Chemical Therapeutics

NEO212, Temozolomide Conjugated to Perillyl Alcohol, Is a Novel Drug for Effective Treatment of a Broad Range of Temozolomide-Resistant Gliomas

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Abstract
Patients with glioblastoma multiforme (GBM), a malignant primary brain tumor, inevitably develop resistance to standard-of-care chemotherapy, temozolomide. This study explores the effects of the novel agent NEO212, a conjugate of temozolomide to perillyl alcohol, on temozolomide-resistant gliomas. NEO212 was tested for cytotoxic activity on three human temozolomide-resistant glioma cell lines, which were resistant to temozolomide based on overexpression of the base excision repair (BER) pathway, mismatch repair (MMR) deficiency, or overexpression of O6 methyl-guanine-DNA methyltransferase (MGMT). BER expression was evaluated by Western blotting and PARP activity. MMR deficiency was determined by Western blotting and microsatellite instability. MGMT overexpression was evaluated by Western blotting and O6-benzyguanine (O6BG) inhibition. For in vivo evaluation of NEO212, temozolomide-resistant glioma cells were implanted into immune-incompetent mice, and NEO212 was administered. NEO212 at equimolar concentrations of temozolomide was more cytotoxic for temozolomide-resistant cells than temozolomide and not toxic to normal cells. NEO212-induced cell death in temozolomide-resistant glioma cells was independent of such mechanisms of resistance as high levels of MGMT, MMR deficiencies, or overexpression of BER proteins. NEO212 functions as a DNA alkylating agent, similar to temozolomide; however, this novel conjugate is unique for it may induce endoplasmic reticulum (ER) stress and inhibits autophagy. In vivo studies show that NEO212 reduces intracranial tumor growth and increases animal survival without significant toxicity. These results demonstrate that NEO212 is an effective drug against malignant gliomas that can be used for a broad range of newly diagnosed and temozolomide-resistant gliomas. Mol Cancer Ther; 1–14. ©2014 AACR.

Introduction
Glioblastoma multiforme (GBM) is the most common and malignant primary brain tumor. Despite therapies consisting of surgery, radiotherapy, and chemotherapy, median survival is only 15 to 18 months. The current standard of care for chemotherapy in GBM is the DNA alkylating agent temozolomide, which is administered in conjunction with radiation therapy and as a stand-alone chemotherapy (1). In more than 90% of patients with GBM, tumors recur and become unresponsive to temozolomide (1, 2). At this point, further treatment options are limited. Repeat surgery is invasive and associated with potential morbidity and has limited efficacy because of the intrinsic invasive nature of GBM (3). Furthermore, repeat radiation is associated with damage to normal brain functions and radiation necrosis (4). Thus, a need for a better-tolerated chemotherapy may be one of the more productive avenues for improvement. This study is the first to use the novel drug, NEO212, a conjugate of temozolomide to perillyl alcohol (POH), to address the critical medical need for patients with temozolomide-resistant, malignant brain tumors.

Recent studies from this laboratory have shown that POH, a naturally occurring monoterpene, has significant anti-glioma activity (5). This unique compound is cytotoxic for temozolomide-resistant as well as temozolomide-sensitive glioma cells; however to be effective, POH must be administered at relatively high doses. The rationale for covalently linking temozolomide to POH is that temozolomide has several drawbacks, despite being used as standard of care for GBM. For maximum activity,
temozolomide is given at fairly high doses, often associated with deleterious effects such as myelosuppression (6), as well as the development of drug resistance (7, 8). The choice of POH as a linkage partner for temozolomide was based on the knowledge that this agent has recognized anticancer activity and is cytotoxic to temozolomide-resistant gliomas (5, 9–11). Furthermore, preliminary data using the blood–brain barrier (BBB) prediction program demonstrated that conjugating POH, which is amphophilic, to temozolomide would increase the lipophilicity of this new compound. This would potentially increase penetration of the conjugate into the brain through the BBB and thereby support the use of lower drug doses to minimize toxicity (12). Thus, this novel drug, NEO212, the conjugate of temozolomide to POH, emerged as a potential therapeutic agent for temozolomide-resistant GBM.

Temozolomide causes cytotoxicity by spontaneously converting to the reactive methylation agent 3-(3-methyltriazen-1-yl) imidazole-4-carboxamide (MITIC), which then degrades to the methyl diazonium cation. The final degradation product, 5-aminoimidazole-4-carboxamide (AIC), is excreted by the kidneys (6). The methyl diazonium cation reacts with DNA to form methyl adducts, such as N\(^2\)-methyladenine (N\(^2\)-meA), N\(^7\)-methyl-guanine (N\(^7\)-meG), and O\(^6\)-methyl-guanine (O\(^6\)-meG), resulting in DNA strand breaks and subsequent cell death (13). While temozolomide is used as standard of care in treating newly diagnosed GBM, treatment with this agent eventually results in temozolomide resistance (14, 15). One mechanism of this resistance involves the overexpression of the repair enzyme, O\(^6\)-methyl-guanine-DNA methyltransferase (MGMT), which restores structural integrity of the O\(^6\)-meG bases by transferring the methyl group to a cysteine residue within its own active site. Although O\(^6\)-meG only accounts for a minor proportion of temozolomide-induced base lesions (6%), O\(^6\)-meG lesions are the most cytotoxic, severely disrupting DNA replication. MGMT is highly expressed in 40% to 50% of GBMs contributing to temozolomide resistance (15–17). In the absence of MGMT, O\(^6\)-meG mispairs with thymidine during DNA replication, resulting in DNA mismatch in replicating cells, leading to cellular apoptosis.

Although MGMT expression is a clinically important prognostic marker linked to temozolomide resistance and patient survival, it is not the only DNA repair mechanism that is associated with temozolomide resistance. The mismatch repair (MMR) pathway removes thymidine but is unable to repair the original O\(^6\)-meG lesion. This results in repeated unsuccessful MMR attempts to repair the DNA, which ultimately leads to double-strand breaks, replication arrest, and cell death (18). MMR thus plays a crucial role in temozolomide-induced cytotoxicity. A deficiency in the MMR pathway would result in the failure to recognize and repair at this position, thus resulting in continued DNA replication past the O\(^6\)-meG block, thereby producing no double-strand breaks, no cell death, and ultimately resistance to temozolomide (19).

Another mechanism of temozolomide resistance involved the base excision repair (BER) pathway. This pathway consists of several DNA repair proteins that cooperate in the removal of damaged or inappropriate DNA bases, such as N\(^2\)-meG (20). Once these methyl groups are removed, the cells replicate and survive; therefore, relatively high levels of these BER proteins are responsible for temozolomide resistance (21, 22). Thus, low levels of BER proteins result in cytotoxicity, but overexpression of these proteins is protective and induces temozolomide resistance (23). PARP cooperates with the BER system to ensure genomic stability by repairing single-stranded DNA breaks (23, 24). If PARP activity is inhibited, these single-stranded DNA breaks become double-strand breaks leading to induction of cell death. Thus, PARP contributes to temozolomide resistance (20), and inhibition of PARP enhances temozolomide-induced cytotoxicity.

As is apparent from several studies, there is a wide variety of mechanisms causing temozolomide resistance (20, 23). The major problem in identifying potential targets to eliminate temozolomide-resistant cells is that there are multiple pathways of temozolomide resistance. Thus, a single drug that can effectively target a wide range of temozolomide-resistant tumors at clinically achievable doses is of major importance.

Studies reported here show that the newly designed NEO212 reagent has the capacity to be cytotoxic to several types of temozolomide-resistant glioma cells, including MGMT positive and MGMT-negative gliomas. This unique reagent is far more active than the mixture of its components and is cytotoxic for several different temozolomide-resistant glioma cell lines and primary GBM cells, but not to normal astrocytes or endothelial cells. In vivo orthotopic GBM tumor studies show that systemic administration of NEO212 is effective in reducing intracranial temozolomide-resistant tumor growth, with no significant organ or bone marrow toxicity. Thus, these studies demonstrate that NEO212 is a strong candidate for the treatment of newly diagnosed GBMs, which are MGMT-positive and temozolomide-resistant GBMs derived as a result of different mechanisms of temozolomide resistance.

Materials and Methods

Cells and reagents

All human tissue specimens were obtained in accordance with the Institutional Review Board guidelines established at Keck School of Medicine, University of Southern California (Los Angeles, CA). Human glioma cell lines, U251, U251 temozolomide-resistant (TR), LN229, LN229TR2, and T98G were cultured in 10% FCS in DMEM supplemented with 100 U/mL penicillin and 0.1 mg/mL streptomycin at 37°C and 5% CO\(_2\). Glioma cell lines were originally
purchased from ATCC; the cell lines have not been authenticated. Temozolomide-resistant cells were developed as described previously (5, 25). Briefly, cell lines were incubated with increasing doses of NEO212 ranging from 10 to 100 μmol/L for approximately 6 months. Once the cells became temozolomide-resistant, these cell lines were treated with 100 μmol/L temozolomide every other week. This method of developing temozolomide-resistant cell lines was designed to parallel the development of temozolomide resistance in the clinic, where GBM patients are initially sensitive and then develop resistance. This phenotype has been stable for over 3 years.

Primary glioma cultures were obtained from GBM tissues acquired post-surgery and rinsed in sterile PBS, minced with surgical blades, and homogenized with a cell douncer. The homogenate was then centrifuged at 800 rpm for 5 minutes and the pellet resuspended in red blood cell lysis buffer for 5 minutes. The mixture was then centrifuged, and the pellet was resuspended in tumor cell culture media, DMEM with 10% FBS. After 2 to 3 days, viable cells were counted and used for cytotoxicity studies. Normal astrocytes were purchased from ATCC and cultured in astrocyte medium (ScienCell). Endothelial cells (EC) were isolated and characterized as previously described (26). These cells were cultured in RPMI containing 10% FCS supplemented with Endogro (Millipore) and used only until passage 6.

In performing the experiments, EC growth supplement was removed from the medium.

NEO212 was provided by NeOnc Technologies, Inc. The structure of this reagent is presented (Supplementary Fig. S1). This reagent was prepared as 100 mmol/L stock solution in DMSO and stored at −20°C. POH (NeOnc Technologies, Inc.) was purified and provided as 6,500 mmol/L stock solution and stored at room temperature (5). Temozolomide (Merck) was prepared as 50 mmol/L stock solution in DMSO, and O6-benzylguanine (O6BG; Sigma Aldrich) was prepared as 100 mmol/L stock solution in DMSO. All drugs were stored at −20°C. PARP inhibitor ABT-888 (Santa Cruz Biotechnology) was prepared as 50 mmol/L stock in DMSO. The above drugs were added to culture medium achieving the final concentration of DMSO less than 0.4%; controls comparing media controls with or without DMSO showed no effect of DMSO alone.

**MTT assay**

Glioma cells (5,000 cells/well), astrocytes or EC cells (10,000 cells/well) were seeded in 96-well plates. After 24 hours, temozolomide or NEO212 was added to the cells at different concentrations and incubated for 72 hours. The MTT assay was performed according to the manufacturer’s protocol (Sigma Aldrich). Absorbance was measured using a microtiter plate reader (Molecular Devices) at 490 nm. Percentage viability was calculated relative to untreated control cells. All experiments were performed in triplicate.

**Colony-forming assay**

Glioma cells were seeded in 6-well plates at 200 cells per well and allowed to adhere overnight. Subsequently, cells were treated with temozolomide, POH, a mixture of temozolomide and POH, and NEO212 for 48 hours; the medium was then removed and fresh medium (no drug) added. Cells were incubated for an additional 7 to 10 days. At the termination of the assay, colonies were visualized by staining with 1% methylene blue in methanol for 4 hours. Dyes were washed out with water and the air-dried plates were quantified. Groups were plated in triplicate. Cells were pretreated with 25 μmol/L of O6BG for 2 hours and then treated with temozolomide or NEO212 for 48 hours for the colony-forming assay (CFA). For PARP inhibition, cells were pretreated with ABT-888 (1 μmol/L) for 1 hour and then drugs (temozolomide or NEO212) were added for an additional 48 hours and finally the CFA was performed as described above.

**Western blot analysis**

Total cell lysates were prepared by disrupting cells with RIPA buffer; protein concentrations were determined using the BCA protein assay reagent (Pierce). Fifty micrograms of total cellular lysates was added to each lane; 10%, 12.5%, or 15% of SDS-PAGE gels were used according to the size of interested proteins. Trans-blot (BioRad) was used to semi-dry transfer. Antibodies to actin and MGMT were purchased (Santa Cruz Biotechnology), as well as antibodies to MSH2 and MSH6 were purchased from Cell Signaling Technology. DNA glycosylase (AAG) and DNA polymerase β (β-Pol) antibodies were purchased from Abcam. Antibodies to GRP78 and actin were obtained from Santa Cruz Biotechnology, Inc. and used according to manufacturer’s recommendations. Antibodies to CHOP, cleaved caspase-7, PARP, p62, and LC3B were purchased from Cell Signaling Technology. DNA damage markers such as p-ATM, p-Chk2, and p-H2AX were purchased from Cell Signaling Technology. Horse-radish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology) were used for detection. Western blot band density was evaluated using ImageJ analysis.

**Microsatellite instability**

The gDNA isolated from pairs of drug-resistant and -sensitive cell lines derived from the same individuals was amplified by PCR using primers for NR-21, Bat-26, Bat-25, and NR-24 loci, which are the recommended mononucleotide repeat polymorphisms in the revised Bethesda guidelines for detection of microsatellite instability (27). The primer sequences were as published in the same guidelines (27). The Penta-C and Penta-D pentanucleotide repeat polymorphisms were also used to verify the common ancestry of cell lines in each pair. The PCR products were electrophoresed on an ABI3900 capillary electrophoresis instrument purchased from Applied Biosystems. Electrophoretic mobility tracings from cell line within a given pair were superimposed for
each locus to examine mobility shifts indicative of microsatellite instability in samples from resistant cell lines.

**Alkaline comet assay**

Comet assays were performed under alkaline conditions according to the vendor’s protocol and using the vendor’s reagents (Trevigen). Briefly, cells were pretreated with indicated doses of temozolomide or NEO212 for 72 hours. Treated cells were then suspended in low-melting agarose (LMA). Seventy-five microliters of the mixture was applied to the comet slide and kept at 4°C in the dark for 10 minutes. The slides were immersed in prechilled lysis buffer to lyse the cells. The slides were washed with TAE buffer and placed flat onto a gel tray in a horizontal electrophoresis apparatus. Electrophoresis was carried out by applying 16 V for 25 minutes. The slides were washed with water and immersed in 70% ethanol for 5 minutes. The samples were dried overnight. Fifty microliter of SYBR green was placed onto each sample. Images of the comet assay were photo-graphed using fluorescent microscopy. Tail lengths were calculated from more than 100 cells using the ImageJ program. Experiments were performed in duplicate.

**Cell death ELISA**

Cells were seeded at a density of $1 \times 10^4$ cells per well in 96-well plates and allowed to adhere overnight; cells were then treated with different concentrations of temozolomide and NEO212 for 24 hours. Attached cells were collected analyzed for apoptosis using commercially available ELISA kit per the manufacturers’ instructions (Cell Death Detection ELISA PLUS, Roche Applied Science).

**In vivo intracranial glioma rodent model**

All animal protocols were approved by the University of Southern California, Department of Animal Resources, and animals maintained according to approved guidelines. Intracranial implantation of tumor cells was performed as previously described (28). Briefly, athymic nude mice were anesthetized and fixed onto a stereotactic head frame. A 2-mm diameter hole at the coordinates A: 1.5 mm, L: 1.5 mm, and V: 2.5 mm was drilled through the skull; $2 \times 10^5$ luciferase-positive temozolomide-resistant human glioma cells (U251TR) were injected into the frontal lobe. Mice were imaged 13 days after implantation. Once tumors appeared in all the mice, the animals were randomly placed into different groups and treatment was started. NEO212 at 5 and 37.2 mg/kg was diluted in a 50% ethanol/50% glycerol formulation and administered subcutaneously or orally by gavage in a volume of 30 µl. Temozolomide (5 mg/kg) was diluted in the same ethanol/glycerol formulation and administered by gavage in a volume of 30 µl. Drugs and vehicle were delivered daily for 10 days; subsequently, the treatment was halted for 7 days and then again initiated for another 10 days, followed by 7 days off.

For pathology analysis, organs (liver, kidney, intestines, heart, and lung) were fixed in 10% formalin, paraffin-embedded, sectioned, and stained with hematoxylin and eosin. Bone marrow specimens were removed from the bone, smeared on the slide, and stained with Hemacolor stain (EMD Millipore).

**Statistical analysis**

Statistical significance was evaluated using the Student 2-tailed test for all *in vitro* experiments. The log-rank test was used to evaluate significance for the survival curve; $P < 0.05$ was considered significant.

**Results**

**NEO212 is cytotoxic to temozolomide-sensitive and temozolomide-resistant glioma cells**

To determine the cytotoxic effects of NEO212 and temozolomide on glioma cells, both drugs were tested on 5 different human glioma cell lines: U251, U251TR, LN229, LN229TR2, and T98G. Using the CFA, the results in Fig. 1 demonstrate that temozolomide-sensitive glioma cells are sensitive to both temozolomide and NEO212 (Fig. 1A). The results presented are calculated based on 3 independent experiments ($n = 3$). The results show that NEO212 is 2- to 3-fold more effective than temozolomide on temozolomide-sensitive cells; the IC$_{50}$ results are summarized in Table 1. Because temozolomide-resistant tumors are difficult to treat, the challenge was to identify an agent that was cytotoxic for chemoresistant cells. Therefore, NEO212 was tested *in vitro* on temozolomide-resistant glioma cells and assayed using the CFA. The data show that NEO212 was highly cytotoxic to U251-resistant (TR) and LN229TR2 (Fig. 1B) as compared with temozolomide and cytotoxic to T98G, an established MGMT-positive temozolomide-resistant cell line (Fig. 1C). The IC$_{50}$ values for these experiments are summarized in Table 1. The cell death ELISA was also used to quantify cytotoxicity following drug treatment; the results show that NEO212 causes significantly more cell death than temozolomide in the temozolomide-resistant populations of GBM using the Cell Death ELISA technique (Supplementary Fig. S2).

**NEO212 is cytotoxic for primary tumors but not for normal brain cells**

To test whether primary gliomas were also responsive to this drug, 4 specimens of primary tumors were tested for responsiveness to NEO212 and temozolomide. Cells were exposed to the drugs for 72 hours. The results show that primary tumor cell populations were also more responsive to NEO212 than to temozolomide (Fig. 1D; Supplementary Fig. S3). These primary gliomas were obtained from newly diagnosed GBMs. To determine whether normal, nontransformed cells were affected by NEO212, normal astrocytes and brain endothelial cells (BEC; Fig. 1E) were exposed to equimolar doses of NEO212 and temozolomide and evaluated using the MTT
The results show that both these drugs did not cause major cytotoxicity to these control cells; even at the highest drug doses tested (100 μmol/L), there was greater than 50% cell survival. Thus, NEO212 and temozolomide are not significantly cytotoxic to normal cells at doses toxic to glioma cells.

![Graph showing cytotoxic effects of NEO212 on temozolomide (TMZ)-sensitive and -resistant glioma cell lines, primary tumor cells, and normal cells.](image-url)

Figure 1. Cytotoxic effects of NEO212 on temozolomide (TMZ)-sensitive and -resistant glioma cell lines, primary tumor cells, and normal cells. Glioma cells were treated with a range of doses of NEO212 or temozolomide for 48 hours and then cultured for another 7 to 10 days in fresh medium in the CFA; wells were plated in triplicate. The data are expressed as percentage of colonies compared with vehicle controls. Three independent experiments were performed and data were combined into one graph. A, temozolomide-sensitive glioma cell lines (U251 and LN229) respond more effectively to NEO212 than temozolomide: U251 at 10 μmol/L (\*P = 0.0002) and LN229 at 10 μmol/L (\*P = 0.0001). B, NEO212 is more effective as a cytotoxic agent than temozolomide in temozolomide-resistant glioma cell lines (U251TR and LN229TR2): U251TR at 40 μmol/L (\*P = 0.003) and LN229TR2 at 60 μmol/L (\*P = 0.003). C, the MGMT-positive temozolomide-resistant glioma cell line T98G was more responsive to NEO212 than to temozolomide (\*P = 0.001 at 50 μmol/L). D, primary tumor cells (GBM-18 and GBM-47) obtained from patient specimens were treated with a dose range of NEO212 and temozolomide for 3 days. Subsequently, cultures were evaluated for cell viability using the MTT assay. E, normal BEC and astrocytes were cultured with NEO212 or temozolomide for 3 days. Survival was evaluated using the MTT assay and expressed as percentage survival compared with untreated cells.
NEO212 is more cytotoxic than temozolomide, POH, or the mixture of these two drugs

To determine whether NEO212 is more effective than each of its components, cells were treated with equimolar concentrations of each drug alone, the mixture of temozolomide plus POH, as well as the temozolomide–POH conjugate NEO212; cytotoxicity was subsequently monitored using CFA. The results show that for temozolomide-sensitive U251, temozolomide-resistant U251TR, or temozolomide-resistant T98G cells, NEO212 is considerably more effective in causing cytotoxicity than either temozolomide, POH, alone or the mixture of temozolomide and POH (Fig. 2A–C, respectively). It should be emphasized that at equimolar doses of NEO212 and POH, only NEO212 is active. The data in Fig. 2 were calculated based on 3 independent experiments (n = 3). The data indicate that at equivalent concentrations, NEO212 is significantly more potent than the individual components and simple mixture of the 2 agents. This demonstrates that NEO212 is a novel entity, with unique capabilities (see Supplementary Fig. S1 for structure).

NEO212 induces glioma cell cytotoxicity through several pathways

Temozolomide is a well-known DNA alkylating agent, causing cell death through DNA damage. To determine whether NEO212 also induces cell death through this mechanism, we performed the alkaline comet assay to detect single- and double-stranded DNA breaks after exposure to each drug. Undamaged DNA is supercoiled and does not migrate far out of the nucleus under the influence of an electric current, whereas cells that have accumulated DNA breaks exhibit a DNA fragment tail (29). The length of the migrating fragment (comet tail) as measured from the nucleoid (comet head) to the tip of the comet tail is correlated with amount of DNA damage. We exposed U251 and U251TR cells to NEO212 and temozolomide for 72 hours and monitored DNA migration (Fig. 3A; additional photographs are presented in Supplementary Fig. 5A). Tail lengths were compared for NEO212 and temozolomide treatment (Fig. 3B). NEO212 caused significantly larger tail lengths than temozolomide in both sensitive and resistant cells, demonstrating that NEO212 does cause DNA damage and is more effective in causing DNA damage than temozolomide at equimolar concentrations.

To further examine the DNA damage response to NEO212 as compared with temozolomide, several kinases comprising the signaling cascade for DNA damage were examined. These proteins include phosphorylated ATM and the downstream effector kinase Chk2 (30). Phosphorylated histone variant H2AX identifies DNA damage and the site of DNA repair. Figure 3C shows that both NEO212 and temozolomide induce DNA damage in both temozolomide-sensitive and -resistant glioma cells. However, at equimolar concentrations (e.g., 10 or 30 µmol/L, respectively), NEO212 induces higher levels of p-ATM and p-Chk2 than temozolomide.

A potential mechanism of drug-induced cell death is activation of the endoplasmic reticulum (ER) stress pathway. Studies have shown that high doses of temozolomide (1 mmol/L) and POH (1 mmol/L) activate cell death through ER stress pathway (5,31). To compare the efficacy of NEO212 and temozolomide, equimolar concentrations of these drugs were analyzed for expression of GRP78, CHOP, and cleaved PARP, proteins responsible for cytotoxicity initiated by ER stress. The results (Fig. 3D) show that at 50 and 100 µmol/L, NEO212 may activate cell death in temozolomide-sensitive and -resistant cells respectively, through CHOP and cleavage of PARP, whereas temozolomide does not exhibit such activity.

Autophagy can be activated during a time of cell stress and serves as an escape mechanism for cells exposed to cytotoxic agents, hypoxia, or a reduced nutrient environment (32). To test whether NEO212 regulates autophagy, glioma cells were treated with this drug or temozolomide (Fig. 3E) at equimolar doses and evaluated for LC3 conversion and p62 accumulation, markers of autophagy (32). These data show that treatment with NEO212 converts LC3I to LC3II, suggesting that NEO212 may induce autophagy. However, the accumulation of p62 with NEO212 treatment also suggests that autophagy may ultimately be inhibited, potentially removing this protective mechanism. Treatment of cells with temozolomide at equimolar doses

<table>
<thead>
<tr>
<th>Cells lines</th>
<th>U251</th>
<th>LN229</th>
<th>U251TR</th>
<th>LN229TR2</th>
<th>T98G</th>
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<tbody>
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<td>18</td>
<td>10</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>NEO212 IC₅₀, µmol/L</td>
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<td>5</td>
<td>40</td>
<td>45</td>
<td>50</td>
</tr>
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NOTE: Cells were treated with NEO212 and temozolomide and analyzed for cytotoxicity using the CFA. The IC₅₀ was determined using the values obtained in Fig. 1. These values were based on the combined data of the 3 experiments performed. The IC₅₀ for temozolomide treatment of temozolomide-resistant glioma cells could not be calculated at the doses tested; a minimal decrease in colony numbers was detected at the highest dose of temozolomide (100 µmol/L). Abbreviation: NA, not attainable (no IC₅₀ was reached at temozolomide 100 µmol/L).
exhibited no apparent effect on autophagy. Thus, both NEO212 and temozolomide stimulate DNA damage, but NEO212 may further induce ER stress and inhibit autophagy at equimolar drug concentrations.

NEO212 is cytotoxic for MGMT-positive glioma cells
MGMT overexpression is thus far the best described mechanism of temozolomide resistance in patients with glioma (23). Therefore, the relationship between MGMT status and sensitivity to NEO212 was examined. T98G glioma cells overexpress MGMT, whereas the other cell lines used in this study do not (Supplementary Fig. S5). As expected, T98G exhibited a limited cytotoxic response to temozolomide (Fig. 1C). This was in sharp contrast to these cells exposed to equimolar doses of NEO212 (Fig. 1C). To explore whether the cytotoxic effects observed with NEO212 was dependent on the activity of MGMT, O\textsuperscript{6}BG, an MGMT-blocking agent, was tested (14). O\textsuperscript{6}BG blocks MGMT enzymatic activity, thereby reducing the protective effects of MGMT when exposed to temozolomide (14). T98G cells were exposed to O\textsuperscript{6}BG for 2 hours and tested for response to temozolomide and NEO212 (Fig. 4A). Results showed that O\textsuperscript{6}BG caused a dramatic increase in sensitivity to temozolomide in MGMT-positive T98G as expected. Treatment of cells with NEO212 plus O\textsuperscript{6}BG also increased cytotoxicity suggesting that NEO212 increases the chemosensitivity of MGMT-positive cells. To determine whether NEO212 can directly regulate MGMT, T98G were treated with NEO212 and tested for MGMT expression. These results show that this agent inhibited MGMT expression (Fig. 4B); temozolomide alone reduced MGMT to a limited extent and POH had no effect at this dose. Importantly, a mixture of unconjugated temozolomide and POH had effects not significantly different from temozolomide alone. Thus, in MGMT-positive tumors, NEO212 functions as a cytotoxic agent by regulating MGMT activity.

NEO212 cytotoxicity is independent of molecular temozolomide resistance mechanisms
NEO212 was effective in killing U251TR and LN229TR2 cells, cells that do not express MGMT (Supplementary Fig. S5). Thus, NEO212 is cytotoxic for temozolomide-resistant cells that do not express MGMT. In the absence of MGMT, the MMR pathway attempts to correct the O\textsuperscript{6}-meG methylation but is unable to do so, resulting in cell death. However, if the MMR pathway is not functioning, O\textsuperscript{6}-meG is skipped, no DNA double-strand breaks occur, and the cells remain alive and resistant to temozolomide (33). Thus, gliomas with low levels of MMR enzymes are resistant to temozolomide. To determine whether these temozolomide-resistant cell lines have a deficiency in the MMR pathway, cells were analyzed for the proteins MSH2 and MSH6. The results showed that LN229TR2 cells exhibit reduced levels of MMR proteins, MSH2, and MSH6 (Fig. 4C), as compared with their temozolomide-sensitive cell counterparts. The mobility electrophoretic profiles of PCR products from 5 different mononucleotide polymorphisms in each pair of resistant and sensitive cell lines was compared to determine whether acquisition of drug resistance was associated with microsatellite instability in any of those lines. The
Figure 3. NEO212 uses various mechanisms to induce cytotoxicity. A, to test whether NEO212 induces DNA damage, the Comet assay was used. Temozolomide (TMZ)-sensitive (U251) and temozolomide-resistant (U251TR) cells were treated with equimolar concentrations of NEO212 and temozolomide for 72 hours and tested for DNA damage using the Comet assay. B, tail length values were calculated by measuring the lengths of the migrating fragment (comet tail) from the nucleoid (comet head) to the tip of the comet tail. Each specimen was evaluated in triplicate. C, DNA damage in temozolomide-sensitive and -resistant glioma cells was further examined by analyzing DNA damage markers (p-ATM, p-Chk2, p-H2AX) using Western blot analysis. Cells were treated with different doses of NEO212 or temozolomide for 24 hours, and DNA damage markers were evaluated. Both NEO212 and temozolomide induced DNA damage; however, NEO212 was effective at lower doses. D, to determine whether NEO212 induced ER stress, U251-sensitive and -resistant glioma cells were treated with different concentrations of NEO212 or temozolomide for 24 hours and tested for the induction of specific ER stress markers GRP78 and CHOP. E, to determine whether autophagy plays a role in NEO212-induced cytotoxicity, autophagy markers were evaluated. U251-sensitive and U251TR-resistant cells were treated for 24 hours with NEO212 or temozolomide and analyzed for the conversion of LC3I to LC3II and the expression of p62.
The electrophoretic mobility tracing of the resistant LN229TR2 cell clone, superimposed on that of the sensitive clone from the same individual, is shown in Fig. 4D (top tracing) for the BAT-26 locus. The figure clearly shows a mobility shift in the resistant cells, indicative of microsatellite instability at this locus. In contrast, similar tracings from U251-resistant and -sensitive clones are identical (Fig. 4D, bottom tracing), indicative of microsatellite stability.

Figure 4. NEO212 is cytotoxic in a variety of temozolomide (TMZ)-resistant cells. A, T98G cells, which express high levels of MGMT, were preincubated with or without O6BG (25 μmol/L) for 2 hours and then treated with NEO212 or temozolomide for another 48 hours. CFA was performed as described above. B, to determine whether NEO212 regulates MGMT as a mechanism of activity, T98G cells were treated with equimolar concentrations (100 μmol/L) of NEO212, the mixture of temozolomide + POH, temozolomide alone, or POH alone for 24 hours, and monitored for MGMT levels using Western blot analysis. C, Western blot analysis for mismatch repair proteins (MSH2, MSH6) in sensitive (U251, LN229) and resistant cells (U251TR, LN229TR2) cells was performed. D, gDNA extracted from sensitive (blue) and resistant (red) subclones of LN229 and U251 was amplified by PCR using primers for the BAT-26 locus and subjected to capillary electrophoresis. The mobility tracings for the resistant and sensitive clones in each pair are superimposed to facilitate comparing their profiles. E, Western blot analysis of the BER proteins, the DNA glycosylase AAG and DNA polymerase Pol β, was examined in sensitive (U251, LN229) and resistant cells (U251TR, LN229TR2) cells after 24-hour incubation. F, the effects of combination treatment with the PARP inhibitor (ABT-888) and NEO212 or temozolomide were tested. Cells were pretreated with the PARP inhibitor (1 μmol/L) for 1 hour, followed by drug treatment for 48 hours; the CFA was subsequently performed.
Evidence of microsatellite instability was also seen in LN229TR2 cells at the NR-21 and NR-24 loci (not shown), implying that such instability was present in 60% of the 5 microsatellite loci examined.

The BER pathway is involved in the repair of $N^3$ and $N^7$ lesions which constitute more than 80% of damage induced by temozolomide (18). To determine whether BER pathway protein overexpression was responsible for temozolomide resistance in these cell lines, Pol $\beta$ and AAG proteins were analyzed in Western blotting ($n = 2$). The results (Fig. 4E) show that Pol $\beta$ and AAG are overexpressed in U251TR as normalized to actin and compared with U251: Pol $\beta$ (50%), and AAG (30%). Elevated PARP expression has also been shown to be associated with temozolomide chemoresistance (24). Using the PARP inhibitor ABT-888, the results (Fig. 4F) show that PARP inhibition increased cytotoxic activity of temozolomide in U251TR cells by approximately 7-fold. The PARP inhibitor increased NEO212 cytotoxicity by approximately 2-fold. These data together with the overexpression of BER proteins suggest that BER is likely to be a mechanism of temozolomide resistance in U251TR cells. Control experiments showed that the PARP inhibitor alone did not induce nonspecific cytotoxicity (Supplementary Fig. S6).

**NEO212 is effective on temozolomide-resistant glioma cells in the in vivo intracranial rodent tumor model**

To determine whether NEO212 is effective on temozolomide-resistant cells in vivo, the intracranial athymic nude mouse model was used. Temozolomide-resistant glioma cells (U251TR), labeled with luciferase, were implanted intracranially; treatment with NEO212 and temozolomide began at the initiation of tumor growth based on imaging information. NEO212 was administered subcutaneously at 5 mg/kg (low) and 37.2 mg/kg (high) doses. Animals were treated with temozolomide (5 mg/kg), by oral gavage, to verify that the intracranial tumors were indeed temozolomide-resistant. The animals ($n = 4$) were imaged periodically at 1- to 2-week intervals. The imaging data (Fig. 5A) show an example of animals treated with vehicle or temozolomide exhibited tumor progression and eventually died by day 42. In contrast, animals treated with NEO212 (5 mg/kg) demonstrated significantly increased survival compared with temozolomide (5 mg/kg; $P < 0.02$). The high-dose (37.2 mg/kg) NEO212 significantly ($P < 0.007$) increased survival compared with vehicle; the Kaplan–Meier plot showing these data are presented in Fig. 5B. In a second in vivo experiment, NEO212 at 5 mg/kg ($n = 6$) was administered subcutaneously. Once again, NEO212 demonstrated a significantly increased survival as compared with temozolomide (5 mg/kg; $n = 6$) administered by gavage (as shown previously; $P = 0.0201$) and temozolomide (5 mg/kg; $n = 6$) administered subcutaneously ($P = 0.0388$). There was no significant difference between temozolomide administered subcutaneously and via gavage ($P = 0.354$; Fig. 5C). These data indicate that treatment with NEO212 delayed tumor growth and increased survival time over 50%, from 25 to 37 days. To determine the potential toxic effects of long-term treatment with NEO212, pathology studies were performed on animals treated with NEO212 for 20 days at 37.2 mg/kg. At time of euthanasia, the following organs were obtained from the high-dose treated mice: liver, heart, intestine, lungs, and kidneys. Specimens were formalin fixed and stained with hematoxylin/eosin. Bone marrow smear specimens were also stained and evaluated. The results show no significant pathologic changes in the organs examined or the bone marrow specimens (Supplementary Fig. S7). These data indicate that NEO212 is effective for intracranial brain tumors with no apparent toxicity to normal tissues, including myelotoxicity.

**Discussion**

This study demonstrates the effects of the novel drug, NEO212 on GBM in vitro and in vivo. Our data show that NEO212, the conjugate of temozolomide and POH, is far more potent than each agent alone or the mixture of these agents (Fig. 2), indicating that NEO212 is a novel and powerful drug with unique characteristics. Previous studies from this laboratory had shown that POH was effective only at high doses, with an IC$_{50}$ value ranging from 1.5 to 1.8 mmol/L for temozolomide-resistant cells (5), and required localized intranasal administration. In contrast, NEO212 is an effective cytotoxic agent with an IC$_{50}$ of 40 to 50 $\mu$mol/L. Because only high doses of POH are effective for anticancer treatment, localized administration of POH (i.e., intranasal administration) is necessary. In contrast, NEO212 is effective at low, nontoxic doses and can be administered systemically that is, subcutaneously or by gavage. The NEO212 is a novel agent with activity different from the simple mixture of temozolomide and POH; at doses tested, there are no effects of the single agents compared with NEO212. Differences in activity between NEO212 and temozolomide are currently under investigation.

The DNA alkylation agent temozolomide is effective in killing temozolomide-sensitive cells but relatively ineffective for temozolomide-resistant glioma cells. In vitro studies using temozolomide at doses up to 100 $\mu$mol/L exhibited no significant response on these cells (Fig. 1). At cytotoxic doses, both NEO212 and temozolomide induce DNA damage, as the primary mode of action in drug-sensitive and -resistant cells, as shown in Fig. 3. However, at equimolar concentrations, the levels of DNA damage are significantly lower in temozolomide-resistant glioma cells treated with temozolomide, as compared with these cells treated with NEO212. These data indicate that NEO212 is an especially effective cytotoxic agent targeting temozolomide-resistant cells.
NEO212 was also tested on primary GBM tumor cells that were derived from patient specimens that did not undergo extensive tissue culture procedures. The specimens used here were obtained from newly diagnosed patients; material obtained from temozolomide-resistant, recurrent tumors did not survive in culture long enough to test. This may be the result of extensive radiation therapy given to the patients at this late stage. In contrast to GBM cells, NEO212 was not cytotoxic to normal, human brain-derived endothelial cells (BEC), and}

![Figure 5. NEO212 is effective in an orthotopic temozolomide (TMZ)-resistant glioma in vivo. A, athymic nude mice were implanted intracranially with luciferase-positive U251 temozolomide-resistant glioma cells. Treatment was begun when tumors became visible; animals were treated with vehicle, temozolomide (5 mg/kg), NEO212 (5 mg/kg), or NEO212 (37.2 mg/kg) daily for 10 days and 7 days off, for 2 rounds. Animals were imaged on days 23, 29, 36, and 42 postimplantation. Images demonstrate decreased tumor progression and increased survival with NEO212 treatment. B, survival data are presented as a Kaplan–Meier plot. C, the U251 temozolomide-resistant intracranial glioma model was used as described above. Groups (n = 6) were treated with vehicle (subcutaneous), NEO212 (5 mg/kg subcutaneous), temozolomide (5 mg/kg subcutaneous), and temozolomide (5 mg/kg gavage). NEO212 treatment significantly increased survival as compared with temozolomide (P = 0.0201; P = 0.0388) by gavage and subcutaneous administration, respectively. There were no significant differences between temozolomide administered subcutaneously or via gavage (P = 0.354).]
normal human astrocytes (Fig. 1E). These data correlate with the pathology evaluations which indicate that NEO212 is relatively nontoxic to nontumor organs in vivo. Tissues evaluated from the different organs following NEO212 long-term treatment (20 day) at 37.2 mg/kg exhibited no significant abnormalities in the liver, kidneys, heart, lungs, intestines, and, most importantly, the bone marrow. Thus, the in vivo studies presented here indicate that at effective doses, NEO212 has no discernibly toxic effects on normal tissues in the animal model.

This study specifically emphasizes the clinical relevance of NEO212. The in vivo experiments presented here clearly demonstrate that NEO212 crosses the blood–tumor barrier and enters the tumor. In vivo studies show that NEO212 when administered subcutaneously, effectively reduced tumor growth and significantly increased survival in the intracranial model. Thus, there is no need for intratumoral delivery, resulting in easier drug administration. We have no direct evidence that the drug crosses the BBB; however, the brain regions around the tumors, where tumor expansion occurs do have an intact BBB (34, 35), suggesting that NEO212 may cross the BBB.

These studies have shown that NEO212 has a cytotoxic advantage over temozolomide in temozolomide-resistant glioma cells. The mechanism causing this advantage is not well understood. NEO212 and temozolomide both induce DNA strand breaks resulting in cell death following cell replication; however, at clinically relevant equimolar concentrations, NEO212 has considerable more activity. A possible mechanism of this enhanced activity is that low-dose NEO212 induce such processes as ER stress. Pathologic conditions that disrupt the ER leading to ER stress cause the accumulation of aberrant proteins, the unfolded protein response, and the transcriptional upregulation of C/EBP-homologous protein (CHOP), which downregulates Bcl2 resulting in apoptosis (32, 36, 37). Prolonged ER stress leads to cell death. Our preliminary evidence suggests that the induction of ER stress by NEO212 may have contributed to the observed enhanced cytotoxicity. Autophagy is an essential prosurvival activity which is upregulated by ER stress (38). This process functions to eliminate aberrant misfolded proteins and damaged organelles, thereby relieving ER stress and enabling the cells to reach homeostasis (39). Autophagy is a process involving the formation and fusion of structures resulting in the formation of autolysosomes where protein degradation takes place. The conversion of LC3II to LC3I is usually involved in the elongation phase of the autophagosomes and therefore provided preliminary evidence that autophagy may have begun with the treatment of NEO212. During the later stage of autophagy, p62 may be highly expressed and then degraded along with the degraded protein (40). Our observation that treatment with NEO212 causes an accumulation of p62 is consistent with the ideas that autophagy is inhibited. Because autophagy is a rescue system for prolonged ER stress, inhibition of autophagy can enhance cell death. The use of the autophagy inhibitor to chemosensitize glioma cells has been reported (41). Our preliminary evidence suggests that NEO212 may stimulate ER stress and inhibit autophagy in temozolomide-sensitive and -resistant glioma cells; however, other mechanisms such as inhibition of mTOR and ras pathways may also play a role in NEO212 activity (42, 43).

This novel drug, NEO212, which is effective on temozolomide-resistant glioma cells with different mechanisms of temozolomide resistance is especially clinically relevant. Studies over the years have shown that temozolomide resistance can be attributed to several different mechanisms, including activation changes in membrane transporter proteins, overexpression of antiapoptotic proteins, as well as abnormalities in DNA repair mechanisms (33, 44). DNA repair enzyme that is thought to play a major role in temozolomide resistance is MGMT. Our data demonstrate that although NEO212 is effective in both MGMT-positive and -negative tumor cells, MGMT levels do play a role in NEO212 activity. The results in Fig. 4B show that the addition of O6BG improves the IC50 of NEO212 from 45 to 25 μmol/L; by sharp contrast, no IC50 can be reached using temozolomide in the absence of O6BG. MGMT expression in GBM occurs in approximately 40% to 50% of patients with GBM, yet temozolomide resistance is almost universal, demonstrating that other mechanisms of temozolomide resistance are operating (17, 45, 46). MMR deficiencies are also documented in recurrent patients with GBM (19, 20, 47). As much as 25% of recurrent GBMs exhibit MMR deficiencies (48). In this study, we show that NEO212 is a safe and effective cytotoxic agent for a wide range of temozolomide-resistant, recurrent tumors regardless of the mechanism of resistance.

Clinically, NEO212 may be used in the treatment of malignant gliomas in 2 potential settings. In the upfront setting, many neuro-oncologists are now screening their patients for MGMT expression in the resected tumor tissue (16). If the tissue is MGMT positive, they have advocated giving another chemotherapy agent instead of temozolomide (17). NEO212 may be effective as upfront therapy for these MGMT-positive patients because of its ability to affect a number of repair mechanisms. In the recurrent setting, after temozolomide failure, NEO212 may be used for these patients, as it is effective in temozolomide-resistant gliomas. Therefore, NEO212 would then provide a viable alternative to patients that would normally have gone to intravenous bevacizumab as their second line of therapy (49).

In summary, our data demonstrate that NEO212 is potent for temozolomide-sensitive and -resistant gliomas, has minimal organ and bone marrow toxicity, and effectively enters the tumor. Thus, NEO212 may provide a viable therapy for patients with malignant glioma in the future.
Disclosure of Potential Conflicts of Interest

T.C. Chen is the CEO/Chairman of, has a commercial research grant from, has ownership interest (including patents) in, and is a consultant/advisory board member for NeOnc Technologies, Inc. No potential conflicts of interest were disclosed by the other authors.

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References


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