Featured Article

Novel Poly(ADP-ribose) Polymerase-1 Inhibitor, AG14361, Restores Sensitivity to Temozolomide in Mismatch Repair-Deficient Cells

Nicola J. Curtin,[1] Lan-Zhen Wang,[1] Anthie Yiakouvaki,[1] Suzanne Kyle,[1] Christine A. Arris,[1] Stacie Canan-Koch,[2] Stephen E. Webber,[2] Barbara W. Durkacz,[1] Hilary A. Calvert,[1] Zdenek Hostomsky,[2] and David R. Newell[1]

[1]Northern Institute for Cancer Research, University of Newcastle upon Tyne, Medical School, Framlington Place, Newcastle upon Tyne, United Kingdom, and [2]Pfizer Global Research and Development/Agouron Pharmaceuticals, Inc., La, Jolla, California

Abstract

Purpose: **Mismatch repair (MMR) deficiency confers resistance to temozolomide, a clinically active DNA-methylating agent. The purpose of the current study was to investigate the reversal mechanism of temozolomide resistance by the potent novel poly(ADP-ribose) polymerase (PARP)-1 inhibitor, AG14361, in MMR-proficient and -deficient cells.**

Experimental Design: **The effects of AG14361, in comparison with the methylguanine DNA methyltransferase inhibitor, benzylguanine, on temozolomide-induced growth inhibition were investigated in matched pairs of MMR-proficient (HCT-Ch3, A2780, and CP70-ch3) and -deficient (HCT116, CP70, and CP70-ch2) cells.**

Results: **AG14361 enhanced temozolomide activity in all MMR-proficient cells (1.5–3.3-fold) but was more effective in MMR-deficient cells (3.7–5.2-fold potentiation), overcoming temozolomide resistance. In contrast, benzylguanine only increased the efficacy of temozolomide in MMR-proficient cells but was ineffective in MMR-deficient cells. The differential effect of AG14361 in MMR-deficient cells was not attributable to differences in PARP-1 activity or differences in its inhibition by AG14361, nor was it attributable to differences in DNA strand breaks induced by temozolomide plus AG14361. MMR-deficient cells are resistant to cisplatin, but AG14361 did not sensitize any cells to cisplatin. PARP-1 inhibitors potentiate topotecan-induced growth inhibition, but AG14361 did not potentiate topotecan in MMR-deficient cells more than in MMR-proficient cells.**

Conclusions: **MMR defects are relatively common in sporadic tumors and cancer syndromes. PARP-1 inhibition represents a novel way of selectively targeting such tumors. The underlying mechanism is probably a shift of the cytotoxic locus of temozolomide to N^7-methylguanine and N^3-methyladenine, which are repaired by the base excision repair pathway in which PARP-1 actively participates.**

Introduction

Temozolomide (Temodal) is a DNA-methylating agent used for the treatment of glioma, astrocytoma, and melanoma (1). The most mutagenic and cytotoxic lesion caused by temozolomide is O^6-methylguanine, which accounts for 5% of the total adducts (2). Methylguanine DNA methyltransferase (MGMT) repairs O^6-methylguanine, and high MGMT levels confer resistance to temozolomide. One strategy to reverse the therapeutic resistance to temozolomide is inactivation of MGMT with O^6-benzylguanine (BG; Refs. 3 and 4). However, MGMT activity is low in bone marrow cells, and BG substantially increases the sensitivity of these cells to temozolomide (5); it is possible that hematological toxicity may limit temozolomide–BG combinations in the clinic. Furthermore, analysis of the pretreatment levels of MGMT in melanoma biopsies failed to predict response to temozolomide (6).

One possible reason for the poor correlation of MGMT levels with temozolomide sensitivity involves the mismatch repair (MMR) system, because defects in MMR result in cellular resistance to temozolomide (7). Correspondingly, in patients with malignant glioma, there was a relationship between MMR deficiency, as well as high MGMT activity, and poor response to temozolomide (8). The function of the mammalian MMR system [comprising five proteins: hMLH1, hPMS2, hMSH2, hMSH3, and hMSH6] is to correct errors in DNA that arise during replication. Defects in MMR reduce the fidelity of DNA replication by 100-1000-fold and are associated with a strong predisposition to tumor development (9). Defects in the MMR genes or their expression are associated with cancer susceptibility syndromes and sporadic cancers of the colon and ovary (10). A study of the 60 cell lines in the NCI anticancer drug screening panel also revealed that 5 (1 leukemia, 2 colon, and 2 ovarian cancer cell lines) were deficient in hMLH1, and these were all resistant to temozolomide (11).

Loss of MMR results not only in increased tumor susceptibility but also in resistance to many useful anticancer agents: DNA-methylating agents; 6-thioguanine, 5-fluorouracil, cisplatin, etoposide, doxorubicin, and ionizing radiation (reviewed in Ref. 12). It is thought that resistance is mediated through tolerance to fraudulent bases in DNA. In cells that are MMR defective, and hence tolerant to O^6-methylguanine, inactivation of MGMT is ineffective (7), and alternative strategies to modulate temozolomide resistance are needed.

The most frequent sites of alkylation by temozolomide, N^7-methylguanine and N^3-methyladenine (which account for 70

Received 8/1/03; revised 10/20/03; accepted 10/22/03.
Grant support: Cancer Research–United Kingdom.
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Requests for reprints: Nicola J. Curtin, Northern Institute for Cancer Research, University of Newcastle upon Tyne, Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH United Kingdom. Phone: 44 191 222 7133; Fax: 44 191 222 7556; E-mail: n.j.curtin@ncl.ac.uk.

and 10% of the total DNA methylation, respectively; Ref. 13), do not cause mispairing. Nevertheless, these lesions are cytotoxic (14) and repaired by the base excision repair (BER) pathway (reviewed in Ref. 15). The abundant nuclear enzyme, poly(ADP-ribose) polymerase-1 (PARP-1), is activated by DNA strand breaks and cooperates with the BER complex, comprising XRCC1, DNA polβ, and ligase 3, to facilitate repair (16). PARP-1 has been strongly implicated in the repair of methylated DNA, and inhibitors of PARP-1 enhance the cytotoxicity of such agents (reviewed in Ref. 13).

In MMR-deficient cells, interference with BER by inhibition of PARP-1, which could increase the cytotoxicity of N^7-methylguanine and N^3-methyladenine lesions, offers a potential approach to enhance the cytotoxicity of temozolomide in MMR− cells. Consistent with this approach, inhibition of PARP-1 by 3-aminobenzamide can sensitize MMR-deficient cells to temozolomide, whereas the MGMT inhibitor, BG, was ineffective (3). However, in this study, genetically matched MMR+ and MMR− cells were not used to confirm the impact of PARP-1 inhibition, in relation to MMR status, against a common genetic background. Moreover, 3-aminobenzamide is a weak PARP-1 inhibitor that also has other cellular effects (17) and can modulate the cytotoxicity of anticancer agents by PARP-independent mechanisms (18).

Two recent advances have made it possible to define further the role of PARP-1 inhibition in the enhancement of temozlomide cytotoxicity in MMR-deficient cells: firstly, we have developed a novel PARP-1 inhibitor, 1-(4-dimethylaminomethyl-phenyl)-8,9-dihydro-7H-2,7,9a-triaza-benzo[cd]azulen-6-one, AG14361 (Fig. 1), which is >1000-fold more potent than 3-aminobenzamide (Ki < 5 nM; Ref. 19) and enhances the cytotoxicity and antitumor activity of temozolomide *in vitro* and *in vivo* (20); and secondly, genetically matched cell lines with defined MMR phenotypes have been developed using microcell transfer techniques. The MMR defect of cells lacking *hMLH1* can be corrected by transfer of chromosome 3, on which the gene is located (21). In the experiments described here, AG14361 and BG have been used to investigate the role of PARP-1 and MGMT in temozolomide-induced growth inhibition in matched pairs of MMR-proficient and -deficient cell lines. The possibility of a direct interaction of PARP-1 with the MMR pathway was investigated by determining the effect of PARP-1 inhibition on cisplatin-induced cell growth inhibition in these cell lines. Lastly, given the ability of PARP-1 inhibitors to potentiate the cytotoxicity of topoisomerase I inhibitors (20, 22, 23), paired cell lines were used to investigate whether the MMR status influenced the ability of AG14361 to sensitize cells to topotecan.

Materials and Methods

Reagents. All reagents, unless stated otherwise, were obtained from Sigma-Aldrich Co., Ltd. (Poole, United Kingdom) or BDH Ltd. (Poole, United Kingdom). Alcohol dehydrogenase and proteinase K were purchased from Boehringer Mannheim Biochemical (Mannheim, Germany). [2-^{14}C]-Thymidine (specific activity = 1.96 GBq/mmol) and [methyl-^{3}H]-thymidine (specific activity = 1.85 TBq/mol) were purchased from Amersham International (Amersham, United Kingdom). DNA containing O^6-[Methyl-^{3}H]methylguanine, prepared as described previously (24), was kindly provided by Dr. G. N. Major (University of Newcastle upon Tyne, United Kingdom). Temozolomide (gift from Cancer Research United Kingdom, London, United Kingdom), *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG; Sigma), topotecan (SmithKline Beecham Pharmaceuticals, Philadelphia, PA), cisplatin (gift from Johnson Matthey, Reading, United Kingdom), BG (gift from Dr. M. E. Dolan, University of Chicago, Chicago, IL), and AG14361 (synthesized by Pfizer GR&D/Agouron Pharmaceuticals, Inc.) were dissolved in dry DMSO to give 150, 20, 2.2, 10, and 10 mM stock solutions, respectively, and stored at −20°C.

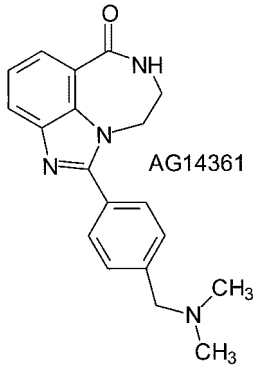

Fig. 1 The structure of AG14361.

Cell Lines. HCT116 MMR-deficient (hMLH1 deficient) human colon adenocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA), and their MMR-proficient counterpart HCT+Chr3 generated by microcell chromosome 3 transfer (Ref. 21; *hMLH1* is located on chromosome 3p23–21 so this corrects for hMLH1-deficiency) was a gift from Dr. R. Boland (Department of Medicine, University of California, San Diego, CA). A2780 human ovarian carcinoma MMR-proficient cells, their hMLH1-deficient variant, CP70 (25, 26), a chromosome 3 transferrant of the CP70 cells, CP70-ch3, which are MMR proficient, and a chromosome 2 transferrant of the CP70 cells, CP70-ch2, which remains MMR deficient (27), were all gifts from Professor R. Brown (Beatson Institute, Glasgow). The hMLH1 status of all cells was confirmed by Western blotting using mouse monoclonal anti-human MLH1 (PharMingen, BD United Kingdom Ltd., Oxford, United Kingdom), horseradish peroxidase-conjugated goat anti-mouse polyclonal antibody (BD PharMingen), and enhanced chemiluminescence.[3] All cells were grown in RPMI medium (Sigma) supplemented with 10% FCS (Globepharm, Cranleigh, United Kingdom), the medium for the HCT+Chr3 was supplemented with geneticin (G418; Sigma; 400 μg/ml), and that for the CP70-ch3 and CP70-ch2 was supplemented with hygromycin B (200 μg/ml; Life Technologies, Inc.). Cell lines were verified as *Mycoplasma* free as described previously (28).

Determination of MGMT Activity. Exponentially growing cells were harvested by trypsination, resuspended in

[3] E. Matheson and L. Wang, unpublished data.

medium, and centrifuged for 5 min at 1750 × *g* at 4°C. The pelleted cells were washed twice, resuspended in medium, and disaggregated, and pellets of 10^7 viable cells were snap frozen in liquid nitrogen and stored at −80°C. Extracts were prepared by homogenizing cell pellets in 5 ml of ice-cold extraction buffer [50 mM Tris-HCl (pH 8.3), 0.5 mM EDTA disodium salt, 1 mM DTT, and 200 mM NaCl] with a T25 Ultra-Turrax homogenizer (SH Scientific, Northumberland, United Kingdom) fitted with an 8-mm head, then centrifuging for 5 min at 1750 × *g*, 4°C; the supernatant was snap frozen and stored at −80°C. The MGMT activity was measured as described previously (24) by incubating cell extracts, diluted in assay buffer [50 mM Tris/HCl (pH 8.3), 0.5 mM EDTA, and 1 mM DTT], with DNA containing O^6-[Methyl-^{3}H]methylguanine (1500 dpm) at 37°C for 90 min, and the reaction was terminated by chilling on ice and adding 200 μg of calf thymus DNA in 300 μl of 80 mM EDTA (pH 6.0). The [Methyl-^{3}H] that had been transferred to protein was solubilized by 10 μl of 20 mg/ml proteinase K in 1 mM $CaCl_2$) for 60 min at 37°C. The DNA was removed by precipitation with 3% cetyltrimethylammonium bromide and centrifugation. The radioactivity in the supernatant was determined by liquid scintillation counting, and the fmol of MGMT per cell was calculated from the stoichiometry of the transfer of methyl-^{3}H groups to the protein and specific activity of the substrate.

Growth Inhibition Assays. Cells were seeded into 96-well plates at a density shown previously to give exponential growth throughout the exposure period, *i.e.*, HCT116 and HCT+Chr3 10^3; A2780, 2 × 10^3; CP70, 500; CP70-ch3, 10^3 and CP70-ch2 1.5 × 10^3 cells/well in 100-μl tissue culture medium. After attachment overnight, the cells were exposed to varying concentrations of temozolomide, topotecan, or cisplatin in the presence or absence of 400 nM AG14361 and/or 10 μM BG for 5 days, then fixed and stained with sulforhodamine B as described previously (29). The concentration required to inhibit cell growth by 50% (GI_{50}) was calculated from point-to-point graphs using GraphPad Prism (San Diego, CA) software. The potentiation factor at 50% growth inhibition was calculated from the ratio of the GI_{50} of the cytotoxic drug (temozolomide, topotecan, or cisplatin) alone to the GI_{50} of the drug plus resistance modifier (BG or AG14361)

DNA Strand Break Assay by Alkaline Elution. The alkaline elution technique was used for the quantitative analysis of DNA single-strand breakage in which fragments of DNA were separated on the basis of size using polycarbonate filters, which are neither protein nor DNA adsorbent, as described by Kohn *et al.* (30). The alkaline elution assay has been shown previously to have a sensitivity of 1 DNA lesion/10^7 nucleotides (30). To increase the precision of the assay, samples were coeluted with an internal standard consisting of irradiated DNA. Exponentially growing CP70-ch3 or CP70-ch2 cells were labeled with [2-^{14}C]-thymidine (0.74 KBq/ml) for 24 h, followed by 4 h in fresh medium, then treated for 4 h with temozolomide (500 μM) with or without AG14361 (400 nM). Cells were lysed on the polycarbonate filters, and DNA was coeluted with [methyl-^{3}H]-thymidine (3.7 KBq/ml)-labeled DNA from internal standard cells exposed to 3 Gy γ-radiation (using a ^{137}Cs source, Gammacell 1000 elite; Nordion International, Inc., Kanata, Canada). Relative elution values of treated samples compared with controls were calculated as: [log retention of control] − [log retention of treated sample], with retention values measured when the internal standard retention was 0.6, as described previously (31).

Estimation of Cellular NAD^+. Exponentially growing cells (8×10^5-1×10^6) were exposed to MNNG in the presence or absence of AG14361 as described in "Results." Cells were chilled and washed with ice-cold PBS, and the cellular macromolecules were precipitated with ice-cold trichloroacetic acid (20% w/v) for 1 h at 0°C-4°C. The precipitate was pelleted by centrifugation, and the supernatant, containing the cellular NAD^+, was extracted with diethyl ether until it reached pH 4.0. Cellular NAD^+ concentration was determined by the microtiter plate assay described by Jacobson and Jacobson (32), in which oxidation of ethanol linked to the reduction of NAD^+ to NADH by alcohol dehydrogenase is coupled to chemical reduction of the yellow reagent 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide to the green colored product mediated by phenazine ethosulphate. Color change, which is proportional to the NAD^+ concentration, was monitored at 570 nm. The sample readings were compared with an ether-extracted 20% (w/v) trichloroacetic acid blank, and the assay was calibrated using a linear standard curve generated using standard NAD^+ concentrations of 0–200 μM.

Results

Temozolomide-Induced Growth Inhibition. The sensitivity to temozolomide-induced growth inhibition was determined in all three pairs of cell lines, and pooled GI_{50} data are given in Table 1. The MMR-deficient HCT116 and CP70-ch2 cells were 2- to 2.5-fold resistant to temozolomide compared with their chromosome 3 transferrant partners HCT+Chr3 and CP70-ch3, respectively. Coincubation with 400 nM AG14361 resulted in a 3.7- to 5.2-fold enhancement of temozolomide-induced growth inhibition in the three MMR-deficient cell lines but only a 1.5- to 3.3-fold sensitization in the three MMR-proficient cell. Because of the greater sensitization in the MMR-deficient cells compared with the MMR-proficient cells, there was no significant difference in the GI_{50} values for temozolomide plus AG14361 in HCT116 *versus* HCT+Chr3 and CP70-ch3 *versus* CP70-ch2. Thus, the PARP-1 inhibitor overcame resistance to temozolomide mediated by MMR deficiency in HCT 116 and CP70-ch2 cells. However, the MMR-deficient CP70 cells, which are >6-fold resistant to temozolomide compared with the parental A2780 cells, were still 3-fold less sensitive to the combination of temozolomide and AG14361 than the A2780 cells. CP70 cells, which show evidence of loss of p53 functions (33), were recently found to have a heterozygous GTT→TTT (Val→Phe) mutation in codon 172 of p53 (34), which may have contributed to the resistance of these cells. Another factor that may contribute to the resistance of CP70 cells was their MGMT content (768 ± 136 fmol/mg cellular protein), which was significantly higher ($P < 0.05$) than that of A2780 cells (476 ± 143 fmol/mg cellular protein). Because of the possible contribution of mutant p53 and elevated MGMT levels in CP70 cells to temozolomide resistance, the A2780–CP70 pair of cells was omitted from additional studies on the

Table 1 Growth inhibition induced by TM in the presence and absence of AG14361 in human colon and ovarian cancer cell lines in relation to MMR status[a]

	Genotype		GI_{50} (μM)[b]		
Cell line	MMR	p53	TM	TM + AG14361	PF_{50}[c]
HCT + Chr3	+	wt	319 ± 80[d]	192 ± 40	**1.5 ± 0.5**[NS]
HCT116	−	wt	841 ± 128	225 ± 69	**3.7 ± 1.2***[e]
Fold resistant[a]			**2.5 ± 0.7***	**1.0 ± 0.3**[NS]	
A2780	+	wt	154 ± 23	62.5 ± 10	**2.5 ± 0.6***
CP70	−	wt/mut	1012 ± 153	194 ± 33	**5.2 ± 1.2***
Fold resistant[e]			**6.6 ± 1.3***	**3.1 ± 0.7***	
CP70-ch3	+	wt/mut	609 ± 102	185 ± 16	**3.3 ± 0.6***
CP70-ch2	−	wt/mut	1241 ± 93	243 ± 38	**5.1 ± 0.9***
Fold resistant			**2.0 ± 0.4***	**1.3 ± 0.3**[NS]	

[a] TM, temozolomide; MMR, mismatch repair; wt, wild type; mut, mutant; *, significant resistance or potentiation ($P < 0.05$: paired Student's *t* test); [NS], not significant.
[b] GI_{50} is the concentration of TM ± 400 nM AG14361 required to inhibit growth by 50% after 5 days of continuous exposure.
[c] PF_{50} is calculated as GI_{50} temozolomide alone ÷ GI_{50} temozolomide + AG14361.
[d] Data are mean ± SD of GI_{50} values from at least three independent experiments.
[e] Fold resistance was calculated as the ratio of the GI_{50} in MMR− cells ÷ GI_{50} in MMR+ cells.

relative contributions of PARP-1 and MGMT to temozolomide resistance in MMR-proficient and -deficient cells.

Potentiation of Temozolomide-Induced Growth Inhibition by BG and AG14361. Representative growth inhibition curves of HCT116, HCT+Chr3, CP70-ch3, and CP70-ch2 cells exposed to increasing concentrations of temozolomide in the presence or absence of 10 μM BG and/or 400 nM AG14361 are shown in Fig. 2. Pooled GI_{50} data from three independent experiments, together with the MGMT content and MMR and p53 status of the four cell lines, are given in Table 2. The MGMT levels were similar within the paired cells but different between the pairs, *i.e.,* the MGMT levels were approximately four times higher in the CP70-ch3 and CP70-ch2 cells than HCT116 and HCT+Chr3 cells. HCT116 cells were ~2.3-fold resistant to temozolomide compared with HCT+Chr3, and CP70-ch2 were >2-fold resistant to temozolomide than CP70-ch3 cells. Coincubation with BG caused a 3-fold potentiation of temozolomide in the HCT+Chr 3 cells ($P < 0.05$), but there was no significant potentiation in the MMR-deficient HCT116 cells. There was a much greater enhancement of temozolomide-induced growth inhibition by BG in the CP70-ch3 cells (~10-fold) compared with the HCT+Chr3 cells, which may reflect the higher levels of MGMT in the CP70-ch3 cells.

AG14361 significantly potentiated temozolomide in the MMR-deficient HCT116 cells but not the MMR-proficient HCT+Chr3 cells, overcoming the resistance mediated by MMR deficiency. In these experiments, temozolomide alone failed to inhibit the growth of the CP70-ch2 cells by 50%, and so GI_{50} and potentiation factor at 50% growth inhibition values could not be calculated. However, at the maximum temozolomide concentration achievable (1500 μM), cell growth was 71 ± 9% of control, and this was reduced to 54 ± 10% by BG to 10 ± 3% by AG14361 and 11 ± 3% by the combination of BG and AG14361; all reductions were significant ($P < 0.01$, paired Student's *t* test). The effects of the combination of BG and AG14361 were similar to those seen with each agent individually in all cell lines, *i.e.,* there was no marked antagonism or synergy.

DNA Damage-Induced PARP-1 Activation and Inhibition by AG14361 in Whole Cells. To determine whether differences in PARP-1 activation could be responsible for the greater potentiation of temozolomide in MMR-deficient compared with MMR-proficient cells, the NAD^+ content of cells was measured after methylating agent-induced DNA damage. Temozolomide undergoes chemical activation to release the active methylating species 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide with a $t_{1/2}$ of 1–2 h (35), and so DNA breaks accumulate gradually. As an alternative, the methylating agent MNNG was used to induce DNA breaks more rapidly, such that consequent PARP-1 activation would induce a rapid depletion of NAD^+. After 20-min exposure to MNNG, cellular NAD^+ was reduced by varying degrees in the cell lines (Table 3). MMR status did not affect the MNNG-induced NAD^+ depletion, *i.e.,* there was no significant difference in the NAD^+ depletion in HCT+Chr3 compared with HCT116 nor in A2780 compared with CP70 cells or CP70-ch3 compared with CP70-ch2 cells. To investigate whether AG14361 had differential effects on PARP-1 activity in MMR-proficient and -deficient cells, cells were exposed to MNNG in the presence of the PARP-1 inhibitor. AG14361 alone had no effect on NAD^+ levels (data not shown). Coincubation with 400 nM AG14361 ameliorated the NAD^+ depletion in all of the cell lines (Table 3). Thus, PARP-1 inhibition by AG14361 was equally effective in MMR-proficient and -deficient cells.

Effect of AG14361 on Temozolomide-Induced DNA Strand Breaks. To investigate whether the greater sensitization of temozolomide-induced growth inhibition by AG14361 in MMR-deficient cells compared with MMR-proficient cells was attributable to a greater accumulation of genotoxic damage, DNA strand breaks were measured in CP70-ch3 and CP70-ch2 cells exposed to temozolomide with or without AG14361. Such strand breaks arise during the processing of N^7-methylguanine and N^3-methyladenine lesions. Maximum DNA strand break induction by temozolomide, and the maximum effect of PARP-1 inhibition on DNA strand break levels, has been shown previously to occur at ~4 h in CHO cells (36), and this was con-

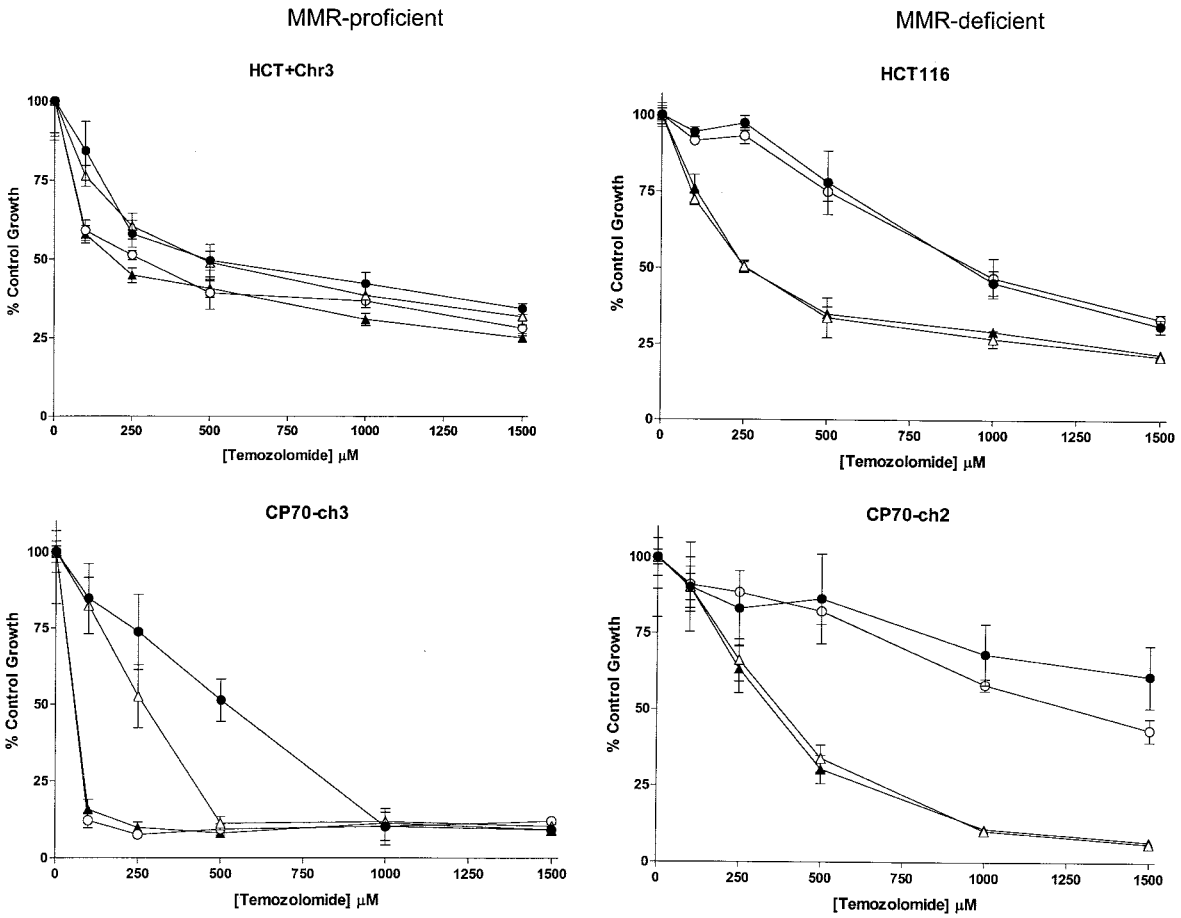

Fig. 2 Growth inhibition by temozolomide in MMR+ and MMR− cells: effect of BG and AG14361. Cells, seeded in 96-well plates, were exposed to temozolomide alone (●), temozolomide + 10 μM BG (○), temozolomide + 400 nM AG14361 (△), or temozolomide + 400 nM AG14361 + 10 μM BG (▲). After 120 h, plates were fixed stained with sulforhodamine B, and the absorbance was determined as described in "Materials and Methods." Data were normalized by comparison with drug-free controls (1% DMSO), 10 μM BG or 400 nM AG14361 as appropriate.

firmed in the CP70-ch3 and CP70-ch2 cells (data not shown). Cells were exposed to 500 μM temozolomide in the presence or absence of 400 nM AG14361 for 4 h. A representative elution profile is shown in Fig. 3*A*, and pooled relative elution data from three independent experiments are given in Fig. 3*B*. After exposure to temozolomide alone, there was a greater accumulation of DNA strand breaks in the CP70-ch2 cells compared with the CP70-ch3 cells. However, coincubation with AG14361 increased the relative elution 3-fold in CP70-ch3 cells but only 2-fold in CP70-ch2 cells, such that there was no significant difference between the relative elution of CP70-ch3 and CP70-ch2 cells exposed to the combination of temozolomide and AG14361. Thus, the greater enhancement of temozolomide-induced growth inhibition by AG14361 in the CP70-ch2 cells could not be attributed to an increased number of DNA strand breaks.

Investigation of the Potentiation of Topotecan by AG14361 in MMR-Proficient and -Deficient Cells. It has been shown previously that PARP-1 inhibitors are effective enhancers of topotecan-induced growth inhibition and cytotoxicity (20, 22, 23). To investigate whether AG14361 also caused a greater sensitization to topotecan in MMR-deficient cells, topotecan-induced growth inhibition was determined in A2780, CP70, CP70-ch3, and CP70-ch2 cells in the presence and absence of 400 nM AG14361 (Table 4). The p53 mutant CP70 cells were more resistant to topotecan than the p53 wild-type A2780 cells. However, because there was no significant difference in the GI_{50} values in CP70-ch3 and CP70-ch2 cells, the MMR status of the cells does not appear to influence sensitivity to topotecan. Potentiation of topotecan by AG14361 was greater in the MMR-proficient (2.8- and 2.4-fold in A2780 and CP70-ch3 cells, respectively) than -deficient cells (2.1- and 1.4-fold in CP70 and CP70-ch2 cells, respectively). Evidently, the greater potentiation of temozolomide-induced growth inhibition by AG14361 in MMR-deficient cells compared with MMR-proficient cells does not extend to a greater sensitization to topotecan.

Investigation of the Potentiation of Cisplatin by AG14361 in MMR-Proficient and -Deficient Cells. In addition to being resistant to alkylating agents, MMR-deficient cells

Table 2 Potentiation of TM-induced growth inhibition by AG14361 and BG in human colon and ovarian cancer cell lines matched for p53 status and MGMT activity[a]

	HCT + Chr3	HCT116	CP70-ch3	CP70-ch2
p53	wt	wt	wt/mut	wt/mut
MMR	+	−	+	−
MGMT (fmol/mg prot.)	209 ± 71[b]	201 ± 95	778 ± 74	833 ± 66
GI_{50} TM (μM)[c]	402 ± 85	938 ± 15	692 ± 92	>1500
GI_{50} TM (μM) + BG	157 ± 59	996 ± 54	69 ± 7	≥1394
PF_{50}[d]	**3.1 ± 1.4**[e]	**0.95 ± 0.05**	**9.8 ± 0.7**[e]	**≥1.1**
GI_{50} TM (μM) + AG14361	317 ± 80	257 ± 27	284 ± 48	503 ± 102
PF_{50}	**1.3 ± 0.5**	**3.7 ± 0.5**[e]	**2.5 ± 0.7**[e]	**>3.2**
GI_{50} TM (μM) + BG + AG14361	124 ± 32	226 ± 14	61.8 ± 1.7	474 ± 97
PF_{50}	**3.5 ± 1.3**[e]	**4.2 ± 0.4**[e]	**11 ± 1.8**[e]	**>3.4**

[a] BG, benzylguanine; MGMT, methylguanine DNA methyltransferase; wt, wild type; mut, mutant; MMR, mismatch repair; TM, temozolomide.
[b] Data are mean ± SD of values from three independent experiments.
[c] GI_{50} is the concentration of TM, ±10 μM BG, ±400 nM AG14361 required to inhibit cell growth by 50% after 5 days of continuous exposure.
[d] PF_{50} is calculated as the ratio of the GI_{50} for TM + AG14361 and/or BG to the GI_{50} for TM alone.
[e] Significant potentiation ($P < 0.05$: paired Student's t test).

are resistant to cisplatin; indeed, the CP70 cells were selected by cisplatin resistance (25). To investigate whether the greater potentiation of temozolomide by AG14361 in MMR-deficient cells extended to a greater sensitization to other drugs to which MMR-deficient cells are resistant, growth inhibition after cisplatin exposure, in the presence and absence of AG14361, was measured. The MMR-deficient CP70 cells were 6- to 7-fold resistant to cisplatin compared with the MMR-proficient A2780 cells, consistent with published data (27). However, the MMR–CP70-ch2 cells were only ~2-fold resistant compared with the CP70-ch3 cells, and this was not statistically significant. This suggests that the resistance of the CP70 cells to cisplatin, compared with the A2780 cells, may be more of a function of loss of p53 rather than MMR. There was no potentiation of cisplatin-induced growth inhibition by AG14361 in any of the cell lines (Table 5)

Discussion

The use of matched cells with different MMR status together with a novel and very potent PARP-1 inhibitor has allowed the investigation of the role of PARP-1 in the resistance to temozolomide mediated by MMR defects. Consistent with data published previously, the MMR-deficient cell lines were 2–7-fold resistant to temozolomide, and the MGMT inhibitor, BG, failed to modulate this resistance (Tables 1 and 2). PARP-1 inhibition by AG14361 resulted in a 1.5–5-fold enhancement of temozolomide-induced growth inhibition in all cell lines, which is again consistent with data obtained previously with unmatched cell lines using other PARP-1 inhibitors: 3-aminobenzamide, PD128763, and NU1025 (3, 20, 37, 38). Using AG14361–temozolomide combinations in the paired cell lines, greater potentiation of temozolomide in MMR-deficient cells was demonstrated, with complete restoration of sensitivity to temozolomide (Table 1). AG14361 was not able to completely overcome temozolomide resistance in CP70 compared with A2780 cells. However, this pair of cells is not strictly isogenic in that CP70 cells were derived by continuous exposure of A2780 cells to cisplatin and are thus likely to have acquired other resistance mechanisms. Indeed, they were recently found to have a heterozygous p53 mutation (34). The data reported here (Table 1) would support the view that PARP-1 inhibition can overcome MMR deficiency-mediated resistance to temozolomide but not resistance that is mediated by other mechanisms, such as loss of p53.

The reason for the greater sensitization of MMR-deficient cells to temozolomide by AG14361 was investigated further. The possibility that there were differences between MMR-deficient and -proficient cells in PARP-1 activity, or its sensi-

Table 3 NAD^+ depletion by MNNG in MMR-proficient and -deficient cells and inhibition of depletion by AG14361[a]

		% Control [NAD+]	
Cell line and MMR status	Control [NAD^+] pmol/10^6 cells	MNNG	MNNG + AG14361
HCT + Chr3 MMR+	1946 ± 700[b]	65 ± 22	ND[c]
HCT116 MMR−	1651 ± 561	47 ± 24	ND
A2780 MMR+	530 ± 127	24 ± 6	97 ± 3
CP70 MMR−	576 ± 81	25 ± 9	91 ± 1
CP70-ch3 MMR+	941 ± 174	61 ± 10	110 ± 12
CP70-ch2 MMR−	1040 ± 390	56 ± 10	107 ± 7

[a] MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; MMR, mismatch repair.
[b] Data are mean ± SD of values determined in more than or equal to three independent experiments. Cells were exposed to 25 μM MNNG (HCT 116, HCT + chr3, CP70-ch2, and CP70-ch3) or 50 μM MNNG (A2780, CP70), ±400 nM AG14361 for 20 min before estimation of NAD^+ content as described in "Materials and Methods."
[c] ND, not determined.

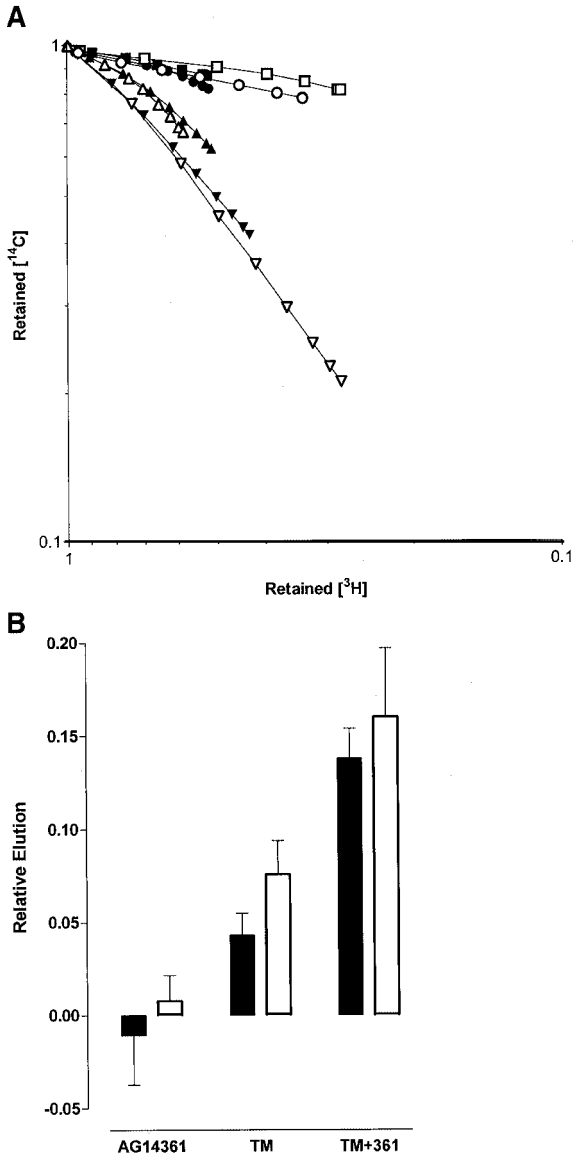

Fig. 3 Effect of AG14361 on temozolomide-induced DNA strand breaks in MMR+ and MMR− cells. In *A*, representative elution profile CP70-ch3 cells (*solid symbols*) and CP70-ch2 cells (*open symbols*) were exposed to 400 nM AG14361 (■, □), 500 μM temozolomide (▲, △), or both drugs together (▼, ▽), and their elution was compared with that of untreated controls (●, ○). *B*, relative elution of DNA from MMR-proficient and -deficient cells exposed to temozolomide in the presence and absence of AG14361 The elution of treated cells, relative to that of untreated controls, was calculated from data, as illustrated in *A* for CP70-ch3 (*filled bars*) and CP70-ch2 (*open bars*) after exposure to 400 nM AG14361, 500 μM temozolomide (*TM*), or temozolomide + AG14361 (*TM + 361*).

tivity to inhibition by AG14361, was excluded by showing that there were no differences in NAD^+ content, MNNG-induced NAD^+ depletion, or inhibition of MNNG-induced NAD^+ depletion by AG14361. Similarly, the greater potentiation of temozolomide-induced growth inhibition by AG14361 in MMR-deficient cells could not be attributed to a greater induction of DNA strand breaks. Furthermore, AG14361 did not potentiate cisplatin activity in any of the cell lines, indicating that PARP-1 inhibition does not reverse MMR-mediated resistance to cisplatin. It is unlikely, therefore, that PARP-1 interacts directly with the MMR-mediated processing of DNA lesions.

We have shown previously that PARP-1 inhibitors enhance the cytotoxicity of topoisomerase I inhibitors (20, 22, 23). To investigate whether the greater enhancement of temozolomide in MMR-defective cells extended to a greater sensitization to topoisomerase I poisons, the combination of AG14361 and topotecan was investigated in the matched cell lines. There was no difference in sensitivity to topotecan between the CP70-Ch2 and CP70-Ch3 cells. However, CP70 cells were less sensitive to topotecan than A2780 cells, which may be a reflection of difference in p53 status, which is known to affect sensitivity to topoisomerase I inhibitors (39). Inhibition of PARP-1 did not cause a greater sensitization of the MMR-deficient cells to topotecan; on the contrary, the MMR-proficient cells were sensitized to a greater extent.

The most probable explanation for the differential effects of temozolomide–AG14361 combinations in the paired cell lines is that in MMR-deficient cells, which are tolerant to O^6-methylguanine adducts, the other more numerous *N*-methylpurine adducts (N^7-methylguanine and N^3-methyladenine) become the primary cytotoxic locus. Increased DNA single-strand breaks, presumably as a result of reduced BER of *N*-methyl purines, after PARP-1 inhibition by AG14361, as reported here, or PD128763 as reported elsewhere (37), result in an increase in the cytotoxicity of these lesions. Our data indicate that there was not a differential effect of AG14361 on temozolomide-induced DNA breaks that could account for the greater potentiation of temozolomide-induced growth inhibition in MMR-deficient cells. Instead, the greater impact of these lesions on cell growth is attributable to a qualitative shift in the primary cytotoxic locus between MMR-proficient and -deficient cells rather than a differential effect on DNA damage and repair. In MMR-proficient cells, O^6-methylguanine is the primary cytotoxic locus, with lesions at other sites on the DNA being less important, and hence, inhibition of the repair of N^7-methylguanine and N^3-methyladenine by AG14361 does not have such a great impact on cell growth.

Table 4 Cell growth inhibition induced by topotecan in the presence or absence of AG14361

Cell lines	GI_{50} TP (μM)[a]		
	TP alone	TP + AG14361	**PF_{50}**
A2780	7.8 ± 1.0[b]	2.8 ± 0.5	**2.8 ± 0.3**[c]
CP70	14.0 ± 5.4	6.6 ± 1.3	**2.1 ± 0.5**[c]
Fold resistant	**1.8 ± 0.7**	**2.4 ± 0.7**[c]	
CP70-ch3	20.5 ± 0.2	8.5 ± 0.6	**2.4 ± 0.1**[c]
CP70-ch2	21.9 ± 0.2	16.2 ± 2.1	**1.4 ± 0.2**[c]
Fold resistant	**1.1 ± 0.01**	**1.9 ± 0.2**[c]	

[a] GI_{50} is the concentration of topotecan (TP) ±400 nM AG14361 required to inhibit growth by 50% after 5 days of continuous exposure.

[b] Data are mean ± SD of IC_{50}s from three independent experiments.

[c] Significant resistance or potentiation ($P < 0.05$: paired Student's *t* test).

Table 5 Cell growth inhibition by cisplatin in the presence or absence of AG14361

	GI_{50} Cis Pt (μM)[a]		
Cell lines	Cis Pt alone	Cis Pt + AG14361	**PF_{50}**
A2780	0.72 ± 0.05[b]	0.70 ± 0.07	**0.98 ± 0.13**
CP70	4.8 ± 2.8	4.7 ± 2.7	**0.96 ± 0.1**
Resistance factor	**6.7 ± 3.7**[c]	**6.5 ± 3.5**[c]	
CP70-ch3	8.0 ± 4.8	8.8 ± 3.9	**1.4 ± 0.8**
CP70-ch2	12.3 ± 6	12.2 ± 6.0	**0.99 ± 0.11**
Resistance factor	**1.8 ± 0.8**	**1.4 ± 0.5**	

[a] GI_{50} is the concentration of cisplatin (Cis Pt) ± 400 nM AG14361 required to inhibit growth by 50% after 5 days of continuous exposure.
[b] Data are mean ± SD of four independent experiments.
[c] Significant resistance ($P < 0.05$: unpaired Student's *t* test).

The importance of N^7-methylguanine and, in particular, N^3-methyladenine and their repair by the BER pathway has been confirmed using methoxyamine, which inhibits BER by preventing AP endonuclease-mediated DNA cleavage (37), and Me-Lex, an agent which produces almost exclusively N^3-methyladenine (14). Methylation at the N^3-position of adenine by Me-Lex was shown to be clastogenic and cytotoxic (14). The cytotoxicity of Me-Lex, and its potentiation by the PARP-1 inhibitor, 3-aminobenzamide, have been demonstrated in MMR-deficient cells (40).

MMR defects are a relatively common occurrence in sporadic cancers, as well as in the cancer syndrome hereditary nonpolyposis colorectal carcinoma. MMR defects, which can also be acquired during treatment, may be a significant cause of clinical drug resistance. Loss of MMR in sporadic colon cancers is associated with hypermethylation of *hMLH1* (10), and evidence suggests loss of hMLH1 expression arises in a similar manner after chemotherapy in ovarian (26) and breast (41) cancer. Furthermore, loss of MLH1 after chemotherapy was an independent predictor of poor prognosis in breast cancer patients (41). Thus, overcoming chemotherapeutic resistance caused by MMR defects could have significant therapeutic potential, particularly because MMR defects have not been reported in normal tissues. The epigenetic silencing of *hMLH1* by hypermethylation can be reversed using 2′-deoxy-5-azacytidine (10), and this can restore sensitivity to cisplatin and temozolomide in CP70 xenografts (42). However, this strategy will be ineffective in tumors, such as HCT116, which lack MLH1 expression because of a mutation in the *hMLH1* gene (42). Furthermore, 2′-deoxy-5-azacytidine inhibits DNA methylation by incorporation into the DNA and is carcinogenic. An alternative approach, based on the observation that replication bypass may be responsible for drug tolerance in MMR-deficient cells, is to use the DNA polymerase inhibitor, aphidicolin. Aphidicolin sensitized MMR-deficient CP70-ch2 cells to a greater extent than the MMR-proficient CP70-ch3 cells to both cisplatin and methylnitrosourea (43). However, aphidicolin inhibits not only DNA polymerases δ and ϵ, involved in replicative bypass, but also DNA polymerase α, which is essential for DNA replication, and aphidicolin is therefore cytotoxic in its own right.

PARP-1 inhibition may represent a more generally applicable approach to overcoming MMR deficiency-mediated methylating agent resistance than either DNA demethylation or DNA polymerase inhibition. Inhibition of PARP-1 is a strategy that is neither inherently cytotoxic nor mutagenic and should be equally effective in tumors deficient in MMR by virtue of either mutation or epigenetic silencing of MMR genes. PARP-1 inhibitors are effective resistance modifiers in preclinical models (20), and the potency and low intrinsic toxicity of the novel inhibitors, such as AG14361, allow the exploration of their clinical potential for the first time.

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