Poly(ADP-ribose) Polymerase Inhibitors Sensitize Cancer Cells to Death Receptor-mediated Apoptosis by Enhancing Death Receptor Expression*

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Background: Factors affecting death ligand sensitivity are incompletely understood.

Results: PARP inhibitors increased CD95 and TNFRSF10B expression as well as death receptor-mediated killing in multiple cancer cell lines and clinical acute myelogenous leukemia samples in vitro.

Conclusion: PARP inhibitors increase death receptor signaling.

Significance: These observations add another dimension to the action of PARP inhibitors and death ligands in the clinic.

Recombinant human tumor necrosis factor-α-related apoptosis inducing ligand (TRAIL), agonistic monoclonal antibodies to TRAIL receptors, and small molecule TRAIL receptor agonists are in various stages of preclinical and early phase clinical testing as potential anticancer drugs. Accordingly, there is substantial interest in understanding factors that affect sensitivity to these agents. In the present study we observed that the poly(ADP-ribose) polymerase (PARP) inhibitors olaparib and veliparib sensitize the myeloid leukemia cell lines ML-1 and K562, the ovarian cancer line PEO1, non-small cell lung cancer line A549, and a majority of clinical AML isolates, but not normal marrow, to TRAIL. Further analysis demonstrated that PARP inhibitor treatment results in activation of the FAS and TNFRSF10B (death receptor 5 (DR5)) promoters, increased Fas and DR5 mRNA, and elevated cell surface expression of these receptors in sensitized cells. Chromatin immunoprecipitation demonstrated enhanced binding of the transcription factor Sp1 to the TNFRSF10B promoter in the presence of PARP inhibitor. Knockdown of PARP1 or PARP2 (but not PARP3 and PARP4) not only increased expression of Fas and DR5 at the mRNA and protein level, but also recapitulated the sensitizing effects of the PARP inhibition. Conversely, Sp1 knockdown diminished the PARP inhibitor effects. In view of the fact that TRAIL is part of the armamentarium of natural killer cells, these observations identify a new facet of PARP inhibitor action while simultaneously providing the mechanistic underpinnings of a novel therapeutic combination that warrants further investigation.

Despite being responsive to chemotherapy, acute myelogenous leukemia (AML)3 remains a deadly disease. In 2012, an estimated 13,780 new cases were diagnosed and 10,200 patients died of this disease in the United States (1). Accordingly, there remains an ongoing need for improved treatment of this disorder, especially therapies that will be active when cells are resistant to other agents.

One of the investigational strategies for eliminating cancer cells involves induction of apoptosis by ligation of the death receptor DR5, which is expressed predominantly on neoplastic cells (2, 3) and tumor blood vessels (4). According to current understanding (3, 5–7), binding of this receptor to its natural ligand TRAIL, agonistic antibodies, or agonistic small molecule activators (8) leads to a conformational change in the death receptor cytoplasmic domain, allowing the adaptor protein FADD to bind and initiate formation of the so-called death inducing signaling complex (DISC). FADD in turn binds and activates caspase 8 or caspase 10, initiating a cascade of cleavages that results in the apoptotic phenotype (9, 10). Based on the somewhat selective expression and action of DR5 in tumor cells leading to their enhanced TRAIL sensitivity (11), recombinant versions of TRAIL and agonistic anti-DR5 antibodies continue to be tested as potential antineoplastic agents (www.clinicaltrials.gov).

A parallel series of studies have brought PARP inhibitors into the clinic as well (12–16). PARP1, the founding member of the PARP family (17, 18), is an abundant nuclear enzyme that is activated 10–500-fold by binding to damaged DNA. Once activated, PARP1 not only modifies a wide range of substrates (19, 20) and participates in DNA repair (13, 21–24) but also

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3 The abbreviations used are: AML, acute myelogenous leukemia; APC, allopurinol; CI, combination index; DEVD-fmk, N-carboxyethyl-Asp-Glu-Val-Asp-fluoromethylketone; DISC, death-inducing signaling complex; DR, death receptor; FADD, Fas-associated protein with death domain; FBS, heat-inactivated fetal bovine serum; HSP90, heat shock protein 90; pADPr, poly(ADP-ribose) polymer; PARP, poly(ADP-ribose) polymerase; PBS, Dulbecco’s phosphate-buffered saline; qRT-PCR, quantitative RT-PCR; Q-V-D-OPH, N-(2-quinoilyl)valaspartyl-(2,6-difluoroxygenyl)methyl ketone; TRAIL, tumor necrosis factor-α-related apoptosis inducing ligand.
causes a variety of transcriptional changes within cells (25, 26). In addition to PARP1, PARP2 also responds to DNA damage (24, 27–29). The remaining 15 members of the PARP family share the ability to bind NAD and transfer ADP-ribose groups, although some are mono(ADP-ribosyl) transferases (13, 18).

In view of the involvement of PARP1 in the DNA damage response, PARP inhibitors were initially explored for their ability to sensitize cells to ionizing radiation and other DNA damaging chemotherapy (13–15). More recent studies have demonstrated that these agents are selectively toxic to breast and ovarian cancer cells lacking BRCA1, BRCA2, or other components of the Fanconi anemia/homologous recombination pathway. As a consequence, several of these agents are undergoing phase II and phase III clinical trials in cancers with homologous recombination deficiencies (12–16). There is, however, substantial interest in identifying opportunities to successfully use these agents in cancers that lack homologous recombination deficiency.

In the present study we report that PARP inhibitors synergize with death ligands. In particular, inhibition or knockdown of PARP1 and/or PARP2 results in enhanced expression of DR5 and Fas as well as enhanced sensitivity of cancer cell lines and clinical AML specimens to treatment with multiple death ligands, including agonistic anti-Fas antibody, recombinant human TRAIL, and agonistic anti-DR5 antibody. These results raise the possibility that responses to DR5 agonists might be enhanced by the addition of a relatively nontoxic and increasingly widely available new class of anticancer agents.

**Experimental Procedures**

**Materials**—Reagents were purchased from the following suppliers: olaparib (AZD2281) from ChemieTek (Indianapolis, IN); veliparib (ABT-888), N-((N-acetylaspartylglutamylvalinylamino)aspartate fluoromethyl ketone (DEVD-fmk), murine monoclonal antibody to poly(ADP-ribose) (pADPr, clone 10H), and adenosine 5'-diphosphate (hydroxymethyl)pyrrolidinediiodo from Enzo Life Science (Farmingdale, NY); CH.11 monoclonal IgM anti-human DR5, and agonistic anti-DR5 antibody. These results were performed using PARP1 and PARP2 knockdown.

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The targets of Sp1 shRNAs were 5'-GCCACTCXAAGTG-3' (NM_00103931.2:1481s) and 5'-CTTGGG-3' (NM_006437.3:1123s21c1). The targets of Sp1 shRNAs were 5'-GCCAGATCG-3' (NM_138473.2:2143s21c1, #2).

After viral infection on two successive days, cells were cultured in medium containing 10 μg/ml puromycin for at least a week before knockdown efficiency was examined by Western blot or quantitative RT-PCR (qRT-PCR) as described below. As an alternative, short oligonucleotides targeting human PARP1 (nucleotides 2242–2262 of coding sequence, GenBank accession number NM_001618.2) from Ambion (Austin, TX) were also used to assess the impact of PARP1 knockdown on TRAIL-induced apoptosis. In brief, 1 x 10<sup>7</sup>log phase K562 cells growing in antibiotic-free medium were suspended in 400 μl of medium containing 5 μg of pEGFP-histone H2B and 10 nm concentrations of control siRNA or PARP1 siRNA. After incubation for 5 min, cells were subjected to electroporation using a BTX830 square wave electroporator (BTX, San Diego, CA) delivering a single 10-ms pulse at 280 V. After a 24-h incubation, TRAIL was added for an additional 24 h before apoptosis was assessed.

**qRT-PCR**—RNA was extracted using an RNeasy Mini kit (Qiagen, Valencia, CA). qRT-PCR was performed in triplicate using 100 ng of RNA and TaqMan One-Step RT-PCR Master Mix (Invitrogen) per the supplier’s instructions. Using PARP1 (Hs00242303_m1), PARP2 (Hs00193931_m1), PARP3 (Hs00193946_m1), PARP4 (Hs00173105-m1), Fas (Hs00533110-m1), DR4 (Hs00269492_m1), DR5 (Hs00366278-m1), and GAPDH (4352934E) probe sets from Invitrogen, RT-PCR was performed on a ABI Prism 7900HT Real-time system using a program consisting of 15 s and 60 °C for 1 min. Data analysis was performed using the following equations: ΔCt = Ct(sample) − Ct(endogenous control).
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Assays for Apoptosis—After the indicated treatment, cells were stained with APC-conjugated annexin V in 140 mM NaCl, 2.5 mM CaCl₂, and 10 mM HEPES (pH 7.4) as previously described (30). Samples were analyzed on a BD Biosciences FACSCanto II flow cytometer using CellQuest software, and cells binding annexin V were regarded as apoptotic. Alternatively, cells were stained with propidium iodide (31) and subjected to flow microfluorimetry to detect cells with <2n DNA content (subdiploid population).

Analysis of Cell Surface Fas, DRS, and CD71—Aliquots containing 5 x 10⁵ cells were washed twice with ice-cold PBS containing 2% (v/v) FBS (PBS/FBS). Cells were incubated with the antibodies to Fas (APO-1-1) or CD71 or with APC-conjugated anti-TRAIL R2 (DR5) on ice for 60 min. To prevent any possible effect of caspase-8 activation due to incubation with the anti-death receptor antibodies, the pan-caspase inhibitor Q-VD-OPh (5 μM, Ref. 32) was included in the incubation. After 2 washes, cells stained with APC-conjugated anti-TRAIL R2 were fixed with 4% (w/v) formaldehyde for 30 min, whereas cells stained with APO-1-1 or anti-CD71 antibody were incubated with APC-coupled anti-mouse IgG for 45 min, washed, then fixed with 4% (w/v) formaldehyde for 30 min before flow microfluorimetry.

Chromatin Immunoprecipitation (ChIP)—ChIP was performed as described previously (39). In brief, after treatment with diluent or olaparib, ML-1 cells were cross-linked in medium containing 1% formaldehyde for 15 min. 2 x 10⁷ cells were washed in PBS, lysed in buffer containing 1% SDS, and sheared by sonication (Diagenode, Sparta, NJ) to fragment DNA to 200–1000 bp. Precleared chromatin was subjected to ChIP analysis using EZ-ChIP™ Kit regents (Millipore, Billerica, MA). Immunoprecipitation was performed at 4 °C overnight with anti-Sp1 antibody or rabbit IgG as a control. Semi-quantitative PCR was performed using the following primers encompassing the previously reported Sp1 binding site in the DR5 promoter: forward, 5'-AGATTGCCGTGCAGACT-3' and reverse, 5'-CCTGCGGTGCTATTATGTGTCGC-3'. 20 µl of each PCR product was subjected to 1.5% agarose gel electrophoresis.

Immunoprecipitation—ML-1 cells treated with diluent or 0.5 μM olaparib for 48 h were lysed in hot lysis buffer containing 1% (w/v) SDS, 30 mM Tris-HCl (pH 7.4), and 150 mM NaCl. Sam-

Differential SDS-PAGE—48 h after treatment with PARP inhibitor, cells were sedimented at 200 x g for 10 min, washed once with ice-cold RPMI 1640 medium containing 10% FCS (pH 7.4 at 4 °C), solubilized in buffered 6 M guanidine hydrochloride under reducing conditions, and prepared for electrophoresis (35). Aliquots containing 50 µg of protein were separated on SDS-polyacrylamide gels, electrophoretically transferred to nitrocellulose, and probed as indicated (36).

DISC Analysis—The Fas DISC was immunoprecipitated essentially as described previously (33, 37, 38). In brief, ML-1 cells were treated with DMSO or 0.5 µM olaparib for 48 h followed with 75 ng/ml CH.11 and 5 µM Q-VD-OPh for an additional 16 h. Aliquots containing 4 x 10⁵ cells were harvested, washed, and solubilized at 4 °C for 30 min in DISC buffer consisting of 1% (w/v) Triton X-100, 30 mM Tris (pH 7.4), 150 mM NaCl, 1% (v/v) glycerol, 1 mM PMSE, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, and 20 mM microcystin. After centrifugation at 14,000 x g for 15 min to remove insoluble material, aliquots containing the same amount of protein (27 mg as assessed using the bicinchoninic acid method) from each treatment were added to 10 µg of rabbit anti-mouse IgM that was precoupled to protein A- and G-agarose beads and incubated at 4 °C for 2 h. At the end of the incubation, beads were sedimented at 14,000 x g for 3 min and washed 5 times with DISC buffer. Immunoprecipitated complexes were released from the beads by boiling for 5 min in SDS sample buffer, subjected to SDS-PAGE, transferred to nitrocellulose, and probed with antibody to FADD or antibody to mouse IgM (indirectly reflecting the amount of Fas immunoprecipitated).

Human Fas and DR5 Promoter Luciferase Reporter Assay—Aliquots containing 10 µg of human Fas promoter in pGL-2 (constructed as described previously in Ref. 33) or human DR5 promoter in pGL-3 (from Addgene, Cambridge, MA) were transfected into ML-1 cells by electroporation at 280 V for 10 seconds. Aliquots containing 10⁵ cells were washed twice with ice-cold PBS containing 2% (v/v) FBS (PBS/FBS). Cells were incubated with the antibodies to Fas (APO-1-1) or CD71 or with APC-conjugated anti-TRAIL R2 (DR5) on ice for 60 min. To prevent any possible effect of caspase-8 activation due to incubation with the anti-death receptor antibodies, the pan-caspase inhibitor Q-VD-OPh (5 µM, Ref. 32) was included in the incubation. After 2 washes, cells stained with APC-conjugated anti-TRAIL R2 were fixed with 4% (w/v) formaldehyde for 30 min, whereas cells stained with APO-1-1 or anti-CD71 antibody were incubated with APC-coupled anti-mouse IgG for 45 min, washed, then fixed with 4% (w/v) formaldehyde for 30 min before flow microfluorimetry.

To assess whether PARP inhibitor affects Fas trafficking, cells were pretreated with PARP inhibitor or diluent for 24–48 h before the addition of mouse IgM control or CH.11 agonistic anti-Fas antibody for an additional 16 h in the presence of 5 µM Q-VD-OPh. After 2 washes with PBS/FBS, cells were incubated on ice with APC-coupled anti-mouse IgM for 45 min. After washes, cells were fixed with 4% (w/v) formaldehyde for 30 min and subjected to flow microfluorimetry.

Promoter in pGL-3 (from Addgene, Cambridge, MA) were transfected into ML-1 cells by electroporation at 280 V for 10 seconds. Aliquots containing 10⁵ cells were washed twice with ice-cold PBS containing 2% (v/v) FBS (PBS/FBS). Cells were incubated with the antibodies to Fas (APO-1-1) or CD71 or with APC-conjugated anti-TRAIL R2 (DR5) on ice for 60 min. To prevent any possible effect of caspase-8 activation due to incubation with the anti-death receptor antibodies, the pan-caspase inhibitor Q-VD-OPh (5 µM, Ref. 32) was included in the incubation. After 2 washes, cells stained with APC-conjugated anti-TRAIL R2 were fixed with 4% (w/v) formaldehyde for 30 min, whereas cells stained with APO-1-1 or anti-CD71 antibody were incubated with APC-coupled anti-mouse IgG for 45 min. After washes, cells were fixed with 4% (w/v) formaldehyde for 30 min after flow microfluorimetry.
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FIGURE 1. PARP inhibitors synergistically enhance TRAIL-induced apoptosis in ML-1 cells. A, ML-1 cells were pretreated with the indicated PARP inhibitor for 24 h followed by the addition of diluent or 12.5 ng/ml TRAIL for an additional 24 h. At the completion of incubation, cells were stained with APC-conjugated annexin V and analyzed by flow microfluorimetry. The percentage of annexin V-positive cells is indicated in each histogram. B and D, summary of experiments performed with veliparib (B) and olaparib (D) as depicted in panel A. Left panels show results of single experiments using varying concentrations of TRAIL along with varying concentrations of veliparib (top) or olaparib (bottom). Right panels, CI values calculated by the method of Chou and Talalay (44) from results shown in the left hand graphs. Note that CI < 1.0 indicates synergy. C and E, summarized results obtained with varying concentrations of each PARP inhibitor at 6.25 ng/ml TRAIL. Error bars, ± S.D. of three independent experiments. *, p < 0.01 compared with TRAIL treatment without PARP inhibitor. Values at intermediate PARP inhibitor concentrations approached but did not reach p < 0.05 after Bonferroni correction.

For immunoprecipitation of pADPr, 300 μg of nuclear extract (supernatant) was precleared with protein G-agarose for 2 h at 4°C. After centrifugation, the supernatant was incubated with 4 μg of anti-pADPr antibody (10H) or murine IgG control at 4°C for 2 h followed by incubation with protein G-agarose overnight. After beads were washed with DISC buffer 5 times, immunoprecipitates were processed as described above and probed with antibodies as indicated.

Assessment of Fas Expression in ML-1 Xenografts—Under a protocol approved by the Mayo Clinic Institutional Animal Care and Use Committee, female athymic nu/nu mice (4–5 weeks, Harlan) were implanted with 100 μl of a 1:1 slurry containing Matrigel (BD Bioscience) and 5 × 10⁶ washed, log phase ML-1 cells in the right flank. When the tumors reached an average volume of 100 mm³, mice were randomly given 50 mg/kg olaparib in 20% Trappsol (Cyclodextrin Technologies Development, Inc.) or diluent by intraperitoneal injection at 24 h intervals for 3 doses. 6 h after the third injection, mice were euthanized by CO₂ overdose under isofluorane anesthesia. After a single-cell suspension was prepared by passing the tumor cells through fine mesh screens, Fas expression was assayed as described above by incubating 5 × 10⁵ cells with isotype control, 200 ng of CH.11, or 2 μg of anti-APO-1 at 4°C for 90 min followed incubation with APC-coupled anti-mouse IgM or APC-coupled anti-mouse IgG.

Leukemic and Normal Myeloid Progenitor Clonogenic Assays—After informed consent was obtained under the aegis of Institutional Review Board-approved protocols, 11 AML bone marrow aspirates and two normal samples were accessioned. The diagnosis of AML was established by morphological examination, flow cytometry after staining with a standard panel of lymphoid and myeloid cluster of differentiation antigens, and marrow cytogenetics (41). Immediately after isolation, mononuclear cells from these samples were washed with RPMI 1640 medium, resuspended at 1.5 × 10⁶ cells/ml in Iscove’s modified Dulbecco’s medium containing 20% (v/v) heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin G, 100 μg/ml streptomycin, and 2 mM glutamine, and plated with the indicated concentrations of olaparib and TRAIL at 600,000 cells/plate in MethoCult® methylcellulose (StemCell Technologies, Vancouver, British Columbia, Canada), which contains the growth factors G-CSF, GM-CSF, and interleukin-3. Leukemic colonies (AML samples) or normal myeloid progenitors (normal bone marrows) were counted based on morphological criteria (42) after a 14-day incubation as instructed by the supplier.

Statistics—Unless otherwise indicated, experiments were repeated at least three times. Where results of multiple experiments are summarized, data were expressed as the mean ± S.D. of the indicated number of independent experiments. For experiments with two groups, results were compared using two-tailed paired Student’s t test unless otherwise indicated. For experiments with more than two groups, differences
between treatments were analyzed by analysis of variance using StatView5 (SAS), and p values were subjected to a Bonferroni correction (43) to yield numbers stated in the figure legends. Combination indices (CIs) were calculated according to the method of Chou and Talalay (44) under the assumption that the effects of the two agents are mutually exclusive. According to this method, which is equivalent to isobologram analysis (45), effects of the agents are synergistic if the CI is < 1.0, additive if CI = 1.0, and antagonistic if CI is > 1.0.

RESULTS

PARP Inhibitors Enhance Death Ligand Sensitivity—To assess the potential impact of PARP inhibition on death ligand sensitivity, ML-1 human AML cells were treated for 24 h with the well characterized PARP inhibitors veliparib (46) or olaparib (47), then exposed to varying concentrations of recombinant human TRAIL. Treatment with either veliparib or olaparib by itself had little impact on cell survival yet markedly sensitized these cells to TRAIL (Fig. 1A). These effects were half-maximal at 0.1 μM veliparib (Fig. 1, B and C) or 0.1 μM olaparib (Fig. 1, D and E). Apoptosis was assayed by propidium iodide staining as illustrated in panel D. * p < 0.05 relative to TRAIL treatment without PARP inhibitor. F, HCT116 cells were pretreated with diluent or 0.5 μM olaparib for 24 h followed by the addition of diluent or varying concentrations of TRAIL for additional 3.5 h. Apoptosis was assayed by propidium iodide staining as illustrated in panel D. Bar graph (right) shows summarized results at the indicated TRAIL concentrations. Error bars in A–F, ± S.D. of three (A–C, E, and F) or four (D) independent experiments.
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FIGURE 3. PARP inhibitors do not affect apoptosis triggered by paclitaxel or etoposide. A and B, ML-1 cells were pretreated with diluent, 0.5 μM olaparib, or 5 μM veliparib as indicated for 24 h followed by the addition of varying concentrations of paclitaxel (A) or etoposide (B) for an additional 24 h. Apoptosis was assayed by annexin V binding assay. The right panels show summarized results obtained with 25 nM paclitaxel (A) or 3 μM etoposide (B). Error bars, ± S.D. of three independent experiments. C, ML-1 cells were treated with PARP inhibitors for 48 h. Whole cell lysates (50 μg of protein) were subjected to SDS-PAGE, transferred to nitrocellulose, and probed for the indicated antigens. HSP90β served as a loading control. Numbers at the left of all blots indicate migration of molecular mass markers (kDa).

cells. Similar sensitization was also observed when ML-1 cells were treated with the PARP inhibitors in combination with agonistic anti-DR5 antibody (Fig. 2A) or agonistic anti-Fas antibody CH.11 (Fig. 2B). Moreover, PARP inhibitors enhanced TRAIL-mediated killing in the BCR/ABL-transformed leukemia cell line K562 (Fig. 2C), KRAS-transformed nonsmall cell lung cancer line A549 (Fig. 2D), and BRCA2-mutant ovarian cancer line PEO1 (Fig. 2E), indicating that the results were not unique to ML-1 cells. On the other hand, PARP inhibitors failed to increase TRAIL-induced apoptosis in HCT116 human colon cancer cells (Fig. 2F), indicating that the sensitization is not universally observed.

To rule out the possibility that PARP inhibitors might be sensitizing these cells relatively broadly to all apoptotic stimuli, we examined the impact of combining PARP inhibitors with various chemotherapeutic agents. In accord with earlier results (48–50), PARP inhibitor treatment failed to enhance the induction of apoptosis by paclitaxel (Fig. 3A) or etoposide (Fig. 3B). Consistent with this failure of PARP inhibitors to increase apoptosis universally, Bcl-2 family members were unaltered by the PARP inhibitor treatment (Fig. 3C).

PARP Inhibitors Increase Death Receptor Signaling—To investigate the mechanism by which PARP inhibitors specifically sensitize cells to death ligand-induced apoptosis, we initially examined the expression of components of DISC. Veliparib and olaparib did not alter the expression of FADD, caspase-8, and c-Flip (Fig. 4A).

Further experiments examined signaling downstream of death receptors after death ligand treatment in the absence or presence of PARP inhibitors. Because enhanced procaspase 8 and Bid cleavage can occur either as a direct effect of increased death receptor activation (51) or downstream of increased caspase 3 activation (52), we analyzed cleavage of procaspase 8 and Bid in the presence of the caspase 3 inhibitor DEVD-fmk. This analysis revealed that olaparib and veliparib increased the cleavage of caspase 8 and Bid during TRAIL treatment (Fig. 4B, lanes 4–6). Cleavage of caspase 8 and Bid was also increased when cells were exposed to PARP inhibitor followed by CH.11 agonistic anti-Fas antibody (Fig. 4C, lanes 4–6). The ability of olaparib to increase death ligand-induced cleavage of procaspase 8 and Bid even in the presence of DEVD-fmk (Fig. 4B; Fig. 4C, lane 4 versus 3) suggests that caspase 8 activation upstream of caspase 3 has been enhanced by the PARP inhibitor.

PARP1 Is Not Recruited to the Fas DISC—As the present work was nearing completion, Yuan et al. (53) reported that treatment of pancreatic cancer cells with DR5 agonistic antibody TRA-8 results in recruitment of PARP-1 to the DR5 DISC, which leads to poly(ADP-ribosyl)ation of procaspase-8 and decreased caspase-8 activation. Conversely, PARP inhibition was reported to decrease procaspase-8 poly(ADP-ribosyl)ation and enhance caspase 8 activation. To assess whether this mechanism might contribute to the death ligand/PARP inhibitor synergy described above, we examined the Fas DISC from ML-1 cells after treatment with CH.11 in the absence or presence of olaparib. As indicated in Fig. 5A, left panel, PARP1 was not detectable in the Fas DISC even though it was readily detectable in cell lysates. Moreover, we did not detect any pADPribosylated caspase-8 immunoprecipitates (Fig. 5B). In contrast, pADPribosylated caspase-8 was readily detectable in cells treated with methyl methanesulfonate. Accordingly, we focused on alternative explanations for the increased death receptor signaling as described below.

PARP Inhibitors Increase Death Receptor Expression—Careful examination of the Fas DISC revealed that olaparib treatment increased the amount Fas pulled down by CH.11 and the amount of FADD in the DISC (Fig. 5A, right panel). To deter-
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We compared binding of fluorochrome-labeled anti-receptor antibody to cells under various conditions. An increase in binding was observable when fluorochrome-labeled antibody was bound to accessible cell surface receptors at either 37 °C (Fig. 6, A and B), a temperature that allows trafficking and antibody-induced trapping of receptor on cells, or 4 °C (Fig. 6C), a temperature that precludes trafficking and antibody-induced receptor trapping at the cell surface (33). Further analysis indicated that this effect was seen with death receptors but not with other cell surface proteins such as the transferrin receptor CD71 (Fig. 6C). These results suggest that PARP inhibitors affect death receptor expression rather than trafficking and do so somewhat selectively.

PARP1 or PARP2 Suppresses Death Receptor Expression—When Fas and DR5 expression was examined by qRT-PCR, mRNA encoding both Fas and DR5 was increased by PARP inhibition (Fig. 7A). In contrast, DR4 mRNA did not increase appreciably (data not shown). Further experiments demonstrated that PARP inhibition had no effect on Fas or DR5 mRNA stability (Fig. 7, B and C). Instead, luciferase reporter assays indicated that this increase in mRNA reflected increased activation of the FAS and TNFRSF10B (DR5) promoters (Fig. 7D).

Recent studies have shown that veliparib and olaparib bind to multiple PARP isoforms, including PARP1 to PARP4 (54). To assess which of these might contribute to death receptor up-regulation, ML-1 cells were stably transduced with shRNAs that down-regulate PARP1, PARP2, PARP3, or PARP4 (Fig. 8A). qRT-PCR demonstrated the ability of these shRNA molecules to selectively down-regulate the targeted message without decreasing expression of other PARPs (data not shown). Down-regulation of PARP1 or PARP2 (but not PARP3 or PARP4) increased DR5 and Fas expression (Fig. 8, B and D), demonstrating that both PARP1 and PARP2 suppress death receptor expression. In line with these observations, PARP1 or PARP2 shRNA enhanced sensitivity of these cells to TRAIL (Fig. 8C) as well as CH.11 agonistic anti-Fas antibody (Fig. 8E), whereas PARP3 or PARP4 shRNA had no effect on death ligand-induced apoptosis (Fig. 8, C and E). Additional experiments demonstrated similar results with PARP1 siRNA (Fig. 8F) as well as multiple PARP2 shRNAs (data not shown). This increase in Fas and DR5 protein expression (Fig. 8, B and D) and death ligand sensitivity (Fig. 8, C and E) reflected that ability of PARP1 or PARP2 shRNA to increase Fas and DR5 mRNA (Fig. 8G).

To further confirm the role of PARP1 and PARP2 in the sensitization, ML-1 cells were treated with olaparib and CH.11. As anticipated from the results above, PARP1 plus PARP2 shRNA increased the sensitivity of the cells to CH.11 agonistic anti-Fas antibody (Fig. 8H). Importantly, olaparib did not further enhance the sensitivity of these cells, indicating that PARP1 and/or PARP2 were required for the effects of olaparib and ruling out participation of some other target (Fig. 8H).

Sp1 Plays a Critical Role in PARP Inhibitor/TRAIL Synergy—It has previously been reported that the transcriptional factor Sp1 binds to and activates death receptor promoters (40, 55, 56). To investigate whether Sp1 contributes to DR5 up-regulation by PARP inhibitors, ML-1 cells were stably transduced with shRNAs that down-regulate Sp1 (inset, Fig. 9A). Sp1 down-regulation diminished the olaparib-induced increase in
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A

Olaparib - + + + + + +
CH.11 - - - - - - -

Fas (ng/mL)
FADD
PARP1
PARP1 (long exp)

DISC Cell lysate

Relative Signal

1 2 3 4 5 6

Olparib

2 5 10

Fas (ng/mL) FADD

Relative Signal

1 2 3 4 5 6

Olparib

B

pADPβ

Procasp-8

C

Negative Control

APO-1

DR5

Cell number

APC fluorescence

D

APO-1

Corrected APO-1 MFI

Veliparib (μM)

200 300 400 500 600 700 800 900

0 0.2 0.4 0.6 0.8 1

Corrected APO-1 MFI

Olparib (μM)

DMSO Veliparib Olparib

E

DR5

Corrected DR5 MFI

Veliparib (μM)

10 20 30 40 50

0 0.2 0.4 0.6 0.8 1

Corrected DR5 MFI

Olparib (μM)

DMSO Veliparib Olparib

F

IgM

CH.11

Corrected CH.11 MFI

0 10 20 30 40

Vehicle Olparib

G

IgG

APO-1

Corrected APO-1 MFI

0 20 40 60 80 100 120 140

Vehicle Olparib
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FIGURE 6. Increased detection of cell surface Fas does not require ligand-induced receptor retention on the cell surface. A, histograms show the CH.11 binding at 37 °C from ML-1 cells that were pretreated with diluent or 0.5 μM olaparib for 48 h followed by the addition of IgM control or 200 ng/ml CH.11 in the presence of 5 μM Q-VD-OPh for an additional 16 h at 37 °C. B, CH.11 binding was assayed as shown in A after ML-1 cells were pretreated with diluent or 0.5 μM olaparib for 48 h followed by varying CH.11 concentrations for an additional 16 h in the presence of 5 μM Q-VD-OPh to prevent CH.11-induced apoptosis. The bar graph (right) shows the summarized results (mean ± S.D.) from three independent experiments. *, p < 0.03 and **, p = 0.003, respectively, relative to diluent-treated cells. C, after pretreatment with DMSO, 5 μM veliparib or 0.5 μM olaparib for 48 h, ML-1 cells were assayed for binding of CH.11 (200 ng/5 × 10^5 cells) or CD71 (1 μg/5 × 10^5 cells) at 4 °C. Error bars, ± S.D. of three independent experiments. *, p < 0.002 relative to diluent-treated cells. MFI, mean fluorescence intensity.

FIGURE 7. PARP inhibition increases Fas and DR5 promoter activity. A, after ML-1 cells were treated with 0.5 μM olaparib for 48 h, FAS and DR5 mRNA levels were quantified by qRT-PCR and normalized to GAPDH mRNA. B and C, ML-1 cells were treated with DMSO or 0.5 μM olaparib for 48 h followed by the addition of 2.4 μM actinomycin D in the presence of 5 μM Q-VD-OPh for the indicated lengths of time. Fas (B) and DR5 (C) mRNA were quantified by qRT-PCR and normalized to GAPDH mRNA. D, 12 h after transfection with Fas or DR5 promoter-luciferase reporter plasmid (10 μg) in the presence of 5 μM Q-VD-OPh, ML-1 cells were treated with 0.5 μM olaparib for additional 20 h. Luciferase activity was assayed and normalized to protein concentrations. Error bars in A and D, ± S.D. of at least four independent experiments.
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DR5 expression (Fig. 9A) as well as the olaparib-induced increase in TRAIL sensitivity (Fig. 9B).

To further examine the role of Sp1 in the PARP inhibitor/TRAIL synergy, ChIP was performed to assess the interaction of Sp1 with the TNFRSF10B promoter. This analysis revealed that Sp1 is normally bound to DR5 promoter, and this binding is increased by PARP inhibitor treatment (Fig. 9C, lane 6 versus 5).

Further experiments demonstrated that the increased binding of Sp1 to the TNFRSF10B promoter after olaparib treat-
ment occurred in the absence of any change in total Sp1 levels (Fig. 9D, lanes 1 and 2). Instead, increased Sp1 binding to the TNFRSF10B promoter reflected increased binding of Sp1 to a relatively salt-resistant nuclear fraction (Fig. 9D, lanes 3–6) where active genes are located (57, 58). In turn, this olaparib-induced redistribution of Sp1 between nuclear fractions appeared to reflect diminished binding of Sp1-containing complexes to pADPr (Fig. 9E, upper panel). Collectively, these results demonstrate that Sp1 plays a key role in the transcriptional activation of the TNFRSF10B gene leading to PARP inhibitor/TRAIL synergy.

**PARP Inhibition Enhances TRAIL Sensitivity in Human AML Specimens**—To assess whether PARP inhibitors would have a similar effect in clinical AML, primary human AML specimens (Table 1) were plated in methylcellulose with diluent, olaparib, TRAIL, or the combination. Results of this analysis (Fig. 10) demonstrated that many of the AML specimens were resistant to TRAIL, as manifested by colony formation in the presence of 120 ng/ml TRAIL. Significantly, however, many of these specimens were markedly sensitized to TRAIL by olaparib (Fig. 10, A–C). In aggregate, 6 of 11 AML specimens were sensitized (Table 1), whereas normal marrows showed little sensitivity to TRAIL, olaparib, or the combination (Fig. 10D). Further analysis failed to show any obvious relationship between FLT3 status, karyotypic complexity, or prior treatment and the ability of PARP inhibitors to enhance TRAIL sensitivity.

**DISCUSSION**

Results of the present study demonstrate that PARP inhibition results in increased sensitivity of multiple cancer cell lines to death ligands such as TRAIL and agonistic anti-Fas antibody. These effects reflect increased expression of the DR5 and Fas receptors. Importantly, death receptor up-regulation was observed in xenografts after PARP inhibitor administration in vivo, and synergistic effects of the PARP inhibitor/TRAIL combination were observed in clinical leukemia specimens ex vivo. These observations point to an active signal transduction inhibitor combination that warrants further preclinical and possible clinical testing.

As the present study was nearing completion, Yuan et al. (53) reported that the second generation PARP inhibitor PJ-34 enhances sensitivity of pancreatic cancer cells to the DR5 agonistic antibody TRA-8. Our results extend these recent findings by showing that (i) sensitization is also seen with recombinant human TRAIL and agonistic anti-Fas antibody, (ii) sensitization is observed in a number of different cell types, including clinical samples of AML, and (iii) the sensitization is seen with PARP inhibitors that are undergoing clinical testing. As discussed in further detail below, our additional studies have shown that inhibition or down-regulation of PARP1 or PARP2 is accompanied by increased DR5 and Fas mRNA as well as increased DR5 and Fas receptor levels at the cell surface. These observations provide new mechanistic insight into PARP inhibitor/death ligand synergy.

A number of agents have previously been reported to enhance the sensitivity of various cell lines to death ligands in vitro. These include the protein kinase Cβ inhibitor enzastaurin, which enhances trafficking of intracellular death receptors to the cell surface (38); caspase 8 recruitment to the DISC (59); sorafenib, which down-regulates the antiapoptotic protein Mcl-1 (60, 61); and the topoisomerase I poison SN-38 or its produrg irinotecan (33, 62), which enhances DR5 expression selectively in p53 wild type cells (33, 63, 64). The present study not only demonstrated enhanced killing when selected cell lines are pretreated with PARP inhibitors for 24 h before exposure to death ligands (Fig. 1) but also traced this effect to increased cell surface expression of Fas and DR5 (Fig. 5, C–E). In contrast to enzastaurin (38), PARP inhibitors do not alter the trafficking of death receptors to the cell surface (Fig. 6) but instead up-regulate the DR5 and Fas messages (Fig. 7A). Moreover, PARP inhibitors do not up-regulate CD71 (Fig. 6C), demonstrating specificity of PARP inhibitor treatment for certain death receptors as opposed to other cell surface proteins.

Previous studies have identified Fas and DR5 as p53-responsive genes (64, 65). Although transcriptional activation and increased message was detected in the present study (Fig. 7), these effects appear to be p53-independent. In particular, promoter activation as well as increased message was observed in K562 cells, which are p53-deficient (66, 67), as well as ML-1 cells, which are p53 wild type (68). Conversely, PARP inhibitors failed to sensitize HCT116 cells to TRAIL (Fig. 2E) despite the presence of wild type p53 (69) and the ability of DNA damage to enhance TRAIL sensitivity in these same cells (33, 62). Thus,
FIGURE 9. Sp1 plays a critical role in PARP inhibitor/TRAIL synergy. A, relative cell surface DR5 expression on ML-1 cells with stable Sp1 knockdown assayed using anti-DR5 antibody as a probe. Inset, immunoblot of Sp1 stable knockdown. HSP90 serves as a loading control. B (from top to bottom), after ML-1 cells stably expressing the indicated shRNA construct were treated with TRAIL for 24 h, subdiploid cells were assayed as illustrated in Fig. 2D. Bar graph at bottom, summarized results of three independent experiments with diluent or 12.5 ng/ml TRAIL. Error bars, ± S.D. of at least three independent experiments. C, after ML-1 cells were treated with diluent or 1 μM olaparib for 40 h, ChIP with control IgG or Sp1 antibody and subsequent semi-quantitative PCR for the 165-bp region of the DR5 promoter containing Sp1 binding site were performed as described under “Experimental Procedure.” D, after ML-1 cells were treated with diluent or 1 μM olaparib for 48 h, nuclei were fractionated using 200 mM NaCl into extractable proteins (Supernatant) and salt-resistant proteins (Pellet). 30 μg of whole cell lysates, 10 μg of supernatant, and 20 μg of nuclear pellet were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with the indicated antibodies. Proliferating cell nuclear antigen (PCNA) served as a loading control. WCL, whole cell lysates. E, after ML-1 cells were treated with diluent or 1 μM olaparib for 48 h, immunoprecipitates (IP) prepared with anti-pADPr antibody (clone 10H) or murine IgG control were subjected to SDS-PAGE and immunoblotting with the indicated antibodies. Cells treated with 1 mM methyl methanesulfonate (MMS) for 10 min served as a positive control for pADPr formation.
factors other than p53 status must determine whether Fas and DR5 are up-regulated by PARP inhibitors.

Consistent with this conclusion, our further results identified Sp1, a transcription factor previously implicated in regulation of death receptors (40, 55, 56), as an important participant in PARP inhibitor-induced death receptor up-regulation. In particular, we observed that Sp1 knockdown markedly diminished the effects of olaparib on DR5 expression (Fig. 9A) and TRAIL-induced killing (Fig. 9B). Furthermore, PARP inhibitor treatment decreased the amount of pADPr-bound Sp1 complexes (Fig. 9E), leading to increased accumulation of Sp1 in a salt-resistant chromatin fraction (Fig. 9D) and increased binding to Sp1.

### Table 1

| Patient # | Karyotype  | Prior hematological disorder | FLT3 mutation status | # prior regimens | Response to most recent Rx | Sensitive to TRAIL alone<sup>a</sup> | Sensitive to olaparib alone<sup>b</sup> | Sensitized to TRAIL by olaparib<sup>c</sup> |
|-----------|------------|-----------------------------|---------------------|----------------|--------------------------|----------------------------------|----------------------------------|----------------------------------|
| 1         | 47XX +8<sup>d</sup> | None                        | ITD<sup>d</sup>     | 2              | Refractory               | +                                | -                                | +                                |
| 2         | 46XY       | None                        | ITD<sup>d</sup>     | 0              | +                        | +                                | +                                | +                                |
| 3         | 46XY       | None                        | wt                  | 1              | CR, then relapse         | ++                               | ++                               | +                                |
| 4         | Complex    | None                        | wt                  | 0              | -                        | -                                | -                                | -                                |
| 5         | Complex    | None                        | wt                  | 0              | -                        | -                                | -                                | ++                               |
| 6         | Complex    | None                        | wt                  | 0              | -                        | -                                | -                                | -                                |
| 7         | 47XY +21   | Thrombocytopenia             | wt                  | 0              | -                        | +                                | -                                | -                                |
| 8         | 46XX       | None                        | wt                  | 0              | +                        | +                                | -                                | -                                |
| 9         | Complex    | MDS 2<sup>e</sup> chemo<sup>f</sup> | wt                  | 0              | ++                       | +                                | +                                | +                                |
| 10        | 47XX +8    | None                        | wt                  | 0              | -                        | -                                | -                                | +                                |
| 11        | 45XY t(11;19) dic (12;17) | None                | wt                  | 0              | -                        | -                                | -                                | -                                |

<sup>a</sup> Samples were defined as sensitive to single-agent TRAIL or single-agent olaparib if colony formation was inhibited by ≥50% (+) and very sensitive if inhibited by ≥90% (++).

<sup>b</sup> -, Not sensitized to TRAIL by olaparib. Samples were considered sensitized by olaparib (+) if colony formation in the presence of TRAIL + olaparib was ≥50% of colony formation in the presence of TRAIL or olaparib alone and markedly sensitized (+++) if colony formation in the presence of TRAIL + olaparib was ≥10% of colony formation in the presence of TRAIL or olaparib alone.

<sup>c</sup> + 8 indicates trisomy 8; "Complex" indicates the presence of ≥3 numerical and/or structural abnormalities; t indicates translocation of indicated chromosomes, and dic indicates dicentric chromosome.

<sup>d</sup> ITD, internal tandem duplication mutation (79).

<sup>e</sup> Myelodysplastic syndrome in the setting of prior chemotherapy for breast cancer.

**Figure 10.** PARP inhibitor sensitizes AML samples to TRAIL-induced reduction of colony formation. Three different AML samples (A–C) and normal marrow (D) were plated in Methocult<sup>®</sup> methylcellulose containing 120 ng/ml TRAIL, 0.5 mM olaparib, or 120 ng/ml TRAIL + 0.5 μM olaparib. Leukemic (A–C) and normal myeloid colonies (D) were counted (42) at 14 days and compared with samples containing diluent. Numbers above each bar graph correspond to sample numbers in Table 1. Note that the ability of olaparib to sensitize to TRAIL without having any effect by itself meets the definition of synergy (45). CFU-L, leukemic cell colony-forming unit.
the DR5 promoter (Fig. 9C). Our finding that Sp1 is present in a salt-resistant nuclear fraction is consistent with previous studies showing that this transcription factor can be recovered with the nuclear matrix (70, 71). To our knowledge the present studies provide the first evidence that PARP inhibitors affect Sp1 partitioning and function within the nucleus. Whether PARP inhibitors similarly affect other transcription factors requires further investigation.

Recent studies have demonstrated that PARP inhibitors such as olaparib and veliparib bind at least four members of the PARP superfamily, PARP1-PARP4 (54). RNA interference experiments demonstrated that the effects of these PARP inhibitors can be reproduced by down-regulation of PARP1 or PARP2 (Fig. 8, B–G). Although the effects of PARP inhibition have in some circumstances been attributed to conversion of PARP1 into a poison that prevents access of the repair machinery to the DNA (50, 72–74), our observation that PARP1 shRNA or siRNA recapitulates the effects of the PARP inhibitor (Fig. 8) argues that sensitization to death ligands described in the present study results from the catalytic inhibition of PARP1. The similar effect of PARP2 shRNA (Fig. 8) does not result from accidental down-regulation of PARP1 by the PARP2 shRNA (data not shown) but instead appears to reflect cooperation between these two related enzymes (24, 28). Moreover, PARP1 and PARP2 down-regulation prevents further sensitization by olaparib (Fig. 8H), suggesting that these two isoforms are the pertinent PARP inhibitor targets for death ligand sensitization.

Because the chemotherapy literature contains a number of examples of biochemical effects that are observed in vitro but cannot be reproduced in vivo due to drug binding or toxicity (75), further studies examined the ability of the PARP inhibitor olaparib to enhance death receptor expression after administration to mice. After three doses of the PARP inhibitor, increased Fas receptor was readily detected in ML-1 xenografts in vivo (Fig. 5, F and G).

Further experiments examined the effects of PARP inhibitor on death ligand sensitivity in primary AML specimens ex vivo. Results of this analysis revealed that 6 of 11 specimens exhibited increased TRAIL sensitivity in the presence of olaparib (Fig. 10 and Table 1).

At the present time, PARP inhibitors are undergoing extensive testing as potential agents for the treatment of homologous recombination-deficient tumors (12–16). Although death receptor activators such as Fas ligand and agonistic anti-Fas antibodies cannot be developed because of profound hepatotoxicity (76), TRAIL, agonistic anti-DR5 antibodies, and small molecule inhibitors targeting death receptors 4 and 5 continue to undergo preclinical and early clinical development (reviewed in Refs. 3, 6–8, and 77). Given the very modest toxicities of PARP inhibitors such as veliparib and olaparib (for review, see Refs. 13–16), results of the present studies suggest that it might be possible to enhance the efficacy of DR5-directed therapy using PARP inhibitors. In view of the promising activity of PARP inhibitors in cancers with homologous recombination deficiency and reports of severe toxicities when PARP inhibitors are added to DNA damaging chemotherapy in this setting (78), a PARP inhibitor/DR5 agonist might be an alternative that is less toxic to normal tissues. Further preclinical and possible clinical studies of this approach appear warranted.

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