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.
Gene expression and mutation-guided synthetic lethality eradicates proliferating and quiescent leukemia cells

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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) (D-NHEJ) in proliferating cells, whereas D-NHEJ plays a major role in quiescent cells (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 [RAD51B], 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).

Collectively, these data strongly suggest that PARP1i selectively inhibited B-NHEJ that promoted accumulation of lethal DSBs, resulting in dual cellular synthetic lethality through the eradication of BRCA/DNA-PK–deficient proliferating and quiescent cells.

PARP1i eradicated BRCA/DNA-PK–deficient proliferating and quiescent immature leukemia cells from individual AMLs and ALLs identified by gene expression analysis. To identify BRCA/DNA-PK–deficient individual leukemias we examined the expression of (a) HR genes, that when absent/mutated caused synthetic lethality with PARP1i (6, 7, 12), and (b) key D-NHEJ genes (13).

Our hypothesis was supported by detection of wide-range expression levels of BRCA1 and PRKDC (encodes for DNA-PK catalytic subunit = DNA-PKcs) in existing databases of individual patient leukemia samples (Supplemental Figure 3, A–C). Moreover, meta-analysis of 166 acute myeloid leukemia (AML) patient 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 [RAD51B], 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-
BRCA/DNA-PK–deficient Lin–AML primary cells from individual patients were more sensitive to BMN673 and BMN673 + 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+ CTV0 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).

Next, we sought to validate the results of these experiments by RT-qPCR analysis of 26 therapy-related myelodysplastic syndrome (t-MDS)/AML samples. We identified 3 samples (12%) that displayed BRCA/DNA-PK deficiency (Z score < –1.5 of at least 1 gene in BRCA and DNA-PK pathways) and 11 that 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 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, $2 \times 10^6$ bone marrow cells (BMCs) from untreated and DA + BMN673-treated individual mice bearing BRCA/DNA-PK-deficient PLX (AML and ALL) were transplanted to secondary recipient mice. All mice injected with cells from untreated donors died within 27 (AML) and 21 (ALL) days, whereas 6 of 8 (AML) and 4 of 8 (ALL) recipients of cells from DA + BMN673-treated donors survived 100 days or more (Supplemental Figure 7). Recipients of AML/ALL cells from mice treated with DA + BMN673 demonstrated significantly prolonged survival, suggesting that LSCs were targeted.

PARPi eradicated BRCA1/DNA-PK-deficient proliferating and quiescent immature leukemia cells identified by gene mutation analysis. BCR-ABL1 oncogenic tyrosine kinase causes downregulation of BRCA1 and DNA-PKcs protein expression (16, 17). In concordance, we detected downregulation of DNA-PKcs and/or BRCA1 in BCR-ABL1–positive chronic myeloid leukemia (CML) in chronic phase (CML-CP) Lin–CD34+Ki67– quiescent and Lin–CD34+Ki67+ proliferating immature cells, respectively (Figure 8A). These observations combined with high levels of ROS-induced DSBs in untreated and tyrosine kinase inhibitor (TKI)–treated CML-CP quiescent and proliferating LSCs and LPCs (18) create an opportunity to trigger dual cellular synthetic lethality by PARP1i.

BCR-ABL1–transformed Parp1−/− murine BMCs (mBMCs) formed approximately 2.5-fold fewer colonies than their Parp1+/+ counterparts (Figure 8B). To determine the role of PARP1 in disease-mimicking settings, a tet-off SCLTαTA p210BCR-ABL1–inducible mouse model of CML-CP was employed (19). CML-CP–like disease latency was prolonged in SCLTαTA p210BCR-ABL1 Parp1−/− mice when compared with SCLTαTA p210BCR-ABL1 Parp1+/+ mice (Figure 8C). Altogether, it appears that PARP1 plays an important role in BCR-ABL1–mediated leukemogenesis.

Olaparib selectively reduced clonogenic activity of Lin–CD34+ CML-CP immature cells in a dose-dependent manner (Figure 8D).

**Figure 5.** BRCA/DNA-PK-deficient AMLs were sensitive to dual cellular synthetic lethality exerted by BMN673. (A) Lin− cells from BRCA/DNA-PK-deficient (blue, n = 8) and BRCA/DNA-PK-proficient (red, n = 4) samples treated with BMN673 (left) and DNR + BMN673 (right). (B) γ-H2AX immunofluorescence in BRCA/DNA-PK-deficient (blue) and BRCA/DNA-PK-proficient (red) cells (n = 3–5) untreated (control) and treated with 1 μg/ml DNR, 12.5 nM BMN673, or DNR + BMN673. *P < 0.05 in comparison with DNR-treated counterparts by Student’s t test. (C) Number of proliferating Lin CD38 CTV+ and quiescent Lin CD38 CTV− cells from individual BRCA/DNA-PK-deficient (blue) and –proficient (red) samples treated with DNR, BMN673, or DNR + BMN673. (D) Cumulative percentages from samples examined in C. *P < 0.02, **P < 0.05 in comparison with corresponding BRCA/DNA-PK-proficient samples using Student’s t test. **P < 0.03 in comparison with DNR- or BMN673-treated BRCA/DNA-PK-deficient samples using 2-way ANOVA. nt, not tested.
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 γ-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 Parp1+/+ counterparts (Supplemental Figure 9B). AML1-ETO–transformed Parp1–/– mBMCs were highly sensitive to olaparib 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 CFSEmax proliferating and Lin−CD34+CD38 CFSEmax quiescent 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-
line therapy drug DNR exerted anticolonogenic effects against cells expressing AML1-ETO, but not against those expressing HOXA9-MEIS1 or GFP (Supplemental Figure 9E).

PARP1i exerted an antileukemic effect in vivo against BCR-ABL1-positive CML. To test the therapeutic potential of PARP1i 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 9, B 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 leukemia 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 dependencies on BRCA dysfunction.
mal 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), imatinib, 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 (BCRA1−/−, 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 PARPi 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 PARPi inhibitors radiosensitized cells (29) and that PARPi inhibitor–treated Lig4−/− and Deleter−/− 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, PARPi 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 PARPi 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 PARPi 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 PARP inhibitor–treated BRCA/DNA-PK-deficient cells in S/G2/M of the cell cycle, and in PARPi inhibitor–treated DNA-PK-deficient cells in G0/G1 phase. However, it is plausible that BRCA/DNA-PK-deficient cells with PARPi-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 PARPi (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 PARPi. This inference is supported by this work and other reports that BCR-ABL1 leukemia cells, which display BRCA1 and DNA-PKcs deficiencies (16, 17), are highly sensitive to PARPi, and that hyperactive NHEJ caused resistance to PARPi in BRCA-deficient Fanca−/− and Fancc−/− hematopoietic stem and progenitor cells (36).

PARPi-induced dual cellular synthetic lethality in BRCA/DNA-PK-deficient cell lines prompted us to test PARPi in BRCA/DNA-PK-deficient individual tumors identified by our personalized medicine strategy referred to as GEMA. Leukemias were used as testing models because their hierarchical compartments of quiescent LSCs and proliferating LSCs/LPCs are well characterized (37). Our report suggests that PARPi eliminated not only proliferating LSCs/LPCs, but also therapy-refractory quiescent LSCs if they displayed BRCA/DNA-PK-deficient status detected by GEMA. The lethal effect of PARPi was associated with the accumulation of DSBs and apoptosis, and was selective for BRCA/DNA-PK-deficient leukemia cells, because normal cells and BRCA/DNA-PK-proficient leukemia cells were mostly spared.

Gene mutation profiling showed that leukemias expressing BCR-ABL1 (100% of CMLs and 2%-7% of ALLs in pediatric age and 20%-40% of ALLs in adults) and AML1-ETO (4%-12% of adult and 12%-30% of pediatric AMLs) are BRCA/DNA-PK deficient. The mechanisms responsible for the downregulation of genes in the BRCA/DNA-PK pathways in leukemia cells expressing BCR-ABL1 and AML1-ETO are characterized only partially. For example, BCR-ABL1 kinase inhibits BRCA1 and DNA-PKcs protein expression by suppression of translation and/or enhanced protein degradation (16, 17). While expression of these proteins is partially restored in imatinib-treated cells, other key members of HR (RAD51) and D-NHEJ (LIG4, DCLRE1C, NHEJ1) are strongly downregulated by imatinib, thus promoting PARPi-mediated synthetic lethality (38, 39). The BRCA2-deficient phenotype of AML1-ETO–positive cells may depend on lack of stimulation of HOXA9 (22).

GEMA, with emphasis on gene mutation analysis, may also be applied to identify additional oncogenes that sensitize cells to PARPi inhibitor treatment, for example TMPRSS2-ERG–positive prostate carcinomas (DNA-PKcs deficiency), EGFR mutant–positive lung carcinomas (Fanconi anemia deficiency), EWSR1-Fli1–positive Ewing’s sarcomas, and KRAS mutant–positive leukemias (40–43).

Moreover, using gene expression profiling we determined that 9%-25% of AMLs/ALLs that did not contain these oncogenes also displayed BRCA/DNA-PK deficiency. It is also plausible that additional BRCA/DNA-PK-deficient AMLs/ALLs could be identified if the list of target genes were to be expanded. For example, inclusion of Fanconi anemia genes (FANCA, FANCC, FANCG, FANCI) in addition to FANCD1 (also known as BRCA2), increased the pool of predicted BRCA/DNA-PK-deficient samples in TCGA database from 9% to 12%.

BRCA/DNA-PK deficiency in individual AMLs/ALLs detected by gene expression profiling may be due to epigenetic alterations. Analysis of methylome arrays revealed distinct BRCA and DNA-PK pathway methylome profiles in individual patients, which could be associated with reduced expression of methylated genes, resulting in sensitivity to PARPi (44). Differences in expression of heterogeneous nuclear ribonucleoproteins (e.g., hnRNP C) and miRNAs (e.g., miR-101 and miR-182) may also contribute to a BRCA/DNA-PK-deficient phenotype of individual AMLs/ALLs (45–47).

In conclusion, we postulate that PARPi-mediated dual cellular synthetic lethality may simultaneously eradicate BRCA/DNA-PK-deficient quiescent and proliferating LSCs/LPCs (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 PARPi-induced dual cellular synthetic lethality. GEMA-guided PARPi-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. BRCAl-mutated and BRCAl-reconstituted MDA-MB-436 human breast carcinoma cells were obtained from Neil Johnson (Fox Chase Cancer Center). BRCAl−/− V-C8 and BRCAl+/− V79 hamster cell lines carrying the DR-GFP reporter cassette were obtained from Maria Jasin (Memorial Sloan Kettering Cancer Center, New York, New York, USA) (48). XRCC6+/− and parental mESC lines with EJ2-GFP and EJS-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-ABL1 mice (19) were cross-bred with Parp1−/− mice (provided by Roberto Caricchio, Temple University School of Medicine) to generate SCLtTA p210BCR-ABL1 Parp1−/− and SCLtTA p210BCR-ABL1 Parp1+/− animals. Transgenic/knockout mice were identified by PCR of tail snip DNA. DNA isolation and purification from mice tails were performed using the RNeasy Plus Micro Kit (Qiagen). Genotyping for the SCLtTA and p210BCR-ABL1 transgenes and Parp1 was performed using transgene/knockout-specific primers (Operon) and 2× GoTaq polymerase Master Mix (Promega). BCR-ABL1-specific primers (forward, 5′-GAGCGTGCAGAGTGGAGGGAGAACA-3′; reverse, 5′-GGTACCAGGAGTGTTTCTCCAGACTG-3′) amplified a 750-bp product using amplification conditions of 40 cycles at 94°C for 30 seconds, 60.5°C for 1 minute, and 72°C for 1 minute.

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 SYBR GreenER 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 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 EXPO 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, ab133736), rabbit polyclonal anti-licigase IV (Abcam, ab26039), mouse monoclonal IgG2b anti-BRCAl (R&D Systems, MAB22101), mouse monoclonal IgG1 anti-BRCAl (R&D Systems, MAB2210), rabbit polyclonal anti-PALB2 (Bethyl Laboratories, A301-246), rabbit polyclonal anti-KU80 (Bethyl Laboratories, A302-627A), rabbit polyclonal anti-KU70 (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 (R&D Systems, MAB2193) was used. Flow cytometric analysis was performed using the LSRFortessa (Becton Dickinson) equipped with lasers 355, 405, 488, and 635 nm.

Transfection of Parp1−/− and Parp1+/− cells. mBMCs were infected with MSCV retroviruses 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 dataset 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.

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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, ab13376), 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).

Immunocytofluorescence. 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 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. Xrc6−/− 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-SceI) 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 DSBR 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 = human hematopoietic cells were incubated in IMDM supplemented with 10% FBS, 100 ng/ml stem cell factor (SCF), and 20 ng/ml IL-3, 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 CFSE, cell trace violet (CTV), or Cell Proliferation Dye eFluor 670 (CPD) (eBioscience) and incubated for 5 days in StemSpan SFEM medium (Stem Cell Technologies) supplemented with the cocktail of growth factors (see above) and inhibitors where indicated. In addition, quiescent/proliferating cells were detected by immunostaining with anti-Ki67 antibody. Quiescent (CFSE−/−, CPD−/−, or Ki67−) and proliferating (CFSE−/−, CPD−/−, or Ki67+) leukemia and normal hematopoietic stem and progenitor cells were detected by flow cytometry using fluorochrome-conjugated 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 × 107 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, 2.5 × 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. Murine 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 PARPi 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 expertise in measuring HR and NHEJ 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 PARPi 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. MDMA provided AML samples. NZ performed statistical analyses. VG provided materials and expertise in measuring HR and NHEJ in primary cells. SMS supervised EM and DDM. T. Skorski designed the studies, supervised the experiments, and wrote the manuscript.

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