MGMT Expression Predicts PARP-Mediated Resistance to Temozolomide

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Abstract
Melanoma and other solid cancers are frequently resistant to chemotherapies based on DNA alkylating agents such as dacarbazine and temozolomide. As a consequence, clinical responses are generally poor. Such resistance is partly due to the ability of cancer cells to use a variety of DNA repair enzymes to maintain cell viability. Particularly, the expression of MGMT has been linked to temozolomide resistance, but cotargeting MGMT has proven difficult due to dose-limiting toxicities. Here, we show that the MGMT-mediated resistance of cancer cells is profoundly dependent on the DNA repair enzyme PARP. Both in vitro and in vivo, we observe that MGMT-positive cancer cells strongly respond to the combination of temozolomide and PARP inhibitors (PARPi), whereas MGMT-deficient cells do not. In melanoma cells, temozolomide induced an antiproliferative senescent response, which was greatly enhanced by PARPi in MGMT-positive cells. In summary, we provide compelling evidence to suggest that the stratification of patients with cancer upon the MGMT status would enhance the success of combination treatments using temozolomide and PARPi. Mol Cancer Ther; 14(5) May 2015. 10.1158/1535-7163.MCT-14-0810 doi: 10.1158/1535-7163.MCT-14-0810

Introduction
Melanoma is a form of skin cancer notoriously resistant to current therapies. Before the arrival of targeted therapies against the BRAF and MEK components of the hyperactivated MAPK pathway, alkylating agent-based chemotherapy was the first-line treatment for decades. In around 40% of all cases, patients qualify for BRAF and MEK inhibitors and targeted therapies provide a good clinical response. However, after 2 to 8 months, the vast majority of patients relapse (1–3). For these patients, their hopes lie in chemotherapy or immunotherapy. The only FDA-approved chemotherapeutic agent is the intravenously administered prodrug dacarbazine, which is metabolically converted in the liver to MTIC, a toxic monofunctional DNA-alkylating agent (4, 5). An alkylating agent related to dacarbazine but with excellent oral bioavailability is temozolomide, which can cross the blood brain barrier and therefore represents an alternative to dacarbazine for melanoma with brain metastases (6–8). Temozolomide is an attractive chemotherapy agent for patients with unresectable metastatic melanoma. However, single-agent escalated dose temozolomide compared with single-agent dacarbazine treatment did not improve overall survival or progression-free survival (9, 10), and overall response rates for both drugs were 15% to 20% (5). Temozolomide is FDA approved for anaplastic astrocytoma and glioblastoma, but again with poor overall responses of around 26.5% (11). This suggests that to improve the efficacy of temozolomide, we need to enhance our understanding about the mode of action of temozolomide as a DNA-damaging and cytotoxic drug.

Temozolomide methylates DNA bases predominantly at oxygen in position 6 and nitrogen at position 7 in guanine and nitrogen at position 3 in adenine (O6-metG, N7-metG, and N3-metA, respectively). O6-metG is the most toxic and mutagenic DNA modification produced by temozolomide and is quickly repaired by the enzyme O6-methyl-guanine DNA methyl transferase (MGMT). MGMT incorporates the O6-methyl adduct into a cysteine and the enzyme is then degraded by proteolysis (4). On the other hand, N3-metA and N7-metG repair are mediated by the base excision repair (BER) machinery in a process that involves PARP, which ADP-ribosylate DNA and proteins. When acting on DNA, PARP proteins create a docking site for the incorporation of other components of the BER machinery, which complete the repair process (4).

The most evident mechanism of resistance to both DTIC and temozolomide is MGMT activity, and MGMT downregulation sensitizes cancer cells to temozolomide (12). Moreover, in elderly...
glioblastoma patients MGMT status predicts clinical response to temozolomide (13). Unfortunately, therapies combining MGMT inhibitors with temozolomide in melanoma or colorectal cancer patients have so far failed to improve outcome due to exacerbated treatment-related hematologic toxicity (14–16). More recently, several small-molecule inhibitors have been developed to target other DNA repair mechanisms, with the aim to use them as synthetically lethal single agents or in combination therapies with chemotherapeutic agents such as temozolomide. Notably, current clinical trials are testing PARP inhibitors (PARPi) as monotherapy in BRCA-deficient breast cancer patients or in combination with DNA-alkylating agents (17, 18). Combination therapies of DTIC or temozolomide and PARPi are better tolerated than those with MGMT inhibitors, but myelosuppression and liver toxicity still represent clinical concerns (19, 20), and overall survival rates were not improved compared with patients treated with alkylating alone (21–23). Therefore, further research is warranted to identify markers that help stratify patients for temozolomide-based therapies to improve clinical outcome. Our study suggests that MGMT-mediated resistance to temozolomide in melanoma and other cancer types requires PARP and that MGMT expression correlates with improved response to combination of temozolomide with PARPi.

Materials and Methods

Cell culture
Melanoma cell lines were provided by Prof. Richard Marais (Cancer Research UK, Manchester Institute, The University of Manchester, Manchester, United Kingdom) and Dr. Adam Hurlstone (Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom) and have been described recently (24). Colorectal carcinoma cells were obtained from Prof. Stephen Taylor, originally from ATCC. Glioblastoma cell lines have been described recently (25). Cell stocks were expanded and vials kept in liquid nitrogen. New aliquots were thawed every 5 to 7 weeks. Melanoma cell lines were authenticated in house by short tandem repeat profiling. Cells were cultured in DMEM or in RPMI-1640 medium (SIGMA) as previously indicated, supplemented with 0.5% penicillin and streptomycin (SIGMA) and 10% bovine calf serum (PAA). Cells were grown at 37°C in a 5% CO2 environment. WM98.1-MGMT–expressing cells were established by transfection of a pcDNA3 vector encoding the cDNA for human MGMT. MGMT-expressing cells were selected using G418 as pcDNA3/Km and stained with the Neomycin resistance gene.

Reagents
Temozolomide and methylmethanesulfonate (MMS) were from SIGMA. Olaparib, veliparib (ABT-888), selumetinib (AZD-6244), and lomeguatrib were from Selleck Chemicals.

Proliferation assays
Cells were plated in 96-well plates and treated with serial increasing concentrations (range, 0.15 μmol/L to 1 mmol/L) of drugs as indicated. After 5 days, cells were fixed and stained with toluidine blue, the concentration necessary to inhibit cell growth to a 50% (GI50) was calculated as previously described (24).

Colony formation assays
Cells were plated in 12-well plates and treated, from the top-left well, with DMSO, 5, 25, 75, 250, or 1000 μmol/L temozolomide for 24 hours (Supplementary Fig. S1). Then cells were washed and fresh, drug-free medium was replaced. Cells were left to form colonies for 10 to 15 days before being fixed and stained with a solution of 4% Formaldehyde (Fisher Scientific) and 0.5% Crystal violet (SIGMA) in PBS. Remaining dye was solubilized in PBS, 1% SDS (Fisher Scientific).

Xenograft assay
Animal studies described within were approved by The University of Manchester (Manchester, United Kingdom) Ethical Review Board and performed according to UK Home Office regulations. Briefly, GFP-expressing human melanoma cells were injected into the pericardial cavity of 48 hours post-fertilization zebrafish embryos. Embryos were treated at 1 day after injection with DMSO (SIGMA), temozolomide, olaparib, or the combination of both. On days 1 and 5, pictures were taken and relative growth quantified as previously described (26).

Western blotting
Cell lysates were prepared using RIPA buffer and analyzed as described (27). Primary antibodies used were: MGMT (Thermo Fisher Scientific), phospho-Thr668-CHK-2 and Ser345-CHK-1, MLH-1, MSH-2, MSH-6, and cleaved caspase-3 (Cell Signaling Technology), ERK2, and β-tubulin (Santa Cruz Biotechnologies).

MGMT activity assays
Briefly, increasing amounts of PARP or MGMT inhibitors were incubated with recombinant MGMT (~6 fmoles) for 1 hour at room temperature, then substrate DNA was added and incubation continued for 1 hour. After incubation, assay samples were processed as described previously in (ref. 28) and radioactivity quantified by scintillation counting.

FACS analysis
One lakh cells were treated as indicated for 3 days, fixed in 80% ice-cold ethanol in PBS. Cells were then washed in PBS and incubated in a solution containing PBS, RNase A, and propidium iodide (SIGMA) at 37°C for 1 hour. The analysis was performed using FACS-Calibur (Becton Dickinson).

Senescence-associated β-galactosidase (SA-β-gal) assay
After 5 days of treatment, cells were fixed in 0.5% glutaraldehyde and stained overnight with X-gal solution: 0.12 mmol/L 5-Bromo-4-chloro-3-indolyl-β-D-galactopiranoside in PBS pH 6.0. Cell nuclei were stained with DAPI. Bright field and UV images were taken using an inverted fluorescence microscope. The percentage of SA-β-Gal–positive cells is determined upon counting of at least 100 cells.

High content analysis of the senescent response
For the high content analysis, cells were plated in 96-well plates and stained by immunofluorescence. Two fluorescence images corresponding to DAPI (to identify individual cells) and gene-specific antibody/AlexaFluor488-secondary antibody were acquired and analyzed as previously described (29). Each well was scored a percentage positive of reference channel to the total cells. Antibodies used were: anti-γ-H2AX (Millipore), anti-BrdU (Invitrogen), anti-p21Cip1 (Sigma), anti-p53 (Santa Cruz Biotechnology), and anti-phospho-(Ser/Thr)Q (Cell Signaling Technology).
MGMT is active in temozolomide-resistant cells

To establish whether MGMT expression correlated with its activity, we used the MGMT inhibitor lomeguatrib. We treated cell lines for 5 days with temozolomide in the absence or presence of 20 μmol/L lomeguatrib (LOM), which only sensitized MGMT-expressing melanoma cells to temozolomide. Accordingly the average PF$_{50}$ for MGMT$^+$ cells was significantly higher than in MGMT$^-$ cells (Fig. 2A–C). As expected, the GI$_{50}$ for MGMT$^+$ cells was significantly higher than for MGMT$^-$ cells. Colony formation assays provided very similar results (Fig. 2D–F). These data confirmed that MGMT expression and activity are correlated in our panel of melanoma cell lines, demonstrating that MGMT activity provides resistance to temozolomide and correlates with temozolomide and PARPi synergy. Importantly, every melanoma cell line expressed detectable levels of the mismatch repair (MMR) proteins MSH-6, MSH-2, and MLH-1 (Supplementary Fig. S3). This result, together with the responses to temozolomide and olaparib or lomeguatrib previously observed, strongly suggested that all our melanoma cells are MMR proficient and the observed differences are not related to MMR deficiency.

The MGMT-dependent response to temozolomide–PARPi treatment is also seen in other cancer types

To determine whether the impact of MGMT expression on the PARPi response is specific for melanoma cells or cancer type independent, we assessed the response to temozolomide and olaparib in cells from three cancer types where temozolomide is used as a therapeutic option: glioblastoma, pancreatic neuroendocrine tumors (NET), and colorectal carcinoma (11, 33, 34). We treated seven glioblastoma cell lines (4 MGMT$^+$ and 3 MGMT$^-$ cell lines) known to be MMR$^+$ (25) and observed that MGMT expression correlated with the response to temozolomide combined with olaparib (Fig. 3A–C). Similarly, although the NET cell line BON, which expresses high levels of MGMT responded to olaparib (and lomeguatrib) cotreatment, QGP-1 NET cells, which lack MGMT, were unaffected by PARP inhibition (Fig. 3D and E). Again, both NET cell lines were found to be MMR$^+$ (Supplementary Fig. S4A). Furthermore, a panel of seven MGMT-expressing colorectal cancer cell lines of known MMR status (35) responded to olaparib cotreatment (Fig. 3F and G). Importantly, when grouped by MMR status, the PF$_{50}$ for olaparib was similar for both groups (Fig. 3H; details of GI$_{50}$ and PF$_{50}$ values for each cell line can be found in Supplementary Fig. S4B).

MGMT overexpression confers PARP-dependent resistance to temozolomide

Our data suggest that MGMT expression provides resistance to temozolomide in a PARP-dependent manner. To further confirm these findings, we ectopically expressed MGMT in MGMT$^-$ melanoma cells (WM98-1; Fig. 4A). As expected, MGMT overexpression conferred resistance to temozolomide but lomeguatrib sensitized WM98-1-MGMT clones to temozolomide-induced growth inhibition (Fig. 4B). Crucially, the MGMT-produced resistance could be overcome by treatment with olaparib (Fig. 4C). Similar results were obtained using veliparib (Supplementary Fig. S5A and S5B). Furthermore, we observed that when MGMT activity is inhibited, PARPi no longer sensitized to temozolomide (Fig. 4D–F). We show that PARPi is required for the MGMT-mediated resistance to temozolomide. As mentioned above, temozolomide produces N3- and N7-methyl adducts, which are
repaird by PARP-mediated BER. To assess the requirement of PARP in this repair, we used the alkylating agent MMS, which exclusively produces N3- and N7-methyl adducts (36). Both olaparib and veliparib significantly sensitized WM98-1 and WM98-1-MGMT cells to MMS (Fig. 4G and Supplementary Fig. S5C). Similar results were obtained in a panel of melanoma cells (Fig. 5A and B). Although PARP inhibition did not enhance the activation of CHK2 by

PARP inhibition potentiates temozolomide-induced cell-cycle arrest in MGMT-proficient cells

To further understand the action of temozolomide and PARP inhibition in the context of the MGMT expression status, we studied the induction of DNA single- and double-strand breaks measuring histone H2AX phosphorylation (γH2AX) after a 16-hour treatment. PARP inhibition only synergized with temozolomide to induce DNA damage in MGMT+ cells (Fig. 5A and B). Analysis of CHK-1 and 2 phosphorylation, (Thr68 and Ser345, respectively), at 24 hours of treatment revealed that in MGMT+ WM266-4 cells, temozolomide activated CHK1/2 at lower concentrations than in MGMT– cells (Fig. 5C and D). Although PARP inhibition did not enhance the activation of CHK2 by

Figure 1.
PARP inhibition synergizes with temozolomide (TMZ) only in MGMT+ melanoma cells. A, melanoma cell lines were treated with temozolomide with or without 0.25 μmol/L olaparib (OLAP). The graph shows the effect of PARP inhibition on the average GI50 for temozolomide. Each point corresponds to an individual cell line. B, top, Western blot analysis for MGMT expression in melanoma cell lines. ERK2 was used as a loading control. B, bottom, methylation-specific PCR for unmethylated (U) and methylated (M) promoter sequences. C and D, cell lines were grouped upon their MGMT status, GI50 results from A reanalyzed lines were plotted. C and D, cell lines were grouped upon their MGMT status, GI50 results from A reanalyzed cell lines were plotted. E, Potentiation Factor results from colony formation assays on MGMT+ and MGMT− cells treated as indicated. Student t test was used to compare the effect of each treatment. F, pictures from A375 and WM266-4 cells injected in zebra fish embryos and treated as indicated for 5 days. G and H, quantification of the in vivo effect of PARP inhibition on temozolomide (200 μmol/L)-treated xenografts. One-way ANOVA was used to compare the effect of each treatment with DMSO (D)-treated animals. *, P < 0.05; **, P < 0.01; n.s, not significant; ***, P < 0.001.
temozolomide in WM266-4 cells, in A375 cells temozolomide-induced CHK-2 phosphorylation was potentiated by olaparib. On the other hand, CHK-1 phosphorylation did not correlate consistently with olaparib cotreatment.

We next analyzed the cell-cycle profile of A375 and WM266-4 cells after 72 hours of treatment. In WM266-4 cells, suboptimal concentrations of temozolomide (5 μmol/L, unable to affect cell-cycle progression) failed to cooperate with olaparib to promote a cell-cycle arrest while 20 μmol/L temozolomide (GI50 = 73.26 μmol/L), produced a marked G2-M arrest (Fig. 5E). In contrast, in A375 cells (GI50 = 439.65 μmol/L) combining olaparib with a suboptimal concentration of temozolomide (50 μmol/L) produced a dramatic arrest in the G2-M phase of the cell cycle (Fig. 5E). Strikingly, we were not able to detect the appearance of a sub-G1 phase, which is associated with cell death (37). In agreement with the absence of a sub-G1 population, we did not observe any caspase-3 cleavage in response to temozolomide in melanoma cells, whereas this was detectable in colorectal cancer cells (Fig. 5F). Moreover, cotreatment of A375 cells with concentrations of temozolomide and OLAP that potently affect cell proliferation failed to induce caspase-3 cleavage (Fig. 5F, bottom). On the other hand, we observed that melanoma cells treated with temozolomide displayed a very flat appearance with a marked increase in cell size/surface (Fig. 5G). Because these are common traits of a senescent response, we decided to gain more insight into the role of senescence in the response of melanoma cells to temozolomide (38).

Characterization of the senescent response induced by the combination of temozolomide and PARP inhibition

A 5-day treatment with growth-inhibitory concentrations of temozolomide induced SA-β-gal staining in MGMT+ WM98-1 as well as MGMT+ A375 and WM98-1-MGMT cells (Fig. 6A–C), confirming previous observations suggesting that temozolomide induces senescence rather than apoptosis in melanoma cells (39). However, in MGMT+ cells treated with concentrations of temozolomide that induced only a weak SA-β-gal activity, PARP inhibition strongly enhanced the senescent response (Fig. 6B and C). High content analysis of A375 cells, used to assess the expression of several known markers and mediators of senescence (29), confirmed that the combination of temozolomide and olaparib induced a strong senescent response.
PARPi induced a potent DNA damage response (DDR, maintained over 5 days of treatment), as indicated by ATM and ATR phosphorylation (ST/Q) and a γH2AX signal (Fig. 6D). The activation of the DDR was accompanied by a significant increase in p53 levels and the cell-cycle inhibitor p21 (Fig. 6E). Finally, as expected from a long-term cell-cycle arrest, we observed a dramatic reduction in the number of cells undergoing DNA synthesis, as revealed by bromodeoxyuridine (BrdUrd) incorporation assay (Fig. 6F). In summary, when PARP is inhibited in MGMT-expressing melanoma cells, temozolomide-induced DNA damage triggers a senescence response that correlates with p53-mediated upregulation of p21.

**Discussion**

Because melanomas are notoriously resistant to chemotherapy, we assessed the potential of combining temozolomide and PARP inhibition in melanoma cells. Strikingly, we observed that PARP, required for BER, plays a role in MGMT-mediated resistance to temozolomide. In MGMT/C0 cells, PARP function appears to be dispensable for the repair of temozolomide-induced DNA damage, because olaparib does not enhance the temozolomide-induced effects on growth. On the other hand, in MGMT+ cells, the MGMT-mediated resistance to temozolomide-induced DNA damage is dependent on PARP, as evidenced by the synergy observed between PARPi and temozolomide to inhibit proliferation.
We suggest that, upon temozolomide treatment, a threshold of N3-, N7-methylation needs to be reached before PARP-dependent DNA repair becomes relevant to the antiproliferative effect of temozolomide. In the absence of MGMT, 6-meG is not repaired (4), and the immediate toxicity of 6-meG prevents this threshold to be reached. Alternatively, tolerance to 6-meG due to deficient MMR might allow to reach this threshold, which would explain previous results, obtained in MMR−/MGMT− leukemia cells (40).

So far the combination of MGMT inhibitors with dacarbazine or temozolomide has failed in the clinic, but our data suggest that the MGMT status could be a predictive marker for response to therapies combining temozolomide and PARPi. To date, early-phase clinical trials using temozolomide and veliparib in CNS and prostate cancer patients have shown modest activity (21, 41). On the other hand, one phase II trial in patients with melanoma showed an increase in progression-free survival over historical...
MGMT-Mediated Response to Temozolomide and PARP Inhibitors

Figure 5. Melanoma cell response to temozolomide (TMZ) and PARP inhibition. A and B, quantification of γH2AX expression in WM266-4 and A375 cells treated as determined by immunofluorescence. C and D, Western blot analysis of phospho Thr68-CHK1 (ppCHK1), phospho Ser345-CHK2 (ppCHK2) in WM266-4 and A375 cells treated as indicated. E, effects on the cell cycle: distribution in G0, S, and G2-M phases of the cell cycle of WM266-4 and A375 cells treated for 3 days with temozolomide with or without olaparib. F, top, Western blot analysis of cleaved caspase-3 expression in A375 cells treated as determined using ImageJ. F and G, induction of β-galactosidase activity upon treatment of WM98-1 (F) or A375 cells treated as indicated for 3 days. **P < 0.01; ***P < 0.001; ns, not significant.

Although trials are in progress, it is clear that there is still a need for biomarkers predicting response to specific therapies to increase clinical response. Indeed the upcoming clinical trial in glioblastoma combining temozolomide and veliparib in patients with MGMT promoter methylation (A071102, NCT02152982, www.clinicaltrials.gov) will hopefully shed more light on the role of MGMT as a marker.

Because we observe a role for MGMT in the temozolomide–veliparib mediated growth inhibition in cell lines from four different cancer types, our data suggest a common mechanism of response to temozolomide. Indeed Palma and colleagues observed that a first cycle of temozolomide and veliparib was most effective in MGMT− tumors from different origin. Initially, MGMT− tumors did not benefit from PARPi cotreatment while a second cycle demonstrated efficacy in tumors that underwent reexpression of MGMT (42). On the other hand, cancer-type specific responses cannot be ruled out as it has been suggested that only MGMT− leukemia and glioblastoma multiforme cells would respond to temozolomide combined with the PARPi veliparib (40, 43, 44). It is nevertheless important to consider that variables such as differences in PARPi action, bioavailability, or the application of particular treatment regimes need to be taken into account to correlate in vitro and in vivo efficacy of temozolomide and PARPi combinations as well as clinical efficacy (31, 42–45). Indeed, to synergize with temozolomide, both drugs work through catalytic PARP inhibition and trapping PARP–DNA complexes. Although olaparib has been shown to be more effective to trap PARP–DNA complexes than veliparib, we show evidence that the role of PARP in MGMT-induced resistance can be overcome by both inhibitors (31). This could be explained by the different concentrations at which olaparib and veliparib were used (0.25 and 5 μmol/L, respectively).
Our data provide compelling evidence of MGMT as a potential marker for temozolomide + PARPi-based therapies. If confirmed clinically, this has the potential for improving clinical responses and reducing toxicity (41).

Importantly, MGMT status assessment remains a challenge and although MGMT promoter methylation has already entered clinical guidelines as predictive biomarker in elderly patients with glioblastoma, it has not been fully validated across cancer types (13, 46). We observed that some MGMT-expressing melanoma cell lines and, notably, also some MGMT-expressing glioblastoma cell lines are in fact positive for promoter methylation [Fig. 1B; ref. 25], suggesting that a positive signal for promoter methylation does not always correlate with MGMT expression. Thus, the MGMT promoter status alone might not be sufficient as marker in a clinical setting. As already described for glioblastoma and NET tumors, we propose that this issue could be overcome by combining promoter methylation with IHC (47, 48).

Notably, we observed that temozolomide and olaparib failed to induce apoptosis in melanoma cells, but rather induced senescence, which is most probably regulated through the p53/p21 axis (39, 49). Whether treatment with monoalkylating agents inhibits mechanisms of cell death, or whether the apoptotic machinery is turned off as cells get arrested in the G2–M phase of the cell cycle warrant further investigation.

In summary, our data provide strong evidence that, specifically in cells expressing MGMT, the combination of temozolomide and PARPi might be of benefit and improve responses.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: I. Arozarena

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