Research Square

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Nuclear Receptor 4A1 (NR4A1) and NR4A2 Regulate Expression of O6-Methylguanine-DNA-Methyltransferase (MGMT) in Glioblastoma and Can Be Targeted by NR4A1/2 Ligands

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Abstract

Purpose

Glioblastoma (GBM) is highly malignant and aggressive with limited treatment options. Resistance to the standard of care temozolomide (TMZ) is associated with expression of O^6-methylguanine-DNA-methyltransferase (MGMT). This study investigates whether orphan nuclear receptor 4A1 (NR4A1) and NR4A2 regulate MGMT expression in GBM cells and whether DIM-3,5 compounds decrease MGMT expression and could be used to enhance TMZ efficacy in GBM cells.

Methods

Human and mouse GBM cell lines were used. Cells were treated with DIM-3,5 compounds and TMZ to evaluate cell proliferation. RNA interference was used to knockdown NR4A1/2, Sp1, or MGMT. MGMT mRNA and protein expression was measured using qrt-PCR and Western blotting. Luciferase reporter assays examined MGMT promoter activity, while chromatin immunoprecipitation (ChIP) assays investigated transcription factor interactions on the MGMT promoter.

Results

DIM-3,5 compounds significantly decreased GBM cell proliferation in all cell lines. DIM-3,5 compounds or knockdown of NR4A1/2 or Sp1 significantly decreased MGMT mRNA, protein, and luciferase promoter expression in all cell lines. ChIP analysis demonstrated that NR4A1 and NR4A2 regulate MGMT through interactions with a NBRE motif and Sp1 regulates MGMT through GC-rich promoter regions. Additionally, MGMT knockdown increased TMZ-induced cytotoxicity in GBM cells.

Conclusion

This study shows that NR4A1/2/Sp1 are involved in MGMT regulation through complex mechanisms that include regulation by Sp1 alone and by direct interaction of NR4A1 and NR4A2 as monomers with an NBRE motif. DIM-3,5 ligands act as inverse NR4A1/2 agonists and downregulate MGMT expression in GBM cells.

1. INTRODUCTION

Glioma is the most common and aggressive intrinsic tumor of the adult central nervous system and glioblastoma multiforme (GBM) comprises approximately 57% of patients with glioma [1]. GBM is a highly malignant and aggressive tumor and patients with GBM have poor prognosis with a median survival time of 12–18 months post-diagnosis [1–6]. Current standard of care (SOC) treatments for GBM include temozolomide (TMZ) administered concurrently and after radiation (XRT) [1] or in combination

with tumor treating fields [7]. TMZ is an orally administered alkylating agent that readily crosses the blood-brain barrier [1], **to** induce DNA methylation including formation of the highly toxic O^6-methyl-guanine adduct. The effectiveness of TMZ is modified by O^6-Methylguanine-DNA-methyltransferase (MGMT), a methyltransferase suicide enzyme, which catalyzes removal of the cytotoxic methyl group transferred to a cysteine residue [1, 6]. For combined SOC TMZ + XRT treatment, the median survival time (MST) of patients with methylated versus unmethylated MGMT promoter was 21.7 versus 12.7 months [13]. Despite improved outcomes consistently and strongly linked to increased MGMT gene promoter methylation [8–13], the ~ 50% of GBM patients with unmethylated MGMT promoters continue to receive TMZ based SOC treatments. This clinical practice reflects the lack of proven alternative treatments for unmethylated GBM and the complex, poorly understood functional relationships between degree of methylation, MGMT gene/protein expression and TMZ activity [14]. However, a recent study that demonstrated enhanced TMZ responses in GBM using EGFR inhibitors supports the potential clinical value of approaches that inhibit MGMT expression and which may have particular relevance for MGMT promoter unmethylated patients [15].

Alternative therapies for GBM including immunotherapy [16–21] and various anti-angiogenic, kinase targeted and combined molecular therapies [22–26] have all failed to achieve outcomes superior to current TMZ based SOC. Therefore, identifying approaches that overcome mechanisms of TMZ resistance could provide more immediate and meaningful improvements in GBM outcomes. Research in our laboratories [27, 28] has been focused on development and applications of novel ligands that target the pro-oncogenic orphan nuclear receptor 4A1 (NR4A1) and NR4A2 (NR4A1/2) that are overexpressed in GBM patients [27–29]. The ligands are bis-indole derived compounds (CDIMs) and include 1,1-bis(3'-indolyl)-1-(3,5-disubstitutedphenyl)methane (DIM-3,5) analogs that bind both NR4A1 and NR4A2 and act as dual receptor inverse agonists to inhibit NR4A1/2-regulated pro-oncogenic genes/pathway in solid tumor-derived cancer cells including glioblastoma cells [28, 30–32]. For example, DIM-3,5 analogs inhibited GBM cell and tumor growth and decreased expression of the NR4A1/2-regulated TWIST1 gene that plays a role in epithelial-mesenchymal transcription (EMT) [28]. Of importance,

EMT associated signaling is implicated in resistance to TMZ in GBM [33]. In addition, compounds like resveratrol and other polyphenolics that exhibit activities similar to DIM-3,5 ligands in GBM cells [34–36], function as NR4A1 ligands [37–40] and previous studies have reported that they downregulate MGMT expression [34–36].

Given the central importance of MGMT in TMZ chemosensitivity, here we used genetic and pharmacologic loss of function approaches to determine whether NR4A1 and NR4A2 regulate MGMT expression in GBM cells. Our study showed for the first time that MGMT expression is regulated by both receptors that can be targeted by DIM-3,5 dual NR4A1/2 ligands. Further, inhibition of MGMT gene expression enhanced TMZ cytotoxicity in GBM cells. Unlike our prior examination of TWIST1 regulation which demonstrated direct NR4A1/2 interactions with promoter DNA-bound Sp1, here we observed independent regulation of MGMT expression by NR4A1/2 and Sp1 through distinct NBRE and GC-rich DNA binding motifs, respectively. Together these results establish NR4A1 and NR4A2 as candidate

therapeutic targets to overcome TMZ resistance through inhibition of MGMT expression in GBM. These studies provide strong rationale for establishing the clinical relevance of targeting NR4A1/2 as an adjunct to potentiate TMZ responses and better define the role of MGMT expression and other drug-resistant genes on TMZ resistance.

2. MATERIALS AND METHODS

2.1. Cell lines, reagents, and ligands

Human GBM cell lines T98G and A172, together with the mouse glioma cell lines CT-2A and Ha-Ras/AKT, used in this study have been described previously [28]. Human T98G, A172, and mouse CT-2A cells were cultured in DMEM (Gibco; Cat. No. 11-965-175) supplemented with 10% FBS (Gibco; Cat. No. A4736301). Ha-Ras/AKT cells were maintained in serum-free DMEM/F12 (Corning; Cat. No. 10-090-CV) proliferation medium supplemented with 1X N2 Supplement, 2 mM glutamine, 50 µg/mL heparin, and the growth factors EGF and bFGF. All cell lines were maintained at 37°C in a humidified incubator containing 5% CO_2. The bis-indole-derived DIM-3,5 analogs, including DIM-3,5-Cl_2, DIM-3,5-$(tBu)_2$, and DIM-3-Cl-5-CF_3, previously described by our group, were used in this study [28–32]. Temozolomide (TMZ) was purchased from MedChemExpress (Cat. No. HY-17364). The final concentration of DMSO in the culture medium was maintained below 0.15% (v/v) for all treatments. All treatments were performed in at least three independent experiments.

2.2. Cell proliferation assay

For cell proliferation studies, human and mouse GBM cells were seeded at a density of 2×10^4 cells per well in 96-well plates containing the appropriate culture medium as previously described [28]. Cells were allowed to adhere overnight and were subsequently treated with increasing concentrations of bis-indole compounds (1, 2.5, 5, 7.5, 10, 15, and 20 µM) or temozolomide (TMZ; 125, 250, 500, and 1000 µM) for 24 hours. Cell viability was then determined using the XTT Cell Viability Kit (Cell Signaling Technology, Cat. No. 9095) according to the manufacturer's instructions. Cell survival was quantified by measuring absorbance at 450 nm using a microplate reader (BioTek Cytation 5, Agilent) and expressed as the percentage of viable cells relative to untreated control. Each treatment was performed in three independent experiments. Cell cultures were routinely tested for mycoplasma contamination.

2.3. Western blotting

Cells were seeded at a density of 3×10^5 cells per well in 6-well plates. Following 24-hour treatment with bis-indole compounds or TMZ or 72-hour treatment with siRNAs, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (ThermoFisher Scientific; catalog #89901) containing protease and phosphatase inhibitors (GenDEPOT; catalog #P3100 and #P3200). Whole-cell lysates were analyzed using western-blotting as previously described [28, 31]. A list of antibodies used in this study is provided in **Supplemental Table 1**.

2.4. Small RNA interference assay

Transient transfections were performed using Lipofectamine RNAiMAX according to the manufacturer's protocol. Twenty-four hours after cell seeding, transfection complexes containing small interfering RNA (siRNA) or endoribonuclease-prepared RNAs (esiRNA), Lipofectamine RNAiMAX (Invitrogen; catalog #56531), and Opti-MEM (Gibco; catalog #31985-062) were prepared and added to the cells following the manufacturer's instructions. Cells were harvested 48–72 hours after transfection for protein and RNA analyses. The cross-reactive siRNAs used for this study are siNR4A1 (SASI_Mm01_00077215- NR4A1_C, SASI_Mm01_00077216- NR4A1_D), siNR4A2 (SASI_Hs02_0034_1056-NR4A2-A; referred to as NR4A2-1, SASI_Hs02_0034_1057-NR4A2-B; referred to as NR4A2-2) and siSp1 (SASI_Hs02_00333289-Sp1-A; referred to as Sp1-1, SASI_Hs02_00070994-Sp1-B; referred to as Sp1-2). The esiRNAs used in this study were MISSION esiRNA targeting human MGMT and MISSION esiRNA targeting mouse MGMT (Sigma-Aldrich; catalog number EHU033391 and EMU041001 respectively; both referred to as siMGMT).

2.5. Quantitative real-time polymerase chain reaction

For gene expression studies, three independent biological experiments were performed, and total RNA was isolated using the Macherey-Nagel NucleoSpin RNA Plus Kit (catalog #740984.50). RNA samples were treated with TURBO DNA-free (Invitrogen) to eliminate residual genomic DNA contamination. First-strand cDNA was synthesized using MultiScribe Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed using amfiSure qGreen Q-PCR Master Mix (GenDEPOT) on a Bio-Rad CFX384 Real-Time PCR System. Relative gene expression was determined using the ΔΔCt method. β-Actin served as the reference gene for normalization of cDNA input in all cell lines. Each qRT-PCR assay included at least three biological replicates and three technical replicates. Primer sequences are listed in **Supplemental Table 2**.

2.6. Plasmid Transfection and Luciferase assay

Cells were seeded in 24-well plates at a density of 2×10^4 cells per well and allowed to attach for 24 hours. The promoter constructs hMGMT-(luc) (VB250311-1422zaz) and mMGMT-(luc) (VB250807-1251gds) were purchased from VectorBuilder and transfected using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions. 8 hours after transfection, the medium was replaced, and cells were allowed to recover for an additional 16 hours. Cells were then treated with DMSO or 10 μM CDIM in DMSO for 24 hours or transfected with small interfering RNAs (siRNAs) for 48 hours. Cells were lysed using a freeze-thaw protocol, and luciferase activity was measured with the Firefly Luciferase Assay Kit 2.0 (Biotium, Cat. No. 30085-2) according to the manufacturer's instructions. Luciferase activity was normalized to total protein content. Each experiment was performed in triplicate.

2.7. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed using the ChIP-IT Express Kit (Active Motif, Cat. No. 53008). Human T98G or mouse CT-2A cells were seeded at a density of 3×10^7 cells per plate. 24 hours after seeding, cells were treated with DMSO or 10 μM DIM-3-Cl-5-CF_3 for 6 hours and processed according to the manufacturer's protocol. Cells were fixed, washed, and lysed, and nuclei were isolated prior to sonication to obtain chromatin fragments of the desired size. Sonicated chromatin

was immunoprecipitated overnight using protein G-conjugated magnetic beads. IgG, NR4A1, NR4A2, and Sp1 antibodies were used for immunoprecipitation. Following reverse cross-linking and proteinase K digestion, DNA was purified using the Chromatin IP DNA Purification Kit (Active Motif, Cat. No. 58002). Purified DNA was analyzed by quantitative real-time PCR using amfiSure qGreen Q-PCR Master Mix (GenDEPOT). Potential NR4A and Sp1 binding sites were identified using JASPAR (https://jaspar.elixir.no/) and eukaryotic promoter database (https://epd.expasy.org/edp/) and unique primers were used for the two isolated regions along the promoter. The antibodies and primer sequences used for amplification of MGMT promoter regions are listed in **Supplemental Table 1** and **Supplementary Table 3**.

2.8. DNA Methylation analysis

96-well microplate-based Matrix methylated DNA immunoprecipitation (Matrix-MeDIP) of ssDNA was done as previously described where 5mC antibody was attached to wall wells via Protein A. Well walls were blocked with 5% BSA. PIXUL (A 96-well plate sample preparation sonicator for multi-omics applications (Matchstick Technologies, Inc, Kirkland, WA and Active Motif, Carlsbad, CA [41]) fragmented ssDNA was used as input. After washes, immunocaptured ssDNA was recovered by incubation with Proteinase K (200ng/μL) in 100μL/well of elution buffer (55°C for 45min, 95°C for 10min, then 4°C). qPCR was then performed with the appropriate primers [42, 43]. All buffers, primers, and antibodies used for analysis can be found in **Supplementary Tables 1 and 4** and the results are found in **Supplementary Fig. 1.**

2.9. Statistical analysis

All of the experiments were carried out in triplicate. Data are expressed as the mean ± SD. For statistical significance, One-way analysis of variance (ANOVA with Dunnett's) was used for experiments with multiple groups and multiple-unpaired t-tests (Holm-Šídák method) was used for two-group comparisons. IC50 values were calculated using a non-linear regression model with variable slope, commonly referred to as the 4-parameter logistic (4PL) equation. P-values ≤ 0.05 were considered statistically significant.

3. RESULTS

The major objectives of this study are to determine the molecular mechanisms of NR4A1/2-dependent regulation of MGMT gene expression in GBM cells and determine whether DIM-3,5 ligands downregulate MGMT expression and if the loss of MGMT affects the cytotoxicity of TMZ in GBM cells. Therefore, we initially investigated the growth inhibitory effects of DIM-3,5 analogs that are dual NR4A1/NR4A2 ligands and the cytotoxicity of TMZ in GBM cells. Results in Figs. 1a and 1b summarize the growth inhibitory effects of DIM-3,5-Cl_2, DIM-3-Cl-5-CF_3 and DIM-3,5-$(tBu)_2$ on human T98G and A172 GBM cell lines. GBM cells were treated with 1–20 μM of the DIM-3,5 ligands and cell growth was inhibited at concentrations ≥ 7.5 μM and IC_{50} values for each compound in T98G and A172 cells were 11.87 to 20.47 μM, respectively. Using the same approach, we observed that the DIM-3,5 analogs also inhibited growth of mouse CT-2A

and Ha-Ras/AKT GBM cells (Figs. 1c and 1d) and IC50 values were 6.97 to 13.15 μM, respectively. In contrast, concentrations from 125 to 1,000 μM of TMZ only significantly inhibited growth of the human cell lines at the highest concentrations (Figs. 1e and 1f). Similar results were observed in mouse CT-2A and Ha-Ras/AKT GBM cells except that there was a non-significant decrease in cell growth in mouse GBM cells using 500 μM TMZ.

The effectiveness of DNA alkylating agents such as TMZ in GBM is attenuated by development of drug resistance genes and this includes enhanced expression of the functional non-methylated DNA repair enzyme MGMT. Several studies report that MGMT expression in GBM cells is downregulated by resveratrol and other polyphenolics [37–39] and a subsequent study reported that resveratrol was an NR4A1 ligand [36]. Therefore, we further investigated the role of NR4A1 and NR4A2 in regulating MGMT expression and the inhibitory effects of DIM-3,5 ligands. Results illustrated in Figs. 2a and 2b show that DIM-3,5 ligands significantly inhibited expression of MGMT protein in human T98G and A172 GBM cells and DIM-3,5-Cl_2 was the most potent ligand significantly inhibiting MGMT expression at a concentration of 5 μM in both cell lines. The DIM-3,5-ligands also decreased levels of MGMT protein in mouse CT-2A and Ha-Ras/AKT GBM cells, and the Ha-Ras/AKT cells were slightly more sensitive to the effects of DIM-3,5-Cl_2 and DIM-3-Cl-5-CF_3 with DIM-3,5-$(tBu)_2$ being the least potent ligand. We also investigated the effects of DIM-3,5 analogs on MGMT mRNA levels and Figs. 2e and 2f show that after treatment with the DIM-3,5 analogs for 4 and 8 hours MGMT mRNA expression is decreased in human T98G and A172 GBM cells. Using the same protocol, the DIM-3,5 ligands also decreased MGMT mRNA levels in mouse CT-2A and Ha-Ras/AKT cells after treatment for 4 and 8 hours.

The TWIST1 gene is regulated by NR4A1, NR4A2 and Sp1 in GBM cells [28] and it was previously reported that Sp1 also regulated MGMT expression in GBM cells [44–46]. Figures 3a and 3b illustrate that NR4A1, NR4A2 and Sp1 are expressed in human T98G and A172 cells and oligonucleotides targeting these receptors decreased their expression. Figures 3c and 3d show that knockdown of NR4A1, NR4A2 and Sp1 by RNA interference also significantly decreased levels of MGMT protein in both human cell lines. In addition, we also observed that knockdowns of NR4A1, NR4A2 and Sp1 in T98G (Figs. 3e-3g) and A172 (Figs. 3h-3i) cells decreased MGMT mRNA levels. The effects of NR4A1, NR4A2 and Sp1 knockdown on MGMT expression were also investigated in mouse CT-2A and Ha-Ras/AKT GBM cells. NR4A1, NR4A2, and Sp1 are expressed in mouse CT-2A (Fig. 4a) and Ha-Ras/AKT (Fig. 4b) cells and decreased after knockdown with receptor-targeting oligonucleotides. These oligonucleotides also decreased levels of MGMT protein in CT-2A (Fig. 4c) and Ha-Ras/AKT cells (Fig. 4d). Furthermore, we also observed that knockdown of NR4A1, NR4A2 and Sp1 in CT-2A (Figs. 4e-4g) and Ha-Ras/AKT (Figs. 4h-4j) decreased MGMT mRNA levels similar to the results observed in the human GBM cells (Fig. 3).

Figures 5a and 5b illustrate the human (pMGMT^{h}-luc) and mouse (pMGMT^{m}-luc) constructs containing the − 970 to 0 and − 1050 to 0 regions of the MGMT promoters respectively. Both constructs contain similar GC-rich and NBRE motifs in their respective promoters and in human T98G and A172 cells transfected with pMGMT^{h}-luc the DIM-3,5 ligands significantly decreased luciferase activity (Fig. 5c and

5d). The DIM-3,5 ligands also significantly decreased luciferase activity in mouse CT-2A and Ha-Ras/AKT GBM cells transfected with the pMGMT^{m}-luc construct (Fig. 5e and 5f). Knockdown of NR4A1, NR4A2 and Sp1 in human T98G cells followed by transfection with the pMGMT^{h}-luc construct also resulted in decreased luciferase activity compared to cells transfected with the siControl (non-specific oligonucleotide) (Fig. 5g). Similar results were observed in mouse CT-2A cells (Fig. 5h) confirming that the responsiveness of the human and mouse MGMT promoter constructs to treatment with DIM-3,5 ligand and knockdown of NR4A1, NR4A2 and Sp1 was similar to that observed for their effects on expression MGMT mRNA and protein (Figs. 2–4).

ChIP assays were used to investigate the association of NR4A1, NR4A2 and Sp1 with the NBRE and GC-rich regions of the MGMT promoter in human T98G (Fig. 6a) and mouse CT-2A (Fig. 6b) cells and primers targeting both motifs are indicated (→ ←). The results showed that NR4A1, NR4A2 and Sp1 were associated with both the NBRE (Fig. 6c) and GC-rich (Fig. 6d) motifs in T98G cells treated with DMSO (control). Treatment with DIM-3,5-Cl_2 did not decrease Sp1 association with the NBRE region of the promoter but significantly decreased levels of NR4A1 and NR4A2 suggesting that NR4A1 and NR4A2 may be independently regulating MGMT through interactions with the NBRE motif (Fig. 6c). Results of the ChIP assay in the GC-rich region of the human MGMT promoter showed that treatment with DIM-3,5-Cl_2 decreased Sp1 but not NR4A1 and NR4A2 association with this region of the MGMT promoter. The treatment related effects on the association of NR4A1, NR4A2 and Sp1 with the NBRE and GC-rich regions of the mouse MGMT promoter (Fig. 6d) were similar to those observed for the human MGMT promoter (Fig. 6c). Previous studies on several genes regulated by NR4A1/Sp and NR4A2/Sp including TWIST1, MerTK, G9a and β1-integrin show that in the ChIP assays on the GC-rich Sp1 binding promoter region that DIM-3,5 ligands decreased both NR4A1 and NR4A2 promoter interactions [28, 31, 32] and this was not observed in this study for MGMT (Figs. 6c and 6d) suggesting the regulation of MGMT expression by Sp1 may not require NR4A1 or NR4A2 as cofactors.

The functional relationship between the degree of MGMT methylation and gene expression remains poorly understood, limiting the ability to develop treatments for patients of varying methylation status [14]. Therefore, we assessed methylation across selected MGMT promoter regions in T98G and A172 cells using previously described primer targets (**Supplementary Fig. 1a**) [42, 43]. Matrix-MeDIP analysis demonstrated that both cell lines exhibited methylation at − 363 bp (**Supplementary Fig. 1b**). In contrast, the cell lines differed in methylation at enhancer site 1 and exon 5 (**Supplementary Figs. 1c–d**), while exhibiting negligible methylation at enhancer site 3 (**Supplementary Fig. 1e**). These findings demonstrate several differences in MGMT methylation in our human T98G and A172 GBM cells, however the expression of MGMT remained detectable and resistance to TMZ was still observed (Figs. 1 and 2).

These results uncover a molecular mechanism demonstrating that both NR4A1 and NR4A2 regulate expression of MGMT in GBM cells. Therefore, the functional role of MGMT in TMZ resistance was further investigated in GBMs cell treated with TMZ in the presence or absence of MGMT knockdown by RNA interference. Knockdown of MGMT in T98G cells resulted in a 11.2% decrease in cell growth however the loss of MGMT significantly enhanced the cytotoxicity of 125–500 μM TMZ in this cell line (Fig. 7a).

Similar results were observed in A172 cells except that in untreated cells, the loss of MGMT alone did not significantly decrease cell proliferation (Fig. 7b). The effects of MGMT loss in mouse CT-2A and Ha-Ras/AKT cells did not significantly affect growth of these cells but did significantly enhance the cytotoxicity of TMZ (Fig. 7c and 7d). Figures 7e, 7f, 7g and 7h illustrate the effectiveness of knockdown MGMT in the 4 GBM cell lines used in this study. Thus our results demonstrate that MGMT expression is regulated by both NR4A1 and NR4A2 in both human and mouse GBM cells and MGMT can be downregulated by DIM-3,5 dual NR4A1/2 ligands to enhance the cytotoxicity of TMZ in human and mouse GBM cells.

4. DISCUSSION

NR4A1 and NR4A2 are immediate early genes induced by multiple stressors including inflammation and oxidative stress and there is evidence that NR4A1 plays a role in aging and age-related decreases [47, 48]. For example, NR4A1 expression decreases with age in humans and rodents and loss of NR4A1 in mice decreases lifespan and resistance to tissue injury; in wild type mice synthetic NR4A1 ligands such as cytosporone B and CDIM compounds protect against tissue damage [49]. In contrast, both NR4A1 and NR4A2 are overexpressed in multiple solid tumors and exhibit comparable pro-oncogenic activities and regulate genes associated the tumor growth, survival, EMT and migration/invasion [50]. Studies in our laboratories reported that the EMT gene TWIST1 was regulated by NR4A1 and NR4A2 in GBM cells and this involved both receptors acting as cofactors of DNA-bound Sp transcription factors [28]. The NR4A/Sp transcription factor complex regulates expression of several pro-oncogenic genes in solid tumor cells including G9a, several integrins, survivin, receptor tyrosine kinases and other nuclear receptors regulate gene expression by acting as a cofactor for Sp1 [28, 30–32, 48].

Ongoing studies in our laboratories on glioblastoma are focused, in part, on the potential efficacy of novel DIM-3,5 inverse NR4A1/2 ligands in combination with other therapeutics including TMZ and immunotherapies. The development of drug resistance to TMZ due to MGMT and other genes, coupled with previous reports showing that resveratrol, later identified as an NR4A1 ligand, downregulated MGMT expression in GBM cells, suggested that MGMT may be an NR4A regulated gene. Initial studies with DIM-3,5 ligands showed that these compounds inhibited growth of two human and two mouse GBM cell lines at concentrations ≥ 5–10 μM whereas the growth inhibitory effects observed for TMZ were observed at concentrations ≥ 500-1,000 μM. Subsequent studies show that DIM-3,5 ligands or knockdown of NR4A1 or NR4A2 resulted in decreased expression of MGMT in mRNA and protein in GBM cell lines and similar results were observed using MGMT-luciferase constructs where the same treatments decreased luciferase activity.

Previous reports show the importance of Sp1 in regulating expression of MGMT [44–46, 51] and analysis of the human and mouse gene promoters show that these promoters not only contain GC-rich Sp1 binding sites, but also an NBRE motif that binds NR4A monomers. ChIP analysis of the human and mouse promoters show that NR4A1, NR4A2 and Sp1 are associated with both the GC-rich and NBRE sites but are differentially decreased in cells treated with DIM-3,5-Cl_2. The results clearly demonstrate

that all 3 transcription factors play a role in regulating expression of MGMT however their interactions at both sites may involve both NR4A/Sp whereas NR4A1 and NR4A2 (as monomers) may independently regulate MGMT expression from the NBRE site.

Additionally, previous studies demonstrate that MGMT promoter methylation is region-specific, with methylation in Differentially Methylated Region 1 (DMR1; -250 bp to -130 bp) and particularly DMR2 (+ 90 bp to + 180 bp) more strongly associated with reduced MGMT expressions than other CpG sites [52–53]. Although methylation within the − 395 to − 352 bp region has also been linked to MGMT expression, its association is weaker [54], consistent with the findings of the Matrix-MeDIP assay that both cell lines exhibited methylation at − 363 bp while maintaining MGMT expression and TMZ resistance **(Sup. Figure 1b;** Figs. 1–2**)**. These findings suggest that methylation at this individual site is insufficient to repress MGMT transcription. Thus our study shows DIM-3,5 treatment decreases transcription factor occupancy, promoter activity, and MGMT expression demonstrating that MGMT is an NR4A1/2 regulated gene in GBM **(**Figs. 2, 5–6**).**

In summary, this study shows the NR4A1/NR4A2 coregulate expression of the MGMT gene in GBM cells and not surprisingly knockdown of MGMT by RNA interference enhances the cytotoxicity of TMZ in GBM cells. Currently our ongoing studies are investigating the interactions of DIM-3,5 ligands and TMZ to determine their additive or synergistic cytotoxicity interactions in cell culture and in vivo. In addition, we are investigating the mechanisms of DIM-3,5-mediated enhancement of the cytotoxicity of TMZ by their downregulation of other NR4A1/2-regulated genes such as G9a and β1-integrin which have also been linked to TMZ drug resistance in GBM [50, 55–56].

Declarations

Running Title

DIM-3,5 NR4A1/2 ligands downregulate MGMT in glioblastoma

Declarations

Ethics approval and consent to participate:

Institution approval of the studies was provided by Texas A&M University Institutional Animal Care and Use Committee (IACUC) and recently renewed (IACUC 2026-0052).

Consent for publication:

Not applicable.

Competing Interest:

The authors have no relevant financial or non-financial interests to disclose.

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Author Contribution

Conceptualization, E.F., A.M., R.R., & S.S.; formal analysis, E.F., S.U., A.R.O, G.S., W.N.T.T., S.M., K.B., & D.M.; writing—original draft preparation, E.F., & S.S.; writing—review and editing, E.F., S.A.M, A.M., R.R., & S.S.; supervision, S.S.; project administration, R.R., A.M., & S.S.; funding acquisition, R.R., A.M., & S.S. All authors read and approved of the final manuscript.

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Data Availability

All data generated or analyzed during this study are included in this published article and its supplementary information files. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request (S.S.).

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Unsectioned Paragraphs