we tested their sensitivity to rucaparib in the presence of 5FU. We show that 5FU cooperates with rucaparib to kill both cell lines. Indeed, the percentage of surviving cells after administration of the combination of PARPi and 5FU is significantly lower compared with administration of the single agents (Fig. 2C). Our data suggest that rucaparib inhibits the DDR and in combination with 5FU is able to decrease cell viability; therefore, our combined treatment calls for causing DNA damage and keeping the DDR low so that cells accumulate massive DNA damage without being able to repair it.

**Figure 1.** Effect of the combination of PARPi and 5FU in normal (not leukemic) BM mononucleated cells in vivo. Western blot analysis of phospho-H2AX (γH2AX) after 5 and 24 hours (5 h, 24 h) treatment in vivo with PARP (P, 1 mg/kg), 5FU (150 mg/kg), and PARPi along with 5FU (P+S) compared with untreated control (Ctr). Three mice were analyzed per group. Vinculin is shown as normalization of the loaded protein amount. The figure shows that in presence of PARPi the increased level of γH2AX induced by 5FU is retained during time.
To determine the checkpoint signaling pathways activated following induction of DNA damage by 5FU, we performed an analysis of activation of CHK1 and CHK2 by Western blot on OCI-AML2 and RPMI-8402. Cells were treated in vitro as described above and proteins analyzed 12, 24, 48, and 72 hours after treatment. In cells treated with rucaparib in combination with 5FU, we observed an accumulation of CHK1 expression, as observed for the BM-MNCs. Moreover, we detected mainly activation of the CHK1 checkpoint, measured as increased levels of CHK1 phosphorylation (Fig. 2D). However, while activation of CHK1 in RPMI-8402 appears to be sustained along our time course, in OCI-AML2 we observe a peak of activation at 24 hours followed by a decrease, mirrored by a decrease in the total level of CHK1 (Fig. 2D). The existence of a p53-dependent feedback loop that downregulates CHK1 has been reported (25). The different behavior of OCI-AML2 and RPMI-8402 could be, therefore, explained by the fact that they are p53-WT and p53-mutated, respectively. Notably, an equivalent Western blot analysis performed in vitro on blasts derived from a xenotransplant of a patient affected by AML produced the same results described for both cell lines (Supplementary Fig. S1).

The combined treatment of rucaparib with 5FU is efficient on leukemic blasts in vivo

We decided to test rucaparib activity in enhancing the cytotoxic effect of 5FU in a mouse model of ALL. Leukemic mice were generated by administration of the tumorigenic agent ENU to WT

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**Figure 2.** Myeloid and lymphoid leukemia cells are sensitive to PARP inhibitor in vitro. A, effect of increasing doses of PARPi on survival of leukemia cells: from 0.1 to 10 μmol/L for OCI-AML2 (left); from 10 to 50 μmol/L for RPMI-8402 (right). B, effect of increasing doses of 5FU on survival of leukemia cells: from 0.1 to 10 μmol/L for OCI-AML2 (left); from 10 to 100 μmol/L for RPMI-8402 (right). C, in vitro chemosensitization of OCI-AML2 (top) and RPMI-8402 (bottom) leukemia cells upon administration of PARPi (3 μmol/L for OCI-AML2 cells, 25 μmol/L for RPMI-8402 cells) in combination with 5FU (3 μmol/L for OCI-AML2 cells, 50 μmol/L for RPMI-8402 cells). For all panels, cell survival was measured 48 hours after administration of the drugs by MTT assay and by WST-1 assay for OCI-AML2 and RPMI-8402, respectively. **P < 0.05; ***P < 0.005. Experiments were performed at least in triplicate and the results are representative of three independent experiments. D, treatment of leukemic cells with PARP inhibitor and 5FU induces a DDR response that is mainly mediated through the Check1 checkpoint and results in apoptosis. Leukemic cells from the OCI-AML2 (left) and RPMI-8402 cell line (right) were treated in vitro with PARP (PARP: 3 μmol/L for OCI-AML2, 25 μmol/L for RPMI-8402), 5FU (5FU: 3 μmol/L for OCI-AML2, 100 μmol/L for RPMI-8402), and PARPi along with 5FU (PARP+5FU). Western blot analysis of the indicated proteins was performed 12, 24, 48, and 72 hours (12 h, 24 h, 48 h, 72 h) after treatment and compared with untreated controls (NT). Analyzed proteins are: phospho-Chek1 (Ser317, pCHK1), phospho-Chek2 (Thr68, pCHK2), phospho-H2AX (Ser139, γH2AX), cleaved caspase-3 (CASP3), total amount of Chek1 (CHK1 tot), and Chek2 (CHK2 tot). Vinculin is shown as normalization of the loaded protein amount.
C57BL6/Ly5.1 recipients. Three days after transplantation, time sufficient for the blasts to home in the BM of the hosts, we started treating the animals. We optimized a schedule of treatment based on the rationale suggested by our *in vitro* data. It calls for five doses of rucaparib (1 dose per day) and a single dose of 5FU (at day 2), both administered via intraperitoneal injection. Administration of PARPi for 5 consecutive days does not seem to be toxic for the animals, however, it also does not significantly affect the overall survival of treated animals compared with the untreated ones (PARPi vs. vehicle, Fig. 3A). A single dose of 5FU prolongs the survival of the leukemic mice. Strikingly, the combination of rucaparib with 5FU induces a further significant improvement of survival compared with the treatment with 5FU alone (*P* = 0.05, Fig. 3A).

To define if these results can be translated to the human pathology, we decided to test the effects of this combined treatment on leukemic cells derived from human patients, using xenotransplantation models. Immunocompromised mice were transplanted with blasts derived both from a patient affected by an ALL and a patient affected by an AML. The xenotransplanted mice were treated using the same protocol and rationale described for the murine ALL model *in vivo*. For the human lymphoid leukemia (A) 1 mg/kg rucaparib (PARPi, *n* = 19), 150 mg/kg 5FU (5FU, *n* = 11), or their combination (PARPi+5FU, *n* = 15); for the human lymphoid leukemia (B) 1.3 mg/kg rucaparib (PARPi, *n* = 4), 75 mg/kg 5FU (5FU, *n* = 5), or their combination (PARPi+5FU, *n* = 6); Vehicle, vehicle-treated control animals (*n* = 13 for mouse T-ALL, *n* = 4 for human T-ALL).

To deﬁne these cells as hyper-γH2AX-positive. Hyper-γH2AX-positive cells are exclusively present in samples treated with 5FU and rucaparib plus 5FU (Fig. 5A and 5C). Notably, in animals treated with rucaparib plus 5FU, the percentage of the hyper-γH2AX-positive cells also increased (Fig. 5A). A similar increase in hyper-γH2AX-positive cells is also observed in the human blasts extracted from the BM of animals xenotransplanted with the hAML (Supplementary Fig. S2).

**Combination of rucaparib and 5FU selectively induces massive DNA damage in leukemic cells**

To elucidate the mechanism of the synergies of PARP inhibition with chemotherapy, we concentrated our efforts mostly on the murine ALL model *in vivo*. We first estimated the level of DNA damage in the leukemic blasts calculating number and intensity of γH2AX foci by image-cytometry (23). ALL leukemic mice were treated with rucaparib and, 24 hours later, with one dose of 5FU. Twenty-four hours after treatment, we collected the BM-MNCs and measured γH2AX foci. The PARPi alone has no evident effect on the number and intensity of γH2AX foci, whereas the potent DNA-damaging agent 5FU almost doubles the values of the untreated sample and the combination of rucaparib with 5FU causes a slight increase (2.4 vs. 2 fold) in foci intensity compared with 5FU alone (data not shown). Despite these little changes in the overall number of γH2AX foci among different treatments, we observed the presence of cells that lose the typical foci signal and emit a much higher and diffuse γH2AX signal positivity. We defined these cells as hyper-γH2AX-positive. Hyper-γH2AX-positive cells are exclusively present in samples treated with 5FU and rucaparib plus 5FU (Fig. 5A and 5C). Notably, in animals treated with rucaparib plus 5FU, the percentage of the hyper-γH2AX-positive cells also increased (Fig. 5A). A similar increase in hyper-γH2AX-positive cells is also observed in the human blasts extracted from the BM of animals xenotransplanted with the hAML (Supplementary Fig. S2). To assess the specificity of the

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**Figure 3.**

Chemosensitization of PARP inhibitor and 5FU treatment in both murine and human lymphoblastic leukemias. Effect of PARPi treatment, alone or in combination with 5FU on the overall survival of mice transplanted with a murine T-ALL (A) and a human T-ALL (B). The transplanted animals were treated as follows: for the murine lymphoid leukemia (A) 1 mg/kg rucaparib (PARPi, *n* = 19), 150 mg/kg 5FU (5FU, *n* = 11), or their combination (PARPi+5FU, *n* = 15); for the human lymphoid leukemia (B) 1.3 mg/kg rucaparib (PARPi, *n* = 4), 75 mg/kg 5FU (5FU, *n* = 5), or their combination (PARPi+5FU, *n* = 6); Vehicle, vehicle-treated control animals (*n* = 13 for mouse T-ALL, *n* = 4 for human T-ALL).
synergism observed between rucaparib and 5FU, we treated the leukemia mice with another conventional chemotherapeutic agent, cisplatin (7 mg/kg single i.p., Teva). We chose cisplatin because it acts as a bifunctional alkylator and, differently from 5FU, activates DDR pathways other than BER. As expected and as described for 5FU, cisplatin almost doubles the number of γH2AX foci intensity compared with untreated samples. However, the addition of cisplatin to rucaparib does not cause any change in terms of DNA damage if compared with samples treated with the single agents (data not shown). Moreover, in vitro treatment with rucaparib and/or cisplatin does not affect the percentage of hyper-γH2AX-positive cells, which is almost absent (<2%, Fig. 5A). These data suggest that the synergism of action on leukemia is specific for PARPi and 5FU and that they specifically involve the BER pathway of DDR.

Seen the high intensity of the detected damage, we used a FACS analysis to demonstrate that hyper-γH2AX-positive cells are specifically present in the leukemic compartment of the BM of the mALL. Because we used the congenic strains C57BL/6Ly5.2 and -Ly5.1, all CD45.2-positive cells detected in the recipients belong to the donor and are, therefore, leukemic. We observe that hyper-γH2AX-positive cells belong almost exclusively to the CD45.2 positive population (Fig. 5B), suggesting a higher sensitivity of ALL blasts to the combined treatment. Taken together, our data indicate a specific synergism between 5FU and rucaparib to cause a hyper DNA damage only in a restricted, but significant, population of leukemic cells.

Treatment with rucaparib and 5FU induces apoptosis of leukemic cells

To understand the fate of the hyper-γH2AX-positive cells, we measured by FACS the expression of the apoptosis marker cleaved caspase-3 in the leukemic compartment of treated animals. We show that 93.5% of the leukemic hyper-γH2AX-positive cells are positive for cleaved-caspase3 and, therefore, apoptotic (Fig. 5B). These cells appear apoptotic morphologically as well. Indeed, by immunofluorescence the nuclei show the typical multilobular apoptotic shape (Fig. 5C). Similarly, following a combined treatment with rucaparib and 5FU, by Western blot analysis of γH2AX and cleaved caspase-3, we observe an accumulation of DNA damage and induction of apoptosis in the leukemic cells derived from the OCI-AML2 and RPMI-8402 cells (Fig. 2D) and from the hAML xenograft (Supplementary Fig. S1), underlining how the combined treatment has the same effects on leukemias of different origin.

It has been demonstrated that the PARP inhibitor Olaparib kills ATM-deficient lymphoid tumor cells via mitotic catastrophe (17). We show that the leukemic hyper-γH2AX-positive cells are lamin B positive and phospho-H3 Ser10 negative (Fig. 5C), suggesting that they are not mitotic. Our data provide evidences that the administration of rucaparib plus 5FU induces massive accumulation of DNA damage and consequent cell death in a significant fraction of leukemic blasts.

Rucaparib interferes with DNA replication

To further clarify the mechanism of the synergism between rucaparib and 5FU, we analyzed cell-cycle perturbations at different time points (0, 5, 8, 24, 48, and 72 hours) on OCI-AML2 cells treated in vitro. For each phase, we simultaneously evaluated by image-cytometry the effects on DNA replication, measured by EdU uptake, and on DNA damage, by quantification of γH2AX foci. No dramatic alterations are detected upon administration of rucaparib alone. However, a specific interference with DNA synthesis is detected at late time points leading to the accumulation of a cell population with intermediate DNA content not incorporating EdU (Fig. 6 and Supplementary Fig. S3). The replication arrest correlates with a marked increase in DNA damage in this cell fraction and is in agreement with the expected inhibition of DDR exerted by PARPi. 5FU treatment causes massive cell death in S phase and consequent accumulation of the surviving fraction in G1 phase, with progressive cell-cycle independent accumulation of DNA damage. Strikingly, an increasing fraction of cells with arrested DNA replication can also be detected remarking a 5FU S-phase

Figure 4. The combined treatment with PARP inhibitor and 5FU significantly reduces the leukemic load in xenotransplants of human leukemias. The figure shows the percentage of human blasts circulating in the peripheral blood of NSG mice transplanted with a human T-ALL sample (A) and a human AML sample (B). The percentage of blasts in the PB was measured by FACS analysis of the human CD45+ cells 5 weeks after transplantation. The transplanted animals were treated as follows: for human T-ALL (A) 1.3 mg/kg rucaparib (PARPi, n = 4), 75 mg/kg 5FU (5FU, n = 5) or their combination (PARPi+5FU, n = 6); for human AML (B) 1.3 mg/kg rucaparib (PARPi, n = 8), 150 mg/kg 5FU (5FU, n = 9) or their combination (PARPi+5FU, n = 6). NT, vehicle-treated control animals (n = 4 for T-ALL; n = 8 for AML). Shown are mean and standard error. *, P < 0.05; **, P < 0.005.
speciﬁc activity (Fig. 6 and Supplementary Fig. S3). Therefore, we can hypothesize that the synergistic effect observed by combination of the two drugs is due to their simultaneous targeting of DNA replication. Indeed, this proposed mechanism of action is conﬁrmed by the kinetic analysis. Besides a dramatic mortality, combination of rucaparib with 5FU results in disappearance of replicating DNA, ﬁrst accompanied by a progressive arrest of replication forks progression, evidenced by reduced levels of EdU incorporation already at 24 hours. In the next time points the effects on cell-cycle progression, already detected upon single rucaparib treatment, is markedly enhanced: a relevant accumulation of S-phase EdU-negative cells is associated with a progressive increase in γH2AX total ﬂuorescence intensity (Fig. 6 and Supplementary Fig. S3). These data clearly suggest that the synergistic effect of the two drugs is due to inhibition of repair of the DNA damage induced by replication.

Discussion

Many PARPi have been identiﬁed and have shown a signiﬁcant antitumor potential against a variety of solid tumors (14). In general, usage of PARPi in the clinic follows two different approaches that take advantage of synthetic lethality of genomic instability and blockage of DNA repair. PARPi are used as single agents in patients with intrinsic DDR deﬁciency or as combined therapies along with DNA-damaging agents to potentiate the damaging effects of conventional chemotherapy and breakthrough the ﬁne tuning of DNA-damage-handling by tumor cells. This last approach increases the efﬁcacy of conventional chemotherapies allowing a substantial reduction of their doses and consequently their side effects. Along this line, we decided to test the effects of the combination of the PARP inhibitor rucaparib and 5FU for the treatment of leukemic tumors. Induction of BER by 5FU and its successive blockade...
by the following administration of PARPi should selectively kill 5FU-sensitive cells.

We demonstrate that both myeloid and lymphoid leukemia cell lines are sensitive to administration of rucaparib as single agent, as previously reported for different PARP inhibitors (15–19). Moreover, our in vitro experiments show that PARP inhibition enhances the chemosensitization of both myeloid and lymphoid leukemia cells to 5FU exposure, suggesting a synergistic effect of the combined therapy in killing leukemia cells. Previous studies exploited the synergism of PARPi in killing leukemia cells in combination with mutations in genes involved in DNA repair (17–19), or different agents such as temozolomide, methyltransferase, or histone deacetylase inhibitors (15, 16); however our is the first report of the effectiveness of the combination of PARPi with a conventional chemotherapeutic agent.

Importantly, our in vitro data are supported by our in vivo observations. We tested the efficacy of the combination therapy, following an administration protocol based on the rationale of causing DNA damage (via 5FU administration) and keeping the DDR low (via PARPi inhibition), both in a mouse model of ALL and in xenotransplants of both hALL and hAML. We demonstrated that the combination therapy is effective on both ALL and AML. In particular, the rucaparib and 5FU treatment significantly improves the survival rate of animals affected by leukemia of both human and murine origins compared with mice treated with vehicle or single agents (Fig. 3). To our knowledge, this is the first report of an effective treatment of a combination therapy with PARPi on acute leukemias in vivo.

Analyzing ex vivo leukemic cells obtained from ALL mice, we observed that rucaparib and 5FU synergize to induce a massive DNA damage, measured by expression of γH2AX, in a certain number of leukemic cells. We demonstrated that such hyperdamage is not induced when the PARP inhibition is associated to treatment with cisplatin, suggesting the specificity of the combination of PARPi with 5FU. The "hyper-damaged," alias "hyper sensitive to the combination," cell population is present only in the leukemic compartment of both murine and human leukemias, suggesting a specificity of the treatment for leukemic cells. Moreover, we show that the "hyper-damaged" cells are

Figure 6. Combination of PARPi and 5FU induces massive DNA damage and replication arrest in myeloid leukemia (OCI-AML2) cells in vitro. A, combination of 5FU (5FU, 3 μmol/L) and PARP inhibitor (PARPi, 3 μmol/L) altered cell-cycle progression through interference in DNA replication and selective killing of S-phase cells. B, 5FU (3 μmol/L) and PARPi (3 μmol/L) synergize to induce selective accumulation of damage during DNA synthesis. Cells were treated as described for Fig. 2C. X-axis, hours posttreatment. NT, vehicle-treated control cells.
not mitotic, as previously reported (17), but are apoptotic (Fig. 5). Strikingly, we observe the same biologic effects and outcomes treating in vitro the hAML, the OCI-AML2, and the RPMI-8402 cells. This underlines the existence of a conserved mechanism of action of the combination of PARPi with 5FU in killing leukemic cells of different origin. The specificity of the treatment for the leukemic cells has been further proven by the fact that administration of rucaparib as single agent did not cause any increase of γH2AX compared with basal levels (Fig. 1), most likely because rucaparib inhibits PARP-mediated DDR in cells where DNA damage is already present. Indeed, BM cells have low/absent physiological levels of DNA damage and we observe no effect of rucaparib alone in normal hematopoietic cells, suggesting low levels of toxicity of rucaparib for nontumor cells. This is in agreement with recent data on primary AMLs in vitro. PARPi preferentially kills the blasts sparing the lymphocytes derived from the same patient (19). Notably, normal BM cells exposed to the combination of rucaparib and 5FU retain the DNA damage caused by 5FU administration during time (Fig. 1).

We revealed the mechanism of action by which the combination of 5FU with PARPi drives leukemic cells into apoptosis. We demonstrate that upon PARP inhibition, leukemic cells accumulate a slight DNA damage and slow down the cell cycling. When the damaged leukemic cells stalled in S phase are treated with 5FU accumulate a massive DNA damage that cannot be repaired anymore and they die (Supplementary Fig. S4). This is in line with a recent report that shows that rucaparib predominantly inhibits repair of DNA breaks in S phase (26).

The lowered leukemic load of hAML and hALL cells in mice treated with rucaparib and 5FU indicates that the combinatory therapy may affect the leukemia stem cell compartment; therefore, in the future it would be interesting to assess the effect of our combinatory therapy on leukemia stem cells. Cancer stem cells sustain the tumor, are responsible for reoccurrence of the malignancy and are characterized by unique mechanisms of handling DNA damage. It is envisaged that this study will lead to the development of antileukemic therapies that have the advantage to reduce the side effects of conventional chemotherapies and, hopefully, decrease the risk of relapse. Strikingly, the combined therapy we propose is effective against two biologically and clinically very different types of acute leukemias, suggesting a broad applicability of our therapeutic strategy in hematologic malignancies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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