Sensitization of a human ovarian cancer cell line to temozolomide by simultaneous attenuation of the Bcl-2 antiapoptotic protein and DNA repair by \( O^6 \)-alkylguanine-DNA alkyltransferase

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Abstract

Temozolomide is an alkylating agent that mediates its cytotoxic effects via \( O^6 \)-methylguanine (\( O^6 \)-meG) adducts in DNA. \( O^6 \)-alkylguanine-DNA-alkyltransferase (MGMT) can repair such adducts and therefore constitutes a major resistance mechanism to the drug. MGMT activity can be attenuated in vitro and in vivo by the pseudosubstrate \( O^6 \)-(4-bromothenyl)guanine (PaTrin-2, Patrin, Lomeguatrib), which is clinical trials is in combination with temozolomide. Resistance to cytotoxic agents can also be mediated by the Bcl-2 protein, which inhibits apoptosis and is frequently up-regulated in tumor cells. Attenuation of Bcl-2 expression can be affected by treatment of cells with the antisense oligonucleotide, oblimersen sodium (Genasense), currently in phase III clinical trials in combination with the methylating agent dacarbazine. Using a human ovarian cancer cell line (A2780) that expresses both Bcl-2 and MGMT, we show that cells treated with active dose levels of either oblimersen (but not control reverse sequence or mismatch oligonucleotides) or PaTrin-2 are substantially sensitized to temozolomide. Furthermore, the exposure of oblimersen-pretreated cells to PaTrin-2 leads to an even greater sensitization of these cells to temozolomide.

Thus, growth of cells treated only with temozolomide (5 \( \mu \)g/mL) was 91% of control growth, whereas additional exposure to PaTrin-2 alone (10 \( \mu \)mol/L) or oblimersen alone (33 nmol/L) reduced this to 81% and 66%, respectively, and the combination of PaTrin-2 (10 \( \mu \)mol/L) and oblimersen (33 nmol/L) reduced growth to 25% of control. These results suggest that targeting both Bcl-2 with oblimersen and MGMT with PaTrin-2 would markedly enhance the antitumor activity of temozolomide and merits testing in clinical trials. [Mol Cancer Ther 2004;3(10):1215–20]

Introduction

Temozolomide is an alkylating agent that mediates its cytotoxic effect by forming \( O^6 \)-methylguanine (\( O^6 \)-meG) DNA adducts, which during DNA replication pair preferentially with thymidine. These \( O^6 \)-meG:T mispairs can result in a G-to-A point mutation during a subsequent round of DNA replication but are also substrates for the postreplication mismatch repair pathway, which after a further round of DNA replication leads to apoptosis (1). \( O^6 \)-meG DNA adducts can be repaired by the DNA repair protein \( O^6 \)-alkylguanine-DNA alkytransferase (MGMT), which removes adducts from the \( O^6 \) position of guanine by accepting them onto a cysteine residue within its active site. Furthermore, fibroblasts and bone marrow cells of MGMT knockout mice are significantly more sensitive to the toxic effects of temozolomide than those from MGMT wild-type mice (2). The protective role of MGMT against the cytotoxic effect of temozolomide has been shown in human cell lines (3) and human xenograft models (4).

MGMT can be inactivated by free guanine base derivatives that have alkyl groups at the \( O^6 \) position, which act as "pseudosubstrates." \( O^6 \)-benzylguanine (5) and \( O^6 \)-(4-bromothenyl)guanine (PaTrin-2, Patrin, Lomeguatrib, KuDOS, Cambridge, United Kingdom; ref. 6) have been identified as the most promising MGMT inactivators. Compared with temozolomide used as a single agent, the combination PaTrin-2-temozolomide has been shown to significantly increase tumor growth inhibition in human melanoma xenografts (7). PaTrin-2 and \( O^6 \)-benzylguanine have recently entered phase I/II clinical trials (8, 9).

The Bcl-2 family of proteins regulates the mitochondria-mediated apoptosis pathway, which includes both apoptosis-suppressing (e.g., Bcl-2) and apoptosis-inducing (e.g., Bax) proteins. Overexpression of Bcl-2 occurs frequently in human cancers (10) and is associated with
tumor cell resistance to chemotherapeutic agents by preventing the apoptotic response normally induced by those agents (11). Oblimersen sodium (Genasense, Aventis/Genta, Berkeley Heights, NJ) is a phosphorothioated antisense oligonucleotide complementary to the first six codons of the Bcl-2 mRNA sequence. Hybridization prevents translation of the mRNA by blocking ribosome binding or progression, and the hybrid molecule is recognized by RNase H, which degrades only the RNA portion, sparing the antisense oligonucleotide (12). Thus, oblimersen treatment decreases the expression of the Bcl-2 protein, an activity that has been shown in human cancer cells and tumor xenografts and in patients with hematologic or solid malignancies (13). A randomized phase III trial comparing the standard therapy with the methylation agent dacarbazine (DTIC) with DTIC plus oblimersen in patients with advanced metastatic melanoma has recently completed accrual. Furthermore, several studies are currently under way, examining oblimersen as a treatment for a variety of cancers in addition to melanoma, and it is hoped that, through the specific targeting of Bcl-2, oblimersen will avoid the undesirable side effects of conventional chemotherapy.

Given the roles of MGMT and Bcl-2 in resistance to the alkylating agent temozolomide, we firstly set out to examine if attenuation of MGMT by PaTrin-2 or of Bcl-2 by oblimersen could lead to sensitization to the cytotoxic effect of temozolomide with the human ovarian cancer cell line A2780 that expresses both MGMT and Bcl-2. We then asked if the combination of both PaTrin-2 and oblimersen could further enhance the toxicity of the alkylating agent in this cell line.

We show that attenuation of MGMT or Bcl-2 can be obtained in A2780 cells after treatment with PaTrin-2 or oblimersen, respectively. We also show that A2780 cells treated with either PaTrin-2 or oblimersen are substantially sensitized to temozolomide, whereas the control reverse sequence or mismatch oligonucleotides are ineffective. Furthermore, the incubation of oblimersen-pretreated cells with PaTrin-2 leads to an even greater sensitization of these cells to temozolomide.

**Materials and Methods**

**Cell Line**

A2780 cells (kindly provided by Dr. Robert Brown, Beatson Laboratories, Glasgow, United Kingdom) were cultured in T75 flasks (catalogue no. 353135, Falcon, Becton Dickinson, Franklin Lakes, NJ) containing 10 mL RPMI 1640 (catalogue no. E105-039, PAA, Pasching, Austria) supplemented with 10% FCS (catalogue no. AI5-043, PAA, Pasching, Austria) and 200 mmol/L L-glutamine (catalogue no. G-5763, Sigma Chemical Co., St. Louis, MO). A2780 cells were screened on a weekly basis throughout the experiment for *Mycoplasma* by PCR (VenorGeM PCR-Mycoplasma Detection Kit, protocol detailed in the user manual, pp. 9–12) and always proven to be contamination free.

**Oligonucleotides**

The following fully phosphorothioated oligonucleotides were provided by Genta: oblimersen sodium (5′-TCTCCCAGCGTGCAGCAT-3′), G3622 reversed sequence control (5′-TACCGCGTGCAGCAT-3′), and G4126 2-bp mismatched control (5′-TCTCCCAGCATGTGCAGCAT-3′).

**MGMT Assay**

The preparation of cell extracts for MGMT assay, the MGMT assay itself, and protein estimation were performed following the protocols described by Watson and Margison (14). Briefly, cell-free sonicates were incubated with 3H-methylated DNA substrate under protein-limiting conditions until the transfer reaction was complete. Excess substrate DNA was hydrolyzed to acid solubility, and radioactivity in the residual protein was measured by liquid scintillation counting.

**Inhibition of MGMT Activity by PaTrin-2**

The ability of PaTrin-2 to deplete MGMT activity in A2780 cells was determined as follows: on day 1, an equal number (2 × 10⁶) of A2780 cells was seeded in a series of T75 flasks containing 10 mL RPMI 1640 supplemented with 10% FCS, 200 mmol/L L-glutamine, and 10 μmol/L PaTrin-2. After 2, 24, 48, 72, 96, and 120 hours, cells were rinsed twice with 10 mL EDTA (0.54 mmol/L, catalogue no. 100935V, BDH, Glasgow, United Kingdom), trypsinized [2 mL trypsin-EDTA solution (catalogue no. T3924, Sigma Chemical) per flask for 2 minutes at 37°C], and removed from the culture flasks on dilution with 10 volumes of medium. The resulting cell suspension was centrifuged at 150 × g for 5 minutes. The pellet was then washed once in culture medium to remove excess trypsin, recentrifuged, and processed (as described above) for MGMT assay.

On day 5, the medium was removed and replaced with medium that did not contain PaTrin-2. Cells were harvested for MGMT assay on days 6 to 10.

**Effect of Oblimersen on Bcl-2 Expression in A2780 Cells**

An equal number (0.5 × 10⁶) of A2780 cells was seeded in T75 flasks containing 10 mL RPMI 1640 supplemented with 10% FCS and 200 mmol/L L-glutamine on day 1. On day 2, cells were rinsed twice with 10 mL 0.54 mmol/L EDTA and the medium was changed to one containing 17 or 33 nmol/L oblimersen and 15 or 30 μmol/L jetPEI for oligonucleotides transfection reagent (catalogue no. GDSP10130, Qbiogene, Carlsbad, CA), respectively, or the transfection reagent alone as negative control. This was repeated on days 3 to 6 (following the clinical trial protocol for the phase III melanoma study). On day 7, the culture medium was removed and cell pellets were obtained as described above. Cells were fixed by the addition of 70% ethanol and left overnight at room temperature. Ethanol was then removed, the samples were embedded in paraffin wax, and 4 μm sections were cut and mounted on slides. Fixed cells were immunostained for Bcl-2 using an immunoperoxidase procedure as described previously (15): monoclonal antibodies against human Bcl-2 (sc-7382, Santa Cruz Biotechnology, Santa Cruz, CA) were used as the primary...
antibody. The secondary antibody was a biotinylated rabbit anti-mouse (catalogue no. E0413, DAKO, Carpinteria, CA), which was then followed by streptavidin–horseradish peroxidase incubation (StreptAWComplex/HRP, catalogue no. K0337, DAKO). The samples were counterstained with hematoxylin, dehydrated, mounted, and examined using optical microscopy.

**Effect of Oblimersen, Reversed Sequence, and 2-bp Mismatch Control Oligonucleotides on Sensitization of A2780 Cells to Temozolomide**

An equal number ($0.5 \times 10^6$) of A2780 cells was seeded in 75 flasks containing 10 mL RPMI 1640 supplemented with 10% FCS and 200 mmol/L L-glutamine on day 1. On day 2, cells were rinsed twice with 10 mL 0.54 mmol/L EDTA and the medium was changed to one containing 33 nmol/L oblimersen or 33 nmol/L reverse sequence control oligonucleotide or 33 nmol/L L 2-bp mismatch control oligonucleotide and 30 μmol/L jetPEI transfection reagent or the transfection reagent alone as negative control. This was repeated on days 3 to 6. On day 7, an equal number (250) of vehicle-treated or oligonucleotide-treated A2780 cells was seeded in 96-well plates (catalogue no. 353072, Falcon) with 100 μL medium. On day 8, 100 μL medium containing temozolomide at increasing concentrations (0–30 μmol/L DMSO, catalogue no. D2650, Sigma Chemical) was added to each well. On day 14, the cells were processed as above.

**Cell Growth Assays**

**MTT Assay.** Cells were seeded in T75 flasks and treated with oblimersen or jetPEI transfection reagent or the transfection reagent alone as described above. On day 7, an equal number (250) of vehicle-treated or oblimersen-treated (17 or 33 nmol/L) cells were seeded in 96-well plates, with 100 μL medium containing 10 μmol/L PaTrin-2 or the equivalent concentration of the vehicle (0.05% DMSO) as negative control. On day 8, 100 μL medium containing temozolomide at increasing concentrations (0–10 μg/mL DMSO) was added to each well. On day 14, the cells were processed as above.

**Clonogenic Assay.** The clonogenic assay was performed following the protocol described by Plumb (16). Cells were seeded in T75 flasks and treated with oblimersen and jetPEI transfection reagent or the transfection reagent alone as described above. On day 7, an equal number ($8 \times 10^3$) of vehicle-treated or oblimersen-treated (17 or 33 nmol/L) cells was seeded in T25 flasks (catalogue no. 353014, Falcon), with 5 mL medium containing 10 μmol/L PaTrin-2 or the equivalent concentration of the vehicle as negative control. On day 9, the medium was replaced by one containing 10 μmol/L PaTrin-2 or the equivalent concentration of the vehicle and temozolomide at increasing concentrations (0–10 μg/mL DMSO for the oblimersen-treated cells and 0–100 μg/mL DMSO for the vehicle-treated cells). On day 11, cells were rinsed twice with 10 mL 0.54 mmol/L EDTA, harvested, and counted. An equal number ($10^3$) of cells was seeded in Petri dishes (catalogue no. 353004, Falcon, three dishes per experiment, i.e., 90 dishes in total) with 5 mL medium (no drug addition). On day 18, the medium was removed and 5 mL gentian violet [10% w/v methyl violet 2B (Sigma-Aldrich Co. Ltd., Poole, United Kingdom) in 70% ethanol (Prolabo, Manchester, United Kingdom)] was added to each dish for 5 minutes (cell staining). The dye was then poured off; the plates were rinsed with tap water, inverted, and allowed to dry; and colonies were counted.

**Statistical Analysis**

In each experiment and for each concentration of temozolomide, a series of nine absorbance data measurements (MTT assay) or three cell colony counts (clonogenic assay) was obtained. The mean was taken and expressed as a percentage of the appropriate control. Each set of experimental data was compared using Student’s t test.

**Results**

**Effect of PaTrin-2 on MGMT Activity in A2780 Cells**

The MGMT activity of A2780 cells was $447.2 \pm 45.3$ fmol/mg protein. The MGMT activity became undetectable 2 hours after the addition of PaTrin-2 and remained undetectable for a total of 7 days. When the medium was replaced with medium not containing PaTrin-2, the MGMT activity remained undetectable for 48 hours. Five days after medium replacement, the MGMT activity of the cells had recovered but only to 53% of its initial value (Fig. 1).

**Effect of Oblimersen on Bcl-2 Expression in A2780 Cells**

Immunocytochemical analysis of untreated cells showed extensive expression of Bcl-2 in the majority of the A2780 cells. As expected, expression was predominantly cytoplasmic. After 5 days of 17 or 33 nmol/L oblimersen treatment, expression of Bcl-2 in the cells was extensively decreased,
the effect being greater with the higher dose (Fig. 2). In these experiments, residual faint cytoplasmic staining could be seen, but in the majority of the cells, no staining was evident.

**Effect of Oblimersen and Control Oligonucleotides on Sensitization of A2780 Cells to Temozolomide**

A2780 cells were significantly more sensitive ($P = 0.003$) to the growth inhibition effect of increasing concentrations of temozolomide after 5 days of treatment with 33 nmol/L oblimersen compared with the reverse or mismatch control oligonucleotides. The sensitivity of A2780 cells to temozolomide after treatment with the reverse or mismatch control oligonucleotides was not significantly different from the vehicle control ($P = 0.9$; Fig. 3; Table 1).

**Effect of Combined Oblimersen and PaTrin-2 on Sensitization of A2780 Cells to Temozolomide**

PaTrin-2-treated or oblimersen-treated A2780 cells were significantly more sensitive ($P = 0.02$) to the cytotoxic effect of temozolomide than the control cells (Figs. 4 and 5; Table 1). The combination of oblimersen and PaTrin-2 further enhanced the sensitivity of A2780 cells to temozolomide ($P = 0.01$; Figs. 4 and 5; Table 1). It is noteworthy that PaTrin-2 given alone did not affect cell survival unlike 17 or 33 nmol/L oblimersen given alone, which reduced cell survival to 66.4 ± 1.5% or 40.8 ± 1.7%, respectively, compared with controls.

**Discussion**

Our hypothesis was that simultaneous MGMT and Bcl-2 attenuation would increase temozolomide sensitivity in the ovarian cancer cell line A2780. To test this, we firstly showed that PaTrin-2 would inactivate MGMT and determined that the level of inactivation would remain adequate during the experiment. Having achieved this, we then showed, as anticipated, that MGMT inactivation further enhanced the sensitivity of A2780 cells to temozolomide ($P = 0.01$; Figs. 4 and 5; Table 1). It is noteworthy that PaTrin-2 given alone did not affect cell survival unlike 17 or 33 nmol/L oblimersen given alone, which reduced cell survival to 66.4 ± 1.5% or 40.8 ± 1.7%, respectively, compared with controls.

**Table 1. Effect of PaTrin-2, oblimersen, reverse, and mismatch oligonucleotides on sensitization of A2780 cells to temozolomide (5 μg/mL)**

<table>
<thead>
<tr>
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<th>MTT-Based Assay</th>
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<td>Cell Survival</td>
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<td>(% of Control)</td>
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<tr>
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<tr>
<td>+ 10 μmol/L PaTrin-2</td>
<td>25.1</td>
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<tr>
<td>33 nmol/L oblimersen</td>
<td>66.3</td>
<td>64.7</td>
</tr>
<tr>
<td>+ 10 μmol/L PaTrin-2</td>
<td>25.1</td>
<td>24.5</td>
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**NOTE:** Values were obtained from the MTT-based and clonogenic assays shown in Figs. 3 to 5. Cell survival is expressed as percentage of control. The lowest dose of temozolomide in those experimental conditions was 10 μg/mL. ND, not determined. E, extrapolated values.
increases the sensitivity of A2780 cells to temozolomide. This increase was not extensive, which may have been a consequence of the high levels of expression of Bcl-2 in these cells. We further showed that oblimersen treatment attenuated Bcl-2 expression in A2780 cells and that this resulted in an enhanced sensitivity of the cells to temozolomide. This effect, which was not observed with either of the control oligonucleotides, was even noted at the lowest dose of oblimersen used and the sensitivity of cells was increased further by increasing the oblimersen dose. This may be related to the observation that Bcl-2 staining was visibly less intense after 33 nmol/L oblimersen compared with 17 nmol/L oblimersen, indicating a greater extent of ablation. Finally, we observed using both growth inhibition and clonogenic survival assays that the combination of oblimersen and PaTrin-2 resulted in a further enhancement of the sensitivity of A2780 cells to temozolomide.

The analysis of the data for oblimersen and temozolomide has revealed an interaction between the two drugs. This would suggest that O\textsuperscript{6}-meG DNA adducts induced by temozolomide lead the cell to death via the Bcl-2-controlled mitochondria-mediated apoptosis pathway. Oblimersen, by attenuating the expression of Bcl-2, renders the cell more susceptible to O\textsuperscript{6}-meG-triggered apoptosis, thus enhancing the effect of temozolomide. The key role played by Bcl-2 in the regulation of apoptosis induced by O\textsuperscript{6}-meG DNA adducts has been shown in mammalian cells (17). On the other hand, recent evidence suggests that O\textsuperscript{6}-meG DNA adducts trigger apoptosis in human peripheral lymphocytes by activating the receptor-mediated apoptosis pathway independently of Bcl-2 (18). However, because Bcl-2 and MGMT are frequently overexpressed in human cancers (10, 19) and are associated with tumor cell resistance to chemotherapeutic agents, it is reasonable to suggest that oblimersen and PaTrin-2 may be a useful combination to explore in clinical studies involving alkylating agents such as temozolomide or DTIC.

An important caveat to be addressed when considering the use of combined therapies in clinical trials is the potential for additional toxicity in normal tissues. PaTrin-2 depletes MGMT not only in tumors but also in other tissues such as the bone marrow. The cytotoxic effect of temozolomide combined with PaTrin-2 is therefore enhanced in tumors, but myelosuppression is also increased, forcing a modest reduction of the dose of the alkylating agent in phase I studies. Strategies to protect the bone marrow against such combinations are now being considered. They involve the transduction of hematopoietic stem cell with a pseudosubstrate-resistant mutant MGMT gene, so that tumor sensitization, by inactivation of endogenous MGMT, and bone marrow protection can be achieved simultaneously even in the presence of a MGMT inactivator (20).

Oblimersen has undergone clinical study as a single agent. Dose-limiting toxicities were seen (at doses >3 mg/kg/d) in patients with non-Hodgkin’s lymphoma and chronic lymphocytic leukemia that consisted of fever, thrombocytopenia, fatigue, and hypotension. However, these tumor types seem to be the only ones in which this toxicity is shown (21–23). Toxicities induced by the combination of fludarabine, cytarabine, and filgrastim with oblimersen in refractory or relapsed myeloid leukemias were not dose limiting and were not clearly attributable to oblimersen (24). This was also shown in patients with chemotherapy-refractory small cell lung cancer treated with the combination of oblimersen and paclitaxel (25). Furthermore, toxic side effects observed in patients with advanced malignant melanoma after treatment with the methylating agent DTIC and oblimersen have been shown to be comparable with those observed with single-agent DTIC therapy (26). The lack of increased toxicity when oblimersen is used as part of a combination therapeutic regimen is promising and may be a consequence of the relatively low levels of expression of Bcl-2 in normal cells.
Sensitization of Human Cells to Temozolomide

The results of the recent randomized phase III study in advanced melanoma (27) showed that the combination of DTIC and oblimersen was associated with a significant higher response rate and progression-free survival than the standard therapy with DTIC alone. However, overall survival was not significantly different. This may have been at least partly a consequence of MGMT. The significant sensitization of tumor cells obtained with the addition of PaTrin-2 to the combination of temozolomide and oblimersen in this experimental system provides a reasonable basis for looking at this three-drug combination in future trials. It is reasonable to suggest that the increased apoptotic drive seen with the combination would convert the increased response rate into a survival advantage.

In summary, we show that a significant enhancement of in vitro sensitivity to the cytotoxic effect of temozolomide can be achieved by combining the action of a MGMT inactivator and an agent attenuating the expression of Bcl-2 in the A2780 ovarian tumor cell line. These findings support continued exploration of this phenomenon in vivo models to examine the timing and dosing of the triple combination. Such findings may prove helpful for investigation of this therapeutic combination of oblimersen, PaTrin-2, and temozolomide in clinical trials.

References

Molecular Cancer Therapeutics

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