Manipulation of Base Excision Repair to Sensitize Ovarian Cancer Cells to Alkylating Agent Temozolomide

Melissa L. Fishel, Ying He, Martin L. Smith, and Mark R. Kelley

Abstract

Purpose: To improve the treatment of women with ovarian cancer, we are investigating the modulation of a prominent DNA-damaging agent, temozolomide, by manipulating the DNA base excision repair (BER) pathway via BER inhibitor, methoxyamine, and overexpression of N-methylpurine DNA glycosylase (MPG).

Experimental Design: Enhancement of temozolomide via methoxyamine and MPG overexpression was analyzed using in vitro assays, including 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay, apoptosis via Annexin staining, and Western blotting for H2AX phosphorylation to quantitate DNA damage.

Results: Our data show that we can effectively modulate the activity of the chemotherapeutic agent, temozolomide, via modulator methoxyamine, in three ovarian cancer cell lines, SKOV-3, Ovcar-3, and IGROV-1. This enhancement of temozolomide-induced cytotoxicity is not dependent on p53 status as we transfected an ovarian cancer cell line with a dominant-negative p53-expressing plasmid (IGROV-1mp53) and obtained similar results. Our results show that MPG overexpressing IGROV-1 and IGROV-1mp53 cells are significantly more sensitive to the clinical chemotherapeutic temozolomide in combination with methoxyamine as assayed by cytotoxicity, apoptosis, and levels of DNA damage than either agent alone.

Conclusions: These studies show that although clinical trials in ovarian cancer to determine temozolomide single-agent efficacy are in development, through manipulation of the BER pathway, an increase in response to temozolomide is achieved. The combination of temozolomide plus methoxyamine has potential for second-line therapy for patients who have failed standard platinum plus paclitaxel chemotherapy.

A new and emerging concept designed to sensitize cancer cells to DNA-damaging agents (i.e., chemotherapy and/or radiation) is inhibition of various proteins in the DNA repair pathways (1). We are focusing on inhibition and manipulation of the DNA base excision repair (BER) pathway in ovarian cancer. BER is responsible for the repair of deaminated cytidines and adenines, bases that are oxidatively damaged, and bases that have been alkylated either from endogenous or exogenous alkylating agents, including some chemotherapeutic agents. Repair via BER requires several enzymes to complete the reversion of damaged DNA back to normal DNA. To begin the repair process of alkylation and oxidative lesions, the BER pathway uses two kinds of glycosylases: class I (complex) and class II (simple; ref. 2). Simple glycosylases, including N-methylpurine DNA glycosylase (MPG), remove the alkylated DNA base, creating an apurinic/apyrimidinic (AP) site but not nicking the DNA backbone. Imbalance of the BER pathway via overexpression of MPG is detrimental to breast cancer cells following treatment with chemotherapeutic agents (3, 4). Following the glycosylase reaction, AP endonuclease/redox effector factor-1 (Ape1/Ref-1) cleaves 5’ of the AP site (5). The AP site (5’ phosphoribosyl) that results from Ape1/Ref-1 repair activity is subsequently removed by deoxyribosephosphate hydrolase activity provided predominantly by DNA β-polymerase, which is followed by insertion of a new base by β-polymerase and ligation by DNA ligase I (Fig. 1).

Methoxyamine is an alkoxyamine derivative able to block the single nucleotide BER pathway by reacting with the aldehyde group in the acyclic sugar left in the DNA abasic site following the glycosylase-driven removal of a damaged nucleotide (6, 7). The methoxyamine-adducted AP site is a stable intermediate, refractory to the lyase activity of AP endonuclease (Ape1/Ref-1) cleavage and to polymerase β, downstream members of the BER pathway (8, 9). Cytotoxicity of methyl methanesulfonate and temozolomide were potentiated by methoxyamine in breast cancer cells (4). The mechanism of cytotoxicity enhancement is through an interruption in BER, which sensitizes cells to methylation by stabilizing the cytotoxic AP site intermediate (Fig. 1; refs. 10, 13). Incompletely repaired

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methoxyamine-blocked AP sites lead to increases in single-strand breaks and double-strand breaks (DSB) as well as cell death (4, 11). Chemical inhibition of BER by methoxyamine is a valid pharmacologic strategy to enhance the cytotoxicity of a methylating chemotherapeutic agent, such as temozolomide, or to overcome temozolomide drug-resistance (10, 11, 13).

Using ovarian cancer cell lines, IGROV-1, NIH-Ovcar-3, and SKOV-3x, we investigated whether blocking the DNA BER pathway using small-molecule, methoxyamine enhanced cellular sensitivity to temozolomide. Temozolomide is an alkylating agent that creates DNA lesions that are repaired by the BER pathway (14). Once administered, temozolomide is non-enzymatically hydrolyzed in solution to the active compound 3-methyl-(triazen-1-yl)imidazole-4-carboxamide (MTIC). Activated 3-MTIC methylates DNA primarily at the N^7 and O^6 positions of guanine and the N^3 of adenine (70%, 5%, and 9%, respectively; ref. 14; reviewed in ref. 15). In colon and breast cancer cells, temozolomide-induced cytotoxicity can be increased by blocking the ability of BER to repair these lesions (4, 10, 13). Lesions will then accumulate causing strand breaks and eventually apoptosis (11). Although temozolomide is not currently used to treat ovarian cancer, the standard regimen of paclitaxel and carboplatin treatment is ineffective for an ovarian cancer patient with platinum- or paclitaxel-resistant recurrent disease. Despite therapeutic advancements, the majority of women with advanced epithelial ovarian cancer will ultimately recur and die from malignant disease (16). Furthermore, in breast cancer cells, overexpression of BER glycosylase, MPG, sensitized cells to various alkylating agents, including temozolomide (3, 4). This is not entirely surprising based on the lesions on DNA following temozolomide treatment, N^7-methylguanine and N^3-methyladenine, both of which are recognized and removed by MPG (17). We show that overexpression of MPG in addition to inhibition of BER pathway via methoxyamine dramatically increases the sensitivity of ovarian cancer cells, the percentage of cells undergoing apoptosis, and the amount of DNA DSB following temozolomide treatment. This enhancement of temozolomide-induced cytotoxicity is independent of p53 status in ovarian cancer cells as we used several lines with varying p53 status [IGROV-1, wild-type (WT) p53; IGROV-1mp53, dominant-negative expressing p53; SKOV-3x, p53 null; and NIH-Ovcar-3: mutant p53; refs. 18, 19].

Materials and Methods

Cell lines. Ovarian cancer cell line, IGROV-1, which expresses WT p53, was stably transfected with a dominant-negative p53 mutant-containing plasmid, pcDNA3.1-p53 V143A (20). The cell line expressing dominant-negative p53 is referred to as IGROV-1mp53 in this article. The p53 mutant plasmid has been used extensively to construct
matched isogenic pairs of cell lines with WT or mutant p53 (20–24) and was used to generate an isogenic matched pair of IGROV-1 lines in the present study. SKOV-3x cells were kindly provided by Dr. Robert Bigsby (Department of Obstetrics and Gynecology, Indiana University School of Medicine, Indianapolis, IN), and NIH-Ovcar-3 cells were purchased from American Type Culture Collection (Manassas, VA). All cell lines were maintained at 37°C and 5% CO₂. IGROV-1 cells were grown in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (HyClone; Logan, UT), and SKOV-3x cells were grown in McCoy’s 5a medium (Invitrogen) supplemented with 5% fetal calf serum and sodium pyruvate (final concentration of 1 mmol/L). NIH-Ovcar-3 cells were grown in RPMI 1640 supplemented with 20% fetal bovine serum (HyClone), sodium pyruvate (final concentration of 1 mmol/L), and 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, 5 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1 00 L RIPA assay buffer containing phosphatase and protease inhibitors [150 mmol/L NaCl, 10 mmol/L Tris (pH 7.2), 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, 5 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 100 μmol/L sodium orthovanadate] (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA). Protein was quantified and electrophoresed (20 μg) in SDS gel-loading buffer on a 12% SDS-polyacrylamide gel. Mouse monoclonal antibody D0-1 (1:1,000; Vector Laboratories, Burlingame, CA) recognizes both WT and mutant p53 protein and was used to confirm overexpression of the dominant-negative mutant p53CA. Western blotting for p53. Exponentially growing IGROV-1 and IGROV-1 cells transfected with mutant p53-containing plasmid, pcDNA3.1-p53 V143A, were harvested, washed in cold PBS, and lysed in −100 μL RIPA assay buffer containing phosphatase and protease inhibitors [150 mmol/L NaCl, 10 mmol/L Tris (pH 7.2), 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, 5 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 100 μmol/L sodium orthovanadate] (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA). Mouse monoclonal anti-p53 antibody D0-1 (1:1,000; Vector Laboratories, Burlingame, CA) recognizes both WT and mutant p53 protein and was used to confirm overexpression of the dominant-negative mutant p53.

DNA damage analysis via Western blotting for H2AX phosphorylation. As an indicator of DNA DSBs, we measured the phosphorylation of histone H2AX at Ser139 (27) with a phosphorylation-specific H2AX antibody from Upstate Cell Signaling Solutions (Waltham, MD). Ovarian cancer cells were treated with temozolomide plus methoxyamine or temozolomide alone for 1 h and then methoxyamine was added to the flask for 24 h. Temozolomide was purchased from LKT Laboratories (St. Paul, MN) and prepared fresh for each experiment by dissolving in medium and sonication. Methoxyamine was purchased from Sigma-Aldrich, dissolved in PBS (pH 7.4), and stored as a 1 mol/L stock at −20°C for 1 month.

Western blotting for p53. Exponentially growing IGROV-1 and IGROV-1 cells transfected with mutant p53-containing plasmid, pcDNA3.1-p53 V143A, were harvested, washed in cold PBS, and lysed in −100 μL RIPA assay buffer containing phosphatase and protease inhibitors [150 mmol/L NaCl, 10 mmol/L Tris (pH 7.2), 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, 5 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 100 μmol/L sodium orthovanadate] (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA). Protein was quantified and electrophoresed (20 μg) in SDS gel-loading buffer on a 12% SDS-polyacrylamide gel. Mouse monoclonal anti-p53 antibody D0-1 (1:1,000; Vector Laboratories, Burlingame, CA) recognizes both WT and mutant p53 protein and was used to confirm overexpression of the dominant-negative mutant p53CA.

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay for cell survival. Cells (2–4,000) were aliquoted into each well of a 96-well plate in triplicate and allowed to adhere overnight. Temozolomide and methoxyamine were added simultaneously based on in vitro data showing that treating with methoxyamine in conjunction with temozolomide showed the most effective enhancement of cytotoxicity as opposed to adding methoxyamine before or after temozolomide treatment (data not shown). Due to the short half-life of temozolomide (25), drugs were left on for the duration of the assay. After 24 or 72 h, 0.05 mg/mL MTS reagent was added to each well and incubated at 37°C for 4 h followed by absorbance measurement at 490 nm. The values were standardized to wells containing media alone.

Apoptotic assays via Alexa Fluor 488–conjugated Annexin V (Annexin V)/propidium iodide staining. To analyze the cells for apoptosis, cells were plated and allowed to attach overnight. Cells were treated with temozolomide plus methoxyamine or temozolomide alone for 4 h and then methoxyamine was added to the flasks for 24 h. Temozolomide was assayed 24 h following temozolomide treatment. Cells were trypsinized, pelleted, washed in ice-cold PBS, and resuspended in 1× binding buffer [10 mmol/L HEPES/NaOH (pH7.4), 140 mmol/L NaCl, 2.5 mmol/L CaCl₂]. Apoptosis was analyzed using the Alexa Fluor 488 Annexin V from Vybrant Apoptosis Assay kit in combination with propidium iodide (Molecular Probes, Eugene, OR) as described previously (4). For IGROV-1 and IGROV-1mp53 cells, which expressed MPG and green fluorescent protein, the cells were stained with 7-amino actinomycin D and Annexin-APC (Molecular Probes) as described previously (4). Cells that were strongly Annexin positive were considered positive for apoptosis. The samples were analyzed by flow cytometry in the Indiana University Cancer Center flow cytometry facility.

DNA damage analysis via Western blotting for H2AX phosphorylation. As an indicator of DNA DSBs, we measured the phosphorylation of histone H2AX at Ser139 (27) with a phosphorylation-specific H2AX antibody from Upstate Cell Signaling Solutions (Waltham, MD).

Fig. 2. Effect of methoxyamine on the cytotoxicity of temozolomide in ovarian cancer cell lines. A, MTS assay was used to determine cell survival. Both drugs were added and left on for 72 h. IGROV-1 and IGROV-1mp53 were treated with 30 mmol/L methoxyamine, and SKOV-3x and Ovcar-3 cells were treated with 20 mmol/L methoxyamine. Points, average of at least three experiments; bars, SE. #, P < 0.05; *, P < 0.01; **, P < 0.001, all compared with temozolomide alone at corresponding dose. ●, cells treated with temozolomide alone; ○, cells that had methoxyamine added to the temozolomide treatment. B, Western blot analysis of protein (20 μg) from the whole-cell lysates of IGROV-1 cells with dominant-negative p53 overexpression.

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electrophoresed in SDS gel-loading buffer on a 12% SDS-polyacrylamide gel. Mouse monoclonal anti–phospho-histone H2AX (1:1,000) or anti-actin antibody (1:1,000; as a loading control, LabVision Corp., NeoMarkers, Fremont, CA) was used to probe for protein levels as described previously (28). Bands were detected using a chemiluminescence kit from Roche Applied Biosciences (Indianapolis, IN). The bands were visualized using Bio-Rad Chemidoc XRS (Hercules, CA) and quantitated using Chemidoc software, Quantity One 4.6.1.

**Adenoviral infection.** Production of the MPG adenovirus has been described previously (4). To distinguish exogenous transgene overexpression from endogenous MPG gene levels, a hemagglutininepitope (HA) tag was added to the 5’ terminus of the MPG sequence (4). Briefly, for IGROV-1 ovarian cancer cells, 2 × 10⁶ cells were resuspended in a serum-free medium, and purified adenovirus was added to the suspension at a multiplicity of infection of 20 infectious units per cell, which was used for adenoviral empty vector, and a multiplicity of infection of 10 infectious units per cell, which was used for vector containing HA-MPG, and at a final infection volume of 100 μL. Adenoviral infections were carried out for 1.5 to 2 h at 37°C in a 5% CO₂ incubator and gently agitated every 15 min. Infection efficiencies of transduced cells were determined 24 h after infection by measuring enhanced green fluorescent protein fluorescence intensities of 10⁴ cells using a FACSCalibur flow cytometry (Becton Dickinson, San Jose, CA) and analyzing results with CellQuest software (Becton Dickinson; data not shown). Enhanced green fluorescent protein expression levels were also visualized by fluorescence microscopy using a TS100F microscope (Nikon, Tokyo, Japan) outfitted with a fluorescence system (Nikon). Western blotting was also done to show an increase in MPG expression following similar Western blot protocol as described above. The MPG gene was HA-tagged, such that a HA antibody (1:1,000; Upstate Cell Signaling Solutions) was used to detect transgene expression (4).

**Results**

*Methoxyamine enhances the cytotoxicity of temozolomide in four ovarian cancer cell lines.* The effect of methoxyamine on temozolomide-induced cytotoxicity in ovarian cancer cell lines was evaluated (Fig. 2A). Methoxyamine dramatically enhanced the cytotoxicity of all four cell lines to temozolomide, implying...
that BER is a potential therapeutic target in ovarian cancer. As shown in Fig. 2, methoxyamine enhanced the cytotoxicity of temozolomide in ovarian cancer cell lines, including IGROV-1 (1.8-fold), IGROV-1mp53 (1.6-fold), Ovcar-3 (1.7-fold), and SKOV-3x (1.5-fold). The fold increase is based on evaluation of the ED50. The most dramatic increase in temozolomide-induced cytotoxicity was observed at higher doses of temozolomide (i.e., >2 mmol/L). We speculate that this correlates with the increase in concentration of temozolomide leading to an increase in abasic sites that methoxyamine can potentially bind to. As the dose of temozolomide is increased, the damage on DNA increases, generating more abasic sites for methoxyamine to bind to and thereby blocking repair of more heavily damaged DNA. The dose of methoxyamine required to enhance temozolomide-induced cytotoxicity without causing significant toxicity alone was determined by a dose escalation of methoxyamine with a fixed concentration of temozolomide (data not shown). The p53 status of the three lines differed: SKOV-3x are null, Ovcar-3 have mutant p53, and IGROV-1 have WT (19). In Fig. 2B, we show the overexpression of dominant-negative mutant p53 protein in IGROV-1 cells (IGROV-1mp53). Methoxyamine enhanced temozolomide-induced cytotoxicity in all four lines regardless of the p53 status.

**Addition of methoxyamine to temozolomide treatment increases the amount of apoptosis and DNA damage.** The increase in temozolomide-induced cytotoxicity when methoxyamine is combined with temozolomide also correlates with an increase in apoptosis. As shown in Fig. 3, ovarian cancer cells have statistically significantly greater percentages of cells undergoing apoptosis with the combination of methoxyamine plus temozolomide than temozolomide alone.

Temozolomide-induced AP sites can lead to single-strand breaks and DSBs in the DNA, which are cytotoxic to dividing tumor cells. We observed an increase in DSBs when methoxyamine was added to the temozolomide treatment. Using a phosphorylation-specific antibody to H2AX, we quantitated the amount of DNA damage after treatment with temozolomide and temozolomide plus methoxyamine. As shown in Fig. 4, temozolomide treatment in combination with methoxyamine increases the amount of DNA damage in all four ovarian cancer cell lines, which is quantitated in Table 1. H2AX is phosphorylated in response to DSBs (29); therefore, the increase in H2AX phosphorylation (H2AX-γ) is likely due to an increase in unprocessed AP sites in DNA that in turn caused DSBs.

**Overexpression of MPG in IGROV-1 and IGROV-1mp53 ovarian cancer cells.** To investigate the effect of imbalancing BER in ovarian cancer, we used adenovirus infection to overexpress the class I glycosylase MPG in ovarian cancer cell lines, IGROV-1 and IGROV-1mp53. To achieve similar infection efficiency, a multiplicity of infection of 20 was used for adenoviral empty vector and a multiplicity of infection of 10 was used for vector containing HA-MPG, which our laboratory showed previously has MPG activity (4). This resulted in 75% to 90% of cells being green fluorescent protein positive (data not shown). Using adenoviral infection of IGROV-1 and IGROV-1mp53 cells, we showed a dramatic increase in the level of HA-tagged MPG protein (Fig. 5A).

In IGROV-1 and IGROV-1mp53 cells that overexpress MPG, an increase in sensitivity to temozolomide was observed (3.75- and 3.6-fold enhancement of ED50 in IGROV-1 and IGROV-1mp53, respectively). A similar increase in temozolomide-induced cytotoxicity was observed with MPG overexpression compared with methoxyamine treatment. However, this increase in sensitivity to temozolomide was even more dramatic when methoxyamine treatment was added to the cells that overexpress MPG (Fig. 5B). Overexpression of MPG increased the amount of cell killing in the presence of temozolomide ~4-fold and even more dramatically in the presence of methoxyamine and temozolomide, 12.5-fold enhancement of ED50.

An increase in DSBs measured via H2AX phosphorylation as described above is also observed following MPG overexpression and treatment with temozolomide (Table 1). Again, when combining MPG overexpression with methoxyamine treatment, temozolomide-induced cytotoxicity is most dramatically enhanced and correlated well with an increase in DSB and apoptosis (Figs. 4B and 6). Methoxyamine in combination with temozolomide increased DSB formation in all four lines. Addition of methoxyamine and temozolomide to IGROV-1 and IGROV-1mp53 cells expressing MPG showed approximately 62- and 49-fold increases in DSBs, respectively. These data closely parallel the cell survival data. The results of the apoptotic staining also implicated the importance of BER in the ovarian cancer cellular response to temozolomide, as addition of methoxyamine, MPG overexpression, and methoxyamine plus MPG overexpression all resulted in statistically significant increases in apoptosis.

**Table 1.** Fold enhancement of H2AX phosphorylation following temozolomide or temozolomide plus methoxyamine treatment compared with control cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>TMZ alone</th>
<th>TMZ + MX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovcar-3</td>
<td>1.0 ± 0.1</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>2.5 mmol/L TMZ</td>
<td>4.1 ± 2.7</td>
<td>19.0 ± 8.2*</td>
</tr>
<tr>
<td>5 mmol/L TMZ</td>
<td>31.1 ± 1.2</td>
<td>52.5 ± 11.9*</td>
</tr>
<tr>
<td>SKOV-3x</td>
<td>4.3 ± 4.7</td>
<td>7.8 ± 5.6</td>
</tr>
<tr>
<td>2.5 mmol/L TMZ</td>
<td>10.5 ± 5.4</td>
<td>16.4 ± 1.9</td>
</tr>
<tr>
<td>5 mmol/L TMZ</td>
<td>36.5 ± 4.9</td>
<td>39.8 ± 3.2</td>
</tr>
<tr>
<td>IGROV-1</td>
<td>1.3 ± 0.1</td>
<td>1.40 ± 0.03</td>
</tr>
<tr>
<td>5 mmol/L TMZ</td>
<td>4.1 ± 1.5</td>
<td>16.2 ± 0.5*</td>
</tr>
<tr>
<td>IGROV-1mp53</td>
<td>1.1 ± 0.3</td>
<td>7.0 ± 4.6</td>
</tr>
<tr>
<td>5 mmol/L TMZ</td>
<td>11.9 ± 1.9</td>
<td>27.1 ± 11.9</td>
</tr>
</tbody>
</table>

NOTE: The values are relative comparisons of Western blot band intensities following normalization to actin as a loading control. Ovcar-3 and SKOV-3x cells were treated with temozolomide alone or temozolomide plus methoxyamine (n = 3). IGROV-1 and IGROV-1mp53 cells were treated with temozolomide alone or temozolomide plus methoxyamine in addition to IGROV-1 and IGROV-1mp53 overexpressing MPG (n = 2).

Abbreviations: TMZ, temozolomide; MX, methoxyamine.  
*P < 0.05.  
**P < 0.01.
Discussion

Temozolomide is a new imidazole tetrazine alkylating agent with preclinical and clinical activity against a variety of tumor types and acceptable toxicity in phase I and II clinical trials. Temozolomide is available as an oral drug and is FDA approved for the treatment of adult patients with refractory anaplastic astrocytoma. Raymond et al. (30) showed that temozolomide has significant activity against several human cancer tissue specimens, including ovarian cancer. In a phase II study for the treatment of recurrent or progressive brain metastasis (including patients with endometrial or ovarian cancer), Abrey et al. (31) show that temozolomide achieved disease control (partial response plus stable disease) in 41% of patients with minimal toxicity.

Temozolomide is an alkylating agent that creates DNA lesions, including N7-methylguanine and N3-methyladenine, which are repaired by the BER pathway (14). N7-methylguanine and N3-methyladenine lesions account for ~80% of the lesions created by temozolomide. Therefore, we proposed that blockage of the DNA BER pathway with methoxyamine after treatment with alkylating agent, temozolomide, would lead to an increase in the efficacy of temozolomide treatment in platinum-resistant ovarian cancer cells. Indeed, combination therapy, including temozolomide and methoxyamine, resulted in an increase in cytotoxicity, apoptosis, and DNA damage in human ovarian cancer cells. Overexpression of MPG increased the amount of cell killing in the presence of temozolomide and even more dramatically in the presence of methoxyamine and temozolomide. In Fig. 5, virtually all cells are dead using the combination of MPG overexpression and methoxyamine treatment at concentrations of 2.5 mmol/L temozolomide and greater; however, the cells treated with temozolomide alone show a 75% survival at 2.5 mmol/L temozolomide. Although the percentage of cells that survive following temozolomide plus MPG overexpression and methoxyamine treatment does not seem to increase with increasing concentrations of temozolomide >2.5 mmol/L, we believe that this is a limitation of the MTS assay as we observed increases in the amount of H2AX phosphorylation and apoptosis as the dose of temozolomide increased. This shows the potential reduction in chemotherapeutic agent dosing and therefore peripheral toxicity that may be possible through modulation of the repair of temozolomide-induced DNA lesions. These findings indicate that imbalancing or blocking the BER pathway to correctly process AP sites may have therapeutic implications for treating ovarian cancer especially where resistance to platinum agents is encountered, as well as other cancers (e.g., colon; ref. 13). The idea of BER as a target is especially attractive in platinum-resistant ovarian cancer patients where the conventional platinum agents are ineffective. Our data also show that the effect of temozolomide cytotoxicity on ovarian
tumor cells is not dependent on p53 signaling. There is a report in a glioma cell line that p53 deficiency predicted an increase in sensitivity to temozolomide (32). Based on our data showing that p53 status did not affect the ED50 of temozolomide in the matched IGROV-1 and IGROV-1mp53 lines nor the ability of methoxyamine to enhance temozolomide-induced cytotoxicity, we speculate that p53 status and temozolomide sensitivity must be cell type specific. Furthermore, this treatment combination would be effective in ovarian cancer patients regardless of the p53 status of the patient’s tumor. Other laboratories have shown that the effect of methoxyamine on temozolomide-induced cytotoxicity seems to be independent of the mismatch repair and O6-methylguanine DNA methyltransferase status of the cell (10, 12). Blocking repair of AP sites through methoxyamine treatment is a specific and targeted approach to enhance alkylating agent therapy (4, 10, 13).

Temozolomide-induced AP sites can lead to single-strand breaks and DSBs in the DNA, which are cytotoxic to dividing tumor cells (11, 33). We observed an increase in DSBs when methoxyamine is added to the temozolomide treatment most likely due to unprocessed AP sites in DNA that eventually lead to DSBs. Adenoviral overexpression of MPG renders ovarian cancer cells (both p53 WT and mutant) sensitive to temozolomide due to an imbalance in the BER pathway. Combining MPG overexpression with methoxyamine treatment, which blocks the function of downstream proteins, Ape1/Ref-1 and β-polymerase, dramatically increases the cytotoxicity and level of DNA DSBs following temozolomide treatment in IGROV-1 cells. Levels of apoptosis as determined by Annexin staining show that combining methoxyamine with temozolomide is effective in increasing the percentage of ovarian cancer cells undergoing apoptosis (Figs. 3 and 6). The deleterious effects of MPG overexpression on the ovarian cancer cell lines is shown by the large amount of DNA damage (i.e., DSBs shown in Fig. 4). Cell survival and quantitation of DSBs after the treatment of ovarian cancer cells expressing high levels of MPG with methoxyamine and temozolomide indicate that this treatment is extremely lethal to the cell. We do not observe as dramatically elevated levels of apoptosis in MPG-overexpressing cells after methoxyamine plus temozolomide treatment as we might expect based on cell survival and DSB levels. However, the levels of apoptosis are statistically significant (\( P < 0.001 \) and \( P < 0.05 \), respectively) on comparison of methoxyamine alone versus methoxyamine treatment in addition to MPG overexpression with 2.5 mmol/L temozolomide in both IGROV-1 and IGROV-1mp53 cells, indicating that the overexpression of MPG resulted in an even more dramatic increase in sensitivity to temozolomide. At the higher dose of temozolomide (5 mmol/L), we may be observing more nonspecific effects of such high levels of alkylating agent. In addition, the pathway of inducing apoptosis in these MPG-overexpressing cells may not involve the phosphatidylserine to which Annexin binds (34); therefore, we used all three assays to ensure that we obtained a reliable snapshot of the cytotoxic effects of manipulating the BER pathway. As a future direction, we can investigate the specific pathways of apoptosis that are activated on temozolomide plus methoxyamine treatment in combination with MPG overexpression. Our data show that we can effectively modulate the activity of chemotherapeutic agent, temozolomide, via methoxyamine treatment in IGROV-1, IGROV-1mp53, Ovcar-3, and SKOV-3x ovarian cancer cells. Further manipulation of the BER pathway through overexpression of MPG also increases the ovarian cancer cells to temozolomide. These findings have clinical implications for ovarian cancer patients with cisplatin-resistant disease and further studies are under way to test the combination of methoxyamine plus temozolomide in an ovarian cancer xenograft model. These studies may directly identify a more effective way of treating ovarian cancer with chemotherapy, using a partnering of a specific chemotherapeutic agent (temozolomide) with a BER inhibitor (methoxyamine) along with imbalancing the BER pathway (MPG plus methoxyamine). Future studies may develop other specific BER inhibitors (e.g., inhibitors for Ape1/Ref-1) to enhance the treatment of patients with ovarian cancer that are failing front-line therapy. Effective modulation of chemotherapeutic agents is necessary both to improve efficacy and to attenuate toxicity to effectively treat ovarian tumors where cisplatin is not as effective.
References


Manipulation of Base Excision Repair to Sensitize Ovarian Cancer Cells to Alkylating Agent Temozolomide

Melissa L. Fishel, Ying He, Martin L. Smith, et al.


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