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Therapeutics, Targets, and Chemical Biology

Cancer Research

6-Thioguanine Selectively Kills BRCA2-Defective Tumors and Overcomes PARP Inhibitor Resistance

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Abstract

Familial breast and ovarian cancers are often defective in homologous recombination (HR) due to mutations in the *BRCA1* or *BRCA2* genes. Cisplatin chemotherapy or poly(ADP-ribose) polymerase (PARP) inhibitors were tested for these tumors in clinical trials. In a screen for novel drugs that selectively kill BRCA2-defective cells, we identified 6-thioguanine (6TG), which induces DNA double-strand breaks (DSB) that are repaired by HR. Furthermore, we show that 6TG is as efficient as a PARP inhibitor in selectively killing BRCA2-defective tumors in a xenograft model. Spontaneous BRCA1-defective mammary tumors gain resistance to PARP inhibitors through increased P-glycoprotein expression. Here, we show that 6TG efficiently kills such BRCA1-defective PARP inhibitor–resistant tumors. We also show that 6TG could kill cells and tumors that have gained resistance to PARP inhibitors or cisplatin through genetic reversion of the *BRCA2* gene. Although HR is reactivated in PARP inhibitor–resistant BRCA2-defective cells, it is not fully restored for the repair of 6TG-induced lesions. This is likely to be due to several recombinogenic lesions being formed after 6TG. We show that BRCA2 is also required for survival from mismatch repair–independent lesions formed by 6TG, which do not include DSBs. This suggests that HR is involved in the repair of 6TG-induced DSBs as well as mismatch repair–independent 6TG-induced DNA lesion. Altogether, our data show that 6TG efficiently kills BRCA2-defective tumors and suggest that 6TG may be effective in the treatment of advanced tumors that have developed resistance to PARP inhibitors or platinum-based chemotherapy. *Cancer Res; 70(15); 6268–76. ©2010 AACR.*

Introduction

Breast cancer is the most common cancer in women in the Western world today, and in the United Kingdom, breast cancer incidence rates have increased by more than 50% over the last 25 years. Familial mutations in the breast cancer susceptibility genes *BRCA1* or *BRCA2* are associated with an increased risk of several cancers, particularly breast, ovarian, and prostate cancer (1). The proteins encoded by these genes play important roles in homologous recombination (HR) repair (2–4) and it is likely that their tumor suppressor function is explained by their role in reducing mutation rates (5). This hypothesis is also supported by the observation that proteins with related functions have also been linked with a predisposition to developing breast cancer, i.e., *CHEK2* (6), *ATM* (7), *PALB2* (*FANCN*; refs. 8–10), and *BRIP1* (*BACH1*; ref. 11).

HR-defective cells are characterized by hypersensitivity to cross-linking agents, which is thought to be related to the role of HR in bypassing inter-strand cross-links during DNA replication (12). HR-defective cells are also hypersensitive to poly(ADP-ribose) polymerase (PARP) inhibitors (13, 14). This involves PARP1 having a role in DNA single-strand break repair (15), which results in the suppression of HR (16). PARP inhibitors might increase the amount of single-strand breaks, which collapse into DNA double-strand breaks (DSB) at replication forks, requiring HR for repair (17). In the absence of HR, these DSBs are not repaired, resulting in HR-defective cells such as *BRCA1*- and *BRCA2*-mutated cancers, which are therefore hypersensitive to PARP inhibitors (13). In the clinic, PARP inhibitors efficiently killed *BRCA1*- and *BRCA2*-defective tumors in a phase I/II clinical trial (18). However, acquired resistance to PARP inhibitor is a problem and may involve either

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additional mutations in the *BRCA1* or *BRCA2* genes that result in restoration of the COOH-terminal part of the proteins (19–21) or upregulation of *Abcb1a/b* genes encoding P-glycoprotein efflux pumps (22). Although the extent of resistance that can be acquired by these mechanisms is unclear in humans, it suggests that the discovery of agents that may overcome such resistance mechanisms merit further investigation.

Materials and Methods

Chemicals

All chemicals were obtained from Sigma unless stated otherwise, AG014699 was provided by Pfizer GRD, and KU0058948 and olaparib were from KuDOS Pharmaceuticals. AG014699 and KU0058948 compounds were dissolved at 10 mmol/L in 100% dry DMSO and stored at −20°C, it was diluted in culture medium to give the final desired drug concentration in 1% DMSO with control cultures exposed to 1% DMSO alone. The National Cancer Institute (NCI) diversity and mechanistic set were obtained from NCI (Bethesda, MA) and stocks were maintained in 96-well plates in DMSO [stock concentration 1 mmol/L (mechanistic set) and 10 mmol/L (diversity set)].

Cell culture and isolation of PARP inhibitor–resistant cells. HCT116 and HCT116+Chr3 were obtained from Dr. Bert Vogelstein, U2OS cell line was obtained from American Type Culture Collection, capan-1, and resistant capan-1 clones from Dr. Toshiyasu Taniguchi, AA8, irs1SF, and CXR3 cells were from Dr. Larry Thompson, whereas V-C8 and V-C8B2 cells were previously isolated (4). All cells were grown in DMEM with 10% fetal bovine serum and penicillin/streptomycin at 37°C under an atmosphere containing 5% CO_2.

shRNA depletion of BRCA2

Depletion of BRCA2 expression in U2OS or HCT116 cells was obtained from the stably integrated regulatable expression of BRCA2 short hairpin (shRNA) using the BLOCK-iT Inducible H1 RNAi Entry Vector Kit from Invitrogen, according to the manufacturers protocol (Invitrogen, Sweden). The target sequence introduced was AAC AAC AAU UAC GAA CCA AAC UU (23).

Western blot

Proteins from cell lysates were separated and detected using Western blotting as previously described (24). The primary antibody was an anti-rabbit BRCA2 antibody (Santa Cruz Biotechnology) diluted 1:500 in blocking solution.

Screening procedure

U2OS BRCA2 shRNA cells were grown in the presence or absence of 2 μg/mL of doxycyclin for 2 days, plated in 96-well plates (2,000 cells/well) in the presence or absence of doxycyclin, and the next day, treated with test compounds (at a concentration 10 μmol/L) from the NCI library. After 72 hours, WST-1 cell proliferation reagent was used to determine the cells viability as described earlier (25). Compounds from the library that selectively suppressed the growth of BRCA2-defective cells, but had only modest effects on BRCA2-proficient cells were selected.

Colony formation assay

Cells were plated into six-well plates at a density of 200 cells/well. The next day, the cells were treated with selected compounds at a range of concentrations, for different time periods (from 3 hours up to 6 days). After 1 week [for V-C8 and V-C8+B2 cells and PARP inhibitor–resistant (PIR) clones] or 2 weeks (for U2OS cells), when colonies could be observed, the colonies were fixed and stained with methylene blue in methanol (4 g/L). Colonies consisting of more than 30 cells were subsequently counted. In addition, exponentially growing V-C8, V-C8+B2, and PIR clones 1C and 2B were exposed to varying concentrations of AG014699 for 24 hours or 6-thioguanine (6TG) for 48 hours prior to seeding in 90 mm dishes in drug-free medium for colony formation. Colonies were fixed and stained with crystal violet 10 to 14 days later and counted on an automatic colony counter (Oxford Optronix).

Pulsed-field gel electrophoresis

Cells were treated with 6TG, collected by trypsinization, resuspended in 1% InCert-agarose (in 37°C PBS) to a final concentration of 1.5 million cells/100 μL and agarose plugs were separated by pulsed-field gel electrophoresis as previously described (26).

Immunofluorescence

Cells were grown on coverslips, treated, fixed, immunostained, and analyzed as previously described (24). The primary antibodies used were mouse monoclonal anti-γH2AX at a dilution of 1:1,000 and rabbit polyclonal anti-Rad51 (H-92; Santa Cruz Biotechnology) at a dilution of 1:1,000.

Propidium iodide staining and fluorescence-activated cell sorting analysis

Cells (1×10^6) were treated (or not treated) with compounds (such as B9 or 6TG), collected by trypsinization, and fixed in ice-cold 70% ethanol overnight at −20°C. The cells were then rehydrated in PBS and stained with 50 μg/mL of propidium iodide and 100 μg/mL of RNase A in PBS for 30 minutes at room temperature. Samples were further analyzed on a BD Biosciences FACScan. The data was analyzed with WinMDI software version 2.8.

In vivo experiments

Exponentially growing VC8 or VC8-B2 cells (1×10^7) were injected i.m. into the thigh of each CD1 nude (Charles River) mouse in 50 μL of PBS and handled and analyzed as previously described (13). Tumor growth was assessed using the ratio of the diameter of the right (tumor bearing) to the left (normal) thighs. When thigh ratio reached between 1.3 and 1.5, mice were divided into groups for the following treatments: AG014699 10 mg/kg (made up on day of use at 1 mg/mL in water) or 6TG 1.5 mg/kg (made up on

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day of use at 0.15 mg/mL in PBS) or saline (control) administered daily × 10 i.p.

Generation of mammary tumors

Brca1$^{\Delta5\text{-}13/\Delta5\text{-}13}$*;p53*$^{\Delta2\text{-}10/\Delta2\text{-}10}$ mammary tumors were generated in *K14cre;Brca1*$^{F5\text{-}13/F5\text{-}13}$*;p53*$^{F2\text{-}10/F2\text{-}10}$ mice and genotyped as described (27). Orthotopic transplantations of tumor fragments into syngeneic animals and caliper measurements of mammary tumors have been previously reported (22).

Results

BRCA2-defective cells are hypersensitive to 6TG

Individuals with inherited mutations in either *BRCA1* or *BRCA2* alleles have a high risk of developing breast cancer (1, 28). Here, we wanted to identify novel compounds to selectively kill BRCA2-defective cells. We developed a shRNA system to deplete the BRCA2 protein upon removal of doxycycline in U2OS sarcoma cells, and we assayed the mechanistic and diversity set compound libraries from the NCI for compounds that selectively killed BRCA2-depleted cells. Mercury-(2-amino-1, 9-dihydro-6H-purine-6-thionato-N7,S6)hexyl (B9) was identified in the screen as the most efficient compound to selectively kill BRCA2-depleted U2OS cells (Supplementary Fig. S1). We also found that this compound selectively killed BRCA2-defective V-C8 cells as compared with V-C8+B2, the isogenic cell line expressing BRCA2^{WT} protein (V-C8+B2; ref. 4; Supplementary Fig. S1). The reason for the selective killing of BRCA2-defective cells is likely to be explained by the role of BRCA2 in HR because cells defective in the RAD51 paralogue XRCC3 were similarly sensitive to the B9 compound (Supplementary Fig. S1).

Structural analysis of the B9 compound revealed a strong resemblance to 6TG, which is a well-established chemotherapy used to treat hematologic malignancies in children and adults (29). We therefore decided to test the sensitivity of BRCA2-deficient cells to 6TG and found that survival was significantly lower than that of BRCA2-expressing cells (Fig. 1A). Furthermore, we found that the BRCA2 protein is required to prevent apoptosis induced by 6TG, measured by sub-G_1 population (Fig. 1B) and the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay (Fig. 1C).

6TG selectively kills BRCA2-defective tumors

To test the hypothesis that 6TG is useful for selective treatment of BRCA2-deficient tumors, we treated mice bearing xenografts derived from BRCA2-deficient V-C8 and wild-type BRCA2-complemented V-C8+B2 cells. Consistent with the hypothesis, we found that neither the PARP inhibitor, AG014699, nor 6TG had any effect on the outgrowth of the BRCA2-proficient tumors (Fig. 1D); in contrast, all mice with BRCA2-defective tumors responded to both 6TG and the PARP inhibitor equally, with significant growth delay and three of five complete tumor regressions in both groups. These results suggest that 6TG is as effective as the PARP inhibitor, AG014699, in selectively killing BRCA2-defective tumors. However, it should be noted that 6TG at 1.5 mg/kg caused a greater loss of body weight than AG014699 (Supplementary Table S1) and was not tolerated at a higher dose of 3 mg/kg (data not shown).

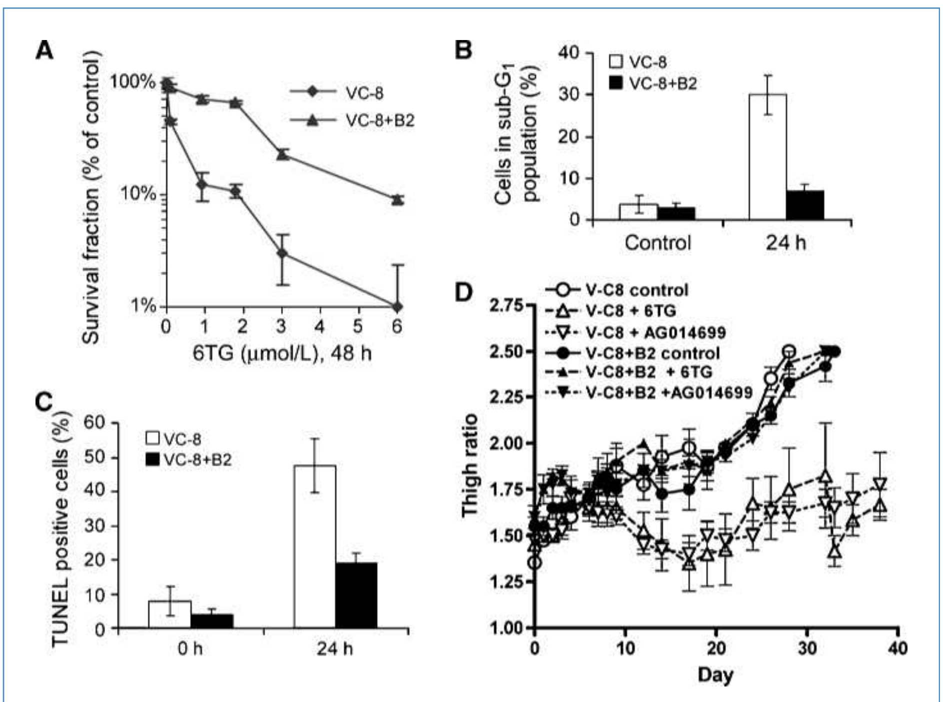

Figure 1. HR-defective cells are hypersensitive to chemical 6TG. A, 6TG selectively kills BRCA2-defective VC-8 cells in a colony formation assay. 6TG induces apoptosis in HR-defective VC-8 cells as measured by fluorescence-activated cell sorting analysis of the sub-G_1 population (B) and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining (C). Columns, averages from at least three experiments; bars, SD. D, tumor outgrowth in xenograft mice (five per group) following injection of V-C8 and BRCA2 complemented V-C8+B2 upon i.p. treatment with 6TG and PARP inhibitor.

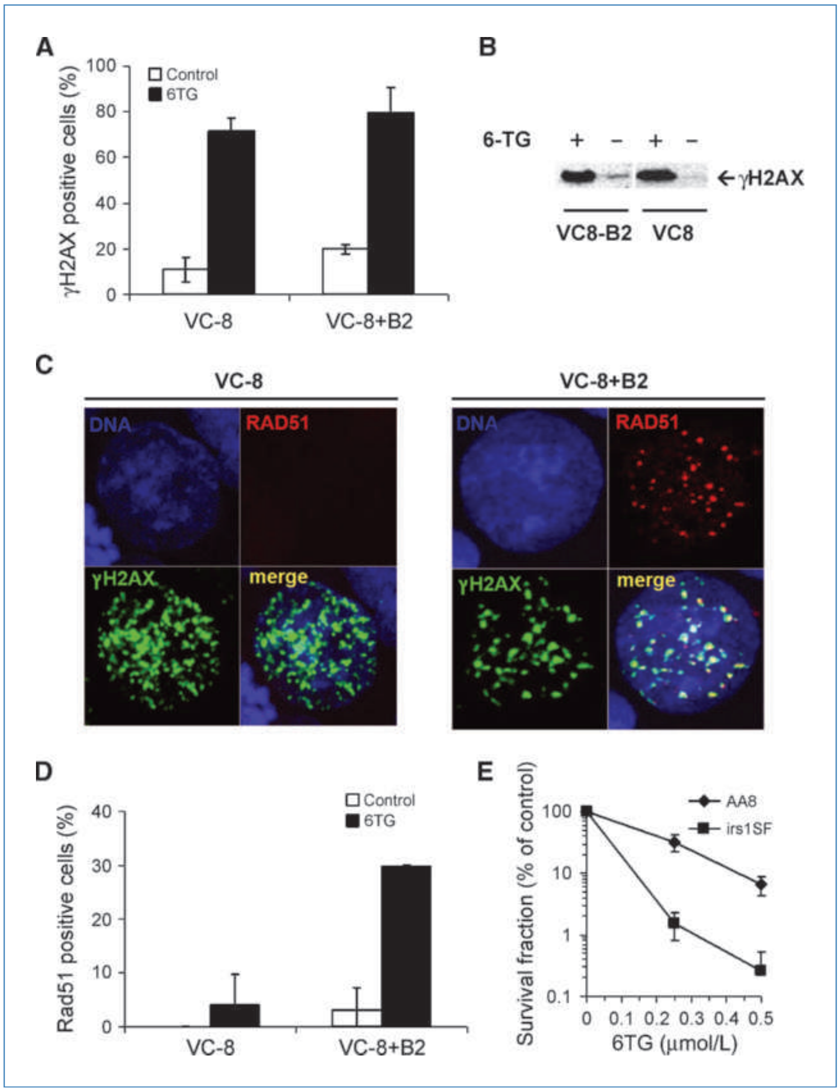

Figure 2. HR is needed to repair the damage induced by 6TG. Equal amounts of DNA damage in V-C8 and V-C8+B2 cells following 6TG treatment, as determined by γH2AX foci (A; cells containing more than 10 foci were scored as positive) or by Western blot (B). C, RAD51 foci do not form in V-C8 cells upon 6TG-induced DNA damage. RAD51 foci do form in V-C8+B2 cells and colocalize with γH2AX foci. D, quantification of RAD51 foci formed in V-C8 and V-C8+B2 cells after 6TG treatment. E, 6TG selectively kills XRCC3 defective irs1SF cells in a colony formation assay as compared with wild-type control (AA8).

6TG-induced DNA DSBs are repaired by HR

6TG is an antimetabolite of purine metabolism and is incorporated into the DNA of mammalian cells in place of guanine during DNA replication (29). The incorporated 6TG (~1 in 10^4 bases) is then methylated *in situ* to 6-meTG by endogenous *S*-adenosylmethionine and becomes a substrate for mismatch repair in the second replication round to mediate its toxicity (30–33). As a measure of formation of DSBs in DNA, we looked at the nuclear levels of γH2AX in cells treated with 6TG and we found that γH2AX foci formed with similar frequency in both BRCA2-deficient and -proficient cells (Fig. 2A and B). This similarity suggests that the hypersensitivity of BRCA2-deficient cells to 6TG is due to an inability to repair DNA damage rather than a difference in the amount of damage introduced. We also found that γH2AX foci colocalized with RAD51 foci after 6TG treatment in BRCA2-proficient cells (Fig. 2C and D, right panels), suggesting that the RAD51 protein is recruited to DSBs to repair the lesion by HR. In contrast, RAD51 foci did not form at 6TG-induced γH2AX foci in BRCA2-defective V-C8 cells, suggesting that DSB repair is deficient in these cells (Fig. 2C and D, left panels). Using pulsed-field gel electrophoresis, we found that BRCA2-defective V-C8 cells were unable to repair 6TG-induced DSBs compared with BRCA2-expressing cells (Supplementary Fig. S2). To further investigate the role of HR in the 6TG-induced DNA damage, we looked at the survival of cells defective in the RAD51 paralogue, XRCC3 (irs1SF), and we found that these cells were considerably more sensitive to 6TG than AA8 control cells (Fig. 2E). Altogether, these findings suggest that the sensitivity of BRCA2-defective cells to 6TG was due to their inability to perform HR repair. HR is important in repairing DNA damage caused by a wide range of mono- or bifunctional alkylating anticancer agents, for example, the commonly used drugs, cisplatin and mitomycin C (34). To our knowledge, these results with 6TG represent the first time HR has been implicated in the

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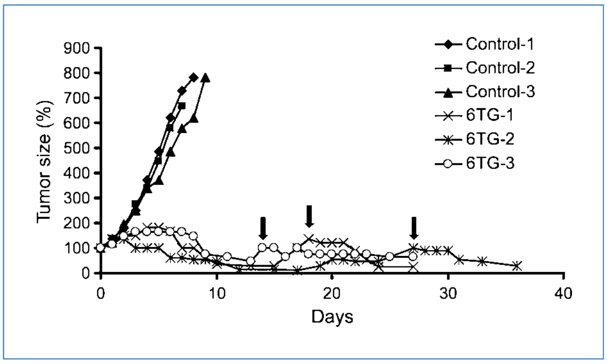

Figure 3. Response of the PIR *Brca1*$^{\Delta5\text{-}13/\Delta5\text{-}13}$;*p53*$^{\Delta2\text{-}10/\Delta2\text{-}10}$ tumor T6-28 to 6TG. Animals carrying orthotopically transplanted tumors were treated with 1.5 mg of 6TG per kg i.p. daily on days 0 to 9 or 50 mg of olaparib per kg i.p. daily when the tumors reached a volume of 150 to 250 mm^3 (100%, day 0). When tumors relapsed back to 100% (arrows), a second treatment of 1.5 mg of 6TG per kg daily for 4 consecutive days was tolerated.

repair of thiopurine antimetabolite drugs. Interestingly, it has previously been shown that HR-defective cells are more sensitive to O^6-methyl guanine lesions than cell lines with defects in other repair pathways (35, 36), a finding which corroborates the importance of HR in repairing lesions on the O^6 position of guanine.

BRCA1-defective tumors that gained resistance to PARP inhibitors through P-glycoprotein expression remain 6TG-sensitive

Increased expression from the *Abcb1a* and *Abcb1b* genes encoding the mouse drug efflux transporter P-glycoprotein explains resistance to the PARP inhibitor olaparib in BRCA1;p53-defective mammary tumors (22). Here, we wanted to determine whether 6TG could target such PIR tumors. To test this, *Brca1*$^{\Delta5\text{-}13/\Delta5\text{-}13}$;*p53*$^{\Delta2\text{-}10/\Delta2\text{-}10}$ mammary tumors derived from *K14cre;Brca1*$^{F5\text{-}13/F5\text{-}13}$;*p53*$^{F2\text{-}10/F2\text{-}10}$ mice were grown out and treated with olaparib as previously described (22). Small tumor fragments of an olaparib-resistant tumor (T6-28) with 80-fold increased expression of the *Abcb1b* gene were transplanted orthotopically into syngeneic wild-type female mice. The animals were then treated with 6TG when the tumor volume reached ~200 mm^3. Interestingly, we found that tumors responded to 6TG (Fig. 3). This shows that spontaneous BRCA1;p53-defective mammary tumors are sensitive to 6TG, and importantly, that PIR tumors in which resistance is caused by increased P-glycoprotein–mediated drug efflux remain

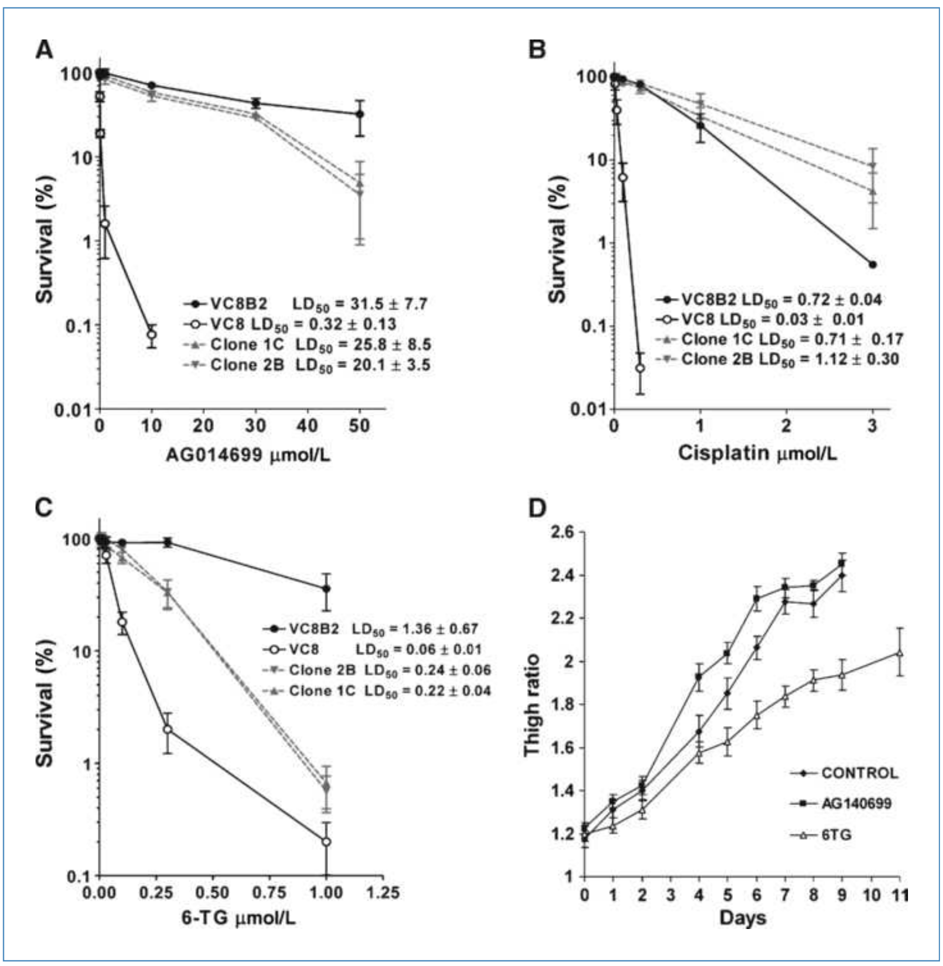

Figure 4. PARP inhibitor and cisplatin-resistant BRCA2-defective cells and tumors respond to 6TG. Clonogenic survival in BRCA2-defective V-C8, BRCA2-complemented V-C8+B2, and V-C8 PIR clones following treatment with PARP inhibitor AG014699 (A), cisplatin (B), or 6TG (C). Columns, averages from at least three experiments; bars, SD. D, tumor outgrowth in xenograft mice following injection of PIR V-C8 clone 2B upon i.p. treatment with 6TG and PARP inhibitor. 6TG retards PIR V-C8 clone 2B tumor outgrowth (statistically significant in Mann-Whitney test, $P < 0.01$). Points, averages from 10 mice in each group; bars, SE.

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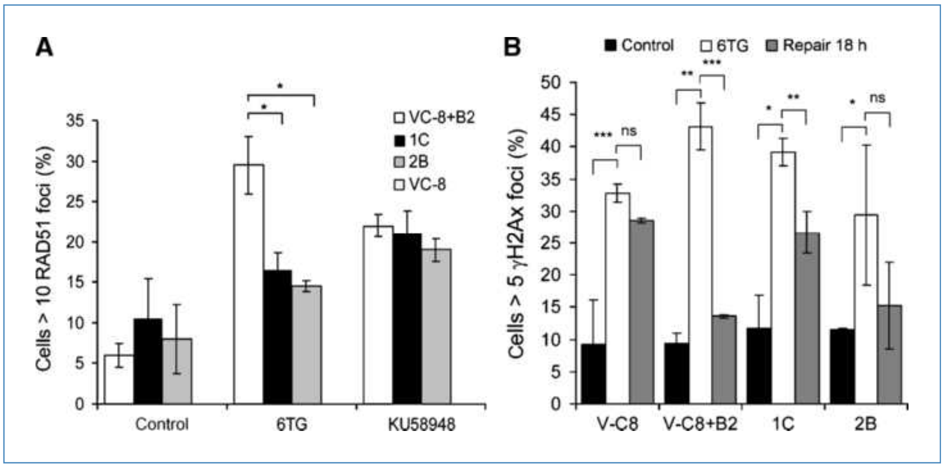

Figure 5. PARP inhibitor–resistant BRCA2-defective cells partially activates HR in response to 6TG. A, quantification of 6TG and PARP inhibitor induced RAD51 foci. BRCA2 complemented V-C8+B2 cells showed a significant increase in RAD51 foci compared with PIR V-C8 cells upon 6TG treatment (24 h after an 18-h treatment with 1 μmol/L of 6TG). B, quantification of repair of 6TG-induced γH2AX foci. HR-defective V-C8 cells fail to repair 6TG-induced γH2AX foci, whereas V-C8+B2 cells repairs a majority of 6TG-induced γH2AX foci. PIR V-C8 clones show an intermediate repair of 6TG-induced γH2AX foci. Columns, averages of three independent experiments; bars, SE. Values marked with asterisks are statistically significant in *t* tests (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$).

sensitive to 6TG. After the 10-day treatment with 6TG, tumors eventually grew back. However, such tumors were still responding to a second line treatment with 6TG, indicating that the tumors did not easily obtain resistance to 6TG.

BRCA2-defective cells and tumors that gain resistance to PARP inhibitors through genetic reversion respond to 6TG treatment

The mechanisms of acquired resistance to PARP inhibitors could also evolve through genetic reversion in BRCA2-defective cancer cells (20, 21). In such cases, a mutation in the *BRCA2* gene results in which the COOH-terminal part of the protein is retained and the protein is overall functional in HR, despite missing a single-stranded DNA (ssDNA) binding domain (20, 21). To investigate this mechanism of resistance further, we used BRCA2-defective V-C8 clones selected for resistance to a PARP inhibitor.[8] All PIR V-C8 clones harbor the same mutation, restoring the correct reading frame for *BRCA2* and at the same time introducing a mutation within a highly conserved region in exon 15. This mutation affects a highly conserved arginine that was also identified in a family with breast and ovarian cancers (37), and was described in mitomycin C–resistant V-C8 cells (38). Thus, the reverted BRCA2 still has a defective ssDNA domain in the COOH-terminal part of the protein, as described earlier (20, 21), which restored HR as indicated by the ability to form RAD51 foci in response to PARP inhibitor treatment (Supplementary Fig. S3). We tested the sensitivity of PIR-resistant clones 1C and 2B to the PARP inhibitor AG014699 and found that both clones had lost their sensitivity to PARP inhibitors (Fig. 4A).

We then tested the sensitivity of PIR V-C8 cells for cross-resistance to cisplatin and 6TG. Parental V-C8 cells are highly sensitive to both cisplatin and 6TG, compared with the BRCA2-expressing V-C8+B2 cells (Fig. 4B and C). As expected from previous studies (20, 21), PIR V-C8 clones exhibited resistance to cisplatin (Fig. 4B). Surprisingly, we found that PIR V-C8 cells had not fully reverted to resistance to 6TG (Fig. 4C), suggesting that 6TG might still kill PIR BRCA2-defective tumors that gained resistance through genetic reversion.

We confirmed that BRCA2 revertant cells, which have acquired resistance to cisplatin, retain sensitivity to 6TG by using BRCA2-defective human pancreatic cancer cell line capan-1 and four different independent cisplatin-resistant capan-1 clones (20). Clones 6 and 12 acquired resistance through an additional mutation in the *BRCA2* gene that corrected the frameshift caused by the 6174delT mutation in capan-1 cells, whereas clones 10 and 11 did not have an additional *BRCA2* mutation, lack BRCA2 protein expression, and are likely to have reverted to cisplatin resistance through other unknown pathways (20). Interestingly, all four cisplatin-resistant clones showed equal sensitivity to 6TG as parental capan-1 cells (Supplementary Fig. S4), providing additional evidence that PIR and cisplatin-resistant BRCA2-defective cancer cells are sensitive to treatment with 6TG.

Next, we wanted to test whether 6TG could also retard the outgrowth of PIR *BRCA2*-defective tumors that have gained resistance through genetic reversion. To test this, we treated mice bearing xenografts derived from PIR clone 2B with a 10-day treatment of AG014699 or 6TG and found that PIR clone 2B tumors only responded to the 6TG treatment and not to the PARP inhibitor (Fig. 4D; statistically significant in Mann-Whitney test, $P < 0.01$).

[8] Gottipati et al. 2010, submitted manuscript.

Differential sensitivity to anticancer drugs in genetically reverted BRCA2-defective cells

To gain further insights into the function of the restored BRCA2 protein carrying a mutation in the ssDNA binding domain, we further investigated the sensitivity of the PIR clones to a range of cytotoxic agents (Supplementary Fig. S5A–E and F). The PIR clones exhibited similar levels of resistance to temozolomide, camptothecin, and ionizing radiation as the V-C8+B2 cells compared with the more sensitive V-C8 cells. Interestingly, the PIR clones were slightly more resistant to doxorubicin than the V-C8+B2 cells, which were in turn more resistant than the V-C8 cells (Supplementary Fig. S5D). Surprisingly, the V-C8 and the PIR clones were less sensitive to gemcitabine and paclitaxel than the V-C8+B2 cells (Supplementary Fig. S5E and F). These data suggest that recombination-defective BRCA1 and BRCA2 tumors would respond poorly to gemcitabine and paclitaxel treatments.

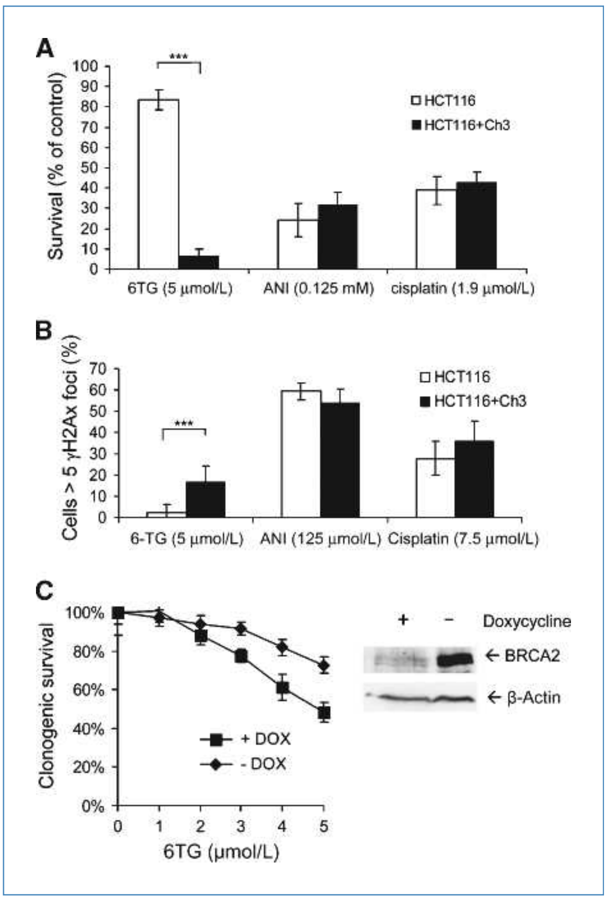

Figure 6. BRCA2 suppresses 6TG toxicity in mismatch repair–deficient cells. A, survival following continuous treatment with PARP inhibitor ANI, 6TG, and cisplatin in HCT116 and HCT116+Ch3 cells. B, γH2AX foci formation in hMLH1-defective HCT116 and hMLH1 complemented HCT116+Ch3 cells after a 24-h treatment with 6TG and cisplatin or a 4-h treatment with PARP inhibitor ANI. C, clonogenic survival in HCT116 and BRCA2 depleted HCT116 to increasing doses of 6TG. Points, average of three independent experiments; bars, SD. Values marked with asterisks are statistically significant in *t* tests (***, $P < 0.001$).

The retained sensitivity to 6TG and resistance to gemcitabine and paclitaxel in the PIR clones is likely to be explained by the reverted *BRCA2* gene which did not revert back to wild-type, but retained a mutation in the ssDNA domain, which may impair HR induced by these agents. This ssDNA domain might be required for the BRCA2 response to 6TG and might prevent the efficient repair of gemcitabine- and paclitaxel-induced lesions.

6TG induces both mismatch repair–dependent and –independent lesions that require HR repair

Next, we wanted to understand the mechanism for PIR cells maintaining their sensitivity to 6TG. We analyzed RAD51 foci and found that PIR V-C8 cells induced RAD51 foci as efficiently as V-C8+B2 cells in response to treatment with a PARP inhibitor, but not in response to treatment with 6TG, suggesting that the BRCA2-reverted protein is not fully proficient for 6TG-induced HR (Fig. 5A). We also investigated the translocation of the RAD51 protein into the chromatin fraction, as this may be associated with the efficiency of RAD51 loading on to DNA and subsequent HR (39). We found that the RAD51 protein is more efficiently recruited to DNA after 6TG in V-C8+B2 cells than in the 1C clone, and conversely, that the RAD51 protein is more efficiently recruited to DNA in the 1C clone than V-C8+B2 following treatment with the PARP inhibitor, KU0058948 (Supplementary Fig. S6). Next, we investigated the repair of 6TG-induced DNA lesions by γH2AX foci formation and find that BRCA2 is required for efficient repair (Fig. 5B). Interestingly, neither the 1C or 2B clones fully repaired the 6TG-induced DNA damage, suggesting that their lack of efficient HR repair is the reason for their 6TG sensitivity.

The reason for the differential response to 6TG and PARP inhibitors might be related to the production of different recombinogenic lesions in DNA. To test this hypothesis, we treated mismatch repair–defective HCT116 colorectal cancer cells and the same cells with restored mismatch repair function (HCT116+Ch3; ref. 40) with 6TG, the PARP inhibitor 4-amino-1,8-naphthalimide (ANI) and cisplatin. We found that only the cytotoxicity of 6TG was dependent on a functional mismatch repair pathway (Fig. 6A; Supplementary Fig. S7). Furthermore, the level of recombinogenic DSBs, measured by γH2AX foci, were dependent on mismatch repair after 6TG treatment, whereas the generation of these lesions was unaffected by PARP inhibitor and cisplatin treatment in these cell lines (Fig. 6B). Our data are in line with evidence showing that toxic DSBs induced by 6TG are mismatch repair–dependent (30), and it has previously been shown that HR induced by O^6-guanine methylating agents depends on mismatch repair (41). This is also in line with our previous observation that 6TG-induced DSBs require HR for repair (Fig. 5B; Supplementary Fig. S2).

There is a possibility that 6TG may produce another mismatch repair–independent HR substrate, given that the PIR V-C8 clones show an intermediate HR response to 6TG (Fig. 5). To test this, we shRNA-depleted BRCA2 in HCT116 cells to test if the absence of 6TG-induced DSBs also abolished the requirements for HR survival. Surprisingly, we find that mismatch repair–defective HCT116 still requires BRCA2 for survival to 6TG, showing that 6TG also produces a recombinogenic lesion that is independent of mismatch repair (Fig. 6C).

Discussion

Here, we report that cells and/or tumors defective in the HR genes *BRCA1, BRCA2*, or *XRCC3* are hypersensitive to 6TG and that, in the case of *BRCA2*, this could be reversed by the introduction of a vector expressing the BRCA2 protein. We show that 6TG induced RAD51 foci at 6TG-induced DSBs and that the DSBs were less efficiently repaired in BRCA2-defective cells, which correlates with increased toxicity in HR-defective cells. This is, to our knowledge, the first time that HR has been implicated in the repair of 6TG-induced DSBs.

Interestingly, the opposite result was previously reported: that expression of $BRCA1^{WT}$ in BRCA1-mutated HCC1937 breast cancer cells increases sensitivity to 6TG (42). However, this is unrelated to any role of BRCA1 in HR (42) and the BRCA1 mutation in HCC1937 cells is unlikely to affect HR, as RAD51 foci are efficiently induced by IR in these cells (43).

Although PARP inhibitors have been shown to efficiently kill both *BRCA1*- and *BRCA2*-defective tumors, resistance to therapy may develop within 18 to 77 weeks (18). Although the exact mechanisms for PIR in patients remains unknown, this might involve the expression of P-glycoprotein efflux pumps as in mammary mice tumors (22) or through genetic reversion of either *BRCA1* or *BRCA2* (19–21). Here, we show that 6TG efficiently kills PIR BRCA1-defective mammary tumors (Fig. 3), which is likely explained by 6TG not being a substrate for P-glycoprotein (44). Furthermore, we show that genetically reverted BRCA2-defective cells and tumors respond to 6TG. Altogether, these findings suggest that 6TG might also be efficient in killing advanced and drug-resistant *BRCA1*- or *BRCA2*-defective tumors.

Here, we find that PIR V-C8 clones do not completely revert back to a functional HR phenotype in response to 6TG (Fig. 5), which likely explains their 6TG sensitivity. This suggests that there may be several lesions formed following 6TG treatment that trigger HR. For instance, we recently showed that HR is involved in restart at stalled replication forks, which does not involve DSB repair (45, 46). Thus, there is a possibility that 6TG might cause replication lesions other than mismatch repair–dependent DSBs that trigger HR. In support for this notion, we find that mismatch repair–defective HCT116 cells are sensitized to 6TG by the depletion of BRCA2 in spite of already being defective in mismatch repair. This shows that both mismatch repair–dependent and –independent HR lesions are formed by 6TG. Also, this would explain the intermediate response in PIR V-C8 clones to 6TG.

In conclusion, we show that 6TG could be efficiently used to selectively kill BRCA2-defective tumors and that 6TG might be used as treatment for *BRCA1* or *BRCA2* mutant tumors which are resistant to cisplatin chemotherapy and/or PARP inhibitor therapy by various mechanisms.

Disclosure of Potential Conflicts of Interest

Two patent applications regarding usage of PARP inhibitors in BRCA1 and BRCA2 mutated tumors were filed in 2003 by Cancer Research Technology Limited and The University of Sheffield (N.J. Curtin and T. Helleday and T. Helleday alone are named inventors on these patents, respectively). The other authors disclosed no potential conflicts of interest.

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Cancer Research

Correction: 6-Thioguanine Selectively Kills BRCA2-Defective Tumors and Overcomes PARP Inhibitor Resistance

In this article (Cancer Res 2010;70:6268–76), which was published in the August 1, 2010 issue of *Cancer Research* (1), the name of the third author is misspelled. The correct spelling is Tatjana Djureinovic.

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1. Issaeva N, Thomas H, Djureinovic T, et al. 6-Thioguanine selectively kills BRCA2-defective tumors and overcomes PARP inhibitor resistance. Cancer Res 2010;70:6268–76.

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