Abstract

Glioblastoma (GBM) is the most common and lethal malignant brain tumor. Current treatment involves surgical resection, radiation, and chemotherapy. Temozolomide (TMZ) is the only FDA approved systemic chemotherapeutic available and many tumors are known to have both extrinsic and intrinsic resistance to the TMZ. Resistance to TMZ has generated a pressing need for novel therapies. ONC201, an Akt/ERK dual inhibitor has shown promising preliminary results in phase II clinical trials and its potent analog, ONC212 is believed to be more clinically efficacious. However, *in vitro* studies with ONC212 are necessary prior to clinical application.

Normal human astrocytes (NHA) and normal human astrocytes with mutant *Ras* (NHA Ras), along with 6 established glioblastoma human cell lines (U251, U87, U373, LN18, LN229, and D54MG) were each evaluated for toxicity with 3 individual drugs (TMZ, ONC201, and ONC212) and IC_{50} values were calculated from toxicity curves. TMZ was found to be less effective in cell lines with high MGMT expression (LN18). ONC212 was found to be more potent than ONC201 and has the potential to be a possible treatment option for GBM. Short-term goals involve repeating the experiment with glioblastoma stem cells which better mimic tumor structure with the potential of beginning *in vivo* animal studies and evaluating efficacy and blood brain barrier penetrance of ONC212, followed by clinical trials. The ultimate goal remains to develop effective novel therapies for patients with GBM.

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Introduction

The majority of malignant brain tumors are gliomas, and most gliomas are astrocytomas. Glioblastoma (GBM), also known as grade 4 astrocytoma, is the most common and lethal malignant brain tumor.[1] The current median survival rate of patients with GBM is approximately 15 months after diagnosis. Standard of care involves surgical resection, radiation, and chemotherapy. However, GBM is diffusely invasive and tumors are molecularly heterogenous, making recurrence inevitable and development of effective therapeutics difficult.[2-4] Additionally, difficulty with central nervous system (CNS) penetrance can make developing adequate drug therapies for GBM difficult.[4] Despite the heterogenous nature of GBM, the current standard of care is the same across patients.[2, 3] Temozolomide (TMZ), a mono-alkylating agent that methylates nitrogenous bases, mainly guanine, is the only FDA approved systemic chemotherapeutic available. However, TMZ treatment often leads to harsh off-target effects including hemotoxicity and cytotoxicity.[2, 5, 6]

Additionally, many tumors also have intrinsic resistance to TMZ or acquire resistance over time. A common form of intrinsic resistance in GBM is obtained by overexpression of DNA repair protein O^6-methylguanine methyltransferase (MGMT), which is capable of removing the lesion conferred by TMZ.[2, 7, 8] MGMT expression is often governed by the methylation of the promoter region of the *MGMT* gene.[9] Furthermore, while the mechanisms are not well elucidated, many tumors acquire resistance to TMZ over the course of treatment. GBM cells have been shown to induce the expression of MGMT protein, decrease Tumor Necrosis Factor-Alpha-Induced Protein 3 (TNFAIP3) expression, and upregulation of Signal Transducer and Activator of Transcription 3 (STAT3) in response to TMZ in order to acquire resistance.[7, 10-13] Due to adverse side-

effects associated with TMZ as well as intrinsic resistance in certain tumor cells, there is a pressing need to develop novel and potent therapies that provide targeted treatment with little to no detrimental secondary response.

To this end, ONC201 is a small molecule inhibitor within the imipridone class that is currently being tested in phase II clinical trials of patients with GBM.[14, 15] ONC201 acts by crossing the blood brain barrier and inhibiting the phosphorylation of the communicating Akt and ERK pathways, as well as inducing the dephosphorylation of Foxo3a. Dephosphorylated Foxo3a translocates into the nucleus and induces transcription of the TNF-related apoptosis inducing ligand (TRAIL). ONC201 also activates EIF2α which stimulates downstream transcription factors ATF4 and CHOP and ultimately upregulates death receptor, DR5. The upregulation of both TRAIL and DR5 leads to apoptosis, regardless of p53 status.[14-16]

Recent studies utilizing ONC201 in breast cancer cell lines point to the direct targeting of mitochondria through suppression of multiple mtDNA genes, independent of TRAIL. ONC201 has been shown to target mitochondrial cellular respiration, making cells reliant on anaerobic respiration resistant to ONC201.[17] However, GBM has specifically been shown to heavily rely on glycolysis for energy production.[18] This is a potential roadblock which may occur when utilizing ONC201 to treat GBM.

ONC212, a recently-derived potent analog of ONC201, has displayed a favorable pharmacokinetic profile in mice against various cancer types, making it an attractive potential drug to treat GBM.[15, 19, 20] ONC212 has been shown to inhibit both glycolysis and oxidative phosphorylation in addition to Akt/ERK inhibition.[21] However, further

investigation regarding the CNS penetrance, potential molecular targets, and toxicity of ONC212 is necessary prior to clinical trials.

Materials and Methods

Tissue culture

Normal human astrocytes (NHA) and normal human astrocytes with mutant *Ras* (NHA Ras) cell lines were established and immortalized by expressing HPV oncogenes E6 and E7 to inhibit the TP53 and Retinoblastoma pathways and hTERT to maintain telomere length.[22] In addition to NHA and NHA Ras, six established GBM cell lines (ECLs) were used: U251, U87, U373, LN18, LN229, and D54MG. Cell lines were maintained at 37°C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin.

Proliferation and cell number optimization

Cell number optimization and proliferation curves were completed prior to treatment to determine plating densities. Optimization was accomplished by plating adherent cells at a range of densities in a 96-well tissue culture treated plate and utilizing MTS Cell Proliferation Assay Kit (Colorimetric) (197010) once a day from day 0 to day 5 to quantify the number cells and calculate doubling times for each cell line. The assay is based on the reduction of MTS tetrazolium compound by the metabolic oxidation of nicotinamide adenine dinucleotide (NADH) in viable cells to formazan, a media-soluble

colored product. MTS was added to cells and plates were incubated for 2 hours at 37°C. Absorbance was recorded at a wavelength of 490 nm in order to quantify cell counts.[23]

Drug treatment

Once cell number was optimized, adherent cells were plated onto a 96-well tissue culture treated plate at the optimized densities to have the same cell counts at time of treatment. Cells were treated with one of nine different concentrations of drug (serially diluted) or DMSO control the following day. Dimethyl sulfoxide (DMSO) was used as a solvent for the drug. Since DMSO is known to be toxic to cells at concentrations greater than 1%, it was also incorporated into the serial dilution.[24] An equivalent concentration of DMSO was used with all cells in order to eliminate its presence as a confounding cause of cellular toxicity. MTS Cell Proliferation Assay Kit (Colorimetric) (197010) was, again, utilized to quantify the number of viable cells five days following treatment. The same procedure involving the MTS Cell Proliferation Assay Kit as conducted in proliferation and cell number optimization experiments was used to quantify cell data.[23]

Statistics

A triplicate blank was averaged in each plate and subtracted from each well's absorbance to eliminate the absorbance due to the background media color. To normalize readings between cell lines, all wells' absorbances were divided by either the average of Day 0 (for proliferation assays) or the DMSO control (for toxicity assays). Error bars on proliferation and toxicity curves (panel **A** in each figure) are standard error

calculations. Error bars on dot plots (panel **B** in each figure) are 95% confidence intervals.

Results

NHA was used as an approximation of a normal human astrocyte, similar to a negative control, while NHA Ras was used as an aggressively oncogenic astrocyte, similar to a positive control. NHA and NHA Ras were compared to the 6 ECLs.

A **B**

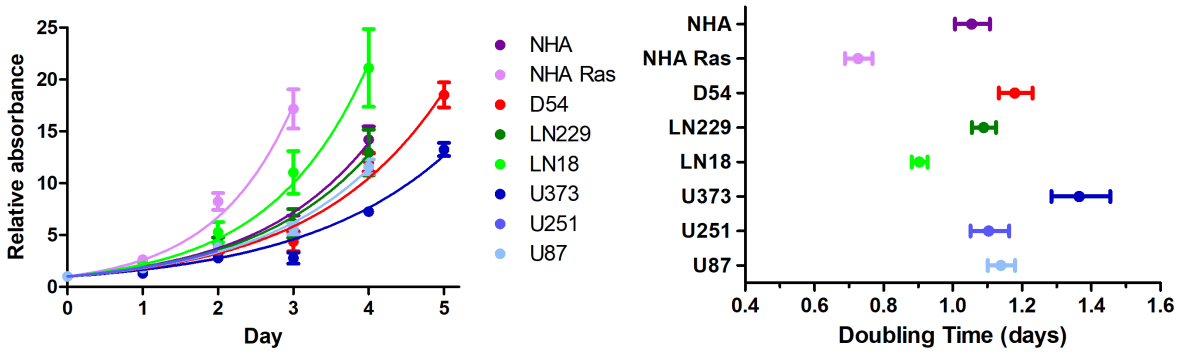

Figure 1 (**A**) Proliferation data over a 5 day time course, measured every 24 hours. Relative absorbance at 490 nm was measured by MTS assay and was plotted against time to measure cell growth. Bars represent standard error. (**B**) Doubling time, which was calculated using proliferation data by determining the time it takes for cells to double. Doubling times were calculated as follows in: NHA (1.054 days), NHA Ras (0.726 days), D54MG (1.179 days), LN229 (1.089 days), LN18 (0.903 days), U251 (1.104 days), U87 (1.139 days), U373 (1.365 days). Bars represent 95% confidence intervals.

These doubling times were used to determine the plating cell densities 24 hours prior to drug treatment. Doubling times for NHA, NHA Ras, and ECLs are summarized in the table below.

Cell line	Doubling time (days)
NHA	1.054
NHA Ras	0.726
D54MG	1.179
LN229	1.089
LN18	0.903
U251	1.104
U87	1.139
U373	1.365

Table 1 Proliferation curves were developed after plotting relative absorbance at 490 nm against time for each of the 8 cell lines (NHA, NHA Ras, U251, U87, U373, LN18, LN229, D54MG). Doubling times were calculated from these plots.

A **B**

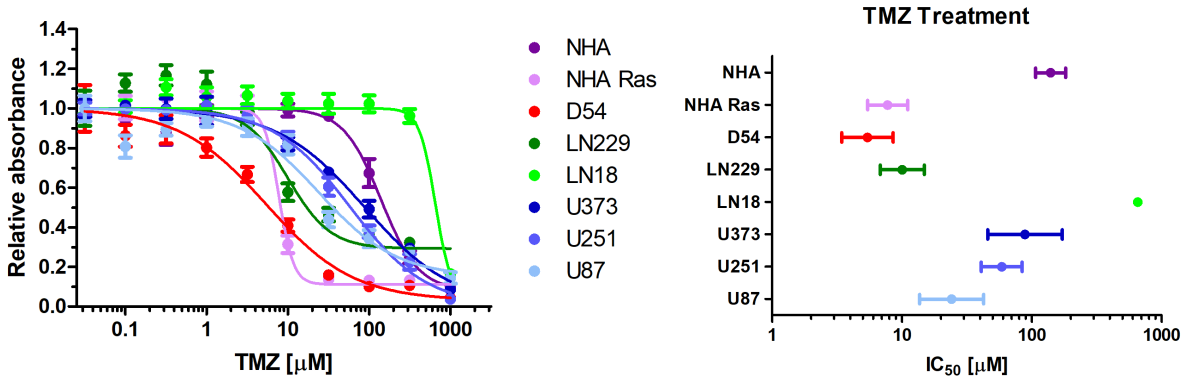

Figure 2 (**A**) Each cell line was treated with varying concentrations of TMZ and was plotted with relative absorbance of light at 490 nm to obtain toxicity graphs. Bars represent standard error. (**B**) IC_{50} values were calculated from the TMZ toxicity curves. IC_{50} values were calculated as follows in: NHA (139 μM), NHA Ras (7.77 μM), D54MG (5.43 μM), LN229 (10.1 μM), LN18 (654 μM), U251 (58.7 μM), U87 (24.2 μM), U373 (88.7 μM). Bars represent 95% confidence intervals.

IC_{50} values for TMZ ranged from minimum of 5.43 μM in D54MG to a maximum of 654 μM in LN18. NHA had an IC_{50} of 139 μM and NHA Ras had an IC_{50} of 7.77 μM.

A **B**

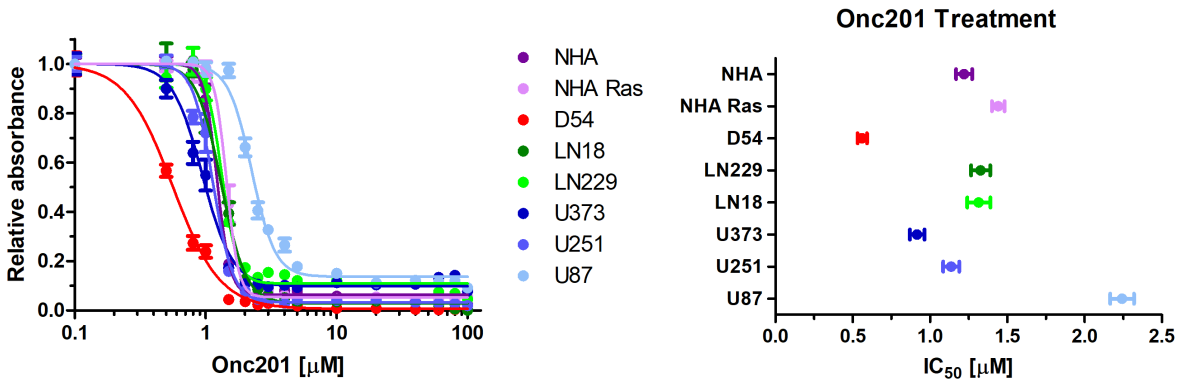

Figure 3 (**A**) Each cell line was treated with varying concentrations of ONC201 and was plotted with relative absorbance of light at 490 nm to obtain toxicity graphs. Bars represent standard error. (**B**) IC_{50} values were calculated from the ONC201 toxicity curves. IC_{50} values were calculated as follows in: NHA (1.22 μM), NHA Ras (1.44 μM), D54MG (0.56 μM), LN229 (1.33 μM), LN18 (1.31 μM), U251 (1.14 μM), U87 (2.24 μM), U373 (0.944 μM). Bars represent 95% confidence intervals.

IC_{50} values for ONC201 ranged from minimum of 0.056 μM in D54MG to a maximum of 2.24 μM in U87. NHA had an IC_{50} of 1.22 μM and NHA Ras had an IC_{50} of 1.44 μM.

A **B**

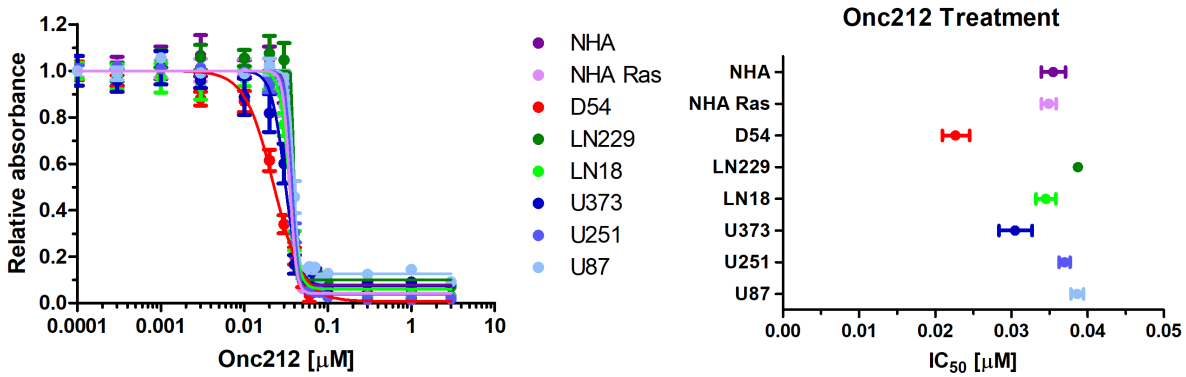

Figure 4 (**A**) Each cell line was treated with varying concentrations of ONC212 and was plotted with relative absorbance of light at 490 nm to obtain toxicity graphs. Bars represent standard error. (**B**) IC_{50} values were calculated from the ONC212 toxicity curves. IC_{50} values were calculated as follows in: NHA (35.5 nM), NHA Ras (34.9 nM), D54MG (22.7 nM), LN229 (38.7 nM), LN18 (34.5 nM), U251 (37.0 nM), U87 (38.6 nM), U373 (30.5 nM). Bars represent 95% confidence intervals.

IC_{50} values for ONC212 ranged from minimum of 22.7 nM in D54MG to a maximum of 38.7 nM in LN229. U87 had the second highest IC_{50} of 38.6 nM. NHA had an IC_{50} of 35.5 μM and NHA Ras had an IC_{50} of 34.9 μM.

IC_{50} values for NHA, NHA Ras, and ECLs following treatment with TMZ, ONC201, and ONC212 are summarized in the table below.

Cell line	**TMZ (μM)**	**ONC201 (μM)**	**ONC212 (nM)**
NHA	139	1.22	35.5

NHA Ras	7.77	1.44	34.9
D54MG	5.43	0.56	22.7
LN229	10.1	1.33	38.7
LN18	654	1.31	34.5
U251	58.7	1.14	37.0
U87	24.2	2.24	38.6
U373	88.7	0.944	30.5

Table 2 Toxicity curves were developed after plotting relative absorbance at 490 nm against drug concentration for each of the 8 cell lines (NHA, NHA Ras, U251, U87, U373, LN18, LN229, D54MG). IC_{50} values were calculated from these plots. IC50 values for each cell lines were calculated in response to TMZ, ONC201, and ONC212.

Discussion

Doubling times vary from 0.726 days to 1.365 days in U373. NHA Ras had a significantly short doubling time due to the oncogenic Ras transformation increasing cell proliferation (**Fig. 1A, 1B, Table 2**). TMZ had high IC_{50} concentrations in comparison to ONC201 and ONC212. Cell lines deficient in MGMT (U251, U87, U373, LN229, and D54MG) responded better to TMZ compared to cell lines which had high MGMT expression (LN18).[25-29] NHA Ras responded better to TMZ than NHA due to its short doubling time. Since TMZ acts on the genetic level via guanine methylation, it is able to cause higher levels of cell death in cells with high proliferation rates (**Fig. 2A, 2B**). ONC201 showed greater efficacy than TMZ in all 8 GBM cell lines and had lower IC_{50} values and narrower 95% confidence intervals when compared to TMZ treatments (**Fig.**

2A, 2B, 3A, 3B). These relatively lower IC_{50} values of ONC201 are clinically promising due to the harsh off-target effects associated with TMZ therapy, such as neutropenia, thrombocytopenia, nausea, and fatigue.[2, 5, 6, 30] A recent phase II clinical trial with ONC201 displayed promising results, reporting a median overall survival of 41.6 weeks. Two patients continue to receive ONC201 treatment for greater than 12 months. One of these patients exhibited regression by 85% in one lesion and 76% in another. The second patient remains disease-free following re-resection. The study also reported excellent drug tolerance. [31] Due to the high tolerance of ONC201 at 625 mg every 3 weeks in clinical trials, other studies have begun to explore increased dosage frequencies.[32] It may be worthwhile to consider ONC212, a potent analog of ONC201, as a more effective treatment option.[15, 19, 20]

Certain cell lines were more sensitive to treatments than others. D54MG was the most sensitive cell line in all treatments. U87 was the least sensitive cell line in ONC201 treatments and was the second least sensitive cell lines in ONC212 treatment. Differences in potency between ONC201 and ONC212 range from 58-fold (U87) to 3.4-fold (LN229) (**Table 2**). Both ONC201 and ONC212 had a very narrow range of IC_{50} concentrations across cell lines, indicating increased suitability under one dosage, which is particularly helpful when treating heterogenous tumor types (**Fig. 3A, 3B, 4A, 4B**). Though the exact mechanism of action for ONC212 continues to be evaluated, it is believed to operate as a dual Akt/ERK inhibitor, similar to ONC201.[15, 19, 20]

Conclusion and Future Directions

ONC201 and ONC212 having a narrow range of IC_{50} concentrations for all 8 cell lines can indicate applicability across a wide variety of molecularly variable tumors and the capacity to act as improved therapeutics for GBM in comparison to TMZ. Furthermore, utilizing a combined multi-drug treatment plan can aid in accounting for both intrinsic and extrinsic drug resistance in molecularly heterogenous tumors. Since ONC212 and ONC201 operate as dual Akt/ERK inhibitor, it can be particularly efficacious in tumors with high Akt or ERK expression. Inhibiting both Akt and ERK can help quench potential response that may occur if only one is inhibited due to Akt and ERK being communicating pathways.[15, 19, 20] Future directions include performing similar experiments with glioblastoma stem cells to better emulate tumor structure due to their 3-dimensional shape compared to the adherent cell lines which were used. *In vivo* studies in animals and clinical trials of ONC212, continuing ONC201 clinical trials, as well as multi-drug therapies to evaluate for potential synergistic effects should also be considered. CNS penetrance of ONC212 must also be assessed prior to it being used as a potential treatment option for GBM.

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