Quiescent and proliferating leukemia cells accumulate highly lethal DNA double-strand breaks that are repaired by two major mechanisms: BRCA-dependent homologous recombination and DNA-dependent protein kinase–mediated (DNA-PK–mediated) nonhomologous end-joining, whereas DNA repair pathways mediated by poly(ADP)ribose polymerase 1 (PARP1) serve as backups. Here we have designed a personalized medicine approach called gene expression and mutation analysis (GEMA) to identify BRCA- and DNA-PK–deficient leukemias either directly, using reverse transcription–quantitative PCR, microarrays, and flow cytometry, or indirectly, by the presence of oncogenes such as BCR-ABL1. DNA-PK–deficient quiescent leukemia cells and BRCA/DNA-PK–deficient proliferating leukemia cells were sensitive to PARP1 inhibitors that were administered alone or in combination with current antileukemic drugs. In conclusion, GEMA-guided targeting of PARP1 resulted in dual cellular synthetic lethality in quiescent and proliferating immature leukemia cells, and is thus a potential approach to eradicate leukemia stem and progenitor cells that are responsible for initiation and manifestation of the disease. Further, an analysis of The Cancer Genome Atlas database indicated that this personalized medicine approach could also be applied to treat numerous solid tumors from individual patients.
Quiescent and proliferating leukemia cells accumulate highly lethal DNA double-strand breaks that are repaired by 2 major mechanisms: BRCA-dependent homologous recombination and DNA-dependent protein kinase–mediated (DNA-PK–mediated) nonhomologous end-joining, whereas DNA repair pathways mediated by poly(ADP)ribose polymerase 1 (PARP1) serve as backups. Here we have designed a personalized medicine approach called gene expression and mutation analysis (GEMA) to identify BRCA- and DNA-PK–deficient leukemias either directly, using reverse transcription–quantitative PCR, microarrays, and flow cytometry, or indirectly, by the presence of oncogenes such as BCR-ABL1. DNA-PK–deficient quiescent leukemia cells and BRCA/DNA-PK–deficient proliferating leukemia cells were sensitive to PARP1 inhibitors that were administered alone or in combination with current antileukemic drugs. In conclusion, GEMA-guided targeting of PARP1 resulted in dual cellular synthetic lethality in quiescent and proliferating immature leukemia cells, and is thus a potential approach to eradicate leukemia stem and progenitor cells that are responsible for initiation and manifestation of the disease. Further, an analysis of The Cancer Genome Atlas database indicated that this personalized medicine approach could also be applied to treat numerous solid tumors from individual patients.

Introduction

Currently available antileukemic treatments often fail to eradicate drug-refractory quiescent leukemia stem cells (LSCs) and drug-resistant proliferating LSCs and leukemia progenitor cells (LPCs). Previous reports suggest that altered DNA repair mechanisms may be responsible for enhanced survival of LSCs and/or LPCs under genotoxic stress caused by reactive oxygen species (ROS) and cytotoxic treatment (1). Thus, leukemia cells may be highly dependent on specific DNA repair mechanisms and targeting these pathways could sensitize LSCs and LPCs to the lethality of DNA damage (2). DNA double-strand breaks (DSBs), the most lethal DNA lesions, are usually repaired by BRCA-mediated homologous recombination (HR) and DNA-dependent protein kinase–mediated (DNA-PK–mediated) nonhomologous end-joining (NHEJ) (3). Poly(ADP)ribose polymerase 1–dependent (PARP1-dependent) NHEJ serves as a back-up (B-NHEJ) pathway in both proliferating and quiescent cells (Figure 1A). In addition, PARP1 may decrease or prevent accumulation of potentially lethal DSBs, either by stimulation of base excision repair and single-strand break repair and/or by facilitation of DSB repair protein MRE11-mediated recruitment of the DNA damage marker RAD51 to promote stalled replication fork restart (4, 5).

Cancer-specific defects in DNA repair pathways create the opportunity to employ synthetic lethality, which has been applied against cancer cells harboring mutations in BRCA1 and BRCA2 by using PARP1 inhibitors (6, 7). This finding initiated more than 100 clinical trials, which indicated that biomarkers of the response to PARP1 inhibitors reach beyond that of BRCA1/2.
status. In addition, PARP1 inhibitor–mediated synthetic lethality would not eradicate BRCA1/2–mutated quiescent cancer stem cells, including quiescent LSCs, which often are responsible for minimal residual disease and disease relapse (8). We hypothesized that PARP1 inhibition (PARPi) can trigger dual cellular synthetic lethality in proliferating LSCs/LPCs and quiescent LSCs that display quantitative deficiencies in BRCA and DNA-PK pathways (Figure 1B).

Since inactivating mutations in BRCA and DNA-PK pathways (e.g., Fanconi anemia D1 = BRCA2 and LIG4, respectively) are rare in leukemias (9), other strategies for identifying patients with leukemias that display BRCA and DNA-PK (BRCA/DNA-PK) deficiency are needed. We developed a comprehensive gene expression and mutation analysis (GEMA) (Figure 1C) that identifies BRCA/DNA-PK–deficient patients using a combination of gene expression (microarrays, reverse transcription-quantitative PCR [RT-qPCR], and flow cytometry) and gene mutation (rare mutations in BRCA/DNA-PK genes and the presence of oncogenes reducing the expression of these genes) analyses to detect insufficient expression of at least 1 gene in each of the BRCA and DNA-PK pathways.

**Results**

PARPi exerted dual cellular synthetic lethality by elimination of BRCA/DNA-PK-deficient proliferating cells and DNA-PK-deficient quiescent cells. HR activity was strongly reduced in the BRCA2−/− hamster cell line V-C8 in comparison with wild-type V79 cells (Figure 2A; DR-GFP), which was accompanied by abundant elevation of γ-H2AX in PARPi inhibitor olaparib-treated Ki67+ proliferating cells, indicating accumulation of DSBs (Figure 2B; BRCA1 panel, Ki67+).

While total NHEJ was reduced, B-NHEJ was activated in the absence of XRCC6 (Figure 2A; EJ2-GFP and EJ5-GFP, respectively). Olaparib exerted strong inhibitory activity against B-NHEJ, and also modestly diminished total NHEJ in XRCC6−/− murine embryonic stem cells (mESCs), but did not affect repair in XRCC6+/+ cells. This effect was associated with elevation of γ-H2AX in olaparib-treated XRCC6−/− Ki67+ and Ki67− cells, indicating accumulation of DSBs in proliferating and quiescent cells, respectively (Figure 2B; XRCC6 panels).

PARPi reduced growth of BRCA1-mutated MDA-MB-436 human breast carcinoma cells (Figure 2C; BRCA1 panels) by elimination of proliferating, but not quiescent cells (Figure 2D; BRCA1 panels). Remarkably, PARPi reduced the number of XRCC6−/− cells more efficiently than XRCC6+/+ counterparts (Figure 2C; XRCC6 panels), but the effect depended mainly on elimination of quiescent cells, with modest impact on proliferating cells (Figure 2D; XRCC6 panels).

To determine the effect of PARPi in cells displaying dual BRCA/DNA-PK deficiency we applied 2 previously characterized models: (a) FANCA−/− XRCC6−/− mESCs (10), and (b) the RAD54−/− LIG4−/− human pre-B leukemic cell line Nalm-6 (11). Cells with BRCA/DNA-PK deficiency were equally or more sensitive to olaparib and BMN673, another PARPi inhibitor, than those displaying individual deficiencies (Figure 3A and Supplemental Figure 1A; supplemental material available online with this article; https://
samples from The Cancer Genome Atlas (TCGA) RNA-seq database revealed that 15 (9%) samples displayed BRCA/DNA-PK deficiency (Supplemental Figure 3D).

Gene expression analysis of our set of 172 cytogenetically normal AML samples revealed a wide range of expression levels of HR and D-NHEJ genes (Figure 4A). AML samples were assigned to the BRCA/DNA-PK–deficient group if a row Z-score value of at least 1 gene in HR (BRCA1, BRCA2, PALB2, RAD51L1 [RAD51], RAD51C, RAD51L3 [RAD51D], XRCC2, and XRCC3) and D-NHEJ (PRKDC [encoding DNA-PKcs], XRCC5 [KU80], XRCC6 [KU70], DCLRE1C, NHEJ1, LIG4, and XRCC4) was less than –1.5. Conversely, BRCA/DNA-PK–proficient samples displayed row Z-score values greater than –0.75 for all genes in HR and D-NHEJ. Using this approach we identified 26 BRCA/DNA-PK–deficient and 16 BRCA/DNA-PK–proficient AMLs (Figure 4B). This finding was supported by detection of BRCA/DNA-PK–deficient AML samples in another microarray database (Supplemental Figure 4).

Downregulation of at least 1 protein in each of the BRCA and DNA-PK pathways was detected in Lin–Ki67+ proliferating cells and Lin–Ki67+/– proliferating/quiescent cells, respectively, from individual BRCA/DNA-PK–deficient AMLs, compared with BRCA/DNA-PK–proficient samples (Figure 4C). The BRCA/DNA-PK-
deficient/proficient status of preselected samples was further validated by direct measurement of HR and NHEJ activities using specific reporter cassettes (Supplemental Figure 5A) (14). Both HR and NHEJ activities were downregulated in representative BRCA/DNA-PK-deficient samples when compared with BRCA/DNA-PK-proficient counterparts (Supplemental Figure 5B).

BRCA/DNA-PK-deficient Lin−AML primary cells from individual patients were more sensitive to BMN673, and BMN673 plus daunorubicin (DNR), in comparison with BRCA/DNA-PK-proficient counterparts (Figure 5A). LSC-enriched Lin−CD38−AML patient cells were used to study the role of PARP1i in quiescent and proliferating LSCs because this subpopulation was the most enriched in LSCs in NSG mice (15). The BRCA/DNA-PK-deficient phenotype was accompanied by accumulation of DSBs in both Lin−CD38−CTVlo proliferating cells and Lin−CD38−CTVmax quiescent cells treated with suboptimal doses of BMN673 and DNR (Figure 5B). These cell subpopulations were much more sensitive to BMN673 alone and in combination with DNR in comparison with BRCA/DNA-PK-proficient counterparts (Figure 5, C and D). Moreover, BMN673 + DNR exerted a synergistic effect against BRCA/DNA-PK-deficient cells.

Next, Fluidigm RT-qPCR analysis of 26 therapy-related myelodysplastic syndrome (t-MDS)/AML samples revealed that 3 samples (12%) displayed BRCA/DNA-PK deficiency (Z score < −1.5 of at least 1 gene in BRCA and DNA-PK pathways) and 11 were BRCA/DNA-PK proficient (Z score > −0.5 of all genes in BRCA and DNA-PK pathways) (Figure 6A). Immunofluorescent staining followed by flow cytometric analysis of BRCA/DNA-PK-deficient and BRCA/DNA-PK-proficient samples (Figure 6A; marked with blue and red squares, respectively) confirmed downregulation of at least 1 protein in each BRCA and DNA-PK pathway in BRCA/DNA-PK-deficient proliferating Lin Ki67+ cells and proliferating/quiescent Lin Ki67+/− cells, respectively, compared with BRCA/DNA-PK-proficient samples (Figure 6B).

Figure 3. PARP1i elevated DSBs and triggered dual cellular synthetic lethality in BRCA/DNA-PK-deficient proliferating and quiescent Nalm-6 leukemia cells. (A) Sensitivity of the indicated cells to olaparib and BMN673. (B and C) Nalm-6 parental cells (gray bars) and RAD54−/−LIG4−/− counterparts (green bars) treated or not with 0.15 μM olaparib for 24 (B) or 48 (C) hours. (B) γ-H2AX-positive Ki67− and Ki67+ cells and (C) dead Ki67− and Ki67+ cells; representative plots are included. Results represent 3 independent experiments. *P < 0.05 in comparison with olaparib-treated parental cells using Student’s t test.

BRCA/DNA-PK-deficient Lin−CD34+ t-MDS/AML primary cells from 3 patients were more sensitive to BMN673 and BMN673 + DNR in comparison with BRCA/DNA-PK-proficient counterparts from 3 patients (Figure 6C). Consistent with this finding, immature Lin−CD34−CTVlo proliferating cells and Lin−CD34−CTVmax quiescent cells from BRCA/DNA-PK-deficient t-MDS/AMLs were much more sensitive to BMN673 alone and in combination with DNR in comparison with the counterparts from BRCA/DNA-PK-proficient samples (Figure 6, D and E). BMN673 + DNR exerted a borderline synergistic effect against BRCA/DNA-PK-deficient cells.

To design a more diagnostically oriented approach for predicting BRCA/DNA-PK deficiency we employed RT-qPCR to quantify expression of DSB repair genes compared with a housekeeping gene such as COX6B. Our data suggest that BRCA1 and LIG4 mRNA levels lower than 1,100% and 2%, respectively, of that encoded by COX6B is predictive of BRCA/DNA-PK deficiency in 2 of 11 (18%) samples, resulting in the highest sensitivity to BMN673-driven synthetic lethality (Supplemental Figure 6, A–C). Meta-analysis of the B cell acute lymphoblastic leukemia (B-ALL) transcriptome database supported our observation that a cohort of B-ALLs were BRCA/DNA-PK deficient and that BRCA1 and LIG4 were frequently downregulated in these cases (Supplemental Figure 6D).

Altogether, gene expression profiling by mRNA microarrays and by RT-qPCR followed by flow cytometry identified individual cases of BRCA/DNA-PK-deficient AMLs/ALLs, in which immature quiescent and proliferating cells were highly sensitive to PARP1i alone or in combination with the approved first-line AML therapy drug, DNR.

PARP1i exerted an antileukemic effect against BRCA/DNA-PK-deficient primary leukemia xenografts. NSG mice bearing BRCA/DNA-PK-deficient or −proficient primary leukemia xenografts (PLXs) were left untreated or treated with vehicle (control), doxorubicin (DA), BMN673, or DA + BMN673 (Figure 7A). Therapeutic effect was measured by detection of hCD45+ cells in...
Figure 4. mRNA microarrays identified BRCA/DNA-PK-deficient AMLs. (A) Gene expression analysis of DSB repair genes in 172 AML samples. (B) BRCA/DNA-PK-deficient and -proficient samples; those used in further analyses are marked by colored squares at the bottom. (C) Flow cytometric analysis of DSB repair proteins in Lin-Ki67+ cells from individual AMLs predicted to be BRCA/DNA-PK deficient (blue) or proficient (red). Each AML sample was measured 3–6 times.* P < 0.001 in comparison with all BRCA/DNA-PK-proficient samples using Student’s t test.
peripheral blood and by median survival time (MST). BRCA/DNA-PK–deficient and –proficient PLXs were preselected based on microarray analysis (Figure 7B), by in vitro sensitivity to BMN673 alone or in combination with DNR (Figure 7C), and by nuclear foci formation or flow cytometric detection of DSB repair proteins (Supplemental Figure 5, C and D).

Control mice succumbed to leukemias characterized by splenomegaly (3- to 15-fold enlarged spleen) and the presence of hCD45+ cells in peripheral blood. Approximately 60% and 79% of these hCD45+ cells were also CD33+ or CD19+, respectively, thus confirming the development of myeloid and lymphoid malignancies. BMN673 alone and a combination of DA + BMN673 reduced the number of BRCA/DNA-PK–deficient hCD45+ AML and B-ALL cells, but did not affect BRCA/DNA-PK–proficient B-ALL cells (Figure 7D).

Moreover, DA + BMN673 exerted synergistic and additive effects against BRCA/DNA-PK–deficient AML and ALL, respectively. Selective elimination of BRCA/DNA-PK–deficient hCD45+ PLX cells by BMN673 and DA + BMN673 was associated with extended disease latency (Figure 7E). A cohort of mice treated with DA + BMN673 survived more than 150 (AML) and 120 (ALL) days and hCD45+ cells were not detected in these mice.

In addition, 2 × 10^6 bone marrow cells (BMCs) from untreated and DA + BMN673–treated individual mice bearing BRCA/DNA-PK–deficient or –proficient AML and ALL, respectively. Selective elimination of BRCA/DNA-PK–deficient hCD45+ PLX cells by BMN673 and DA + BMN673 was associated with extended disease latency (Figure 7E). A cohort of mice treated with DA + BMN673 survived more than 150 (AML) and 120 (ALL) days and hCD45+ cells were not detected in these mice.

In addition, 2 × 10^6 bone marrow cells (BMCs) from untreated and DA + BMN673–treated individual mice bearing BRCA/DNA-PK–deficient or –proficient AML and ALL, respectively. Selective elimination of BRCA/DNA-PK–deficient hCD45+ PLX cells by BMN673 and DA + BMN673 was associated with extended disease latency (Figure 7E). A cohort of mice treated with DA + BMN673 survived more than 150 (AML) and 120 (ALL) days and hCD45+ cells were not detected in these mice.

In addition, 2 × 10^6 bone marrow cells (BMCs) from untreated and DA + BMN673–treated individual mice bearing BRCA/DNA-PK–deficient or –proficient AML and ALL, respectively. Selective elimination of BRCA/DNA-PK–deficient hCD45+ PLX cells by BMN673 and DA + BMN673 was associated with extended disease latency (Figure 7E). A cohort of mice treated with DA + BMN673 survived more than 150 (AML) and 120 (ALL) days and hCD45+ cells were not detected in these mice.

In addition, 2 × 10^6 bone marrow cells (BMCs) from untreated and DA + BMN673–treated individual mice bearing BRCA/DNA-PK–deficient or –proficient AML and ALL, respectively. Selective elimination of BRCA/DNA-PK–deficient hCD45+ PLX cells by BMN673 and DA + BMN673 was associated with extended disease latency (Figure 7E). A cohort of mice treated with DA + BMN673 survived more than 150 (AML) and 120 (ALL) days and hCD45+ cells were not detected in these mice.
The combination of TKI imatinib and PARP1 inhibitor olaparib synergistically abrogated clonogenic potential of Lin–CD34+ immature cells from CML-CP and also from more aggressive CML accelerated phase (CML-AP) patient samples (Figure 8E); the effect was preceded by the accumulation of DSBs measured by $\gamma$-H2AX and induction of apoptosis detected by activation of caspase 3 (Figure 8F).

Olaparib also eliminated Lin–CD34+CTVmax quiescent CML-CP cells (Figure 8G) and imatinib plus olaparib reduced the number of LSC-enriched Lin–CD34+CD38–CTVmax quiescent CML-CP cells (Figure 8H). The latter effect was accompanied by an accumulation of DSBs and annexin V–positive apoptotic cells (Figure 8H). Moreover, olaparib combined with imatinib or ponatinib exerted synergistic effects against BCR-ABL1–positive and BCR-ABL1(T315I)–positive B-ALL, respectively (Supplemental Figure 8).

Meta-analyses of transcriptome databases and other reports suggested that the fusion oncoprotein AML1-ETO, but not the oncogenic partners HOXA9 and MEIS1 or the oncogenic mutant FLT3/ITD, may negatively modulate BRCA and DNA-PK pathways (20–22). In fact, AML1-ETO–transformed mBMCs display decreased expression of BRCA1, BRCA2, and/or KU70 proteins in comparison with HOXA9 + MEIS1–transformed cells (Supplemental Figure 9A). Parp1–/– mBMCs expressing AML1-ETO, but not HOXA9 + MEIS1, demonstrated reduced clonogenic activity in comparison with cells expressing HOXA9 + MEIS1 or GFP only (Supplemental Figure 9C). In addition, olaparib eliminated Lin–CD34+ immature leukemia cells, including LSC-enriched Lin–CD34+CD38 CFSElow proliferating and Lin–CD34+CD38 CFSEquiescent cell populations from AML patient samples carrying t(8;21)(q22;q22) encoding for AML1-ETO (Supplemental Figure 9D). Moreover, the combination of olaparib and low doses of first-
imatinib or BMN673 applied individually reduced the number of LSC-enriched Lin–CD34+CD38– cells and Lin–CD34+CD38+ LPCs (23) by 2.5- to 4-fold, whereas the combination reduced the LSC-enriched population and LPCs by 24- and 13-fold, respectively (Figure 9E). Imatinib + BMN673 exerted borderline synergistic effects against LSC-enriched Lin–CD34+CD38– cells.

In addition, sublethally irradiated SCID mice were injected with GFP+ BCR-ABL1–positive murine leukemia cells followed by imatinib and/or BMN673 treatment (Figure 9F). When applied individually, imatinib or BMN673 reduced the leukemia burden in peripheral blood by 7- to 10-fold, whereas the combination exerted a synergistic effect and caused a 40-fold reduction (Figure 9G). This effect was associated with prolongation of the MST of leukemic mice treated with imatinib or BMN673 by 1.7- to 1.8-fold and by 4.4-fold of those treated with a combination (Figure 9H). Two out of 6 mice treated with imatinib + BMN673 survived more than 150 days and GFP+ cells were not detected in these animals.

PARPi exerted an antileukemic effect in vivo against BCR-ABL1–positive CML. To test the therapeutic potential of PARPi in CML, sublethally irradiated NSG mice were injected with 2.5 × 10⁶ Lin–CD34+ CML-CP cells obtained from 4 individual patients followed by treatment with vehicle, imatinib, olaparib, or imatinib + olaparib (Figure 9A). Since CML-CP cells usually do not induce lethal disease, we monitored leukemia cells in bone marrow 7 days after treatment. Imatinib or olaparib reduced the number of hCD45+ leukemia cells by 2- to 3-fold; in contrast, combination of these drugs caused a greater than 5-fold reduction of leukemia cells (Figure 9B and C).

The above experiments were repeated using 1 × 10⁷ Lin–CD34+ cells from a more advanced CML-AP patient; 1 week following injection mice were treated with vehicle, imatinib, BMN673, or imatinib + BMN673 (Figure 9A). Imatinib or BMN673 diminished the number of hCD45+ cells by 2- to 2.5-fold, whereas imatinib + BMN673 exerted a 7-fold reduction (Figure 9D). Moreover, imatinib or BMN673 applied individually reduced the number of LSC-enriched Lin CD34+CD38– cells and Lin CD34+CD38– LPCs (23) by 2.5- to 4-fold, whereas the combination reduced the LSC-enriched population and LPCs by 24- and 13-fold, respectively (Figure 9E). Imatinib + BMN673 exerted borderline synergistic effects against LSC-enriched Lin CD34+CD38– cells.

In addition, sublethally irradiated SCID mice were injected with GFP+ BCR-ABL1–positive murine leukemia cells followed by imatinib and/or BMN673 treatment (Figure 9F). When applied individually, imatinib or BMN673 reduced the leukemia burden in peripheral blood by 7- to 10-fold, whereas the combination exerted a synergistic effect and caused a 40-fold reduction (Figure 9G). This effect was associated with prolongation of the MST of leukemic mice treated with imatinib or BMN673 by 1.7- to 1.8-fold and by 4.4-fold of those treated with a combination (Figure 9H). Two out of 6 mice treated with imatinib + BMN673 survived more than 150 days and GFP+ cells were not detected in these animals.
The success of the PARP1 inhibitor olaparib in BRCA1- and BRCA2-deficient breast tumors has established a proof of concept for personalized cancer therapy utilizing synthetic lethality (6, 7). Tumors from individual patients predicted to be sensitive to PARP1i are usually identified either by detecting mutations in genes encoding BRCA pathway proteins, epigenetic-mediated downregulation of BRCA pathway protein expression, or by functional tests uncovering synthetic lethality.

Discussion

Quiescent and proliferating tumor cells accumulate spontaneous and treatment-induced DSBs, which are eventually repaired and thereby tolerated (18, 24, 25). Inhibition of DSB repair has emerged as a promising antitumor treatment, especially in the context of synthetic lethality, which targets specific pathways to eradicate tumor cells while sparing normal cells (26).
mal residual disease and disease relapse (8). Therefore, we postulate that a precision medicine–guided PARP1i-mediated dual cellular synthetic lethality approach that simultaneously targets quiescent and proliferating cancer stem and progenitor cells could be curative. In contrast, considering inefficient RAD51 foci formation (26). While these approaches may be effective at targeting proliferating cells, they are not able to simultaneously eradicate quiescent therapy-refractory cancer stem cells (3). However, quiescent LSCs are often responsible for minimal residual disease and disease relapse (8). Therefore, we postulated that a precision medicine–guided PARP1i-mediated dual cellular synthetic lethality approach that simultaneously targets quiescent and proliferating cancer stem and progenitor cells could be curative.

Figure 9. PARP1i exerted a therapeutic effect in mice bearing BCR-ABL1–positive leukemia. (A) Experimental design: sublethally irradiated NSG mice were injected with Lin CD34+ CML-CP cells (B and C) or Lin CD34+ CML-AP cells (D and E) followed by treatment with vehicle (control), imatinib (IM), BMN673, olaparib, IM + BMN673, or IM + olaparib for 14 consecutive days. Leukemia burden was assessed by detection of indicated human cells in mBMCs. (B) hCD45+ CML-CP cells (n = 4–8/group). *P ≤ 0.003, **P < 0.02 in comparison with control and IM or olaparib, respectively, using Student’s t test adjusted for multiple comparisons. (C) Percentage of hCD45+ CML-CP cells (framed) in representative plots. (D) hCD45+ CML-AP cells (n = 4/group). *P < 0.01, **P ≤ 0.001 in comparison with control and IM or olaparib, respectively, using Student’s t test adjusted for multiple comparisons. (E) Percentage of Lin hCD34+CD38+ (red dots) and Lin hCD34+CD38− (blue dots) CML-AP cells; representative plots from 4 mice/group are shown. *P < 0.02, **P < 0.03 in comparison with control and IM, respectively, using Student’s t test. ***P = 0.08 in comparison with individually treated mice by 2-way ANOVA. (F) Experimental design: sublethally irradiated SCID mice were injected with GFP+BCR-ABL1 leukemia cells and 1 week later treated with vehicle (control), IM, BMN673, or IM + BMN673 for 14 consecutive days. GFP+ leukemia cells in peripheral blood leukocytes (PBLs) and survival were scored. (G) Percentages of GFP+BCR-ABL1 leukemia cells (green dots) in representative plots from 3–4 mice/group. *P = 0.01 in comparison with control using Student’s t test. **P < 0.02 in comparison with individually treated mice by 2-way ANOVA. (H) Survival curves and MST (n = 6 mice/group). *P < 0.001, **P < 0.001 in comparison with control and IM or BMN673, respectively, using Kaplan-Meier log-rank test.
As expected, PARP1 inhibitors (olaparib and BMN673) elevated lethal DSBs and promoted synthetic lethality in BRCA-deficient (BRCA1−/−, FANCA−/−, RAD54−/−) proliferating tumor cells. This effect is likely caused by the abrogation of PARP1-mediated stalled replication fork restart and/or inhibition of PARP1-mediated B-NHEJ (27, 28). In addition, we show that PARP1i elevated DSBs, resulting in synthetic lethality also in DNA-PK-deficient (XRCC6−/−, LIG4−/−) cells; this phenomenon is supported by previous reports that combined use of DNA-PK and PARP1 inhibitors radiosensitized cells (29) and that PARP1i inhibitor-treated Lig4−/− and Dclre1c−/− mouse embryonic fibroblasts accumulated DSBs, thereby reducing cell survival (30).

DNA-PK-deficient proliferating cells repair DSBs by HR and/or B-NHEJ (31); thus, PARP1i would abrogate B-NHEJ activity and also delay stalled replication fork restart and HR (32), which is consistent with a modest but significant degree of synthetic lethality. Interestingly, the lethal effect of PARP1i was more pronounced in DNA-PK-deficient quiescent rather than proliferating cells. Quiescent cells accumulate potentially lethal DSBs, which are repaired by NHEJ (D-NHEJ and/or B-NHEJ) (25, 31); thus, targeting PARP1 in DNA-PK-deficient quiescent cells is synthetically lethal. This idea is supported by the observation that radiation-induced DSBs in Lig4−/− cells reduced cell survival independently of DNA replication (30).

We report here that olaparib and BMN673 induced synthetic lethality in both proliferating and quiescent BRCA/DNA-PK-deficient cells. This dual cellular synthetic lethality effect results from the accumulation of lethal DSBs in PARP1 inhibitor-treated BRCA/DNA-PK-deficient cells in S-G2/M of the cell cycle, and in PARP1 inhibitor-treated DNA-PK-deficient cells in G0/G1 phase. However, it is plausible that BRCA/DNA-PK-deficient cells with PARP1i-induced DNA damage/DSBs can continue through the G2/M checkpoint and/or spindle-assembly checkpoint, leading to the accumulation of excessive lethal DNA damage/DSBs in DNA-PK-deficient G0/G1 cells, thus resulting in cell death (33, 34).

It has been reported that BRCA-deficient tumors with low D-NHEJ activity might be less responsive to PARP1i (35). These studies, however, relied on short-term disabling of the DNA-PK pathway by using genetic or pharmacologic approaches. BRCA/DNA-PK-deficient cells tested here harbored long-term DNA-PK deficiency, presumably enforcing substantial remodeling of DSB repair pathways, which promotes sensitivity to PARP1i. This inference is supported by this work and other reports that BRCA1-ABL1 leukemia cells, which display BRCA1 and DNA-PKcs deficiencies (16, 17), are highly sensitive to PARP1i, and that hyperactive NHEJ caused resistance to PARP1i in BRCA-deficient Fanca−/− and Fancc−/− hematopoietic stem and progenitor cells (36).

PARP1i-induced dual cellular synthetic lethality in BRCA/DNA-PK-deficient cell lines prompted us to test PARP1i in BRCA/DNA-PK-deficient individual tumors identified by our TCGA database may display BRCA/DNA-PK deficiency (Supplemental Figure 10). In addition, we have developed a potentially novel clinically oriented personalized medicine approach, which we refer to as GEMA, for identifying patients with AMLs/ALLs displaying BRCA/DNA-PK deficiency, which could benefit from PARP1i-induced dual cellular synthetic lethality. GEMA-guided PARP1i-mediated dual cellular synthetic lethality may have a broad application for cancer treatment considering that numerous individual samples from a variety of tumor types in TCGA database may display BRCA/DNA-PK deficiency (Supplemental Figure 11).
Methods

Primary cells. Peripheral blood and bone marrow samples from patients with newly diagnosed CML-CP, CML-AP, ALL, AML, and t-MDS/AML were obtained from: (a) Stem Cell and Leukemia Core Facility of the University of Pennsylvania, Philadelphia, Pennsylvania, USA; (b) Department of Internal Medicine I, Division of Hematology & Hemostaseology, Medical University of Vienna; (c) Princess Margaret Cancer Centre; (d) Department of Hematology, Institute of Hematology and Blood Transfusion, Warsaw, Poland; and (e) Division of Hematopoietic Stem Cell and Leukemia Research, City of Hope National Medical Center. Clinical annotations for these samples are listed in Supplemental Tables 1–4. Samples of normal hematopoietic cells were purchased from Cambrex Bio Science. Lin CD34+ cells were obtained from mononuclear fractions by magnetic sorting using the EasySep negative selection human progenitor cell enrichment cocktail followed by Human CD34 Positive Selection Cocktail (StemCell Technologies). PLXs containing greater than 80% and greater than 90% of AML and B-ALL cells, respectively, were used.

Cell lines. BRCA1-mutated and BRCA1-reconstituted MDA-MB-436 human breast carcinoma cells were obtained from Neil Johnson (Fox Chase Cancer Center). BRCA2+/– V-C8 and BRCA2+/– V79 hamster cell lines carrying the DR-GFP reporter cassette were obtained from Maria Jasim (Memorial Sloan Kettering Cancer Center, New York, New York, USA) (48). XRCC6+/– and parental mESC lines with EJ2-GFP and EJ5-GFP reporters and FANCA+/– and FANCA+/– XRCC6+/– mESC lines were obtained from Jeremy Stark (Beckman Research Institute of the City of Hope) (10). Nalm-6 parental cells and RAD54+/–, LIG4−/−, and RAD52−/– LIG4+/+ isogenic lines were purchased from Horizon.

Transgenic/knockout mice. SCLtTA p210BCR-ABL mice (19) were cross-bred with Parp1+/– mice (provided by Roberto Caricchio, Temple University School of Medicine) to generate SCLtTA p210BCR-ABL Parp1+/– and SCLtTA p210BCR-ABL Parp1−/– animals. Transgenic/knockout mice were identified by PCR of tail snap DNA. DNA isolation and purification from mice tails were performed using the REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich). Genotyping for the SCLtTA and p210BCR-ABL transgenes and Parp1 was performed using transgene/knockout-specific primers (Operon) and 2× GoTaq polymerase Master Mix (Promega). BCR-ABL1-specific primers (forward, 5′-GAGGTTGCAGAGTGGAGGAGAACA-3′; reverse, 5′-GGTACCGAGGTGTTTCTCCAGACTG-3′) amplified a 500-bp fragment using amplification conditions of 40 cycles at 94°C for 45 seconds, 55°C for 1 minute, and 72°C for 1 minute. SCLtTA-specific primers (tTA, 5′-TTTGTGATCTGGACATGTTGG-3′; SCL, 5′-AGAACAGAATTCAGGGTCTTC-3′) yielded a 750-bp product using amplification conditions consisting of 40 cycles at 94°C for 40 seconds, 60.5°C for 1 minute, and 72°C for 1 minute. Parp1-specific primers used were: forward, 5′-CATGTTTGATGGGAGATTTCC-3′; wild-type reverse, 5′-CAGGCGGAGCTAGAGGACCA-3′; and mutant reverse, 5′-CATGTTTGATGGGAGATTTCC-3′. The primers amplified a 112-bp fragment if wild type, a 350-bp fragment if Parp1 null, and both 112- and 350-bp fragments if heterozygous using amplification conditions consisting of 35 cycles at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 3 minutes. PCR products were run in a 1.5% agarose gel containing ethidium bromide, and visualized using the Gel Doc XR+ Molecular Imager System (Bio-Rad). Mice were provided with drinking water supplemented with 0.5 g/l tetracycline hydrochloride (Sigma-Aldrich) and leukemia was induced by withdrawal of tet-nuclease. CML-CP–like leukemia was characterized by splenomegaly and leukocytosis associated with expansion of mature myeloid cells as described previously (18, 19).

Transfection of Parp1+/– and Parp1−/– cells. mBMCs were infected with MSCV retrovirus containing AML1-ETO9a-ires-GFP, HOXA9-IRES-MEIS1, BCR-ABL1-IRES-GFP, and IRES-GFP as described previously (49). GFP+ cells were sorted and used for experiments.

Microarray analysis. Gene expression data for 15 genes were used to select BRCA/DNA-PK-deficient and –proficient AMLs from a data set of 172 samples arrayed on an Illumina HT-12 (supplemental data: AML gene expression levels). Peripheral blood or bone marrow samples were collected from subjects with AML after obtaining informed consent according to the procedures approved by the Research Ethics Board of the University Health Network (Toronto Princess Margaret Cancer Centre). The AML samples were cytogenetically normal. Bioinformatic analysis was performed in R. The log2 expression values for each gene were centered to a zero mean and Z scores were generated by dividing by the SD.

RT-qPCR

B-ALL. Total RNA from human leukemia cells was extracted using an RNeasy Plus Micro Kit (Qiagen) according to the manufacturer’s instructions. cDNA was generated using a poly(dT) oligonucleotide and SuperScript III Reverse Transcriptase according to the manufacturer’s protocol (Invitrogen). The levels of gene expression were measured using the SYBRGreenER mix (Invitrogen) with primers listed in Supplemental Table 5. The reactions were performed using the ABI 7900HT real-time PCR system (Applied Biosystems) and analyzed with the SigmaPlot version 12.0 software program.

t-MDS/AML. Human CD34+ cells from t-MDS patients and normal controls were isolated by using magnetic bead selection (Miltenyi Biotec). Total RNA was extracted using the RNeasy Plus Micro Kit. First-strand cDNA was synthesized from 200 cells using a Superscript III First-Strand Synthesis System (Invitrogen) and preamplified for 18 cycles, and qPCR analysis performed by multiplex qPCR using the Fluidigm system and primers listed in Supplemental Table 6. Results are expressed as the ratio to β2 microglobulin.

Detection of DSB repair proteins

Flow cytometry. The cells were fixed and permeabilized using a PerFix EXPOSE kit according to the manufacturer protocol (Beckman Coulter) followed by staining with specific antibodies: rabbit polyclonal anti-Ki67 (Abcam, ab15580), rabbit polyclonal anti-XRCC3 (Abcam, ab13736), rabbit polyclonal anti-ligase IV (Abcam, ab26039), mouse monoclonal IgG2b anti-BRCA1 (R&D Systems, MAB22101), mouse monoclonal IgG1 anti-BRCA2 (R&D Systems, MAB2476), rabbit polyclonal anti-PALB2 (Bethyl Laboratories, A301-246), rabbit polyclonal anti-KU80 (Bethyl Laboratories, A302-623A), rabbit polyclonal anti-DNA-PKcs (Bethyl Laboratories, A300-518A). For FACS analysis the antibodies were conjugated with fluorochromes (Alexa Fluor 405, Alexa Fluor 488, or allophycocyanin) using a Zenon labeling kit (Life Technologies). Isotype control antibodies (Abcam) were labeled and used as a background staining control. For DSB detection the rabbit polyclonal antibody against histone H2A.X phosphorylated at Ser193 (Biologend, 613403) was used. Flow cytometric analysis was performed using the LSFRFortessa (Becton Dickinson) equipped with lasers 355, 405, 488, and 635 nm.
Western analysis. Cells were lysed at 95°C in buffer containing 50 mM Tris-HCl pH 6.8, 10% glycerol, and 2% SDS. Cell lysates were analyzed by Western blot using the antibodies detecting BRCA1 (R&D Systems, MAB22101), BRCA2 (R&D Systems, MAB2476), XRCC3 (Abcam, ab133736), RAD51 (Santa Cruz Biotechnology, sc6862), DNA-PKcs (Bethyl Laboratories, A300-518A), Ku70 (Bethyl Laboratories, A302-623A), Ku80 (Thermo Fisher Scientific, MA5-15873), cleaved caspase 3 (Cell Signaling Technology, 9661), PARylated proteins (Trevigen, 4335-MC-100), and β-actin (Sigma-Aldrich, A5316).

Immunofluorescence. Nuclear expression of DNA-PKCs, Ku70, BRCA1, and RAD51 was quantified as previously described with modifications (50). Briefly, Lin CD34+ cells were cytospun followed by co-staining with anti-Ki67 and anti-DNA-PKcs, -Ku70, -BRCA1, or -RAD51 antibodies. Secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 568 were applied (Molecular Probes, A-11034 and A-11004). DNA nuclear staining was visualized using an inverted Olympus IX70 fluorescence microscope equipped with a 100× UPlan Apo lens (numeric aperture 1.35), and a Cooke Sensicam QE camera (The Cooke Company). Images were acquired with Slidebook 3.0 (Intelligent Imaging Innovations). All graphic adjustments were performed using Adobe Photoshop.

HRR and NHEJ assays. Xrcc6−/− and parental mESCs with EJ2-GFP and EJ5-GFP reporter cassettes and Brca2−/− V-C8 and Brca2−/−V79 cells carrying DR-GFP reporter cassette were cotransfected with pCBASce1 (encoding I-Sce1) and pDsRed (transfection efficiency control) plasmids using Lipofectamine 2000 (Invitrogen) as described previously (10, 50). Olaparib (Selleckchem, AZD2281) or vehicle (DMSO) was added immediately after removing the transfection complexes. The percentage of GFP+DsRed+ cells in the DsRed+ population was detected after 72 hours by flow cytometry to assess DSB repair activity.

In vitro treatment. TKIs such as imatinib and ponatinib, cytotoxic drugs (DNR, DA, cytarabine), and PARPi inhibitors olaparib and BMN673 (all from Selleckchem) were added to the indicated cells for 3 to 5 days. Murine hematopoietic leukemia cells were maintained in Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 10% FBS, 100 ng/ml stem cell factor (SCF), and 20 ng/ml IL-3. Primary human hematopoietic cells were incubated in IMDM supplemented with 10% FBS and growth factors (CML, AML, and normal counterparts = 100 ng/ml SCF, 20 ng/ml IL-3, 100 ng/ml Flt-3 ligand, 20 ng/ml G-CSF, 20 ng/ml IL-6; B-ALL = 10 ng/ml IL-7) followed by plating in Methocult (StemCell Technologies) when indicated; colonies were scored after 5 to 7 days. For quiescent/proliferating cells, Lin− cells were stained with ed anti-Lin (catalog 340546), anti-CD34 (catalog 347203 and 555821), and anti-CD38 (catalog 355790 and 555460) antibodies (all from BD PharMingen) as described previously (18). Cell viability was determined either by trypan blue exclusion or by annexin V staining.

In vivo treatment. ICR SCID mice (Taconic Biosciences) were total-body irradiated (250 cGy) and injected i.v. with 1 × 10⁶ BCR-ABL1-transformed GFP+ mBMCs. NSG mice (The Jackson Laboratory) were total-body irradiated (250 cGy) and inoculated i.v. with 1 × 10⁶ AML and ALL PLX cells. 25 × 10⁶ CD34+ CML-CP cells, or 1 × 10⁶ CD34+ CML-AP cells. Seven or 10 days later mice were treated with vehicle (control), BMN673 (0.33 mg/kg/day by oral gavage for 7 or 14 days; see ref. 51), olaparib (50 mg/kg daily i.p. for 14 days; see ref. 52), DA (1.5 mg/kg/day i.v. [days 1-3]) + ara-C (50 mg/kg i.v. [days 1-5]; see ref. 53), imatinib (100 mg/kg twice daily by oral gavage; see ref. 54), and a combination of imatinib + BMN673/olaparib or DA + BMN673. Leukemia burden was analyzed by flow cytometry 7 days after the end of treatment. Mice leukemia cells were GFP+. Human leukemia cells were detected by anti-human CD45 antibody and leukemia stem and progenitor cells were detected by anti-human CD34 and CD38 antibodies as described previously (18). Median survival time was determined.

Statistics. Data are expressed as mean ± SD. When conducting subgroup comparisons between 2 groups, a 2-tailed t test was used. When multiple comparisons were conducted using a t test, Bonferroni’s method was used for adjusting P values for multiple testing. The synergistic effect was evaluated by 2-way ANOVA. MST of the mice ± SEM was calculated by Kaplan-Meier log-rank survival analysis. P values less than 0.05 were considered significant.

Study approval. Human studies were approved by the appropriate IRBs and met all requirements of the Declaration of Helsinki. Animal studies were approved by the Temple University IACUC.

Author contributions

MNS performed in vitro experiments with primary cells and assisted with experiments in mice. KS performed experiments with AML/ALL PLX and BCR-ABL1 leukemia in mice. YD tested dual cellular synthetic lethality in adherent cell lines and examined HR and NHEJ activities in primary cells. PPB analyzed protein expression by immunofluorescence. GH performed in vivo experiments in NSG mice injected with CML cells. SM generated data in NALM-6 cell lines. EM and DDM generated in vitro data with AML1-ETO in Parp1−/− and wild-type BMCs. EBG assisted in analyzing the effect of PARP1i on quiescent and proliferating cells. KCM performed experiments using B-ALL PLX cells. JL analyzed expression of HR and NHEJ genes in B-ALLs by RT-qPCR. KE and LB performed meta-analyses of gene expression patterns in the cohorts of AMLs. AS assessed the expression of DNA repair proteins in CML cells. ML, SB, and RB obtained t-MDS/AML cells and analyzed them by RT-qPCR. DG performed TCGA analyses. SCR, IS, and PV provided primary CML cells. T. Stoklosa contributed to the in vivo studies with CML cells. KP supervised PPB. CIC contributed AML/ALL PLX cells. MM supervised JL, JED, JCYW, MDM, and KE shared data from transcriptome microarrays on AML samples. MDM provided AML samples. HZ performed statistical analyses. HG provided materials and expertise in measuring HR and NHEJ in primary cells. VM supervised EM and DDM. T. Skorski designed the studies, supervised the experiments, and wrote the manuscript.

Acknowledgments

This work was funded by NIH/NCI grants R01 CA134458 and CA186238, Leukemia and Lymphoma Society New Idea Award NIA-89999-14, and Leukemia SPORE Developmental Research Award from MD Anderson Cancer Center (all to T. Skorski); NIH/NCI R00 CA158461, the W.W. Smith Foundation, and the American Society of Hematology Junior Scholar Award (all to S.M.S);
the European Union Innovative Economy Operational Program: Innovative methods of stem cell applications in medicine (grant 01.01.02-00-109/09-00) (to G.H.); the Canadian Institutes of Health Research and Cole Foundation (to K.E.); EU program: FP7-REGPOT-2012-CT2012-316254-BASTION (to T. Stoklosa); Poland National Science Centre grants UMO-2014/15/D/N23/05187 (to P.P.B.) and 2011/01/B/N23/02145 (to K.P.); the Rotary Foundation Global Grant Scholarship in the field of “Disease Prevention and Treatment” (to D.D.M.); and the Austrian Science Funds (FWF) SFB grant F4704-B20 (to P.V.). We thank Jaroslav Jelinek (Fels Institute for Cancer Research & Molecular Biology, Temple University School of Medicine) for help with analysis of TCGA database.

Address correspondence to: Tomasz Skorski, Temple University Lewis Katz School of Medicine, Department of Microbiology and Immunology, 3400 North Broad Street, MRB 548, Philadelphia, Pennsylvania, 19140, USA. Phone: 215.707.9157; E-mail: tskorski@temple.edu.