

Poly(ADP-ribose) polymerase inhibitor ABT-888 potentiates the cytotoxic activity of temozolomide in leukemia cells: influence of mismatch repair status and O^6 -methylguanine-DNA methyltransferase activity

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Abstract

The poly(ADP-ribose) polymerase (PARP) inhibitor ABT-888 potentiates the antitumor activity of temozolomide (TMZ). TMZ resistance results from increased O^6 -methylguanine-DNA methyltransferase (MGMT) activity and from mismatch repair (MMR) system mutations. We evaluated the relative importance of MGMT activity, MMR deficiency, nonhomologous end joining (NHEJ), and PARP activity in ABT-888 potentiation of TMZ. MMR-proficient and MMR-deficient leukemia cells with varying MGMT activity, as well as primary leukemia samples, were used to determine TMZ IC_{50} alone and with ABT-888. ABT-888 effectively inhibited PARP activity and enhanced TMZ growth inhibition in most leukemia cells. ABT-888 potentiation was most effective in MMR-deficient cells with low MGMT activity [potentiation factor (PF) = 21]. ABT-888 also potentiated TMZ activity in MMR-deficient cells with elevated MGMT activity. Unexpectedly, ABT-888 also en-

hanced TMZ activity in MMR-proficient cells (PF = 3–7). ABT-888 potentiation was unrelated to NHEJ activity. ABT-888 potentiated TMZ (PF = 2–5) in two of four acute myeloid leukemia patient samples but showed little potentiation in primary acute lymphoblastic leukemia. In conclusion, although ABT-888 potentiation of TMZ was most pronounced in MMR-deficient cells with low MGMT activity, neither MMR proficiency nor MGMT overexpression completely abrogated ABT-888 potentiation of TMZ. [Mol Cancer Ther 2009;8(8):2232–42]

Introduction

DNA repair systems mediate tumor cell response to DNA-damaging anticancer agents. Temozolomide (TMZ), an alkylating agent active in the treatment of gliomas (1, 2) and some leukemias (3, 4), creates DNA damage by adding methyl adducts to N^7 guanine (70% total adducts), N^3 adenine (9%), and O^6 guanine (5%; Fig. 1; ref. 5). The cytotoxicity of TMZ has been attributed to the creation of O^6 -methylguanine (6), which results in single-stranded DNA breaks, growth arrest, and apoptosis (7, 8). TMZ resistance results from at least two mechanisms: (a) increased levels of the DNA repair protein O^6 -methylguanine-DNA methyltransferase (MGMT), which removes O^6 -methyl adducts from the O^6 position of guanine in DNA, and (b) deficiencies in the DNA mismatch repair (MMR) system, resulting in microsatellite instability (MSI) and tolerance of O^6 -methylguanine adduct DNA mismatches (Fig. 1; ref. 9). Although MGMT inhibitors, such as O^6 -benzylguanine (O^6 -BG), can effectively overcome TMZ resistant in MMR-proficient (MSI stable) cells, they are ineffective in cells with MMR deficiencies (8, 10).

Because TMZ also generates N -methylated bases (N^3 and N^7), which can be removed by the base excision repair (BER) system (5), robust BER activity can result in TMZ resistance (5, 11). Central to BER and the removal of methylated N^3 and N^7 adducts is the enzyme poly(ADP-ribose) polymerase (PARP), an abundant nuclear enzyme that senses both single-stranded DNA and dsDNA breaks. In BER, PARP acts as a nick sensor, catalyzing the cleavage of NAD^+ and attaching PARP to itself, histones, and other target proteins. Negatively charged ADP-ribose polymers create electrostatic repulsions between DNA and histones, opening chromatin for DNA repair; PARP also recruits BER proteins to sites of single-stranded DNA breaks, initiating DNA repair (12). Thus, PARP inhibitors (PARPi) may overcome TMZ resistance in MMR-deficient cells by blocking BER, resulting in cytotoxicity from N^3 - and N^7 -methyl adducts (11, 13).

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PARP inhibitors have been tested in several tumor types and have been shown to enhance the antitumor effects of TMZ in leukemia (13), glioma (14–16), lung (17, 18), and colon carcinoma, both *in vitro* (16, 18–20) and in xenograft models (17, 21). Previous research has shown that the oral PARPi ABT-888 effectively inhibits PARP activity in animals (22, 23). In a phase 0 trial in humans, a single 25 mg dose of ABT-888 resulted in a median plasma ABT-888 concentration of 210 nmol/L, resulting in >92% PARP inhibition (24).

Because MMR status has been well characterized in a wide range of established leukemia cell lines, our objective was to use these cell lines as a model to assess the relative importance of MGMT activity and MMR status on the ability of ABT-888 to potentiate the growth-inhibitory effects of TMZ. ABT-888 has previously been shown to inhibit both PARP-1 and PARP-2 isoenzymes (22). Our goal was to determine (a) whether PARPi potentiation of TMZ was effective in cells with MMR proficiency, (b) whether PARPi potentiation of TMZ was abrogated by elevated MGMT, and (c) whether other mechanisms influence PARPi potentiation of TMZ.

Materials and Methods

Chemicals

RPMI 1640 cell culture medium, PBS, dextrose, sodium pyruvate, sodium bicarbonate, and HEPES were purchased from Life Technologies; fetal calf serum and high-glucose RPMI 1640 cell culture medium were purchased from the American Type Culture Collection; bovine growth serum was purchased from Hyclone; penicillin/streptomycin was purchased from Invitrogen; and Lymphoprep for mononuclear cell isolation was purchased from Greiner Bio-One. ABT-888 was synthesized and kindly provided by Abbott Laboratories. ABT-888 was diluted in DMSO to a stock concentration of 62 mmol/L. *O*⁶-BG (NSC 637037) was provided by the Cancer Therapy and Evaluation Program of the National Cancer Institute. TMZ (Schering-Plough) was purchased and formulated in DMSO according to the manufacturers' recommendations.

Cell Lines

The human T-cell acute lymphoblastic leukemia (ALL) cell lines Jurkat, Molt4, and HSB2; the human pre-B ALL cell lines JM1 and Reh; the B-cell lines Raji and Daudi; the histiocytic cell line U937; and the acute myeloid leukemia (AML) cell lines HL-60 (acute promyelocytic leukemia), KG1, HEL (erythroleukemia), and THP1 (monocytic leukemia) were purchased and cultured as directed by the American Type Culture Collection.

Culture of Primary Leukemia Cells

Leukemia cells were obtained from peripheral blood, leukapheresis, or bone marrow aspirate specimens from children with newly diagnosed acute leukemia before chemotherapy in accordance with Institutional Review Board guidelines. Peripheral blood mononuclear cells were isolated using Lymphoprep and frozen at a cell density of 1×10^7 /mL at -80°C until use. Primary leukemia cells were

cultured in RPMI 1640 supplemented with 20% FCS and penicillin/streptomycin. During drug sensitivity assays, cell viability was determined by trypan blue exclusion at 48 or 72 h and noted to be >90% in the absence of drug.

In vitro Cytotoxicity Assays

The growth inhibition effect of ABT-888 and TMZ was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric dye reduction as previously described (25) or the CellTiter-Glo luminescent cell viability assay (Promega) according to the manufacturer's instructions. Leukemia cell lines were plated at a cell density of 0.5 to 2×10^5 /mL. TMZ was serially diluted over a 10^6 -fold range of concentrations to determine single-agent IC₅₀s as described (4). For assays assessing single drug activity or TMZ in combination with ABT-888, replicates of six-wells were used for each drug concentration and the assay was repeated using two replicate plates. ABT-888 was tested in MMR-proficient U937, THP1, and JM1 and in MMR-deficient HSB2, Molt4, Jurkat, and Reh (Table 1). IC₅₀ values for each cell line were determined in at least three independent experiments using the Hill equation as previously described (25). Primary cells were plated at a concentration of 8 to 40×10^4 /mL and drug concentrations were tested in triplicate. IC₅₀ values for each primary cell sample were determined in at least two independent experiments. In combination experiments, ABT-888 and/or *O*⁶-BG were added 30 min before TMZ. Viability was assessed after 72 h. Representative cell lines combining TMZ with ABT-888 are shown from each MGMT and MSI subgroup (Fig. 3A and B). Combinations between TMZ, *O*⁶-BG, and ABT-888 were tested in the MMR-proficient cell lines JM1 and U937 as well as the MMR-deficient cell lines Jurkat and Molt4. The combination effect of drugs used with TMZ (*O*⁶-BG and ABT-888) was modeled using the universal response surface approach method as described (25, 26).

MGMT Activity, MSI, and PARP Activity Assays

MGMT activity in peripheral blood mononuclear cell or bone marrow aspirate tumor cell lysates was determined by the removal of *O*⁶-[³H]methylguanine from a ³H-methylated DNA substrate and quantified as fmol *O*⁶-[³H]methylguanine/mg total protein as described (27). For MSI determination in four representative primary leukemia cells (patients p115 and p120 with AML and patients p152 and p157 with ALL), genomic DNA was extracted and amplified using three MSI multiplex reaction mixtures containing National Cancer Institute panel markers (BAT-25, BAT-26, D2S123, D17S250, and D5S346; ref. 28), quasimonomorphic mononucleotide markers (NR-21, NR-22, NR-24, BAT-25, and BAT-26; ref. 29), or an alternative panel of markers (D18S35, TP53-DI, TP53-PENTA, D1S2883, and FGA) as described (4). MSI status of established cell lines was obtained from the literature (9, 30–33). PARP activity was measured by the incorporation of biotinylated poly(ADP-ribose) into histones using modifications of a commercially available PARP assay (Trevigen). Cell lysates were prepared from 1×10^7 leukemia cells using 1× PARP buffer (Trevigen) supplemented with 0.4 mmol/L phenylmethylsulfonyl fluoride, one-half complete protease inhibitor cocktail tablet

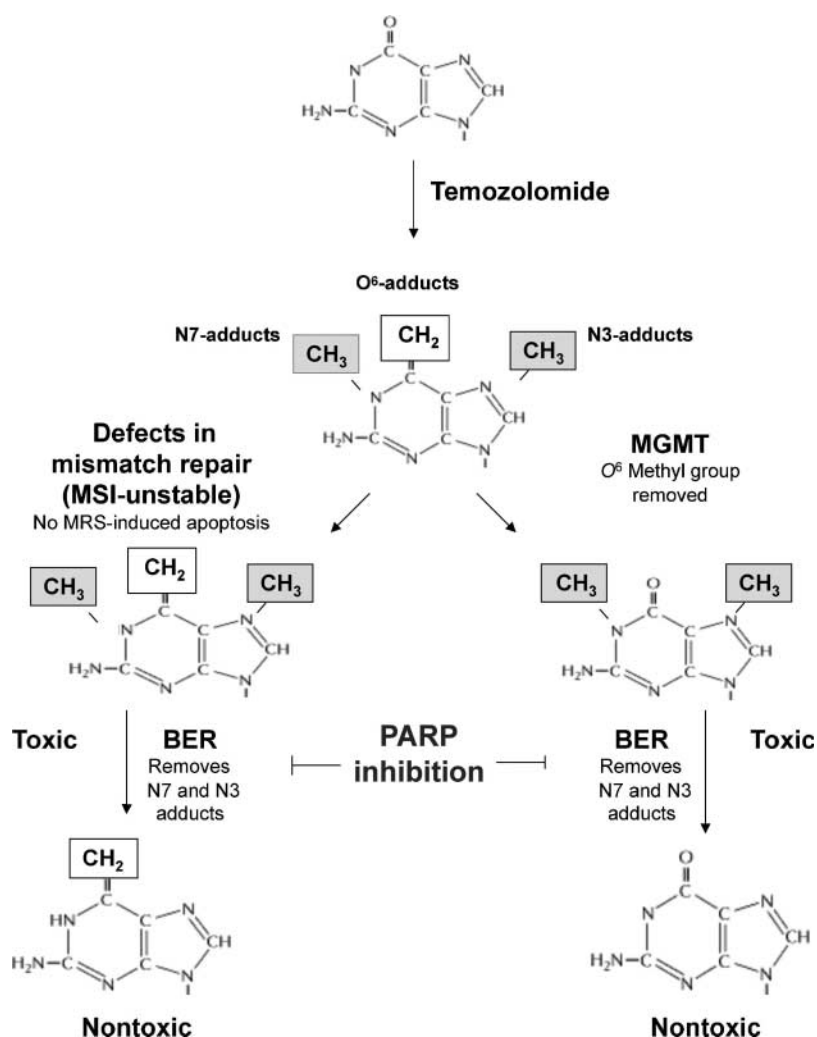


Figure 1. Model of the effects of PARP inhibition on TMZ activity. TMZ creates O⁶-methylguanine adducts (CH₂) and N⁷- and N³-methyl adducts (CH₃ on either adenine or guanine), which are efficiently removed by BER. Model shows guanine as a representative nucleotide. TMZ resistance results from either (a) MMR mutations (*left*), which allow DNA replication in the presence of dinucleotide (mG-T) mismatches, or (b) elevated MGMT activity (*right*), which removes the O⁶-methyl group from guanine. PARP inhibition blocks the repair of N⁷- and N³-methyl adducts, resulting in apoptosis during cell division.

(Roche), 1% NP40, and 0.1% SDS. Each sample was done in triplicate, and recombinant PARP (Trevigen) was used to produce a standard curve for each assay. PARP activity was assessed in either duplicate or triplicate in at least two independent experiments. PARP inhibition with ABT-888 was determined in MMR-proficient cell lines JM1 and U937 as well as MMR-deficient cell lines Molt4, Reh, Jurkat, and HSB2.

Immunoblots

Cell lysates were prepared from 1×10^7 cells as previously described (34). Thirty to 50 μ g of protein extract from representative cell lines (MMR-proficient cell lines JM1, U937, and THP1 as well as MMR-deficient cell lines Molt4, Reh, HSB2, and Jurkat) were run on SDS-PAGE gels and transferred to nitrocellulose membranes (Bio-Rad). Membranes were incubated overnight with MGMT (1:500 dilution; Cell Signaling Technology), 1 μ g/mL anti-human PARP (BD Pharmingen), 4 μ g/mL anti-Ku70 (clone N3H10; Abcam), rabbit anti-human phospho-histone H2AX (1:1,000 dilution; Cell Signaling Technology), or β -actin (1:10,000 dilution; Sigma) antibodies diluted in Odyssey blocking buffer

(Li-Cor). Bound primary antibodies were detected with IR-800 or IR-700 dye-labeled, appropriate species-specific secondary antisera and visualized on a Li-Cor Odyssey IR scanner. The intensity of gel bands was measured using Molecular Dynamics ImageQuant software (version 5.2).

Nonhomologous End-Joining Assay

Small-scale *in vitro* nonhomologous end-joining (NHEJ) assays were done essentially as described (35), except that a 2.9-kb *Pvu*II- and *Hind*III-linearized 5'-³²P-labeled pCDNA3.1 fragment was used. Reactions (10 μ L) were done with 30 μ g of protein extract and 10 ng of ³²P-labeled DNA. Full image is provided in Supplementary Fig. S1.

Transient Transfection Assay

pCDNA3.3 and pCDNA-MGMT plasmids were transfected into MMR-proficient THP1 AML cells and MMR-deficient HSB2 T-cell ALL cells using Nucleofector Kit V from Amaxa, Inc. as directed with minor modifications. Approximately 1.5×10^6 cells were suspended in 100 μ L of Nucleofector Solution V containing 0.5 μ g of plasmid DNA. Plasmid pEGFP was used to monitor transfection efficiency. Following 6 to 8 h of incubation at 37°C, cells

were washed and cultured for another 40 h before protein lysates were made and cytotoxicity assays were done. The transfection efficiency, measured by counting the green fluorescent cells, was estimated to be 70% to 80% for each of the cell lines used (full image in Supplementary Fig. S1)

Statistics

Dose-response curves are shown using mean \pm SD. IC₅₀s were calculated using the Hill equation as previously described (25). MGMT activity and PARP activity are reported as mean \pm SD of each sample done in triplicate. PARP activity in MMR-proficient and MMR-deficient cell lines was compared using a two-sided Student's *t* test. PARP activity, MGMT activity, and potentiation factors (PF) were compared as continuous variables using the Wilcoxon rank-sum test. K₅₀s and PF were determined from at least three independent experiments.

Results

TMZ and PARP Inhibition in Leukemia Cell Lines

The growth-inhibitory effects of TMZ and the PARPi ABT-888 were tested in MMR-proficient and MMR-deficient leukemia cell lines with variable MGMT activity (Table 1). Previous work has shown that plasma TMZ concentrations (C_{max}) reached \sim 62 μ mol/L when given at the maximum tolerated dose (200 mg/m²) over 5 days (36). As shown in Fig. 2A, most leukemia cell lines were resistant to TMZ at this concentration. Although three MMR-proficient cell lines (JM1, HEL, and U937) were more sensitive to TMZ (IC₅₀, 11–50 μ mol/L; Fig. 2A), MMR-deficient cell lines were resistant to TMZ, with an average IC₅₀ of 450 μ mol/L. Leukemia cells with elevated MGMT activity (Jurkat, Reh, and KG1) were also more resistant to TMZ than cell lines with absent (U937 and HEL) or low (JM1) MGMT activity.

ABT-888 also inhibited leukemia cell growth, with IC₅₀s ranging from 20 to 196 μ mol/L (Fig. 2B). These ABT-888 inhibitory concentrations, however, were approximately 5- to 33-fold higher than plasma concentrations achieved in either animals or humans (22, 24). Because steady-state concentrations in animals ranged from 0.35 to 1 μ mol/L, and

the single-dose ABT-888 C_{max} was 0.2 to 11 μ mol/L, further exploration of the ABT-888-potentiating effects was done using concentrations of 0.5 and 5 μ mol/L.

Variable MGMT Activity and MMR Status in Leukemia Cell Lines

MGMT expression is variable in primary leukemia cells, ranging from undetectable to 6,000 fmol/mg protein (4). As shown in Table 1, MGMT activity varied in both MMR-proficient (MSI stable) and MMR-deficient (MSI unstable) leukemia cell lines. In MMR-proficient cells, MGMT activity varied from undetectable in the AML cell lines HEL and U937 to high (1,170 fmol/mg protein) in the AML cell line KG1. In MMR-deficient (MSI unstable) cell lines, MGMT activity ranged from 320 fmol/mg protein in the T-cell ALL line HSB2 to 1,300 fmol/mg protein in the pre-B ALL cell line Reh. To assess the relative contributions of MGMT activity and MMR status to ABT-888 potentiation of TMZ, leukemia cell lines were subdivided into seven groups based on relative MGMT activity and MMR status (Table 1). Because normal adult peripheral blood mononuclear cells express an average of 770 \pm 170 fmol/mg protein MGMT activity (4), MMR-proficient cell lines (MSI stable; S) were divided into those with undetectable (S0), low (S1), average (S2), or high (S3) MGMT activity, corresponding to undetectable, <460 fmol/mg, 460 to 1,100 fmol/mg, and >1,110 fmol/mg MGMT protein, respectively. MMR-deficient cell lines (MSI unstable; U) were similarly classified into groups with low (U1), average (U2), and high (U3) MGMT activity based on the same criteria.

ABT-888 Potentiates TMZ Activity in Cells with Proficient MMR and MGMT Activity

With one exception (U937 AML cells), ABT-888 enhanced the activity of TMZ in MMR-proficient cells from 3- to 7-fold, an effect that seemed to be independent of MGMT activity (Fig. 3A and C). ABT-888 was more effective in potentiating TMZ-induced cytotoxicity in MMR-deficient cells (10- to 21-fold; Fig. 3B) and was most potent in MMR-deficient leukemia cells with low MGMT activity (U1), such as the T-cell ALL line HSB2 (Fig. 3B, left). In this cell line, ABT-888 decreased the TMZ IC₅₀ from 440 to 20 μ mol/L,

Table 1. Summary of leukemia subtype, MGMT activity, and MSI status in leukemia cell lines

| Cell line | Leukemia subtype | MGMT activity (fmol/mg protein) | MSI | Group* |
|-----------|------------------|---------------------------------|----------|--------|
| U937 | AML | Absent | Stable | S0 |
| HEL | AML | Absent | Stable | S0 |
| THP1 | AML | Low (370 \pm 11) | Stable | S1 |
| JM1 | Pre-B ALL | Low (450 \pm 17) | Stable | S1 |
| Raji | B ALL | Average (380 \pm 89) | Stable | S1 |
| HL-60 | AML | Average (750 \pm 94) | Stable | S2 |
| Daudi | B ALL | Average (902 \pm 17) | Stable | S2 |
| KG1 | AML | High (1,170 \pm 34) | Stable | S3 |
| HSB2 | T-ALL | Low (320 \pm 20) | Unstable | U1 |
| Molt4 | T-ALL | Average (560 \pm 37) | Unstable | U2 |
| Jurkat | T-ALL | High (1,290 \pm 36) | Unstable | U3 |
| Reh | Pre-B ALL | High (1,300 \pm 14) | Unstable | U3 |

*Group assignment based on MGMT activity (0 = none, 1 = low, 2 = medium, 3 = high) and MSI status (S = stable, U = unstable).

a 21-fold enhancement. ABT-888 alone had no effect on HSB2 growth at the same concentration (5 $\mu\text{mol/L}$; Fig. 2B). ABT-888 also enhanced the effects of TMZ in MMR-deficient cell lines with average or elevated MGMT activity. In pre-B and T-ALL cell lines with the highest MGMT activity (U3), ABT-888 provided a 10- to 13-fold potentiation (Fig. 3B, right). This suggests that ABT-888 can enhance TMZ-induced cytotoxicity in MMR-deficient leukemia cell lines despite elevated MGMT activity.

Combination of MGMT Inhibitor O^6 -BG with ABT-888 and TMZ

Because MGMT inhibitors have been extensively studied as a means to increase TMZ efficacy (37, 38), it was of interest to determine the relative potentiation of the MGMT inhibitor O^6 -BG and ABT-888. In MMR-proficient cell lines, such as JM1, O^6 -BG seemed to provide more TMZ potentiation than ABT-888 (Fig. 3D) and ABT-888 did not provide any additional TMZ potentiation. In MMR-deficient cell lines, however, such as the T-cell ALL cell lines Molt4 (Fig. 3E) and Jurkat (data not shown), the addition of both O^6 -BG and ABT-888 each potentiated TMZ and the addition of both O^6 -BG and ABT-888 to TMZ was additive when modeled using the universal response surface approach method (26).

Overexpression of MGMT Does Not Prevent ABT-888 Potentiation of TMZ

To determine if MGMT overexpression could overcome ABT-888 potentiation of TMZ growth inhibition, we transfected the MMR-proficient THP1 and MMR-deficient HSB2 cells with a construct constitutively expressing MGMT. By immunoblot densitometry, MGMT expression was increased at least 30-fold (Fig. 4A). Increase in MGMT expression was able to partially, but not completely, overcome ABT-888 potentiation of TMZ cytotoxicity. In the MMR-proficient THP1 cells (Fig. 4B), PF decreased from 3.4 to 1.6, and in the MMR-deficient cell lines HSB2 (Fig. 4C), the PF decreased from 15 to 5.8.

ABT-888 Potentiation of TMZ: Relationship to PARP Activity

To determine whether ABT-888-mediated TMZ potentiation was related to the degree of PARP inhibition, immunoblots and activity assays were done in several of the leukemia cell lines (Fig. 5). PARP protein (Fig. 5A) and

PARP activity levels (Fig. 5B) in MMR-proficient and MMR-deficient cell lines were quite variable and did not seem to correlate with MGMT activity. MMR-proficient cell lines had PARP activity ranging from 45 fmol/ μg protein (AML cells) to 3,430 fmol/ μg protein (HEL AML cells). Although leukemia cells with MMR deficiencies (MSI unstable) tended to have higher PARP activity (510–2,050 fmol/ μg protein) than MMR-proficient cells, the difference between MMR-proficient and MMR-deficient leukemia cell lines was not statistically significant ($P = 0.76$, Student's *t* test).

Overall, ABT-888 inhibited PARP activity by 78% to 97% (Fig. 5B). The degree of PARP inhibition was roughly proportional to the degree of ABT-888 potentiation of TMZ in most leukemia cell lines. For example, ABT-888 induced 90% PARP inhibition in the MMR-deficient cell line HSB2, which showed the greatest degree of ABT-888-induced TMZ enhancement (21-fold). Residual PARP activity in HSB2 was only 60 fmol/ μg protein. In contrast, in the MMR-proficient JM1, which showed less potentiation (3-fold), ABT-888 provided only 78% inhibition of PARP activity and the highest residual PARP activity (360 fmol/ μg protein) was observed.

Notably, ABT-888 did not potentiate TMZ growth inhibition in the AML cell line U937, a MMR-proficient cell line with minimal MGMT activity (Fig. 5D). The U937 cell line expressed very little PARP by immunoblot (Fig. 5A) and had minimal PARP activity (Fig. 5B), suggesting that ABT-888 may be less effective at potentiating TMZ in cells with very low PARP activity.

In summary, when MGMT activity, PARP activity, and the PFs were examined as continuous variables, there were no significant differences in MGMT activity or PARP activity between the MMR-deficient and the MMR-proficient cell lines ($P = 0.37$ and 0.23 , respectively, Wilcoxon rank-sum test). PFs, however, were significantly higher in MMR-deficient cell lines when compared with those with intact MMR ($P = 0.02$, Wilcoxon rank-sum test).

ABT-888 Potentiation of TMZ Is Not Due to NHEJ

PARP also participates in forms of DNA double-strand break (DSB) repair, including both homologous and non-homologous recombination and a backup pathway of NHEJ in cells compromised for classic NHEJ (12, 39, 40).

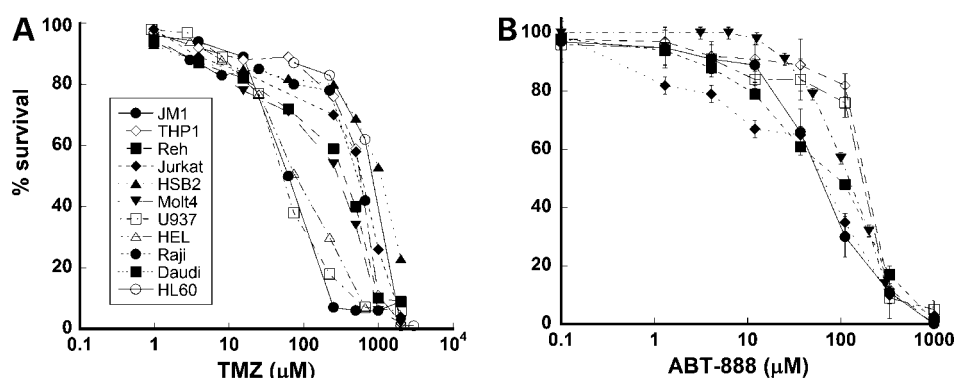
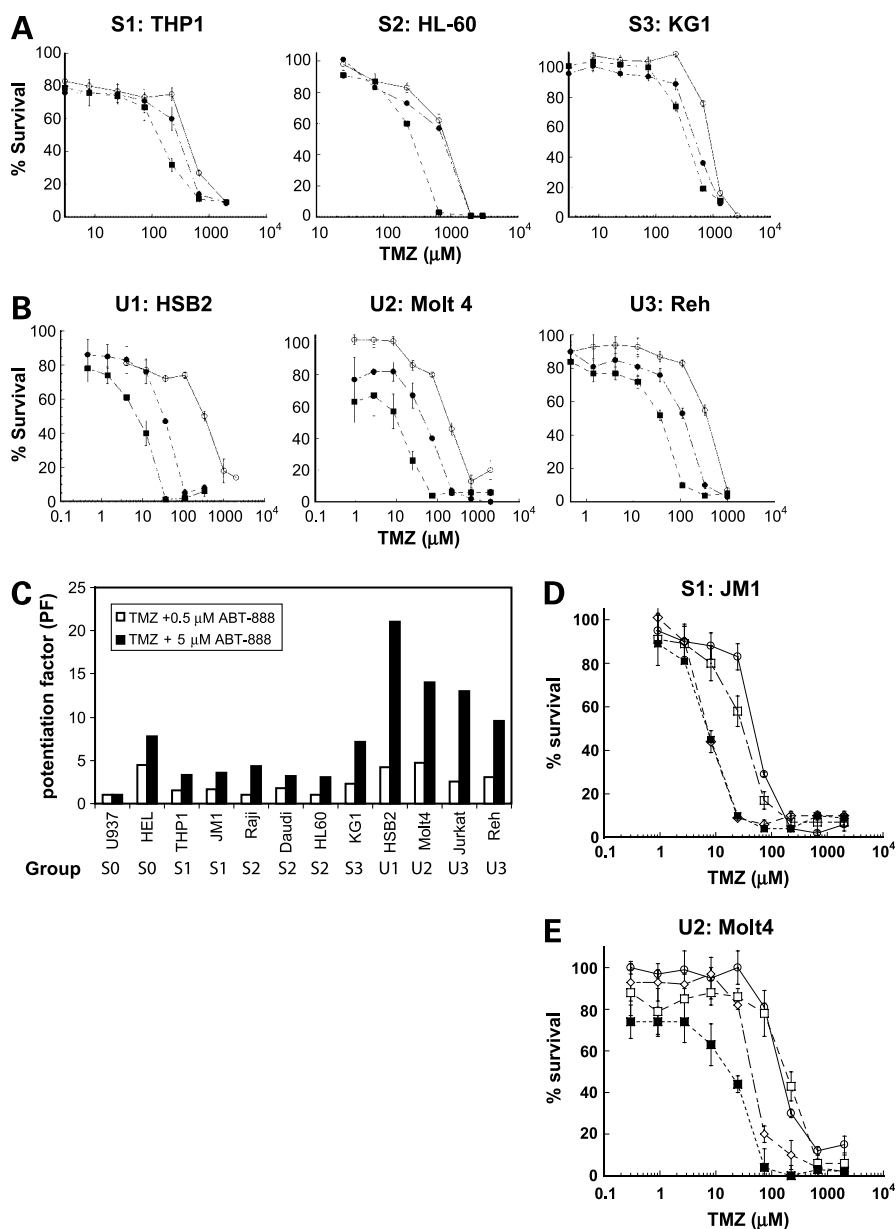


Figure 2. TMZ and ABT-888 growth inhibition in leukemia cell lines. MTT cytotoxicity assays were done with either TMZ (A) or ABT-888 (B) at concentrations ranging from 1 to 2,000 $\mu\text{mol/L}$. Percentage cell survival is plotted as a function of drug concentration. Leukemia cells include the MMR-proficient JM1 (●), U937 (□), THP1 (◇), HEL (△), Raji (●, dotted line), Daudi (■, dotted line), and HL-60 (○) and the MMR-deficient Jurkat (◆), HSB2 (▲), Reh (■), and Molt4 (▼) cell lines. Open symbols, AML cell lines; closed symbols, ALL cell lines.

Figure 3. ABT-888 potentiation of TMZ in MMR-proficient (A) or MMR-deficient (B) cell lines. **A**, AML cell lines THP1 (S1, left), HL-60 (S2, middle), and KG1 (S3, right) treated with TMZ alone (○) or TMZ in combination with 0.5 μmol/L (●) or 5 μmol/L (■) of ABT-888. Similar results were obtained with ALL cell lines JM1 (S1; Fig. 2D), Raji, and Daudi (S2; data not shown). **B**, T-cell ALL cell lines HSB2 (S1), Molt4 (S2), and pre-B ALL cell line Reh (S3) treated as above. Cell lines have low (S1, U1), average (S2, U2), or elevated (S3, U3) MGMT activity. **C**, summary of PFs (IC₅₀ TMZ/IC₅₀ TMZ + ABT-888) in leukemia cell lines that are MMR proficient (MSI stable; S) and MMR deficient (MSI unstable; U) treated with TMZ combined with either 0.5 μmol/L (□) or 5 μmol/L (■) of ABT-888. Coefficients of variation for the modeled IC₅₀s varied from 8% to 29%. **D** and **E**, MMR-proficient pre-B ALL cell line JM1 (D) and MMR-deficient T-cell ALL cell line Molt4 (E) treated with TMZ alone (○) and TMZ in combination with 0.5 μmol/L ABT-888 (□), 1 μmol/L O⁶-BG (◇), or both 0.5 μmol/L ABT-888 and 1 μmol/L O⁶-BG (■).



To determine whether PARP potentiation of TMZ was affected by NHEJ in leukemia cell lines, we examined the expression of Ku70, which was strongly expressed in all leukemia cell lines tested, including U937 (Fig. 5A).

To further determine whether PARPi potentiation of TMZ was affected by NHEJ status, we measured NHEJ activity in leukemia cell lines using an *in vitro* NHEJ assay (35). Leukemia cell lysates were incubated with a radiolabeled probe designed to dimerize as the results of NHEJ as shown in 293T cells (Fig. 5E). MMR-proficient cell lines with similar ABT-888 potentiation of TMZ had different NHEJ activity levels. The AML cell lines THP1 and HL-60 had undetectable NHEJ, whereas in the pre-B ALL JM1 cell line NHEJ was robust. We also examined phosphorylated histone

H2AX, which is increased in response to unrepaired DSBs, and found no correlation between H2AX protein and ABT-888 potentiation of TMZ (Fig. 5A). These data suggest that ABT-888 potentiation of TMZ is not related to its role in NHEJ.

ABT-888 Potentiation of TMZ in Primary Leukemia Cells Is Related to Leukemia Subtype

Because leukemia cell lines have developed multiple adaptations to growth in culture, we also examined the ability of ABT-888 to potentiate TMZ in primary acute leukemia cells obtained directly from patients (Fig. 6). We determined whether ABT-888 potentiation correlated with MGMT activity or, in a subset of patients, with MMR status (Table 2). ABT-888 did not significantly potentiate TMZ

activity in primary ALL leukemia cells (Fig. 6A; Table 2) but enhanced the growth-inhibitory effects of TMZ in two of four AML leukemias (Fig. 6B; Table 2). The lack of ABT-888 potentiation of TMZ in two primary AML specimens did not seem to correlate with MGMT expression, as patient specimen p115, which showed the greatest ABT-888-induced potentiation of TMZ among the primary leukemias (4.6-fold), had elevated MGMT activity (2,580 fmol/mg protein). All tested primary leukemia cells were MMR proficient.

To determine whether ABT-888 potentiation of TMZ was related to baseline PARP activity, we measured PARP activity in 14 primary leukemia samples (Fig. 6C). Similar to leukemia cell lines, primary leukemia cells had widely variable PARP activity, ranging from <40 fmol/ μ g protein (below the level of quantitation) to 1,860 fmol/ μ g protein. There seemed to be little correlation between PARP activity and

ABT-888 potentiation of TMZ. Of interest, one of the two AML primary samples that showed no ABT-888 potentiation of TMZ (sample 4) also showed no PARP activity similar to the U937 cell line.

Discussion

In this study, we used a panel of leukemia cell lines to assess the relative effects of MGMT activity, MMR status, and PARP activity on the ability of ABT-888 to potentiate the antitumor effects of TMZ. Although our data indicate that ABT-888 was most effective in potentiating TMZ in MMR-deficient cell lines with low MGMT activity, ABT-888 also potentiated TMZ in MMR-proficient cell lines. In primary acute leukemia cells, ABT-888 potentiation of TMZ seemed to correlate only with leukemia subtype, providing >3-fold potentiation in two of four primary AML samples.

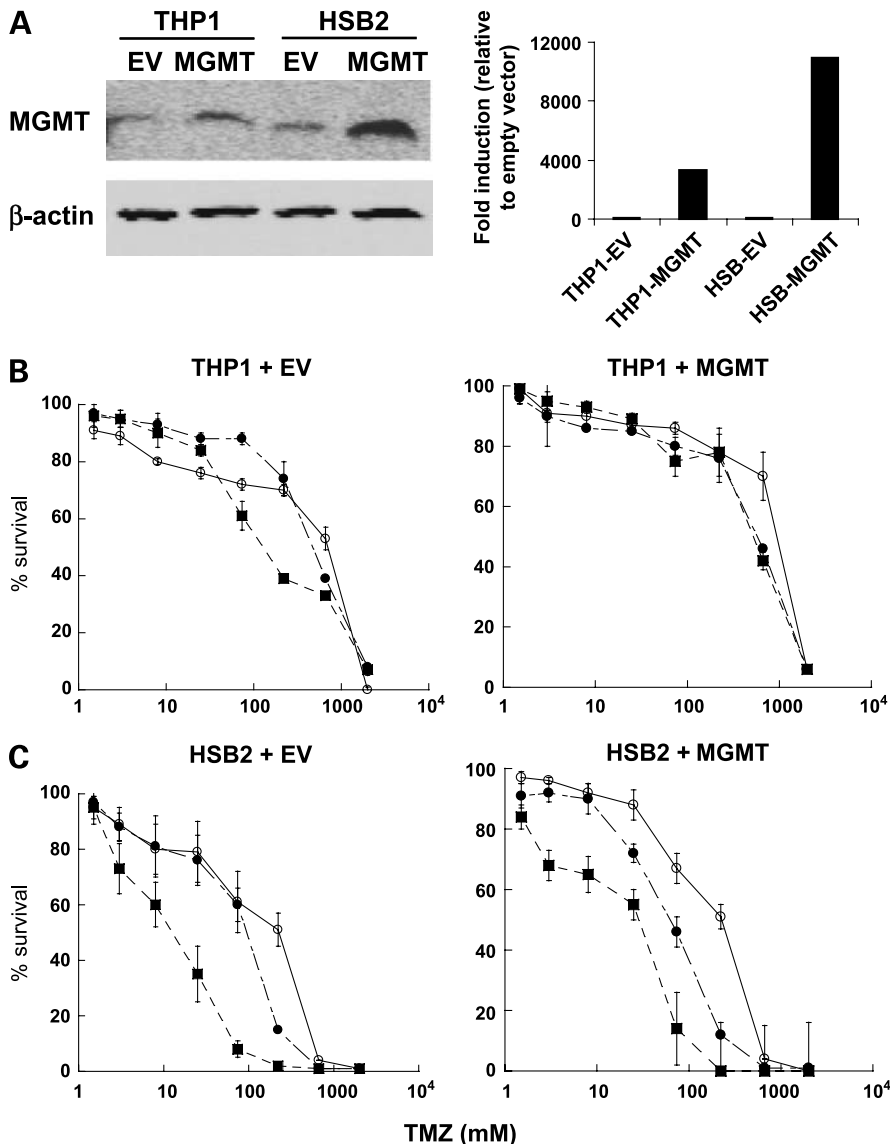
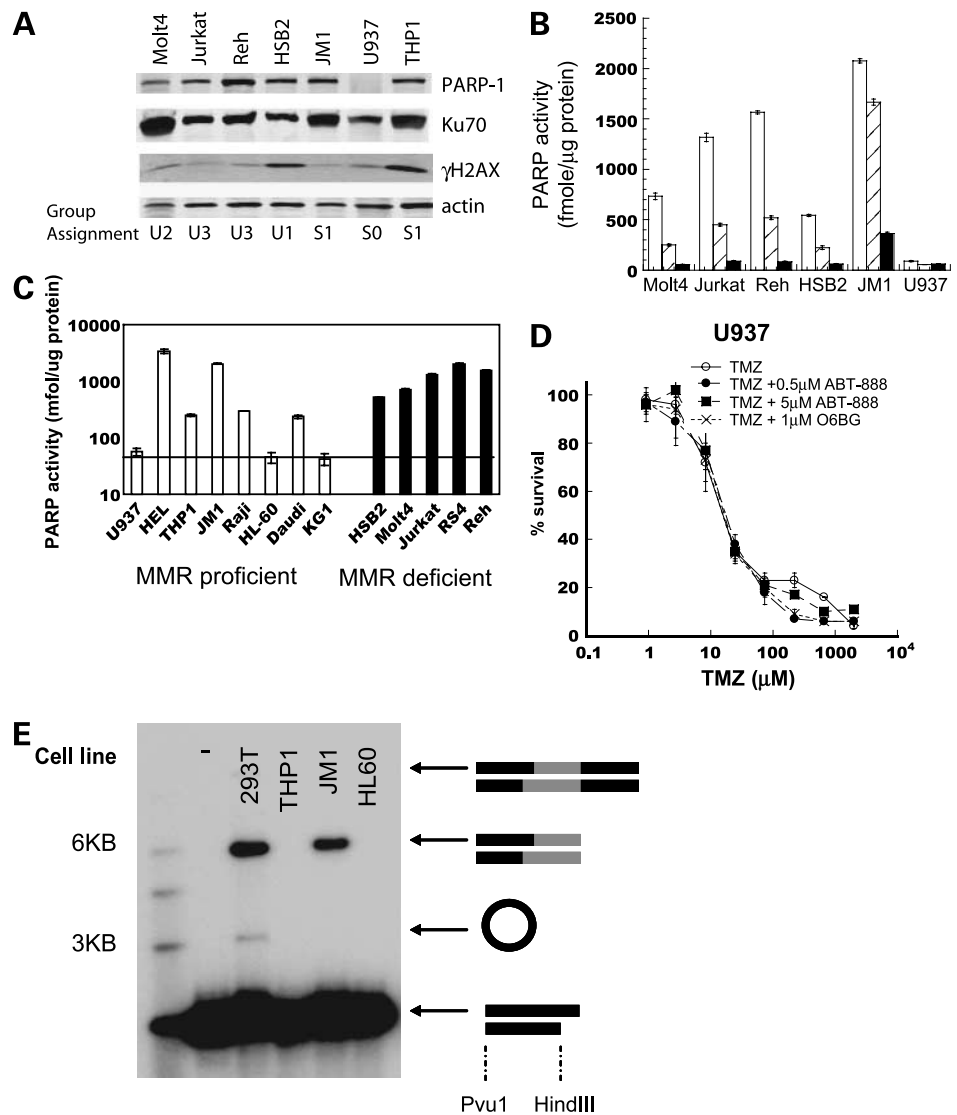


Figure 4. Effect of MGMT overexpression on ABT-888 potentiation of TMZ in MMR-proficient and MMR-deficient leukemia cell lines. **A**, confirmation of increased MGMT expression by Western blot and quantitation of increased MGMT expression by densitometry. The MMR-proficient AML cell line THP1 (**B**) and the MMR-deficient T-cell ALL leukemia cell line HSB2 (**C**) were treated with TMZ alone (○) or TMZ with either 0.5 μ mol/L (●) or 5 μ mol/L (■) of ABT-888. *Left*, transfected with pCDNA vector (EV); *right*, transfected with pCDNA vector containing the full-length coding sequence for MGMT.

Figure 5. Mechanisms of PARP potentiation in leukemia cell lines. **A**, pretreatment PARP, Ku70, and phospho-H2AX protein expression in four MMR-deficient (*U*) and three MMR-proficient (*S*) leukemia cell lines. Expression is normalized to actin expression. **B**, PARP activity for the leukemia cell lines described in **A** untreated (*white columns*) or treated with either 0.5 $\mu\text{mol/L}$ (*gray columns*) or 5 $\mu\text{mol/L}$ (*black columns*) of ABT-888. PARP activity (fmol/ μg protein) was determined as described in Materials and Methods. **C**, PARP activity in established leukemia cell lines. *Horizontal line*, limit of detection for assay. **D**, dose-response curve of TMZ alone (\circ) and TMZ in combination with either 0.5 $\mu\text{mol/L}$ (\bullet) or 5 $\mu\text{mol/L}$ (\blacksquare) of ABT-888 or 1 $\mu\text{mol/L}$ *O*⁶-BG (\times) in U937 AML cells. **E**, small-scale NHEJ *in vitro* assay. A 3-kb probe, designed to favor dimerization over recircularization or multimerization, was incubated with different leukemia cell line cell extracts. The presence of robust NHEJ can be detected by the formation of the 6-kb dimer as shown in the 293T control lane. *Lane 1*, DNA molecular weight ladder, *lane 2*, radiolabeled DNA fragment only (no cell lysate); *lane 3*, NHEJ-proficient control cell line 293T; *lanes 4 to 6*, leukemia cell lines THP1, JM1, and HL-60.



Although Tentori et al. (41) had also noted that leukemia cell lines could be sensitized to TMZ using the PARPi benzamide, this is the first report to examine the effect of MGMT activity on the ability of PARPis to potentiate TMZ in leukemia MMR-proficient and MMR-deficient cell lines. This is also the first reported examination of ABT-888 potentiation of TMZ in primary acute leukemia. Similar to previous reports in leukemia cell lines, colon cancer, and glioma, PARPi potentiation of TMZ was more pronounced in cells with MMR deficiencies (11, 14, 19).

Several PARPis are under investigation in oncology clinical trials. ABT-888 is undergoing phase 1 testing in adults with melanoma. Other PARPis under clinical study include AZD2281 (AstraZeneca), AG014699 (Pfizer) in combination with TMZ for adults with solid tumors and metastatic malignant melanoma (42), INO-1001 (Inotek/Genentech) in combination with TMZ for metastatic melanoma and glioblastoma multiforme (43), KU59436 (KuDOS/AstraZeneca)

in advanced solid tumors (including patients with BRCA1/BRCA2 mutations; ref. 44), and BS201 (BiPar) for refractory solid tumors and lymphomas (12). A better understanding of the factors that govern PARPi potentiation of TMZ may allow for the prospective determination of which tumors most likely to respond to this combination.

Prior clinical studies in both adults and pediatrics have suggested that TMZ may be effective in AML patients with low/absent MGMT tumor activity but is less effective in the presence of MMR deficiency or elevated MGMT activity (3, 4). Our data suggest that PARPi may be effective at potentiating TMZ in AML patients without MMR deficiencies and that this potentiation may be independent of MGMT activity. Although common in adults with treatment-related AML (45), MMR deficiencies are uncommon in pediatric patients with newly diagnosed AML. However, pediatric patients with relapsed leukemia frequently have elevated MGMT (4) and this group of patients would benefit from

potentiating agents that could overcome both MMR deficiencies and elevated MGMT activity.

In MMR-deficient cell lines, we found an inverse correlation between MGMT activity and potentiation of TMZ by ABT-888. MGMT activity seemed less important in determining ABT-888 potentiation of TMZ than MMR status in these leukemia cell lines. First, we observed 3-fold ABT-888 potentiation of TMZ in MMR-proficient leukemia cell lines and 10-fold ABT-888 potentiation of TMZ in MMR-deficient leukemia cells with elevated MGMT activity (Fig. 3C). Second, overexpression of MGMT in both MMR-deficient and MMR-proficient cell lines was unable to completely block ABT-888 potentiation of TMZ (Fig. 4). PARPi potentiation of TMZ in the presence of MGMT was also observed using the PARPi 3AB in colon cancer cell lines (46). These data suggest that neither increased MGMT activity nor MMR proficiency precludes the ability of PARPi to potentiate TMZ.

In some cases, the effects of ABT-888 seemed to depend on a threshold level of PARP activity because ABT-888 did not potentiate TMZ in either the U937 AML cell line or primary leukemia cells with very low PARP activity (Figs. 5C and 6A). However, ABT-888 did not result in TMZ potentiation in many primary ALL samples with high PARP activity, indicating that the presence of robust PARP activity is not sufficient for ABT-888 potentiation. There was also one AML cell line (KG1) with very low PARP activity, which showed 3-fold ABT-888 potentiation of TMZ, suggesting that either ABT-888 potentiation of TMZ may be dependent on nonribosylation functions of PARP. TMZ potentiation effects that are independent of PARP inhibition (i.e., off-target effects) cannot be excluded. Previous data showed that the PARPi GPI-15427 increased the antitumor activity of TMZ and irinotecan in colon cancer xenografts irrespective of PARP activity (16). Although the range of PARP activity

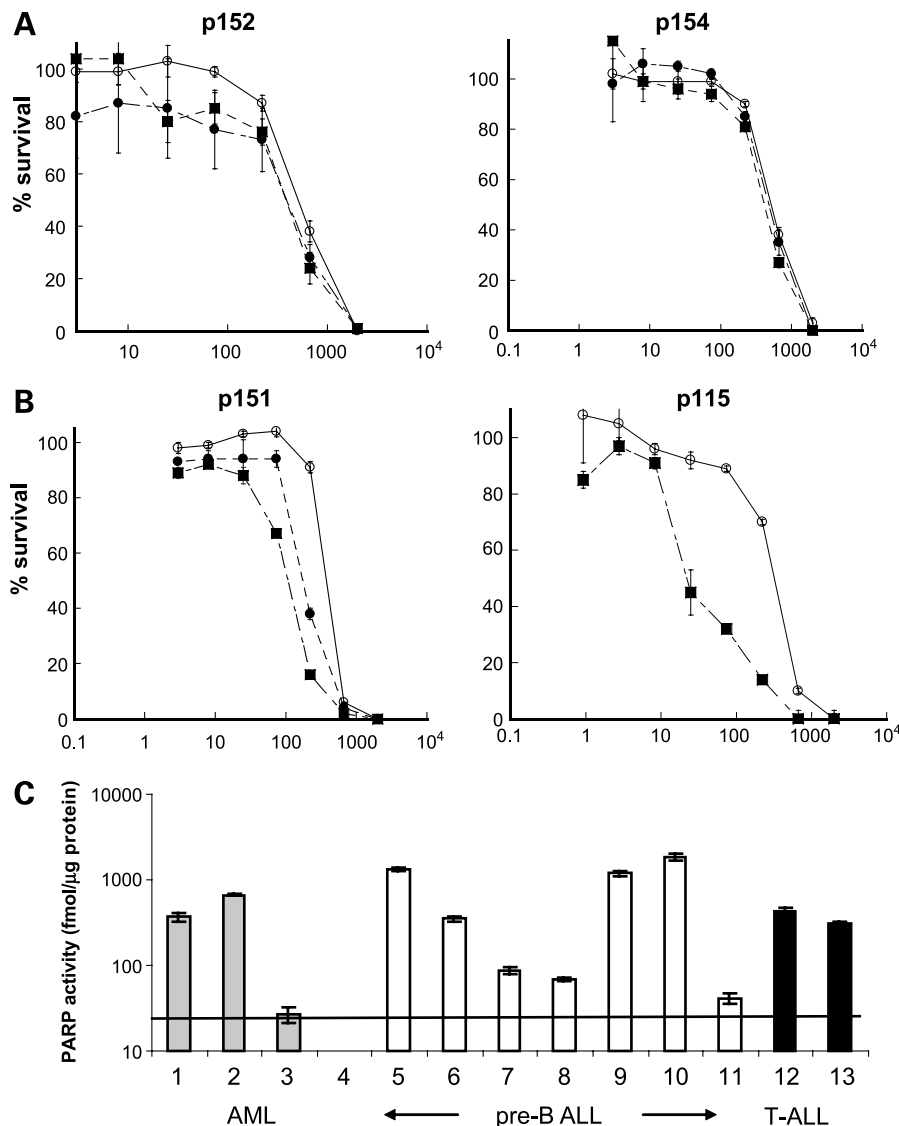


Figure 6. PARP activity and ABT-888 potentiation of TMZ in primary leukemia samples. **A to D**, dose-response curve of TMZ alone (○) or TMZ in combination with either 0.5 μmol/L (●) or 5 μmol/L (■) of ABT-888 in four representative primary leukemia patient samples. **A**, representative ALL patient samples in which ABT-888 did not potentiate TMZ. *Left*, 11-y-old Caucasian female with newly diagnosed pre-B ALL; *right*, 2-y-old Hispanic female with newly diagnosed T-cell ALL. Potentiation factors are listed in Table 2. **B**, AML patient samples in which ABT-888 potentiated TMZ. *Left*, 2-d-old Hispanic male with fulminant AML/myelodysplastic syndrome; *right*, 17-y-old Hispanic male with relapsed MO AML (trisomy 8 translocation). **C**, PARP activity in 14 patient leukemia samples. *Horizontal line*, limit of detection for assay.

Table 2. Summary of MGMT activity, PARP activity, and ABT-888 potentiation of TMZ in primary pediatric cells

| Cell ID | Leukemia subtype | MSI | MGMT activity (fmol/mg protein) | PARP activity (fmol/ μ g protein)* | ABT-888 PF [†] |
|---------|------------------|-----|---------------------------------|--|-------------------------|
| p115 | AML | S | 2,580 | ND | 4.6 |
| p151 | AML | ND | 1,200 | 670 | 3.2 |
| p120 | AML | S | UD | 370 | 1.2 |
| BM1261 | AML | ND | 151 | UD | 1 |
| p145 | Pre-B ALL | ND | 1,690 | 1,320 | 1 |
| p152 | Pre-B ALL | S | 325 | 350 | 1 |
| p157 | Pre-B ALL | S | 1,260 | 1,180 | 1 |
| p158 | Pre-B ALL | ND | 3,250 | 1,860 | 1 |
| BM1169 | Pre-B ALL | ND | 310 | 40 | 1 |
| BM1339 | Pre-B ALL | ND | 1,110 | 90 | 1.2 |
| BM1350 | Pre-B ALL | ND | 550 | 70 | 1.5 |
| p153 | T-ALL | ND | 1,580 | 430 | 1 |
| p154 | T-ALL | ND | 1,760 | 310 | 1 |

Abbreviations: S, MSI stable; ND, not determined; UD, undetectable.

*MGMT activity <100 fmol/mg protein; PARP <40 fmol/ μ g protein.

[†]IC₅₀ TMZ/IC₅₀ TMZ + ABT-888. Value of 1 indicates no potentiation.

in most primary tumors is unknown, Plummer et al. (47) examined PARP activity in adult melanoma specimens and noted that PARP activity was quite variable, ranging from 1.7 to 3,600 pmol PARP/mg protein. Hence, it is likely that the effectiveness of PARPi will vary, reflecting variability in both baseline PARP activity and the efficiency of PARP inhibition.

Although best known for its role in BER, PARP also participates in DSB repair (5). Evidence suggests that PARP competes with Ku for DSBs and PARP can either preserve DSBs for homologous recombination (48) or (in the absence of Ku) initiate the use of a backup NHEJ repair pathway involving DNA ligase III (40). The effects of PARP inhibition could be influenced by the intactness of the DSB repair pathways; for instance, in the absence of Ku or BRCA1/2, tumor cells may be very sensitive to PARP inhibition (49). We have shown here that NHEJ status did not affect the ability of ABT-888 to potentiate TMZ in leukemia cell lines. Further study is needed to determine whether the status of the backup NHEJ repair pathway or homologous recombination influences ABT-888 potentiation of TMZ.

The influence of MGMT activity, PARP activity, and MSI on the ability of ABT-888 to potentiate TMZ is derived from a limited number of leukemia cell lines and therefore should be interpreted with caution. PARPi potentiation of TMZ in MMR-proficient cell lines may also be limited clinically because ABT-888 potentiation is seen only at high TMZ concentrations (>100 μ mol/L). PARPi potentiation of TMZ could also be influenced by factors that were not examined, such as ABT-888 transport or metabolism.

In limited phase 1 studies of TMZ in patients with leukemia, where patients may have either ALL or AML, clinical responses have been observed exclusively in patients with AML. In a phase 1 study of TMZ in pediatric leukemia (4), two of eight patients with AML had objective responses. These patients had absent or low MGMT activity and no MSI. Similarly, in an adult phase 1 study of TMZ

in acute leukemia, the four patients with an objective response to TMZ (complete remission or complete remission with incomplete platelet recovery) had AML (3). In this study, *in vitro* ABT-888 potentiation of TMZ was seen in some primary samples from patients with AML (Table 2). Although the reason for this is unclear, it may be that more complete PARP inhibition can be obtained in patients with AML.

In summary, we have shown that the PARPi ABT-888 can effectively enhance the activity of TMZ, potentiating TMZ growth inhibition 3- to 21-fold in leukemia cell lines. ABT-888 potentiation of TMZ was most effective in cells with MMR deficiencies and low MGMT activity. Unexpectedly, PARP inhibition also potentiated TMZ activity in MMR-proficient leukemia cell lines, providing a 3- to 7-fold enhancement of TMZ growth inhibition independent of MGMT activity. Our data suggest that ABT-888 may function as a useful potentiator of TMZ; further studies, particularly in AML, are warranted.

Disclosure of Potential Conflicts of Interest

M.E. Dolan: coinventor of O⁶-BG, which has been licensed to Access Oncology, Inc. No other potential conflicts of interest were disclosed.

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Poly(ADP-ribose) polymerase inhibitor ABT-888 potentiates the cytotoxic activity of temozolomide in leukemia cells: influence of mismatch repair status and O⁶-methylguanine-DNA methyltransferase activity

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