ATM Regulates 3-Methylpurine-DNA Glycosylase and Promotes Therapeutic Resistance to Alkylating Agents

Sameer Agnihotri1, Kelly Burrell1, Pawel Buczkowicz1, Marc Remke1, Brian Golbourn1, Yevgen Chornenkyy1, Aaron Gajadhar2, Nestor A. Fernandez1, Ian D. Clarke1, Mark S. Barsczyk1, Sanja Pajovic1, Christian Ternamian1, Renee Head1, Nesrin Sabha1, Robert W. Sobol3,4,5, Michael D. Taylor1, James T. Rutka1, Chris Jones6, Peter B. Dirks1, Gelareh Zadeh1,7, and Cynthia Hawkins1,8
ABSTRACT

Alkylating agents are a first-line therapy for the treatment of several aggressive cancers, including pediatric glioblastoma, a lethal tumor in children. Unfortunately, many tumors are resistant to this therapy. We sought to identify ways of sensitizing tumor cells to alkylating agents while leaving normal cells unharmed, increasing therapeutic response while minimizing toxicity. Using an siRNA screen targeting over 240 DNA damage response genes, we identified novel sensitizers to alkylating agents. In particular, the base excision repair (BER) pathway, including 3-methylpurine-DNA glycosylase (MPG), as well as ataxia telangiectasia mutated (ATM), were identified in our screen. Interestingly, we identified MPG as a direct novel substrate of ATM. ATM-mediated phosphorylation of MPG was required for enhanced MPG function. Importantly, combined inhibition or loss of MPG and ATM resulted in increased alkylating agent-induced cytotoxicity in vitro and prolonged survival in vivo. The discovery of the ATM–MPG axis will lead to improved treatment of alkylating agent–resistant tumors.

SIGNIFICANCE: Inhibition of ATM and MPG-mediated BER cooperate to sensitize tumor cells to alkylating agents, impairing tumor growth in vitro and in vivo with no toxicity to normal cells, providing an ideal therapeutic window. Cancer Discov; 4(10); 1198–1213. ©2014 AACR.

See related commentary by Wick and Platten, p. 1120.

INTRODUCTION

Identifying biologic targets and pathways that lead to treatment resistance presents a major hurdle to developing effective cancer therapies. Cancer cells acquire or intrinsically harbor resistance to many first-line treatments (1). A major mechanism of cancer cell resistance is through activation of DNA repair pathways which reverse the cytotoxicity of many clinically used DNA-damaging agents, including alkylating agents. Therefore, targeting DNA repair pathways on which tumor cells, and not normal cells, are dependent could potentially overcome treatment resistance in many cancers.

In adults, the most common primary brain tumor is glioblastoma (GBM). Large clinical trials have demonstrated a survival benefit for patients with GBM with the addition of the alkylating agent temozolomide over radiation alone (2, 3). Temozolomide is an oral alkylating agent that readily crosses the blood–brain barrier, making it an attractive drug to use clinically, and has become part of the standard of care for adult GBM (4, 5). Interestingly, not all patients benefit from temozolomide, and current data suggest that patients with GBM with promoter methylation and lack of O6-methylguanine–DNA methyltransferase (MGMT) expression do better than patients with an unmethylated MGMT promoter and MGMT expression. Alkylating agents, including temozolomide, work by causing DNA damage—namely, alkylation of O6-guanine, N7-guanine, and N3-adenine residues on DNA. However, DNA repair mechanisms exist within cells that can reverse this damage. MGMT repairs the alkylated O6-guanine lesion (6), explaining why its expression may mediate resistance to temozolomide. Importantly, many patients whose tumors do not express MGMT are nevertheless resistant to temozolomide, with the mechanism underlying this resistance remaining unclear.

In children with GBM, the use of temozolomide is more controversial. A recent trial by the Children’s Oncology group demonstrated no survival benefit for patients receiving adjuvant temozolomide compared to children treated in Children’s Cancer Group (CCG) study CCG-945 (7). Thirty percent of high-grade gliomas in this study expressed MGMT, and this was associated with worse progression-free survival. What may have been mediating resistance in the remaining cases is unclear. In other studies of pediatric GBM, temozolomide seems to have little or no effect despite a lack of MGMT expression in many cases (8–10). Overall, the mechanisms of treatment resistance in pediatric GBM are poorly understood. Recent data have highlighted the genetic differences between pediatric and adult GBM, suggesting that novel strategies are required to identify effective therapeutics for children with GBM (11–13). We hypothesized that alternate, non–MGMT-dependent, mechanisms of DNA repair and treatment resistance could explain why pediatric GBM fails to respond to alkylating agents. To identify molecular mechanisms of therapeutic resistance, we performed an siRNA screen targeting DNA damage response genes in the presence of sublethal doses of temozolomide. Our results demonstrate that the 3-methylpurine-DNA glycosylase (MPG), as well as ataxia telangiectasia mutated (ATM), were identified in our screen. Interestingly, we identified MPG as a direct novel substrate of ATM. ATM-mediated phosphorylation of MPG was required for enhanced MPG function. Importantly, combined inhibition or loss of MPG and ATM resulted in increased alkylating agent-induced cytotoxicity in vitro and prolonged survival in vivo. The discovery of the ATM–MPG axis will lead to improved treatment of alkylating agent–resistant tumors.

Note: Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org/).

Corresponding Author: Cynthia Hawkins, The Hospital for Sick Children, 555 University Avenue, Toronto, ON M5G 1X8, Canada. Phone: 416-813-5938; Fax: 416-813-5974; E-mail: cynthia.hawkins@sickkids.ca
doi: 10.1158/2159-8290.CD-14-0157
©2014 American Association for Cancer Research.
glycosylase (MPG)–mediated base excision repair (BER) pathway plays a major role in promoting resistance to temozolomide in pediatric GBM, and that loss of MPG results in an accumulation of unrepaired cytosolic N³ methyladenine residues, which results in stalled DNA replication and cell death (14, 15). Furthermore, we demonstrate that the BER pathway specifically mediated by MPG can be regulated by ataxia telangiectasia mutated (ATM) through direct phosphorylation of MPG. MPG is a DNA glycosylase responsible for initial recognition of the damaged DNA caused by temozolomide, specifically alkylated N³ guanine and alkylated N³ adenine residues (16–18). By linking ATM signaling with MPG, we identified a novel axis that tumor cells may use to repair damage caused by alkylating agents. Importantly, targeting BER and ATM increases sensitivity to temozolomide and prolongs survival in an in vivo model of pediatric GBM. By painting a broader landscape of the role of DNA damage response pathways in pediatric GBM than was previously appreciated, this study suggests novel therapeutic combinations to overcome treatment resistance.

RESULTS

An siRNA Screen Identifies the BER Pathway as the Major Temozolomide Sensitizer in Pediatric GBM

Pediatric GBM lines were screened for temozolomide sensitivity by calculating the IC₅₀ at days 3 and 7. Cells with an IC₅₀ of >100 µmol/L were considered resistant, as such doses are not physiologically achievable in temozolomide-treated patients (19–21). Five of six pediatric GBM lines showed temozolomide resistance, and IC₅₀ values were comparable to those of adult GBM lines (Supplementary Fig. S1A). To identify the mechanism mediating this resistance, we performed a siRNA screen targeting over 240 DNA repair genes in two of the temozolomide-resistant pediatric GBM cell lines, SJG2 and KNS42. Cells were incubated with or without temozolomide (100 µmol/L) in biologic triplicates. Cell viability was assessed 72 hours after siRNA transfection. Data were normalized using the z-score method, and significant temozolomide sensitizers were determined using z-score cutoffs of less than −1.65 (P < 0.05 in all three biologic replicates; Fig. 1A and B and Supplementary Fig. S1B and S1C).

We identified 24 genes whose knockdown resulted in decreased cell viability in response to temozolomide, 13 of which were common to both cell lines (Fig. 1C). Interestingly, this analysis revealed enrichment of genes involved in the BER pathway (MPG, APEX1, APEX2, XRCC1, and POLB), ATM (a master regulator of DNA repair), and LIG4 [involved in double-strand break repair (DSBR) and nonhomologous end joining (NHEJ)]. We confirmed knockdown at the RNA level (Supplementary Fig. S1D) and protein level for our top candidates (MPG, APEX1, LIG4, and ATM; Supplementary Fig. S1E and S1H). We next rescreened our top candidates in pediatric GBM cells as well as fetal normal human astrocytes (NHA) and fetal neural stem cells (NSC) with an initial concentration of 100 µmol/L at day 3 to test for toxicity (Fig. 1D). We also rescreened at a lower dose (25 µmol/L) of temozolomide to evaluate the effect of gene knockdown at a low, clinically achievable dose over a longer period of time (day 7; Supplementary Fig. S2A). ATF, TDG, and DCLRE1 were eliminated as candidates as they were toxic to NHAs and NSCs and loss of these genes, even in the absence of temozolomide, caused significant reduction in viability (Fig. 1D and Supplementary Fig. S2A).

Next, we screened a series of pediatric GBM lines for endogenous expression of our top candidates. All six pediatric GBM lines demonstrated expression of APEX1, LIG4, and PARP1. MPG was not expressed in SF188 and RES259 cells, both of which are sensitive to temozolomide, whereas SJG2 cells expressed high levels of MPG and are temozolomide resistant (Fig. 1E). SJG2 and KNS42 cells expressed nuclear MPG, and SF188 cells had no expression of MPG (Fig. 1F and Supplementary Fig. S2B). In addition to high nuclear MPG, SJG2 cells expressed high MPG glycosylase activity, whereas SF188 cells had no detectable MPG activity (Supplementary Fig. S2C). Furthermore, MPG protein expression measured in our pediatric GBM (pGBM) cell lines correlated with the IC₅₀ of temozolomide measured on days 3 and 7 (Fig. 1G and Supplementary Fig. S2D). MGMT protein expression did not correlate with the IC₅₀ values of temozolomide in pediatric GBM cells and was not expressed in temozolomide-resistant pediatric GBM cells used in the screen (Fig. 1E and G and Supplementary Fig. S2D). In addition to temozolomide, MPG protein expression correlated with IC₅₀ values for other alkylating agents, including bis-chloroethyl nitrosourea (BCNU/carmustine) and methyl methanesulfonate (MMS, Supplementary Fig. S2E and S2F).

MPG Is Strongly Expressed in Pediatric Patients with GBM

To ensure the clinical relevance of our findings, we investigated the expression of MPG, BER proteins (APEX1, APEX2, and XRCC1), ATM, and LIG4 in pediatric GBM operative samples compared with normal brain. Immunohistochemical (IHC) analysis of tissue microarrays containing 72 clinically annotated supratentorial pediatric GBMs from two independent centers demonstrated high nuclear staining in 48 of 72 (67%), cytoplasmic staining in 5 of 72 (7%), and negative staining in 19 of 72 (26%) patients' tumors (Fig. 2A). The Kaplan–Meier survival analysis of the London dataset (King's College, UK) demonstrated that patients with negative MPG had better overall survival (P = 0.02; Fig. 2B). For the entire Toronto dataset, which consisted of 41 heterogeneously treated patients, MPG-negative status showed a trend toward survival benefit but was not statistically significant (P = 0.14; Supplementary Fig. S2G). Interestingly, on a pairwise analysis of the 16 patients in the SickKids cohort treated with an alkylating agent [temozolomide or lomustine (CCNU)], MPG-negative patients had a significantly better median survival than MPG-positive patients [median survival was 3.5 years in the MPG-negative group (n = 4) vs. 1.7 years in the MPG-positive group (n = 12); *, P < 0.038]. However, these numbers are small, and a prospectively temozolomide-treated cohort from a clinical trial is needed to fully evaluate the benefit of using MPG and other targets from our screen as predictors of temozolomide resistance.

We next explored gene expression of MPG and several other components of BER at the mRNA expression level in pediatric GBM compared with normal brain in an additional gene-expression dataset of pediatric GBM (22, 23; Fig. 2C and F). We confirmed that mRNAs encoding BER proteins—MPG,
Figure 1. An siRNA screen identifies BER pathway members as sensitizers to temozolomide (TMZ) in pediatric GBM. 

A and B, SJG2 and KNS42 cells were transfected with a 240 DNA siRNA pool library, and 24 hours after transfections, cells were cultured with or without temozolomide (100 μmol/L). Cell viability was assessed by the AlamarBlue cell viability assay 72 hours after siRNA transfection. Data were normalized using the standard \( z \)-score method by correcting the raw data for plate-to-plate variation. Significance of potential temozolomide sensitizers was determined using \( z \)-score cutoff values of less than −1.65 (dotted line), which corresponded to a \( P \) value of 0.05 in all three biologic replicates (rep. 1–3). 

C, Venn diagram of genes common and unique to both cell lines from the siRNA screen. 

D, Heatmap of cell viability to validate target genes in temozolomide and non-temozolomide conditions using SJG2 cells, KNS42 cells, NHAs, and normal NSCs. Temozolomide dose used was 100 μmol/L, and viability was assessed using almarBlue assay on day 3. *, \( P < 0.05 \) using ANOVA followed by a post hoc Dunnett test. Each heatmap box represents the average of three independent experiments. 

E, Immunoblotting to evaluate the protein expression levels of BER pathway members in pediatric GBM cell lines. 

F, Immunofluorescence of MPG in SJG2 cells showing nuclear expression of the protein. Scale bar, 16 μm. 

G, Pearson correlation plots of MPG (black) and MGMT (red) protein expression quantified by chemiluminescence densitometry versus \( IC_{50} \) values following 7 days of temozolomide in pediatric GBM cell lines from Supplementary Fig. S1A. Strong correlation is seen with MPG protein levels (\( r = 0.78 \) at 7 days) but not with MGMT levels (\( r = 0.03 \)). All experiments were performed in triplicate; error bars, SE of the mean.
BER genes are upregulated in pGBM and adult gliomas. A, immunohistochemistry analysis of 72 pediatric GBM samples demonstrating strong nuclear, cytoplasmic, or negative staining. Scale bar, 20 μm. B, the Kaplan–Meier survival curve analysis of patients staining positive for MPG or negative. Log-rank $P = 0.016$. C–H, RNA gene-expression analysis of BER proteins downstream of MPG in pediatric GBM (pGBM, $n = 53$) compared with normal human brain (NB, $n = 172$). ***, $P < 0.001$. 

**Figure 2.** BER genes are upregulated in pGBM and adult gliomas. A, immunohistochemistry analysis of 72 pediatric GBM samples demonstrating strong nuclear, cytoplasmic, or negative staining. Scale bar, 20 μm. B, the Kaplan–Meier survival curve analysis of patients staining positive for MPG or negative. Log-rank $P = 0.016$. C–H, RNA gene-expression analysis of BER proteins downstream of MPG in pediatric GBM (pGBM, $n = 53$) compared with normal human brain (NB, $n = 172$). ***, $P < 0.001$. I, copy-number alterations identify that 20 of 40 BER DNA repair genes are significantly altered in 47 pediatric high-grade gliomas. Gains were established as 2.5 more copies of tumor DNA per gene compared with matched normal controls, and losses were established as 1.5 copies or fewer of tumor DNA compared with normal control tissue, both corrected at a false discovery rate ($q$ value) of $< 5%$.

APEX1, APEX2, and XRCC1—were significantly upregulated compared with normal brain. ATM and LIG4 were also upregulated in pediatric GBM compared with normal human brain (Fig. 2G and H).

We next explored copy-number alterations for all genes with a DNA repair gene ontology involved in BER (GO term: GO:0006284) on 47 of our genomically characterized high-grade pediatric gliomas (13, 24). Analysis of 40 BER DNA repair genes revealed significant amplifications in 20 of 40 BER genes with MPG amplifications among the top three most amplified BER genes (*, $P < 0.05$; Fig. 2I). In summary, the BER pathway, including MPG, is highly upregulated at the RNA level, exhibits copy-number gains, and has high protein expression in pediatric high-grade gliomas, including pediatric GBM.

**MPG Modulates Resistance to Alkylating Agents in Pediatric GBM**

To further characterize the role of MPG in temozolomide resistance, we generated stable loss of MPG in SJG2 and KNS42 cells by shRNA-mediated gene silencing (Fig. 3A). Temozolomide (50–500 μmol/L) treatment led to a significant decrease in the cell number in SJG2 and KNS42 cells with MPG knockdown compared with scramble control cells (Fig. 3B). In addition to reduced cell count, temozolomide treatment of SJG2 and KNS42 cells with MPG knockdown compared with scramble control cells led to increased DNA damage (increased γH2AX) and reduced DNA repair (increased alkaline comet tail length; Fig. 3C and Supplementary Fig. S3A and S3D). Increased DNA damage and reduced...
Figure 3. MPG loss sensitizes pGBM cells to alkylating agents. A, immunoblotting confirming MPG protein knockdown in pediatric GBM cell lines SJG2 and KNS42 following transfection with pooled stable clones generated from two unique MPG shRNA constructs. B, cell count of pediatric GBM cell lines SJG2 and KNS42 expressing MPG shRNA or shRNA controls (con) exposed to increasing doses of temozolomide (TMZ; 0–500 μmol/L). Cell counts were performed 72 hours after temozolomide treatment. ***, P < 0.001. C, immunofluorescence of γH2AX in SJG2 cells expressing MPG shRNA or control (con) shRNA. Scale bar, 16 μm. D, activated cleaved caspase-3/7 assay of SJG2 and KNS42 cells treated with temozolomide after 24-hour treatment. **, P < 0.01. E, Colony-forming unit (CFU) assay in SJG2 and KNS42 cells cultured with or without 100 μmol/L temozolomide. CFUs were counted after 14 days. **, P < 0.01, knockdown versus control cells. F, immunoblotting of MPG and LIG4 following siRNA treatment showing effective protein knockdown. G and H, plot of cell viability of SJG2 (G) and KNS42 (H) cells transfected with MPG, LIG4, or dual siRNA following exposure to varying concentrations of temozolomide. Viability was measured using the alamarBlue viability assay and quantified after 7 days. Dual siRNA knockdowns were also compared with single knockdowns to evaluate additive effects of double knockdown. *, P < 0.05; **, P < 0.01; and ***, P < 0.001.
DNA repair led to increased apoptosis in MPG knockdown cells compared with shRNA control cells, as measured by activated caspase-3/7 (*, P < 0.05; Fig. 3D) and expression of cleaved PARP (Supplementary Fig. S3E). A colony-forming unit (CFU) assay measured over 14 days demonstrated that both lines with MPG knockdown had reduced colony growth when grown in 100 μmol/L temozolomide (Fig. 3E) or other alkylating agents (200 μmol/L BCNU or 10 μmol/L MMS; Supplementary Fig. S3F and S3G) compared with controls.

Conversely, we reexpressed MPG in two low/negative MPG lines, RES259 and SF188 (Supplementary Fig. S4A). Stable expression of MPG increased resistance to temozolomide at doses from 50 to 250 μmol/L, as measured by cell count (Supplementary Fig. S4B). Stable MPG-expressing cells in the presence of temozolomide had reduced DNA damage, as measured by comet tail assay and γH2AX staining, increased CFU number, and decreased apoptosis, as measured by activated caspase-3/7 and cleaved PARP (Supplementary Fig. S4C and S4D).

Targeting Multiple BER Pathway Members and Non-BER Pathway Members Increases Sensitivity to Alkylating Agents in Pediatric GBM

We next tested whether targeting MPG with genes involved in non-BER pathways or multiple targeting of BER might lead to an additive sensitivity to temozolomide. LIG4 is involved in DSBR and NHEJ. Knockdown of LIG4, as confirmed by Western blotting (Fig. 3F), led to significantly reduced cell survival. The effect was additive with MPG loss at several doses of temozolomide in SJG2 cells (*, P < 0.05; Fig. 3G and H). Combining MPG knockdown with knockdown of ATM, a master controller for cell response to DNA damage and of genome stability, also led to an additive sensitivity to temozolomide in SJG2 cells (*, P < 0.05; Supplementary Fig. S5A and S5B). Furthermore, knockdown of APEX1 or POLB, downstream BER effector proteins, led to a significant decrease in cell viability in response to temozolomide compared with scrambled siRNA controls (*, P < 0.05; Supplementary Figs. S5A, S5C, and S5D). Combined loss of MPG with APEX1, but not POLB, led to a further additive reduction in cell viability (*, P < 0.05; Supplementary Fig. S5C and S5D). Finally, as MGMT is critical for repairing O6-methyl guanine–damaged bases induced by temozolomide, we next wanted to determine whether targeting MPG-mediated BER in MGMT-expressing cells could sensitize them to temozolomide. siRNA-mediated knockdown of either MGMT or MPG in RES186 and UW479 cells (normally expressing both MGMT and MPG) led to reduced cell viability when treated with several doses of temozolomide (*, P < 0.05; Supplementary Fig. S5E and S5G). Combined loss of MPG and MGMT resulted in an additive reduction of cell viability (*, P < 0.05; Supplementary Fig. S5E and S5G).

In summary, targeting MPG-mediated BER with non-BER pathways regulated by LIG4, MGMT, and ATM or additional downstream BER proteins, including APEX1, results in additive cytotoxicity when cells are treated with temozolomide.

MPG Is Phosphorylated and Activated by ATM

Having determined that MPG modulates response to alkylating agents, we next attempted to elucidate novel regulators of MPG. Using an in silico kinase screen of MPG, we identified several phosphorylation motifs (Supplementary Table S1; ref. 25). Interestingly, we identified ATM, a major effector of the DNA damage response, as a putative kinase of MPG. ATM itself was also a top validated target in our siRNA screen (Fig. 1 and Supplementary Fig. S5). In silico analysis demonstrated that the ATM–ATR (ataxia telangiectasia and Rad3-related) phosphorylation site SQ (serine-glutamine, at serine 172) on MPG is highly conserved across several species (Fig. 4A). We observed significant constitutively active phospho-ATM in our adult (T98G) and pediatric GBM cells (SJG2, KNS42, and RES259), which was further enhanced in the presence of temozolomide (Fig. 4B and Supplementary Fig. S6A for densitometric quantification). ATR, on the other hand, was only phosphorylated in the presence of temozolomide and was not identified as a sensitizer to temozolomide in our siRNA screen; thus, we proceeded to further investigate ATM.

We next explored whether ATM and MPG were associated, and whether MPG was a direct target of ATM. Endogenous immunoprecipitations (IP) of MPG from T98G cells (a well-characterized model of temozolomide resistance) and SJG2 cells were enriched for ATM compared with IgG controls, whereas reverse IPs of ATM were enriched for MPG (Fig. 4C). Interestingly, following treatment of the cells with an ATM inhibitor (KU55933), MPG was no longer enriched in IPs of phospho-SQ (ATM substrate site; Fig. 4D), suggesting that the ATM phosphorylation is necessary for the interaction. In keeping with this, phospho-MPG was detected using the ATM phospho-specific SQ antibody after specific IP of MPG from a protein interaction dissociative buffer to enrich for MPG protein only in the absence, but not in the presence, of an ATM inhibitor (KU55933; Fig. 4E).

To complement our endogenous IPs, we transfected MPG-MYC-tagged and ATM-FLAG-tagged constructs into T98G and SJG2 GBM cells and observed exogenous ATM and MPG associated with each other (Supplementary Fig. S6B), similar to our endogenous IP results. Phosphorylation of our MPG-FLAG construct was ablated in cells treated with an ATM inhibitor (Supplementary Fig. S6C). Furthermore, a site-directed mutant MPG-FLAG in which serine 172 (the putative ATM phosphorylation site) was replaced with glycine by site-directed mutagenesis was no longer phosphorylatable by ATM (Supplementary Fig. S6C). Phospho-MPG and phospho-ATM were observed only in normal human astrocytes when subjected to temozolomide or radiation-induced DNA damage (Supplementary Fig. S6D). Using an ATPγ-sial analogue, we performed an in vitro kinase assay with MPG purified using the Glutathione S-transferases (GST) tag system and immunoprecipitated ATM kinase to test whether ATM directly phosphorylates MPG. ATM phosphorylated MPG at serine 172 (Fig. 4F). Removal of ATPγ-sial, use of cold ATP, performing the reaction in the presence of an ATM inhibitor, or use of an S172G MPG-GST mutant caused loss of phosphoserine on MPG (Fig. 4F). Inhibition of ATM by KU55933 (Fig. 4E) directly reduced MPG glycosylase activity (P < 0.05; Fig. 4G) in both T98G and SJG2 cells. Treatment of pediatric GBM primary cultures (pGBM462 and pGBM477) with KU55933 also significantly reduced MPG glycosylase activity (*, P < 0.05; Supplementary Fig. S6E and S6F). In summary,
Figure 4. MPG is a substrate of ATM, and phosphorylation of MPG is essential for function. A, primary amino acid sequence of MPG across species demonstrates that the phospho-(serine/threonine) (pSQ) ATM substrate residue is evolutionarily conserved. B, immunoblotting of ATM, phospho-ATM (pATM), ATR, and phospho-ATR (pATR) in adult GBM (T98G) and pediatric GBM (SJG2, KNS42, and RES259) cell lines in the presence (+) and absence (−) of temozolomide (TMZ). Cells were treated with temozolomide for 48 hours. C, immunoblots demonstrating co-IP of ATM and MPG in adult (T98G) and pediatric (SJG2) GBM cell lines. Both proteins are detected following IP of either ATM or MPG. D, immunoblots demonstrating detection of MPG following IP with a pSQ ATM/ATR substrate antibody in both adult (T98G) and pediatric (SJG2) GBM cell lines in the absence (−) but not in the presence (+) of an ATM inhibitor (ATM inb, KU55933 used at 5 μmol/L). E, denaturing IP of MPG and immunoblotting of pSQ substrate-specific antibody in the presence of an ATM inhibitor (ATM inb, KU55933 used at 5 μmol/L). F, in vitro kinase ATM substrate assay. Immunoblots for thio phosphate ester to detect phosphate incorporation into MPG from a thiol-labeled ATP analogue in the presence of ATM kinase. In vitro kinase assay was performed with wild-type MPG (MPG WT), mutant MPG (MPG S172G) in the presence (+) or absence (−) of cold ATP, ATPγS, p-nitrobenzyl mesylate (PNBM), or with an ATM inhibitor (KUS5933, 5 μmol/L), demonstrating that MPG is directly phosphorylated by ATM kinase at serine 172. G, fresh lysed whole cell lysates from E were used for the MPG molecular beacon assay to confirm that inhibition of ATM resulted in reduced MPG glycosylase activity. RFU, relative fluorescence units.
our results show that ATM and MPG are directly associated in vitro and in vivo, that phosphorylation of MPG at serine 172 is dependent on ATM, and that the loss of phospho-ATM reduces MPG glycosylase activity.

**Phosphorylation of MPG Is Required for Its Optimal Function in DNA Repair**

We next generated wild-type MPG, catalytic-dead MPG (R182A), phospho-dead MPG (S172G), and phospho-mimetic (S172D) mutant constructs (Fig. 5A). Transient transfection of these constructs in RES259 cells (tumor-removal-sensitive cells) demonstrated that wild-type MPG-transfected cells, but not MPG S172G or MPG R182A cells, significantly increased MPG glycosylase activity, increased DNA damage repair (as measured by comet tail assay) and cell viability, and significantly reduced apoptosis when compared with empty-vector controls (Fig. 5B and E). MPG S172D, a phospho-mimetic, was able to partially restore wild-type function (Fig. 5B and E). To test whether ATM-mediated MPG phosphorylation occurs in clinical pediatric GBM patient samples, we immunoprecipitated ATM/ATR substrates from 5 frozen, treatment-naive, pediatric GBM patient samples using a phospho-SQ ATM/ATR substrate antibody. In 3 of 5 cases, MPG protein was detected in ATM/ATR substrate pull-downs (Fig. 5F). Furthermore, we performed a reverse IP from these lysates in 1% SDS to specifically enrich for MPG without binding partners. We were able to immunoprecipitate MPG in all 5 clinical samples, and 3 of 5 exhibited phosphorylated MPG, as detected by the phosphorylated SQ antibody (Fig. 5G). In summary, phosphorylated MPG was detected in 60% (3 of 5) of our clinical pediatric GBM samples.

**Combined Loss of MPG and ATM Cooperates to Sensitize Pediatric GBM Cells to Temozolomide In Vivo**

To determine the effect of single or dual inhibition of ATM and MPG, we generated stable knockdowns using shRNA in SJG2 cell lines (Fig. 6A). The cells were injected into the frontal lobe of immunocompromised mice. After confirming the presence of tumors by MRI, mice were treated with temozolomide (65 mg/kg/5 days) or vehicle (1% DMSO dissolved in PBS). There were no survival differences between control (shRNA scramble), single or dual knockdowns of ATM and/or MPG (Fig. 6B), and the control PBS-treated mice. Similarly, there was no effect of temozolomide treatment on mice harboring tumor cells with normal MPG and/or ATM levels (scramble shRNA control), confirming the resistance of SJG2 cells to temozolomide in vivo (Fig. 6C). Importantly, mice treated with temozolomide harboring tumor cells with loss of MPG or ATM had significantly better survival than control mice. Further, mice harboring tumors with dual knockdown of ATM and MPG had the greatest survival when treated with temozolomide (Fig. 6C). Extended life correlated with reduced tumor cell proliferation, as measured by Ki67 staining (Fig. 6D), and knockdown of ATM and MPG was maintained at the time of sacrifice, as measured by IHC analysis (Supplementary Fig. S7A).

**Methoxyamine, an Inhibitor of BER, Sensitizes Pediatric GBM to Temozolomide In Vitro and In Vivo**

Methoxyamine is a potent inhibitor of BER and works by binding and inhibiting further repair of abasic sites generated by MPG. To complement our genetic manipulation of the BER pathway through knockdown of ATM and MPG, we hypothesized that methoxyamine could also overcame temozolomide resistance of pediatric GBM. Methoxyamine was able to sensitize pediatric (SJG2) GBM cells in vitro to temozolomide and induce apoptosis (Supplementary Fig. S7B and S7C). Knockdown of MPG, ATM, or both in SJG2 cells increased cell death when treated with temozolomide alone, but abolished the ability of methoxyamine to further sensitize cells to temozolomide compared with control siRNA-treated cells, suggesting that the effect of methoxyamine may be mediated through MPG and ATM (Supplementary Fig. S7C). Similar results were observed in adult GBM cell lines U87 and T98G (data not shown). To test the in vivo effect of methoxyamine, we implanted SJG2 pediatric GBM cells into the forebrains of immunocompromised mice. After MRI confirmation of the presence of tumors, mice were randomized into four treatment arms: vehicle, temozolomide (65 mg/kg), methoxyamine (100 mg/kg), or temozolomide + methoxyamine (65 mg/kg temozolomide + 100 mg/kg methoxyamine) for 2 weeks. Compared with vehicle-treated cells, only the mice receiving dual treatment had a significant increase in survival (approximately doubling overall survival, \( P < 0.001; \) Fig. 6E). Increased survival correlated with decreased tumor cell proliferation (\( P < 0.01; \) Fig. 6F). To complement our cell line work, we tested primary low-passage adult (G179) and pediatric (pGBM462 generated from samples in Fig. 5F) GBM patient-derived cultures established from newly resected tumors maintained in defined neural stem cell media. These cells expressed both ATM and MPG (Supplementary Fig. S7D). Methoxyamine sensitized primary adult GBM cultures to temozolomide treatment, leading to reduced cell viability (Supplementary Fig. S7E). Dual loss of ATM and MPG, but not single loss, reduced cell viability in pGBM462 (Supplementary Fig. S8A), which was further enhanced by temozolomide (Supplementary Fig. S8B). To complement our gene silencing of MPG and ATM, dual treatment of temozolomide and methoxyamine, but not vehicle or single agents, reduced pGBM462 cell viability (Supplementary Fig. S8C) and increased apoptosis (cleaved caspase activity; Fig. S8A). Surprisingly, single knockdowns of ATM or MPG or dual knockdowns did not reduce viability in normal neural fetal stem cells in the absence or presence of temozolomide (Supplementary Fig. S8D and S8E). Similarly, dual treatment of methoxyamine and temozolomide did not reduce viability (Supplementary Fig. S8F) or increase apoptosis (cleaved caspase activity; Supplementary Fig. S9A) in normal fetal neural stem cells compared with controls.

We next generated a patient-derived xenograft (PDX) model of pediatric GBM from our primary culture of pGBM462 cells as another in vivo orthotopic model to complement our in vitro work using SJG2 cells. Mice were treated with vehicle, temozolomide, methoxyamine, or dual treatment (temozolomide + methoxyamine) for 2 weeks. Mice receiving dual treatment but not vehicle or single treatments had a significant increase in survival of approximately 40%, with hematolxyn and eosin (H&E) staining, confirming that our xenograft model retained the features of GBM, including hypercellularity and an invasive edge (\( *, P < 0.01; \) Fig. 6G and H).
Figure 5. Phosphorylation of MPG is required for its optimal function in DNA repair. A, immunoblot using anti-Flag antibody demonstrating robust expression of MPG flag–tagged constructs in pediatric GBM cell line RES259. EV, empty vector; WT, wild-type; S172G, MPG mutant in which the serine at residue 172 is replaced by glycine; R182A, MPG mutant in which the arginine at residue 182 is replaced by alanine. B, quantification of molecular beacon MPG activity assay after transfection of empty vector, wild-type, or mutant MPG constructs (R182A, S172G, or S172D) in the presence of temozolomide (TMZ; 100 μmol/L). RFU, relative fluorescence units. C, quantification of comet tail assay (A) after transfection of empty vector, wild-type, or mutant MPG constructs (R182A or S172G) in the presence (+) or absence (−) of temozolomide (100 μmol/L). D, cell viability assay after transfection of wild-type or mutant MPG constructs in the presence or absence of temozolomide at 48 hours. E, activated cleaved caspase-3/7 assay after transfection of wild-type or mutant MPG constructs in the presence of temozolomide (100 μmol/L). F, IP of ATM/ATR pSQ substrates in 5 frozen pGBM operative samples. Immunoprecipitates were additionally probed for MPG and detected in 3 of 5 samples. IgG was used as a negative control. G, IP of MPG is 1% SDS followed by Western blotting of pSQ from the 5 samples used in F to demonstrate that MPG is phosphorylated at the pSQ residue in clinical samples. *, P < 0.05; **, P < 0.01; and ***, P < 0.001. All experiments were performed in triplicate with mean and SEM reported where appropriate.
Figure 6. Combined loss of MPG and ATM sensitizes pGBM cells to temozolomide (TMZ) in vivo. A, immunoblotting of SJG2 cells demonstrating effective MPG, ATM, or dual knockdown. B, the Kaplan–Meier survival curve analysis of intracranially injected SJG2 cells expressing control shRNA, MPG shRNA, ATM shRNA, or dual knockdown into NOD/SCID mice. C, the Kaplan–Meier survival curve analysis of intracranially injected SJG2 cells expressing control shRNA, MPG shRNA, ATM shRNA, or dual knockdown into NOD/SCID mice treated with temozolomide (65 mg/kg/5 days). D, quantification of Ki67 staining in mice (n = 3 mice per group) from C. E, the Kaplan–Meier survival curve analysis of intracranially injected SJG2 cells into NOD/SCID mice treated with vehicle, temozolomide (TMZ; 65 mg/kg/5 days), methoxyamine (MA; 100 mg/kg/5 days), or both temozolomide + methoxyamine. F, quantification of Ki67 staining in mice (n = 3 mice per group) from E. G, the Kaplan–Meier survival curve analysis of an orthotopic PDX mouse model from pGBM462 cells. A total of 20 mice were injected with 5 per each treatment arm: vehicle, temozolomide (65 mg/kg/2 weeks), methoxyamine (100 mg/kg/2 weeks), or both temozolomide + methoxyamine. H, H&E staining of represented tumors mice from each treatment arm confirmed high-grade glioma/GBM morphology. Scale bar, large insets at 500 μm and small magnified insets at 25 μm. N, normal mouse brain; T, xenograft tumor growth. E and G, mice were treated upon confirmation of tumor by 12-MRI. *P < 0.05; **P < 0.01.
Functional Redundancy in DNA Repair Pathways Protects Normal Cells from Damage When Targeting the ATM–MPG Axis

In both our siRNA screen and subsequent functional work, we observed that normal cells, including glial cells and neural stem cells, were resistant to depletion of the ATM–MPG axis. We hypothesized that normal central nervous system (CNS) cells have several intact DNA repair pathways, and therefore functional redundancy protects them, whereas cancer cells by definition have alterations, mutations, and deletions in their DNA repair machinery, making them susceptible. To test this, we generated MPG stable knockdown in normal human astrocytes by shRNA and knocked down ATM by transient siRNA (Fig. 7A). In keeping with our siRNA knockdown data (Fig. 1D), we did not observe any significant decrease in viability in either single or dual knockdown cells, compared with controls, when treated with temozolomide (Fig. 7B). Similarly, cell viability of normal human astrocytes was not affected by treatment with temozolomide and methoxyamine compared with controls (Supplementary Fig. S9B).

To address the issue of functional redundancy, we silenced additional genes redundant to ATM or MPG by siRNA to generate triple knockdowns. Loss of proteins redundant to ATM, namely, ATR/p53 and CHEK2, in addition to ATM and MPG, significantly reduced normal human astrocyte cell viability in the presence of temozolomide (Fig. 7C, column 4 of heatmap). Furthermore, loss of additional proteins in the BER pathway downstream of MPG, namely, APEX1/PARP1/XRCC1 or MGMT (which repairs other DNA lesions induced by temozolomide), significantly reduced normal human astrocyte cell viability when combined with ATM and MPG loss in the presence, but not the absence, of temozolomide (Fig. 7C, column 4). Single loss of ATR, PARP1, CHEK2, XRCC1, MGMT, and APEX1 did not result in reduced viability when treated with or without temozolomide (Fig. 7C, columns 1–2). Interestingly, loss of ATR/CHEK2/p53, but not BRCA1, in combination with temozolomide and methoxyamine significantly reduced the cell viability of normal astrocytes (Supplementary Fig. S9C). In addition, loss of APEX1, PARP1, and XRCC1 downstream of MPG also reduced cell viability when combined with temozolomide and methoxyamine compared with controls (Supplementary Fig. S9C). Finally, loss of MGMT, which repairs cytotoxic lesions distinct from those repaired by MPG, also reduced cell viability when combined with temozolomide and methoxyamine (Supplementary Fig. S9C). In summary, normal human astrocytes have significantly redundant DNA repair pathways protecting them from alkylating agent–induced cell death.

DISCUSSION

Pediatric GBM is a devastating disease with poor survival. Our current approaches have thus far failed to effectively treat these tumors. This may be due, in part, to a flaw in our therapeutic strategies. Many pediatric GBM treatments are based on adult data, and it is now clear that pediatric and adult GBMs are distinct entities (12, 26). Pediatric GBM, in most cases, fails to respond to alkylating agent therapy, leading us to hypothesize that they may express pathways that mediate this resistance. Finding effective sensitizers to temozolomide would allow for more effective treatment and use of less-toxic doses (27). Furthermore, using monotherapies to treat pediatric GBM may be futile, and multimodality therapies, which show synergism and low toxicity, would be advantageous. Here, using a siRNA-screening approach, we identified multiple members of the BER pathway mediating alkylating agent resistance in pediatric GBM. The average plasma concentration of temozolomide in adult patients with GBM is approximately 72 μmol/L and is thought to be similar in pediatric patients (28, 29). We performed our initial screen at 100 μmol/L temozolomide; thus, it is possible that we may have not identified the full spectrum of mediators of temozolomide resistance (19). Nevertheless, when tested with 25 μmol/L temozolomide, a clinically achievable, low dose of temozolomide, inhibition of the BER pathway continued to sensitize pediatric GBM to temozolomide therapy, and the combination of BER inhibition with temozolomide led to increased survival of mice bearing pediatric GBM tumors.

Modulation of the MPG-mediated BER pathway has been shown to promote both sensitivity and resistance to alkylating agents, demonstrating how tissue specificity and cancer type must be carefully evaluated before targeting the BER pathway (30–34). We observed that modulation of MPG, the first step of temozolomide-induced BER, can promote sensitivity to temozolomide when lost, or resistance when reexpressed in pediatric GBM. A similar finding has been seen in adult GBM in cases where MGMT, a common mechanism of temozolomide resistance in these tumors, could not explain resistance to temozolomide in vitro and in vivo (30, 35). However, forced overexpression of MPG in pediatric GBM cells already expressing MPG may be toxic, as it may lead to accumulation of BER toxic intermediates that downstream BER enzymes cannot quickly resolve (31, 36).

The crystal structure of MPG has been identified, but thus far there are no effective small-molecule inhibitors of this glycosylase (17). MPG contains more disordered regions in the active site that are needed for substrate specificity; thus, it may be difficult to develop structure-based rational drug design targeting MPG (37, 38). However, if MPG cannot be targeted directly, the BER pathway may be therapeutically targeted through use of methoxyamine, a small-molecule inhibitor that binds to 3′ hydroxyl groups that are left behind by MPG following excision of the damaged base and thus inhibits BER activity (5, 39, 40). Of great interest is that loss of MPG or other BER pathway members in normal astrocytes and neural stem cells did not promote cytotoxicity. This suggests that targeting BER should have limited toxicity to normal brain. We did not directly test whether targeting BER may be toxic to non-CNS tissues, although mice did not suffer systemic side effects during their treatment in this study. Redundant and proficient DNA repair pathways in normal cells may limit their toxicity while allowing targeting of tumor cells. In accordance with this, we observed that loss of additional DNA repair proteins in normal human astrocytes, including those in the ATM–p53–CHEK2 pathway or the BER pathway (APEX1, PARP1, XRCC1), sensitized these cells to temozolomide and led to reduced cell viability when MPG and ATM were also targeted either by siRNA knockdown or by treatment with methoxyamine. Similar screening strategies were used to identify PARP inhibitors.
Figure 7. Model of ATM-MPG-mediated therapeutic resistance in pGBM. A, Western blot analysis demonstrating MPG knockdown by retroviral shRNA transduction and ATM knockdown by siRNA at 72 hours. Control cells were transduced with shRNA control retrovirus and treated with siRNA scramble controls. B, control or double MPG and ATM knockdown astrocytes were treated with 100 μmol/L temozolomide (TMZ) and assessed for viability at 72 hours. B, nonsignificance. C, control or double MPG and ATM knockdown astrocytes were targeted by siRNA for DNA damage response genes redundant to either ATM or MPG and treated with 100 μmol/L temozolomide and assessed for viability at 72 hours. All experiments were performed in triplicate with the SEM reported. D, summary model of targeting the BER and ATM pathways for sensitization to alkylating agents. MA, methoxyamine.
as effective therapeutics in several cancers where tumor cells with BRCA1/2 mutations were sensitive to PARP inhibition but normal tissue was not (41, 42). Of interest, pediatric GBMs frequently harbor mutations in DNA repair proteins, p53 and ATRX (11, 13), suggesting that targeting of the ATM–MPG axis is of therapeutic relevance. However, it should be noted that patients with underlying germline conditions in which DNA repair proteins are mutated, such as Li-Fraumeni (mutations in p53) or Seckel syndrome (mutations in ATR), may not benefit from targeting MPG and ATM as it may result in severe toxicity to normal tissues or even secondary malignancies.

Cellular responses to DNA damage are mediated by a number of master protein kinases, and at the core of these signaling pathways are ATM and ATR. Constitutively active ATM may arise through persistent double-stranded breaks or oxidative stress observed in many cancers, including pediatric GBM, and can initiate cross-talk between DNA repair pathways (43–46). Interestingly, serine 172, the target of ATM-mediated MPG phosphorylation, is in between tyrosine 162 and arginine 182, both critical amino acids for MPG substrate specificity and catalytic domain function (17). Phosphorylation of serine 172 may be important for maintaining the domain structure or function of MPG-mediated base excision. Tumor cells may become dependent on this pathway for survival in the face of cellular stress, making it a prime therapeutic target. Loss or inhibition of ATM is known to sensitize tumor cells to temozolomide, but the mechanism is unknown. Our results suggest that ATM inhibitors, at least in part, may lead to temozolomide sensitization through BER pathway inhibition. ATM-mediated phosphorylation of MPG and its associated increased activity would be blocked by ATM inhibitors, leading to reduced BER. Combination with downstream BER inhibitors, such as methoxyamine, leads to even greater temozolomide sensitivity in pediatric GBM (Fig. 7D) and potentially other cancers showing alkylating agent resistance (47). In addition to identifying a novel function for ATM, our study demonstrates the role of BER in pediatric GBM resistance to alkylating agents and a direct targetable pathway that may lead to more effective treatments.

METHODS

Human Samples and Study Approval

Pediatric GBM biopsy samples and matching clinical data were obtained from the Hospital for Sick Children following approval by the Institutional Research Ethics Board (48). All samples were deidentified before analysis. Thirty-five of 41 patients were given chemotherapy with 16 of 35 patients having received temozolomide. Additional GBM tissue microarrays were obtained from the London Cancer Institute (Dr. Chris Jones) with a material transfer agreement.
Methods.

M. Remke, Y. Chornenkyy, M.S. Barszczyk, M.D. Taylor, C. Jones, M. Remke was funded by the Dr. Mildred Scheel foundation Research grants (CIHR MOP 102513 and 115004, to C. Hawkins).

Grant Support
obtained by a materials transfer agreement. School of Medicine). Both the MPG-GST and ATM constructs were thank Dr. Michael Kastan for the ATM construct (Duke University generous gift from Dr. Mitsuyoshi Nakao (Institute of Molecular and its affiliated funding agencies. The MPG-GST construct was a for Cancer Research for input and evaluation of the article. For MRI

Acknowledgments

Please refer to Supplementary Data for complete details of Methods.

Disclosure of Potential Conflicts of Interest

R.W. Solob is a consultant/advisory board member for Treven, Inc. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: S. Agnihotri, G. Zadeh, C. Hawkins Development of methodology: S. Agnihotri, I.D. Clarke, R.W. Solob Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Agnihotri, K. Burrell, Y. Chornenky, N.A. Fernandez, I.D. Clarke, M.S. Barszczcyk, C. Ternamian, R. Head, N. Sabha, J.T. Rutka, C. Jones, P.B. Dirks, C. Hawkins

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Agnihotri, P. Buckzowicz, M. Remke, Y. Chornenky, M.S. Barszczyc, M.D. Taylor, C. Jones, G. Zadeh, C. Hawkins

Writing, review, and/or revision of the manuscript: S. Agnihotri, K. Burrell, P. Buckzowicz, M. Remke, Y. Chornenky, J.T. Rutka, G. Zadeh, C. Hawkins

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Agnihotri, K. Burrell, P. Buckzowicz, B. Golbourn, A. Gajadhar, S. Pajovic, C. Hawkins

Study supervision: S. Agnihotri, C. Hawkins

Acknowledgments

The authors thank Dr. Geoff Margison at the Paterson Institute for Cancer Research for input and evaluation of the article. For MRI images, they thank Dr. Warren Foltz and the Spatio-Temporal Targeting and Amplification of Radiation Response (STTARR) program and its affiliated funding agencies. The MPG-GST construct was a generous gift from Dr. Mitsuyoshi Nakao (Institute of Molecular Embryology and Genetics Kumamoto University). The authors also thank Dr. Michael Kastan for the ATM construct (Duke University School of Medicine). Both the MPG-GST and ATM constructs were obtained by a materials transfer agreement.

Grant Support

This study was funded by Canadian Institutes of Health Research grants (CIHR MOP 102513 and 115004, to C. Hawkins). R.W. Solob was supported by a grant from the NIH (CA148629). M. Remke was funded by the Dr. Mildred Scheel foundation (German Cancer Aid).

Statistical Analysis

All experiments were performed in triplicate with mean and SEM reported where appropriate. ANOVA was conducted for multigroup comparisons followed by a post hoc Dunnett test (groups compared with one control group) or a post hoc Tukey test (to identify differences among subgroups). Where appropriate, direct comparisons were conducted using an unpaired two-tailed Student t test (*, P < 0.05; **, P < 0.01; and ***, P < 0.001).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 14, 2014; revised July 31, 2014; accepted August 1, 2014; published OnlineFirst August 6, 2014.

REFERENCES

17. Lau AY, Scharrer OD, Samson L, Verdine GL, Ellenberger T. Crystal structure of a human alkylbase-DNA repair enzyme complexed to...
ATM Regulates 3-Methylpurine-DNA Glycosylase and Promotes Therapeutic Resistance to Alkylating Agents


Cancer Discovery 2014;4:1198-1213. Published OnlineFirst August 6, 2014.

Updated version
Access the most recent version of this article at:
doi:10.1158/2159-8290.CD-14-0157

Supplementary Material
Access the most recent supplemental material at:
http://cancerdiscovery.aacrjournals.org/content/suppl/2014/08/16/2159-8290.CD-14-0157.DC2

Cited articles
This article cites 53 articles, 25 of which you can access for free at:
http://cancerdiscovery.aacrjournals.org/content/4/10/1198.full.html#ref-list-1

Citing articles
This article has been cited by 8 HighWire-hosted articles. Access the articles at:
/content/4/10/1198.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.