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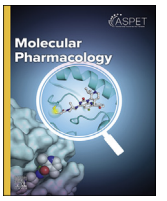

ARTICLE

# Dual nuclear receptor 4A1 (NR4A1/NR4A2) ligands inhibit glioblastoma growth and target TWIST1

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ABSTRACT

1,1-Bis(3′-indolyl)-1-(3,5-disubstitutedphenyl)methane (DIM-3,5) compounds are dual receptor ligands that bind both orphan nuclear receptor 4A1 (NR4A1) and NR4A2. Knockdown of *NR4A1* or *NR4A2* by RNA interference in glioblastoma (GBM) cells decreased growth and induced apoptosis and comparable effects were observed for DIM-3,5 analogs, which exhibit inverse agonist activity and inhibit NR4A1- and NR4A2-mediated pro-oncogenic activity. Knockdown of *NR4A1* or *NR4A2* or treatment with DIM-3,5 analogs also decreased expression of *TWIST1* mRNA and protein in GBM cells by 40%–90%.The proximal region of the *TWIST1* gene promoter contains functional GC-rich binding sites that bind Sp1 and Sp4, and knockdown of these transcription factors also decreased TWIST1 expression in GBM cells. Further analysis by chromatin immunoprecipitation, protein-protein coimmunoprecipitation, and binding assays demonstrated that NR4A1/NR4A2 coregulate *TWIST1* gene expression as ligand-dependent cofactors of Sp1 and Sp4, which interact with cis proximal GC-rich sites in the *TWIST1* gene promoter. In vivo studies show that DIM-3,5 dual NR4A1/2 inverse agonists also reduced intratumoral TWIST1 expression while significantly prolonging survival of mice in a syngeneic mouse model of GBM, demonstrating that these ligands are promising new agents for targeting TWIST1 and treating GBM.

*Significance Statement:* The *TWIST1* gene is a pro-oncogenic factor that regulates epithelial-to-mesenchymal transition in glioblastoma cells. This paper shows that the orphan nuclear receptor 4A1 (NR4A1) and NR4A2 regulate TWIST1 expression, which can be targeted by bis-indole–derived dual NR4A1/2 inverse agonists.



## 1. Introduction

The orphan nuclear receptors NR4A1, NR4A2, and NR4A3 are immediate early genes induced by multiple stressors that play an important role in maintaining cellular homeostasis and pathophysiology. There is increasing evidence for the role of these receptors in metabolic, immune, inflammatory, cardiovascular, and neurological dysfunctions and cancer (Pearen and Muscat, 2010; Safe et al, 2014). NR4A subfamily receptors were initially defined as nerve growth factor–induced-$\beta$ receptors that bind as monomers to an nerve growth factor–induced-$\beta$ response element (AAAGGTCA). NR4A receptors also bind as a homodimer to a Nur-responsive element (TGATATTACCTCCAAATGCCA) that has been characterized in the pro-opiomelanocortin gene promoter, and NR4A1 also forms a complex with RXR to modulate gene expression (Philips et al, 1997; Maira et al, 1999). In addition, studies in this laboratory have shown that NR4A1 also activates gene expression through interactions with the DNA-bound Sp1 and Sp4 transcription factors through formation of an NR4A1/p300/Sp complex. For example, survivin; $\beta$1-, $\alpha$5-, and $\beta$3-integrins; and PD-L1 are regulated by NR4A1/Sp1 in breast cancer cells, whereas $\alpha$6-integrin,

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PAX3-FOXO1, and $\beta$1-integrin are regulated by NR4A1/Sp4, indicating that effects of NR4A1/Sp1 and NR4A1/Sp4 in breast cancer and rhabdomyosarcoma cells, respectively, are both gene-dependent and cell context–dependent (Lee et al, 2010; Lacey et al, 2016; Hedrick et al, 2017; Lacey et al, 2017). NR4A1 is overexpressed in lung, colon, liver, and breast cancer patients and in rhabdomyosarcoma and is a negative prognostic factor for lung, breast, and colon cancer patient survival (Wu et al, 2011; Lee et al, 2012; Muscat et al, 2013; Wang et al, 2014; Zhou et al, 2014).

The functional activity of NR4A1 and NR4A2 in cancer has been extensively investigated in cancer cell lines by either knockdown or overexpression. NR4A1 regulates proliferation, survival, cell cycle progression, migration, and invasion in lung, melanoma, lymphoma, pancreatic, colon, cervical, ovarian, and gastric cancer cell lines, and similar functions have been observed for NR4A2 (Safe et al, 2014; Safe and Karki, 2021). Thus, the pro-oncogenic activities of NR4A1 and NR4A2 indicate that both receptors are potential drug targets. Studies in our laboratories have shown that a series of bis-indole–derived compounds (CDIMs) bind NR4A1 or NR4A2 and act as inverse agonists to inhibit cancer cell and tumor growth (Karki et al, 2021). Structure-activity studies initially identified various CDIM analogs as NR4A1 inverse agonists (Safe and Karki, 2021), and a third generation set of 1,1-bis(3′-indolyl)-1-(3,5-disubstitutedphenyl)methane (DIM-3,5) analogs were the most potent (Upadhyay et al, 2024) and inhibited tumor growth in athymic nude mouse xenograft models at doses <1 mg/kg/day (Karki et al, 2021). CDIMs containing only a 4-chloro or 4-bromo group (DIM-4-CI/DIM12 or DIM-4-Br) interact with a cofactor binding site on NR4A2 and act as inverse NR4A2 agonists (Hammond et al, 2018; Karki et al, 2020), which are defined as a receptor ligands that decrease receptor-regulated constitutive/basal activity.

NR4A1 and NR4A2 are expressed in glioblastoma (GBM) tumors and cells, and recent studies show that DIM-4-CI inhibits GBM cell growth, survival, and migration and tumor growth in vivo (Karki et al, 2020). Studies also show that NR4A1 and NR4A2 are potential druggable targets in GBM; however, the effects of inverse NR4A1 agonists on GBM cells have not been reported. We have recently demonstrated that the potent DIM-3,5 analogs bind both NR4A1 and NR4A2 and are novel dual receptor ligands (Upadhyay et al, 2024). This study investigates the role of NR4A1 and NR4A2 and effects of dual NR4A1/2 ligands on GBM cells and tumors. As we previously demonstrated a critical role for the bHLH transcription factor TWIST1, a key gene that promotes GBM invasion through mesenchymal change, we hypothesized that the tumor suppressive effects of NR4A ligands may also be associated with inhibition of TWIST1 (Elias et al, 2005; Mikheeva et al, 2010; Mikheev et al, 2015, 2017, 2018).

## 2. Materials and methods

### 2.1. Cell Lines, reagents, and ligands

Human GBM cell lines T98G, A172, CT2A, and U87MG were purchased from the American Type Culture Collection and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS (Gibco/Invitrogen). Mouse glioma cell lines Ras/Akt and Ras/shp53 were transformed by overexpression of Ha-RasV12 and constitutively active AKT1 or p53 knockdown, respectively, in mouse neuroprogenitor cells isolated from brains of transgenic mice harboring the *mTmG* transgene. Mouse glioma cell lines Ras/Akt, Ras/shp53, and CT2A used in this study have been previously described (Tanaka et al, 2011; Cilibrasi et al, 2017; Park et al, 2017; Feng et al, 2018). Mouse CT2A cells were maintained in DMEM supplemented with 10% FBS. Ras/Akt and Ras/shp53 cells were grown in serum-free proliferation media DMEM/F12 supplemented with 2 mM glutamine, 50 $\mu$g/mL heparin, and growth factors EGF and bFGF. All cells were maintained at 37 °C in the presence of 5% $CO_2$. Bis-indole–derived compounds DIM-3,5 analogs DIM-3,5-$Cl_2$, DIM-3,5-$Br_2$, and DIM-3-Cl-5-$CF_3$, reported earlier, were used for this study (Karki et al, 2021; Upadhyay et al, 2024). The solvent (DMSO) was always less than 0.15% by volume in the medium. For each treatment, 3 independent experiments were carried out. Antibodies used in cell culture experiments include Sp1 (sc-17824), Sp4 NR4A1 (sc-365113X), NR4A2 (sc-376984X and sc-81345), TWIST1 (sc-81417), IgG (sc-2025) from Santa Cruz Biotechnology; PARP (9532), CPARP (9541S), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5174S), secondary anti-rabbit horseradish peroxidase (707HS and 7076S) from Cell Signaling Technology; IgG (mouse) (AB18413), and NR4A1 (ab109180) antibodies from Abcam.

### 2.2. Cell proliferation assay

For cell proliferation studies, human and mouse glioma cells were seeded at a density of $2 \times 10^4$ cells/well in a 96-well plate with appropriate culture medium. Cells were incubated overnight and then treated with different concentrations of bis-indole compounds (2.5, 5, 10, and 20 $\mu$M) for 24 hours. After 24 hours, the XTT assay was carried out using the XTT Cell Viability Kit (Cell Signaling Technology, catalog number 9095). The percentage of cell survival was calculated after measuring the absorbance at 450 nm wavelength in a plate reader. For each treatment, 3 independent experiments were carried out. Cells were routinely tested for mycoplasma contamination.

### 2.3. Scratch assays

Human U87MG and mouse CT2A cells were seeded at a density of $3 \times 10^5$ cells/well in DMEM supplemented with 10% FBS. After attaining 80% confluency, the medium was removed, and a scratch was made on the surface using a sterile pipette tip. Cells were then treated with the medium that contained either DMSO or the desired concentration of bis-indole compounds. Cell migration into the scratch was determined after 24 hours, with the images taken using the EVOS digital inverted microscope (ThermoFisher) before and after the treatment. Images were analyzed using the ImageJ/Fiji (Wiki-based) wound healing size tool.

### 2.4. Luciferase assay

Cells were plated on 24-well plates at a density of $3 \times 10^4$/well for 24 hours. NR4A1- and NR4A2-dependent transactivation were carried out using GeneJuice as a transfection reagent (Sigma-Aldrich) as previously described (Upadhyay et al, 2024). For NR4A1-dependent transactivation, cells were cotransfected with UAS×5-luc (300 ng), GAL4-NR4A1 (300 ng), and $\beta$-gal (50 ng). Similarly, for NR4A2-dependent transactivation, cells were cotransfected with UAS×5-luc (300 ng), GAL4-NR4A2 (300 ng), and $\beta$-gal (50 ng). After 24 hours, the medium was removed, and cells were treated with DMSO and 15 $\mu$M of CDIM for 24 hours. Cells were lysed using a freeze–thaw protocol, and luciferase assays were performed using the Luciferase Assay System (Promega) according to the manufacturer's instructions and normalized to $\beta$-gal activities. Each experiment was conducted in triplicate. For the Twist-dependent transactivation study, pTWIST1 W(−209/131)-luc (TWwt) and the mutant pTWIST1m(−209/131)-sub-luc (TWmt) with substitution of CT to AG (−88/−87) was obtained commercially (VectorBuilder Inc).

### 2.5. Coimmunoprecipitation assay

The coimmunoprecipitation assay was carried using Dynabeads Protein G and Protein A (Invitrogen: 10003D and 10001D) following the manufacturer's protocol. Dynabeads Protein G was used for coupling with anti-NR4A1 (Santa Cruz Biotechnology, sc-365113X) antibody and Dynabeads Protein A for coupling with anti-NR4A2 antibodies (sc-376984X and sc-81345). Mouse IgG (sc-2025) with beads only was used as control. Protein lysate was extracted from U87MG in lysis buffer (25 mM HEPES, pH 7.5, 300 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride and 1 tablet proteinase cocktail inhibitor/30 mL (Roche; 11836170001). Protein was eluted with 0.1 M glycine HCl at pH 3.5 and immediately neutralized with 0.5 M Tris-HCl pH 7.5. The total cell lysate and coimmunoprecipitated samples were then used to detect SP1and SP4 on SDS gel for western blot analysis using the anti-SP1 and anti-SP4 antibodies.

### 2.6. Western blotting

Cells were seeded on 6-well plates. After treatments, cells were lysed with radioimmunoprecipitation assay buffer that contained protease and phosphatase inhibitors. Equal amounts of protein were loaded on 4%–20% Mini-PROTEAN TGX Gels (Bio-Rad, 4561094). Proteins were then transferred to a polyvinylidene difluoride membrane using Bio-Rad Trans Blot Turbo Transfer System. Blots were blocked in 5% milk followed by primary and secondary antibody incubation and treated with Immobilon Western Chemiluminescence horseradish peroxidase substrates (EMD Millipore) for detection using the ChemiDoc Imaging System (Invitrogen) as described (Lee et al, 2010; Lacey et al, 2016; Hedrick et al, 2017; Lacey et al, 2017).

### 2.7. Small RNA interference assay

Cells ($1.5 \times 10^5$) were seeded on a 6-well plate. Transfection was carried out following the Lipofectamine RNAiMAX reagent protocol. After 24 hours, the transfection mixture was prepared with small interfering RNA (siRNA) along with Lipofectamine RNAiMAX reagent (Invitrogen, 56531) and Opti-MEM (Gibco, 31985-062) following the Lipofectamine RNAiMAX reagent protocol. After 72 hours, cells were harvested for protein and RNA analysis. siRNAs targeting NR4A1, NR4A2, Sp1, and Sp4 were purchased from Sigma-Aldrich. siRNAs used were siNR4A1 (SASI_Mm01_00077215, SASI_Mm01_00077216, NR4A1_C and NR4A1_D), siNR4A2 (SASI_Hs02_0034_1056, SASI_Hs02_0034_1057, SASI_Mm 01_00128029 and SASI_Mm01_00128037, siSp1 (SASI_Hs02_003 33289, SASI_Hs02_00070994, SASI_Mm01_00145222, SASI_Mm 01_00145223), siSp4 (SASI_HS01-00114420, SASI_HS01-00114421, SASI_MS01-00067683 and SASI_MS01-00067684), and Scrambled siRNA (CGU ACG CGG AAU ACU UCG A).

### 2.8. Quantitative real-time polymerase chain reaction

For gene expression studies, 3 independent experiments were performed, and the total RNA was extracted using the Zymo Research Quick-RNA Miniprep kit. The RNA samples were treated with TURBO DNA-free (Invitrogen) to remove any contamination from genomic DNA. The first-strand cDNA synthesis was performed using MultiScribe Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The amfiSure qGreen Q-PCR Master Mix (GenDEPOT) was used for real-time polymerase chain reaction (PCR). Quantitative reverse transcriptase-PCR amplifications were performed using a Lightcycler 480 (Bio-Rad). The fold change was calculated using the ΔΔCt method. A reference gene (GAPDH for human cell lines and TATA binding protein [TBP] for mouse cell lines) was included to normalize for input cDNA. Each quantitative PCR assay was carried out using at least 3 biological and 3 technical replicates. Primer pairs specific for the amplification of each gene are listed below. hGAPDH forward 5′-GTCTCCTCTGACTTCAACAGCG-3′ and hGAPDH reverse 5′-ACCACCCTGTTGCTGTAGCCAA-3′, hTwist forward 5′-GGCTCAGCTACGCCTTCTC-3′ and hTwist reverse 5′-TCCATTTTCTCCTTCTCTGGAA-3′, hNR4A1forward 5′-GGACAACG CTTCATGCCAGCAT-3′ and hNR4A1reverse 5′-CCTTGTTAGCCAGGCAGATGTAC-3', hNR4A2forward 5′-CGACATTTCTGCCTTCTCC-3′ and hNR4A2reverse 5′-GGTAAAGTGTCCAGGAAAAG-3′, mTBP forward 5′-CCTTGTACCCTTCACCAATGAC-3′ and mTBP reverse 5′-ACAGCCAAGATTCACGGTAGA-3′, mTwist forward 5′-GATTCAGACCCTCAAA CTGGCG-3′ and mTwist reverse 5′-AGACGGAGAAGGCGTAGCTGAG-3′, mNR4A1forward 5′- GTGCAGTCTGTGGTGACAATGC-3′ and mNR 4A1reverse 5′-CAGGCAGATGTACTTGGCGCTT-3', mNR4A2forward 5′-CCGCCGAAATCGTTGTCAGTAC-3′ and mNR4A2reverse 5′-TTCGGCTT CGAGGGTAAACGAC-3′.

### 2.9. Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was carried out using the ChIP-IT Express kit (Active Motif, 53008). Human GBM U87MG cells ($3 \times 10^7$) were seeded on a plate. After 24 hours, cells were treated with DMSO or 15 $\mu$M DIM-3,5-$Cl_2$ and processed following manufacturer's protocol. Cells were fixed, washed, and lysed, and nuclei were collected. Collected nuclei were then sonicated to the desired chromatin length. Sonicated chromatin was immunoprecipitated overnight using protein G-conjugated magnetic beads. IgG, NR4A1, NR4A2, Sp1, and Sp4 antibodies were used for the ChIP assay. Eluted chromatin then reverse cross-linked and treated with proteinase K. Samples were then subjected to a DNA clean-up using Chromatin IP DNA Purification Kit (Active Motif, 58002). Purified DNA products were analyzed by using amfiSure qGreen Q-PCR Master Mix (GenDEPOT). The primers used for detection of the *TWIST1* promoter region were ChIPTwistF1 5′-ACCCACTGTGTAGAAGCTGT-3′ and ChIPTwistR1 5′-TCGTCCTCCCAAACCATTCA-3′ and ncTwistF 5′-TTCACTTGCGGTACTGTTCG-3′ and ncTwistR1 5′-TTCATGACCCTGG-TAGCCTT-3′.

### 2.10. In vivo studies

All animal procedures were performed according to protocols approved by the Houston Methodist Research Institute Animal Care and Use Committee, and equal numbers of male and female mice were purchased from the Jackson Laboratory. Ha-Ras/shp53 mouse GBM cells were previously described (Mikheev et al, 2017). Presence of the mTmG transgene facilitated identification of tumor cells. We demonstrated that malignancy of tumors derived from Ha-RasV12/shp53 cells injected into syngeneic hosts is regulated by TWIST1 expression (Mikheev et al, 2017). Ha-RasV12/shp53 cells ($2.5 \times 10^5$) were injected using a stereotaxic frame in syngeneic 4–5-month-old C57Bl/6J animals (Male and female, 6 animals per group) as previously described (Mikheeva et al, 2010; Mikheev et al, 2015, 2017, 2018). DIM-3,5-$Cl_2$ treatment was initiated on day 5 after injection using a daily dose of 7.5 mg/kg/day i.p. in corn oil while controls were injected intraperitoneally with an equivalent volume of corn oil. Animals were monitored for clinical manifestations, and mice were euthanized by $CO_2$ asphyxiation. Terminal tumors were dissected under a fluorescence dissecting microscope and snap frozen in liquid nitrogen. Frozen tumor fragments were ground and lysed in radioimmunoprecipitation assay buffer to extract proteins. Extracted proteins were analyzed by western blotting. Western blot densitometry was performed using ImageJ. Averaged normalized densitometry was plotted as a bar diagram. Antibodies used for

analysis of the tumor lysates included: TWIST1 (Santa Cruz Biotechnology, sc-81417), actin (Sigma-Aldrich, A5441), NR4A1 (Proteintech, 12235-1-AP), and NR4A2 (Proteintech, 10975-2-AP).

### 2.11. Statistical analysis

Statistical analysis was performed using a *t* test. Median survival of tumor-bearing animal cohorts was compared by log-rank (Mantel-Cox) test analysis using Prism 9. All of the in vitro experiments were carried out in triplicate. Data are expressed as the mean ± SD. One-way analysis of variance (Dunnett's) was used to determine statistical significance, and *P* values <.05 were considered statistically significant.

## 3. Results

### 3.1. Cell proliferation and transactivation

In this study, we initially investigated the effects of 3 dual NR4A1/2 ligands, DIM-3,5-$Cl_2$, DIM-3-Cl-5-$CF_3$, and DIM-3,5-$Br_2$ on proliferation of 3 human (U87MG, T98G, and A172) (Fig. 1A) and 3 mouse (CT2A, Ha-Ras/Akt and Ha-Ras/shp53) (Fig. 1B) GBM cell lines. Inhibition of cell proliferation was observed at concentrations of 5–10 $\mu$M for most compounds in all 6 cell lines. The role of NR4A1 and NR4A2 on cell viability using U87MG and CT2A as model human and mouse GBM cells, respectively, was determined by RNA interference in which knockdown of *NR4A1* or *NR4A2* resulted in decreased proliferation of U87MG (Fig. 2C) and CT2A (Fig. 2D) cells. Figure 1E illustrates the knockdown efficiencies of *NR4A1* (siA1-C and siA1-D) and *NR4A2* (siA21 and siA2-2) in U87MG cells. In addition, we also observed that treatment of U87MG cells transfected with GAL4-NR4A1 and GAL4-NR4A2 and a UAS-luc reporter gene with the DIM-3,5 analogs decreased transactivation, consistent with their dual NR4A1/2 inverse agonist activity (Fig. 1F). These results are comparable to those previously observed for an NR4A2 ligand and *NR4A2* knockdown in GBM cells (Karki et al, 2020) and demonstrate that both NR4A1 and NR4A2 have comparable functional effects on proliferation of GBM cells. Structures of the DIM-3,5 analogs are illustrated in Fig. 1G.

### 3.2. Migration, viability, and apoptosis

Previous studies have also shown that *NR4A2* knockdown and NR4A2 ligands inhibit GBM cell invasion/migration (Karki et al, 2020), and results in Fig. 2, A and B, demonstrate that the DIM-3,5 ligands also significantly inhibit U87MG cell migration in a scratch assay. Knockdown of *NR4A1* and *NR4A2* decreased cell viability (Fig. 2C). DIM-3,5 compound (15 μM) had minimal effects on proliferation of NR4A1-deficient cells, and only DIM-3-Cl-5-$CF_3$ significantly inhibited viability of NR4A2-deficient cells. Knockdown of *NR4A1* or *NR4A2* in U87MG cells decreased total PARP and increased expression of cleaved PARP and the survival gene product Bcl-2 (Fig. 2D). Similar results were observed for cleaved PARP and Bcl-2 after treatment with DIM-3,5-$Cl_2$ (Fig. 2E). The results indicate that both NR4A1 and NR4A2 exhibit similar effects on GBM cell growth and survival and DIM-3,5 dual receptor ligands act as inverse NR4A1/2 agonists, and their effects resemble responses observed after knockdown of *NR4A1* or *NR4A2*.

### 3.3. NR4A1/NR4A2 regulation of TWIST1 and effects of DIM-3,5 ligands

It was previously reported that like CDIM compounds, several natural products including flavonoids, resveratrol, and piperlongumine also induce apoptosis, inhibit proliferation, migration, and epithelial mesenchymal transition (EMT) in cancer cell lines, and this was accompanied by downregulation of *TWIST1*, a key gene involved in EMT (Cilibrasi et al, 2017; Park et al, 2017; Feng et al, 2018). Recent studies have also shown that flavonoids such as quercetin, resveratrol, and piperlongumine also bind and act as NR4A1 inverse agonists in cancer cells (Zhang et al, 2022; Lee et al, 2023; Zhang et al., 2023), and ongoing research suggests that they are also dual NR4A1/2 ligands (data not shown). Figure 3A illustrates that treatment of human T98G, A172, and U87MG GBM cells with DIM-3,5-$Cl_2$ (15 $\mu$M) decreased expression of TWIST1 protein. Moreover, decreased levels of TWIST1 protein in mouse Ras/Akt, Ras/shp53, and CT2A GBM cells were also observed after treatment with 15 $\mu$M DIM-3,5-$Cl_2$. Treatment of the 3 human and 3 mouse cell lines with 15 $\mu$M DIM-3,5-$Cl_2$ also decreased *TWIST* mRNA levels (Fig. 3, C and D), suggesting transcriptional and possibly posttranscriptional regulation of TWIST1 and demonstrating that DIM-3,5-$Cl_2$, a dual NR4A1/2 ligand, acts as an inverse agonist in terms of regulation of *TWIST1* gene expression in GBM cells.

Because DIM-3,5 binds both NR4A1 and NR4A2, we used knockdown studies to determine whether NR4A1 and NR4A2 regulate TWIST1 expression. Figure 4A demonstrates that NR4A1, NR4A2, and TWIST1 proteins are expressed in U87MG and CT2A GBM cells and treatment with DIM-3,5-$Cl_2$ decreased levels of TWIST1 protein, and downregulation of NR4A1 and NR4A2 protein was also observed. This response is variable and dependent on the ligand and tumor cell type. Knockdown of *NR4A1* and *NR4A2* by RNA interference in U87MG cells with 2 oligonucleotides for each receptor was effective for at least 1 of the oligonucleotides and decreased levels of NR4A1 or NR4A2, and this was accompanied by decreased expression of TWIST1 protein (Fig. 4B). Similar results were observed in CT2A cells (Fig. 4C), confirming that TWIST1 is coregulated by both NR4A1 and NR4A2, and knockdown of NR4A1 and NR4A2 also decreased levels of *TWIST1* mRNA in U87MG and CT2A cells (Fig. 4D). Together these results suggest transcriptional regulation of TWIST1 downstream of NR4A1/2. It was previously reported that proximal cis-elements in the *TWIST* promoter bind Sp1 and that Sp1 is a major regulator of TWIST expression (Ohkuma et al, 2007; Asanoma et al, 2015). Because NR4A1 acts as a cofactor of Sp1 and Sp4 to enhance expression of several genes including survivin, integrins, and G9a (Lee et al, 2010; Lacey et al, 2016; Hedrick et al, 2017; Lacey et al, 2017), we investigated the role of NR4A/Sp in regulation of TWIST1. Results in Fig. 5A show that knockdown of *Sp1* or *Sp4* (using 2 oligonucleotides) in U87MG cells decreased *Sp1* and *TWIST1* mRNA and protein expression. This suggests that both Sp1 and Sp4, which bind similar promoter sequences, regulate TWIST1 expression. In addition, knockdown of *Sp1* and *Sp4* in CT2A cells decreased expression of TWIST1 protein (Fig. 5B). The human *TWIST1* promoter contains CCT repeats at −92 to −84 that bind Sp1 (Ohkuma et al, 2007). Figure 5C illustrates the 2 synthetic oligonucleotides that contain the CCT repeats (TWwt) and mutations of these repeats (TWmt). The oligonucleotides were cloned into a luciferase vector, and transfection of both oligonucleotides into U87MG cells showed that mutation of the CCT site resulted in a >90% decrease in luciferase activity of the mutant versus wild type construct, indicating the significant contribution of this site to transactivation of TWIST1 in GBM cells. In addition, treatment with DIM-3,5-$Cl_2$, a dual NR4A1/2 ligand, decreased luciferase activity only in U87MG cells transfected with TWwt but not TWmt (Fig. 5D). These results suggest that, like Sp1 and Sp4, NR4A1/2 also play a role in coregulation of expression of TWIST1. This was confirmed in U87MG cells transfected with TWwt and TWmt where *NR4A1* and *NR4A2* knockdown also decreased luciferase activity in cells transfected

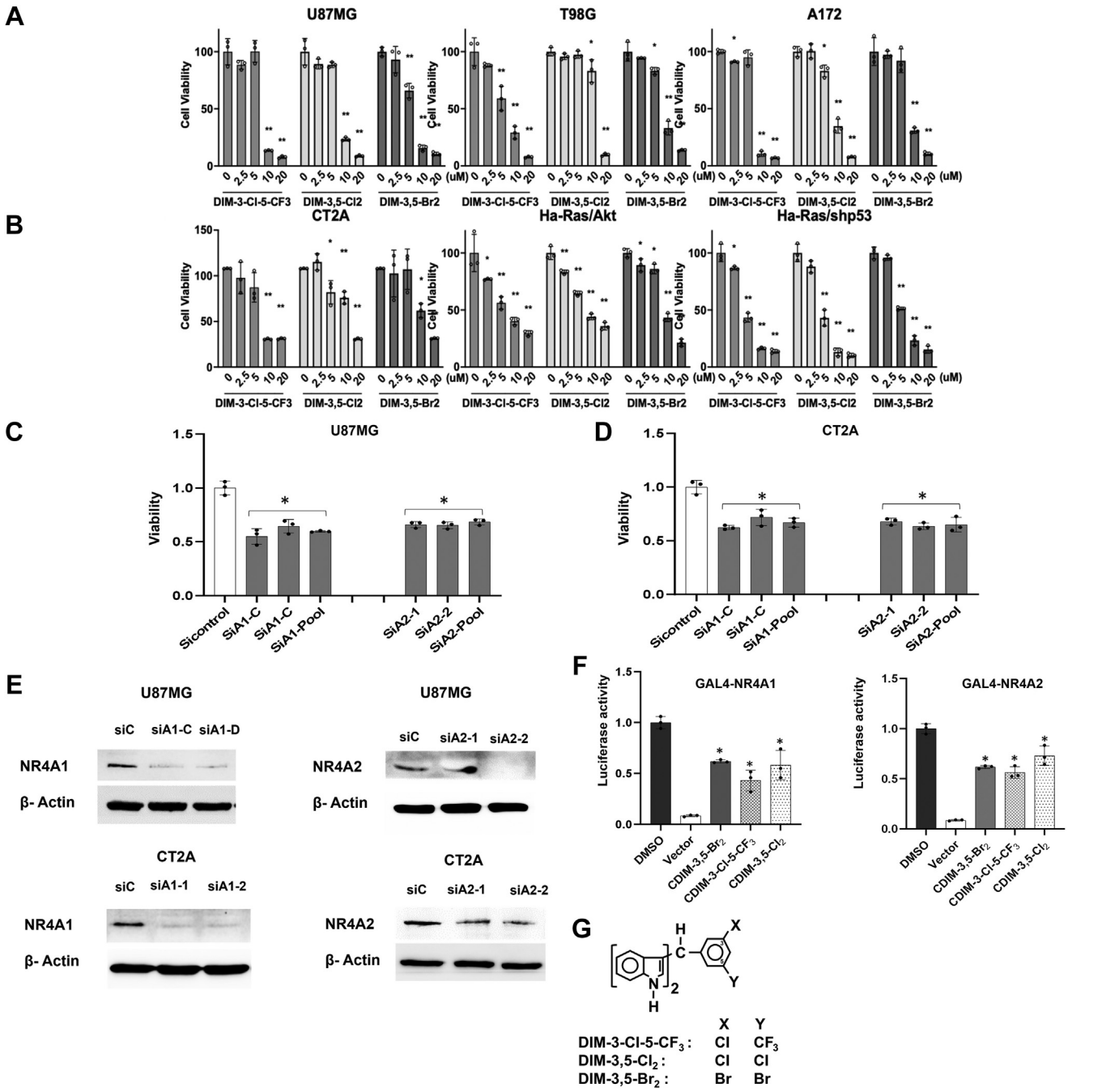


**Fig. 1.** NR4A1 and NR4A2 regulation of cell proliferation and transactivation. Human (A) and mouse (B) GBM cell lines were treated with DMSO and DIM-3,5 analogs for 24 hours, and cell viability was determined using an XTT assay. U87MG (C) and CT2A (D) cells were transfected with a control (nonspecific) oligonucleotide, and oligonucleotides targeting NR4A1 and NR4A2 and cell proliferation was determined using an XTT assay. Percent viability of knockdown was compared to the control (set at 1.0). (E) U87MG and CT2A cells were transfected with 2 oligonucleotides targeting *NR4A1* and *NR4A2*, and knockdown efficiencies were determined by western blotting of cell lysates as outlined in *Materials and methods*. (F) Effects of DIM-3,5 analogs on luciferase activity was determined U87MG and CT2A cells transfected with GAL4-NR4A1 or GAL4-NR4A2 and a UAS-luc construct containing 5 tandem GAL4 response elements. Luciferase activity was determined as outlined in *Materials and methods*. (G) Structures of DIM-3,5 compounds. Results are expressed as means ± SD for at least 3 determinations, and significant ($P < .05$) inhibition is indicated (*).

with TWwt (Fig. 5E). Figure 5F shows that NR4A1 and NR4A2 antibodies coimmunoprecipitated Sp1 and Sp4, and this further demonstrates interactions of these proteins.

The role of Sp transcription factors in regulating TWIST1 expression in U87MG cells was supported by the effects of mithramycin, which is known to inhibit Sp-mediated transactivation by interacting with cis-elements. Using U87MG cells as a model, treatment with mithramycin inhibited expression of TWIST1 protein (Fig. 6A) and mRNA (Fig. 6B). Interactions of NR4A1, NR4A2, Sp1, and Sp4 with the Sp binding site in the proximal region of the *TWIST1* promoter were determined in a ChIP assay using primers targeted to this region (Fig. 6C). All 4 proteins bound to this region in U87MG cells treated with solvent (DMSO) control. Treatment with 15 μM DIM-3,5-$Cl_2$ resulted in decreased association of NR4A1, NR4A2, Sp1, and Sp4 with this region of the promoter.

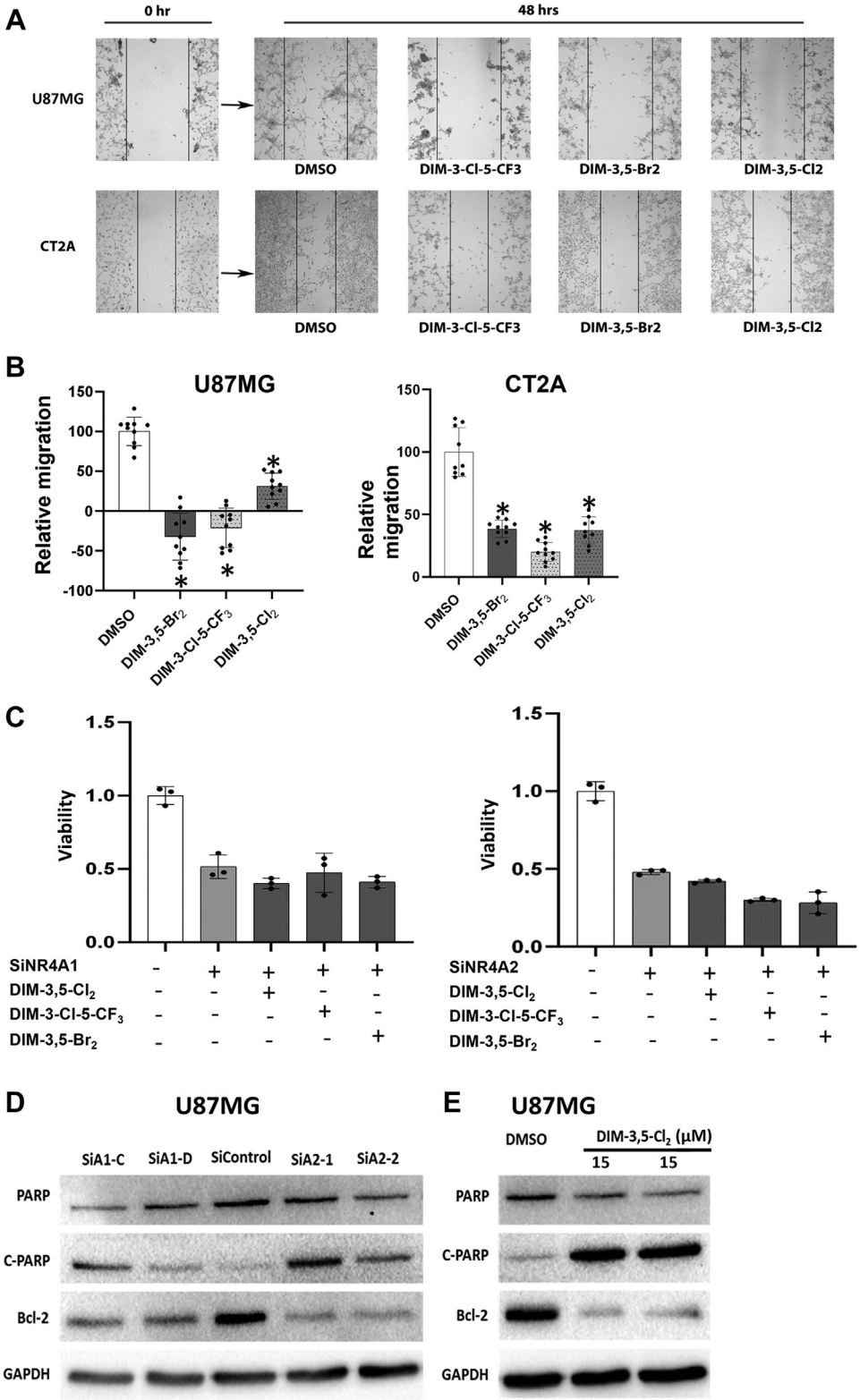


**Fig. 2.** Functional effects of DIM-3,5 analogs and NR4A1/2 knockdown: migration and apoptosis. (A) U87MG and CT2A cells were treated with DIM-3,5 analogs, and their effects on cell migration were determined in a scratch assay, as outlined in *Materials and methods*, and (B) quantitated. (C) U87MG cells were transfected with oligonucleotides targeting *NR4A1* or *NR4A2*, and the viability of the knockdown cells alone or in combination with DIM-3,5 analogs was determined as outlined in *Materials and methods*. U87MG cells were transfected with control or targeted oligonucleotides (D) or treated with DIM-3,5-$Cl_2$ (E) for 24 hours, and whole cell lysates were analyzed by western blotting, as outlined in *Materials and methods*. Results are expressed as means ± SD for at least 3 determinations, and significant ($P < .05$) inhibition is indicated (*). C-PARP, cleaved PARP.

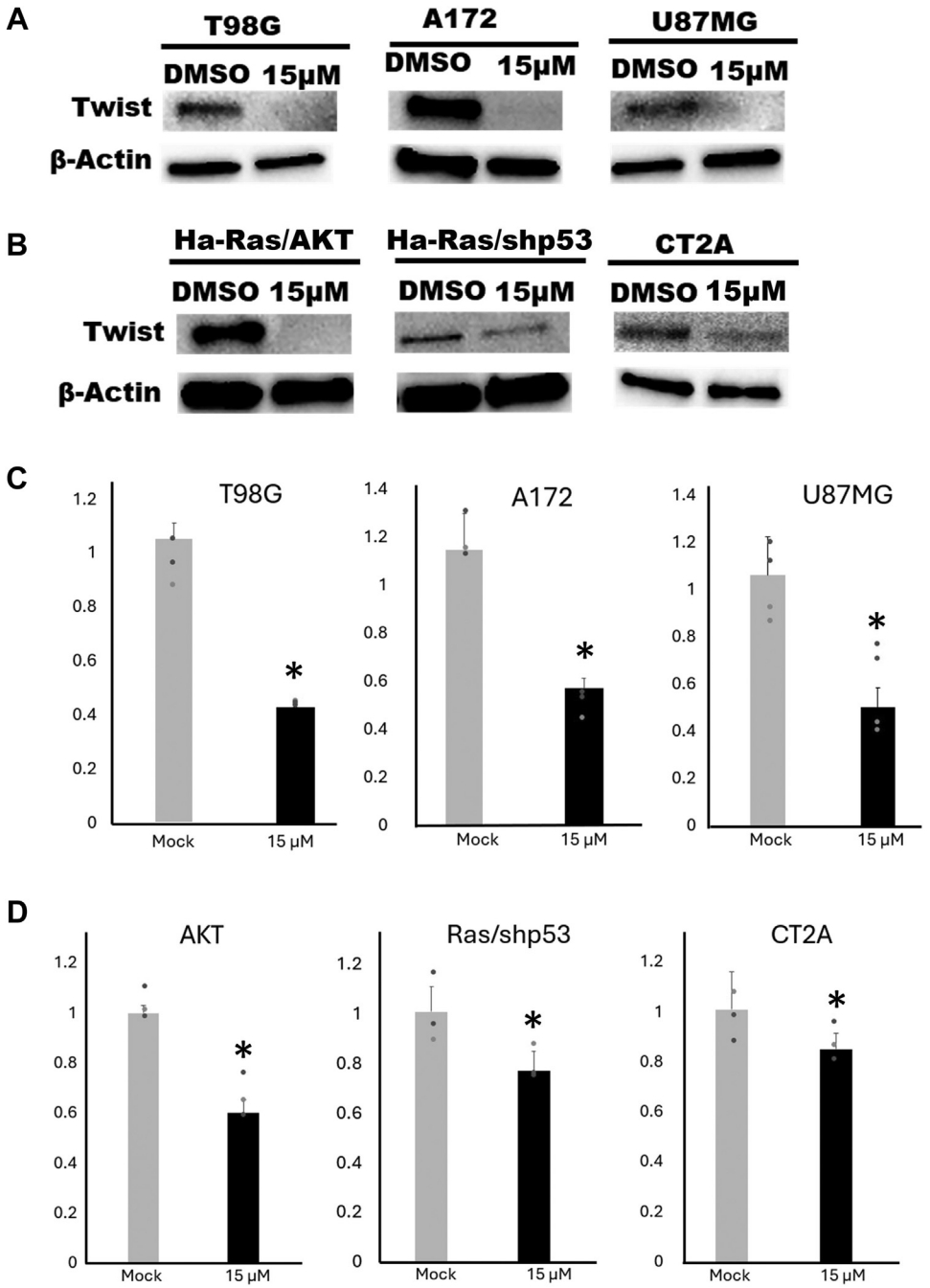


**Fig. 3.** DIM-3,5-$Cl_2$ decreases TWIST1 mRNA and protein expression in GBM cells. Human (A) and mouse (B) GBM cell lines were treated with 15 $\mu$M DIM-3,5-$Cl_2$ for 24 hours, and whole cell lysates were analyzed by western blotting, as outlined in *Materials and methods*. Human (C) and mouse (D) GBM cells were treated with 15 $\mu$M DIM-3,5-$Cl_2$ for 18 hours, and mRNA extracts were analyzed for *TWIST1* mRNA expression, as outlined in *Materials and methods*. Results are expressed as means ± SD for at least 3 replicate determinations and significantly ($P < .05$) decreased *TWIST1* mRNA levels are indicated (*).

### 3.4. *In vivo studies*

The effects of DIM-3,5-$Cl_2$ treatment of an orthotopic syngeneic mouse glioma model generated in C57Bl/6J male and female hosts (6 animals/group) from Ha-Ras/shp53-transformed neural progenitor cells was also investigated as described (Mikheev et al, 2017). Figure 6D illustrates fluorescence imaging of terminal tumors from the untreated group (1–4) and treated mice (5 and 6), and tumors (7–9) are from mice euthanized on day 79. Tumor sizes were not determined due to interference by large hemorrhages. Figure 6E illustrates coronal hematoxylin and eosin staining of vascular tumors and the arrows indicate distinct masses consistent with invasive growth. Statistically significant increases in survival with DIM-3,5-$Cl_2$ treatment (7.5 mg/kg/day) compared with control were observed after 79 days ($P = .0161$) at which time all control animals reached criteria for euthanasia while 3 of 6 CDIM treated animals remained alive and well (Fig. 6F). Enhanced survival was associated with dramatic reduction of TWIST1 mRNA and protein expression in tumors of DIM-3,5-$Cl_2$–treated animals compared with controls harvested at terminal time points (Fig. 6G).

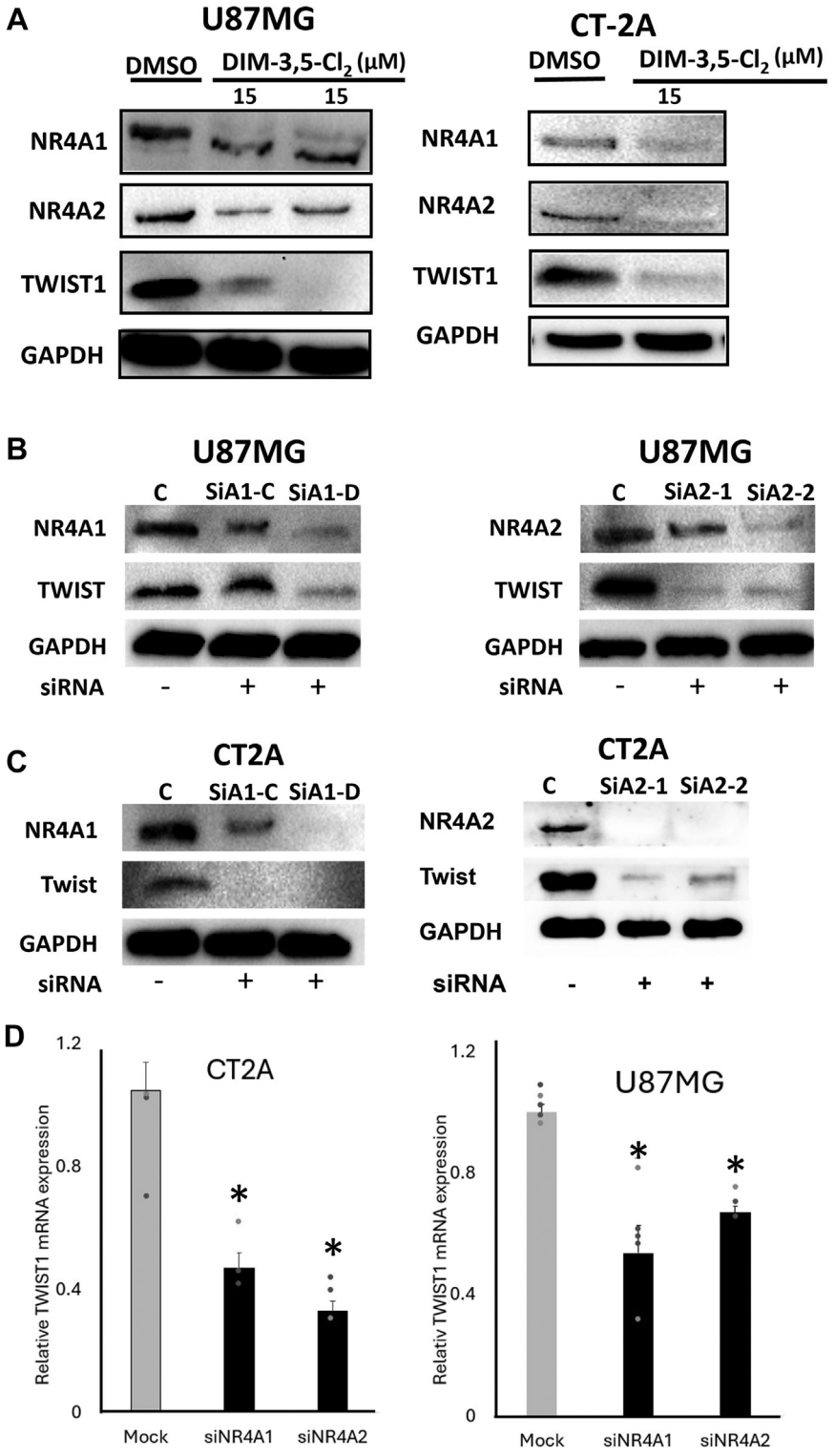


**Fig. 4.** *TWIST1* is an NR4A1/2-regulated gene. U87MG and CT2A cells (A) were treated with DMSO or DIM-3,5-$Cl_2$ for 24 hours. U87MG (B) or CT2A (C) cells were transfected with oligonucleotides targeting *NR4A1* or *NR4A2*, and whole cell lysates were analyzed by western blotting. (D) Knockdown of *NR4A1* or *NR4A2* in U87MG or CT2A cells was carried out, and *TWIST1* mRNA expression (relative to control knockdown) was determined by reverse-transcription PCR. Results are means ± SD for at least 3 replicate determinations, and significant ($P < .05$) reduction of *TWIST1* mRNA levels are indicated (*).

In contrast, levels of NR4A1 and NR4A2 proteins were variable in the tumors. Thus, the dual NR4A1/2 DIM-3,5 ligands act as inverse NR4A1/2 agonists that inhibit GBM cell growth in vitro and in vivo in concert with downregulation of TWIST1, identified here as an NR4A1/2-regulated gene.

## 4. Discussion

GBM is a devastating cancer with a median age of diagnosis of 65 years and a slight male predominance (Ostrom et al, 2018a). GBM is the most common and lethal malignant brain tumor and

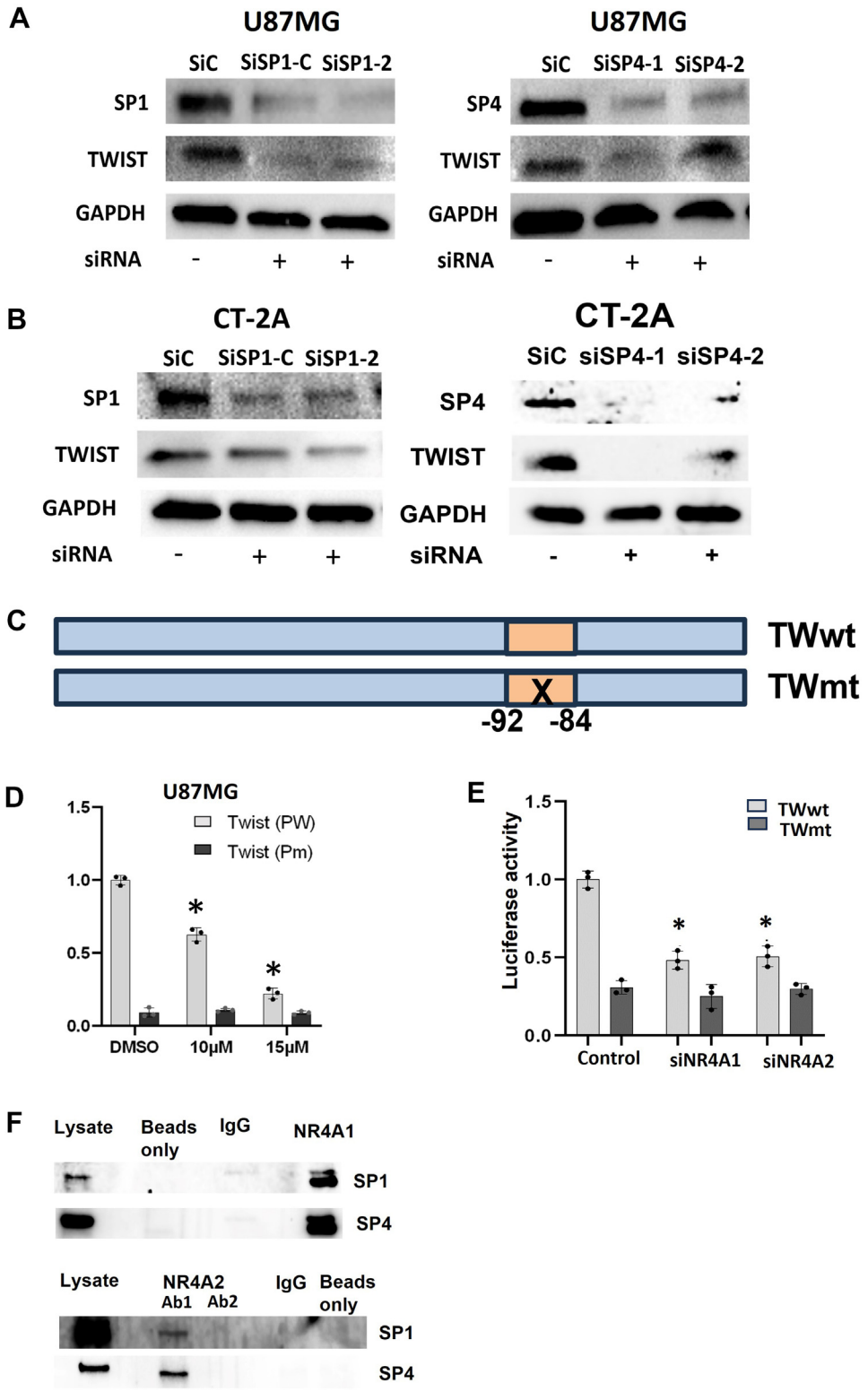


**Fig. 5.** NR4A1/2/Sp regulates TWIST1 expression in GBM cells. U87MG (A) and CT2A (B) cells were transfected with a nonspecific (control) oligonucleotide or nucleotides targeting Sp1 or Sp4, and whole cell lysates were analyzed by western blotting. (C) Synthetic constructs from the *TWIST1* human gene promoter. U87MG cells were transfected with TWwt or TWmt (wild type and mutant constructs, respectively) and treated with 10 or 15 $\mu$M DIM-3,5-$Cl_2$ (D) or transfected with oligonucleotides targeting *NR4A1* or *NR4A2* (E), and luciferase activity was determined as outlined in *Materials and methods*. (F) Cell lysates were immunoprecipitated with NR4A1 and NR4A2 antibodies, and western blot analysis was used to examine coimmunoprecipitation of Sp1 and Sp4 as outlined in *Materials and methods*. Results are expressed as means ± SD for at least 3 replicate determinations, and significantly ($P < .05$) decreased responses compared to control (DMSO) values are indicated (*).

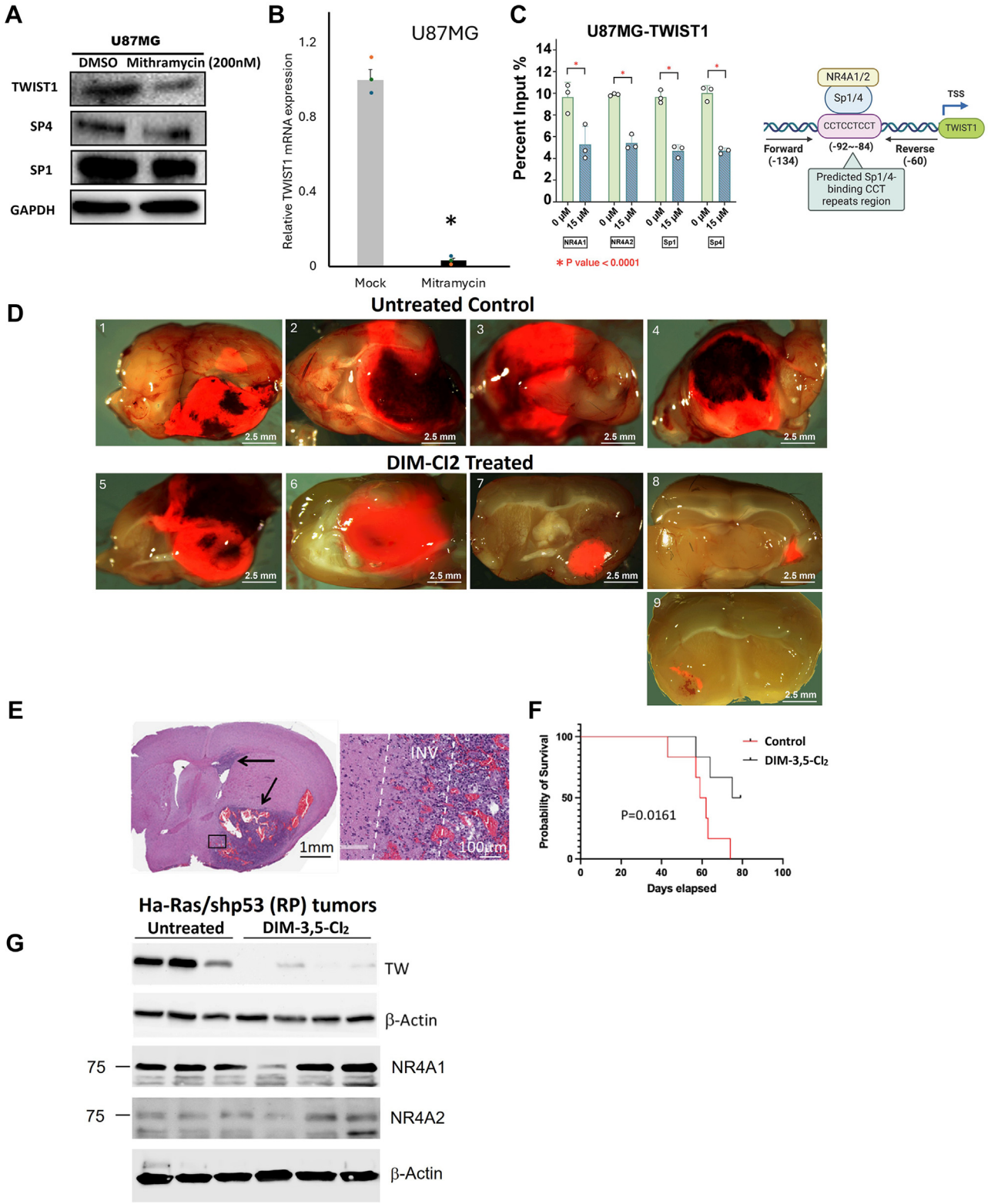


**Fig. 6.** Effects of mithramycin and ChIP assay on TWIST1 and in vivo studies. U87MG cells were treated with DMSO or mithramycin (200 nM) for 24 hours, and both whole cell lysates (A) and RNA (B) were isolated and analyzed by western blotting or mRNA quantification, respectively, as outlined in *Materials and methods*. A ChIP assay (C) was determined using primers targeted to the Sp binding region of the proximal promoter region of the *TWIST1* gene as outlined in *Materials and methods*. Animals harboring Ha-Ras/shp57 (RP) tumors were treated with DIM-3,5-$Cl_2$ for the duration of the experiment. (D) Tumor images from treated (tumors 5 and 6) and untreated (1–4) mice; tumors 7–9 are derived from mice treated for 79 days. Tumor images were obtained using a fluorescence-capable Leica dissecting microscope. (E) Left: representative coronal section stained with hematoxylin and eosin shows a vascular tumor with multifocal growth (arrows). Right: high-power image shows invasive margin (dotted white lines). (F) DIM-3,5-$Cl_2$ treatment (7.5 mg/kg/day) significantly prolonged animal survival (log-rank $P = .0161$). The experiment was terminated on day 79. In the treated group, among 3 euthanized animals, we only found microtumors in 2 animals. (G) Analysis of tumor lysates by western blotting from 3 control and 4 DIM-3,5-$Cl_2$ treated tumors. Experiments were carried out in triplicate, and significant ($P < .05$) differences from control values are indicated (*).

represents approximately 57% of all gliomas and 48% of malignant central nervous system cancers in adults (Stupp et al, 2005; Kudo-Saito et al, 2009, 2013; Ostrom et al, 2015; Brown et al, 2018; Ostrom et al, 2018b; Smita et al, 2018; Cho et al, 2019; Leven et al, 2019; McGranahan et al, 2019; Tan et al, 2020; Zhang et al, 2020). GBM patients are primarily treated with maximal safe surgical resection followed by a combination of radiotherapy and chemotherapy with temozolomide (TMZ), resulting in a relapse-free and median overall survival of only 6.9 and 14.6 months, respectively (Stupp et al, 2005). Treatment of recurrent tumors is also not very effective, and the 5-year survival of GBM patients is <10% (Ostrom et al, 2015, 2018a,b). Since the introduction of TMZ and irradiation (the "Stupp protocol") (Stupp et al, 2005), only 1 treatment, tumor treating fields combined with the Stupp protocol, has shown small but verified additional benefits for GBM patients in randomized controlled trials. The remarkable responses observed in advanced stage melanoma (Leven et al, 2019) and non-small cell lung cancer (Zhang et al, 2020) to immune checkpoint inhibitors heralded a potential breakthrough therapy for GBM. However, clinical trials have been disappointing and underscored the fundamental roadblock posed by robust immunosuppressive GBM (Brown et al, 2018; Cho et al, 2019; McGranahan et al, 2019). Therefore, the limited benefits of genotoxic and immune-based therapies underscore the critical need to identify new therapeutic targets in GBM with potential activity to potentiate standard TMZ/irradiation and/or enable immunotherapy.

The lack of current treatment efficacy for GBM has spurred a significant amount of research on development of alternative therapies including agents/biotherapeutics that target receptor tyrosine kinases, angiogenesis pathways, and other factors; however, at present, these therapies are not routinely used for clinical treatment of GBM either because of lack of efficacy (eg, bevacizumab) or challenges posed by intratumoral cellular and molecular heterogeneity. We have previously reported that the orphan nuclear receptor NR4A2 is a druggable target for treatment of GBM, and there is evidence that NR4A2 is overexpressed in GBM and is a negative prognostic factor for survival (Karki et al, 2020). NR4A1- and NR4A2-mediated activities are functionally similar in solid tumors and their derived cell lines (Karki et al, 2020; Safe and Karki, 2021). However, the effects of NR4A1 ligands on GBM cells has not previously been investigated. Genomic analysis of cerebellar GBM showed that NR4A1 is overexpressed in these tumors and is a potential master regulator of cerebellar GBM and "nuclear NR4A1-mediated transactivation played an oncogenic role in GBM" (Cho et al, 2019). We recently identified a series of highly potent DIM-3,5 analogs that inhibited tumor growth in vivo (Karki et al, 2021) and subsequently demonstrated that these compounds bound not only NR4A1 but also NR4A2 and were dual NR4A1/2 ligands (Upadhyay et al, 2024). We hypothesized that they may be highly effective agents for treating GBM.

Our initial studies focused not only on the activity of prototypical dual NR4A1/2 ligands in GBM cells but also on showing that both NR4A1 and NR4A2 were pro-oncogenic in these cells. DIM-3,5 ligands inhibited GBM cell growth, induced apoptosis, inhibited U87MG and CT2A cell migration, and inhibited both NR4A1- and NR4A2-dependent transactivation. Moreover, knockdown studies showed that both NR4A1 and NR4A2 exhibit comparable functional pro-oncogenic activities, and treatment with DIM-3,5 compounds or NR4A1/2 knockdown resulted in similar effects. The mechanism of NR4A1/NR4A2 regulation of TWIST1 in GBM cells suggests that the receptors may act cooperatively. This is supported by the effects of the dual receptor ligands, which do not significantly affect growth of NR4A1- and NR4A2-deficient cells. The only exception was DIM-3-CI-5-$CF_3$, which inhibited growth of NR4A2-deficient cells, and this may be due to an additional compound-dependent response that we are currently investigating. These results demonstrate that the dual NR4A1/2 ligands act as inverse agonists, which is consistent with the activities of other NR4A1- and NR4A2-active DIM compounds in other solid tumor-derived cell lines (Karki et al, 2021; Safe and Karki, 2021). Recently, activation of EMT in carcinomas has been identified as a driving factor for immune suppression (Kudo-Saito et al, 2009, 2013; Smita et al, 2018; Jiang and Zhan, 2020; Taki et al, 2021). GBMs with mesenchymal features also exhibit a robust and unique immune suppressive tumor microenvironment as well as resistance to standard of care therapy. The *TWIST1* gene is highly expressed in GBM cells and other cancer cell lines and plays an important role in EMT in enhancing cell migration/invasion, tumorigenicity, treatment resistance, and the immunosuppressive tumor microenvironment (Elias et al, 2005; Mikheeva et al, 2010; Tanaka et al, 2011; Mikheev et al, 2015, 2017, 2018). Therefore, development of agents that directly target TWIST1 represents a novel approach for GBM therapy because they target both the tumor and immune cells.

Several studies have shown that natural products that exhibit anticancer activity, such as resveratrol, piperlongumine, quercetin, and other flavonoids, downregulate TWIST1 in cancer cells (Cilibrasi et al, 2017; Park et al, 2017; Feng et al, 2018), and it has recently been reported that these compounds also bind NR4A1 and, like DIM-3,5 compounds, exhibit inverse receptor agonist activities (Zhang et al, 2022; Lee et al, 2023; Zhang et al, 2023). Therefore, we hypothesized that TWIST1 is regulated by NR4A1 and possibly NR4A2 in GBM and is therefore a druggable target for DIM-3,5 dual NR4A1/2 ligands. Treatment of GBM cells with DIM-3,5-$Cl_2$ decreased expression of TWIST1 mRNA and protein and knockdown of NR4A1, NR4A2, Sp1, and Sp4 also decreased TWIST1 mRNA and protein, suggesting that NR4A1/2 acts as a cofactor for Sp1 and Sp4 bound to proximal CCT repeat sites in the *TWIST1* promoter. This is consistent with previous studies showing the importance of Sp1 and the cis-acting site in the proximal region of the *TWIST1* promoter (Ohkuma et al, 2007), and high levels of TWIST1 are related to high expression of Sp1, Sp4, NR4A1, and NR4A2 in many cancer cell lines. ChIP analysis of the *TWIST1* promoter demonstrated association of NR4A1, NR4A2, Sp1, and Sp4 with the proximal region of the promoter that was previously shown to bind Sp1. The results do not define whether NR4A1 and NR4A2 individually or cooperatively interact with DNA-bound Sp1/4. However, it is possible that an NR4A1-NR4A2 heterodimer could be involved because it has previously been reported that NR4A1 and NR4A2 can bind to form a heterodimer (Maira et al, 1999) (Fig. 7). The

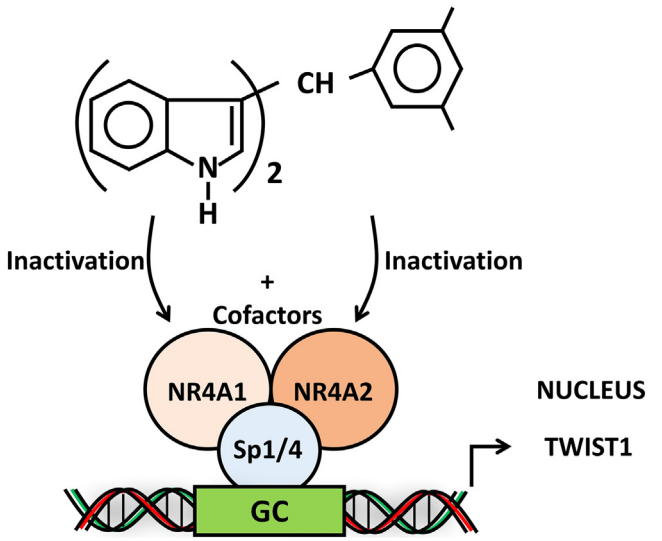


**Fig. 7.** Model of proposed mechanism of action of TWIST1 expression by NR4A1 and NR4A2 interacting with (GC-rich) promoter DNA and inactivation of this response by DIM-3,5 analogs binding both receptors. NR4A1 and NR4A2 regulate TWIST1 expression through interactions with DNA-bound Sp1 and Sp4 transcription factors, and DIM-3,5 analogs bind both receptors to inactivate expression of TWIST1.

putative cofactor activity of NR4A1/2 as an enhancer of Sp-mediated transactivation is not uncommon because many other nuclear receptors interact with and enhance DNA-bound transcription factor (eg, Sp1, AP1)-mediated activity (Safe and Kim, 2004).

The in vitro studies were complemented by in vivo results showing that DIM-3,5-$Cl_2$ enhances survival in a syngeneic mouse model of GBM, and this is accompanied by downregulation of TWIST1 in tumors from the treated animals. In conclusion, our study demonstrates for the first time that NR4A1 and NR4A2 are upstream regulators of TWIST1 expression, and inhibition of TWIST1 by treatment with DIM-3,5 analogs contributes to the inhibition of tumor and cell growth and enhanced animal survival. Previous studies in a syngeneic mouse model of colon cancer showed that DIM-3,5 NR4A1/2 ligand not only inhibits tumor growth but also reverses T cell exhaustion in CD8+ and CD4+ T cells (Mohankumar et al, 2023). Future studies on GBM will investigate effects of these dual NR4A1/2 ligands on T cells and other immune cell types and the role of NR4A1/2-mediated downregulation of TWIST1. In addition, we will also investigate combination therapies to determine whether inactivation of NR4A1 and NR4A2 will also enhance the effects of immune checkpoint inhibitors and decrease immune cell exhaustion and thereby facilitate effective incorporation of immune therapies into GBM treatment protocols.

## Abbreviations

CDIM, bis-indole–derived compound; ChIP, chromatin immunoprecipitation; DIM-3,5, 1,1-bis(3′-indolyl)-1-(3,5-disubstitutedphenyl)methane; DMEM, Dulbecco's modified Eagle medium; EMT, epithelial-to-mesenchymal transition; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GBM, glioblastoma; PCR, polymerase chain reaction; siRNA, small interfering RNA; TBP, TATA binding protein; TMZ, temozolomide; TWmt, mutant pTWIST1m(−209/131) sub-luc; TWwt, pTWIST1 W(−209/131)-luc.

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## Conflict of interest

The authors declare no conflicts of interest.

## Data availability

The data that support the findings of this study are available on request from the corresponding author.

## Authorship contributions

*Participated in research design:* Upadhyay, A.M. Mikheev, Rostomily, Safe.

*Conducted experiments:* Upadhyay, Lee, Zhang, Oany, S.A. Mikheeva, A.M. Mikheev.

*Contributed new reagents or analytic tools:* Upadhyay, S.A. Mikheeva, Safe.

*Performed data analysis:* Upadhyay, Lee, Safe.

*Wrote or contributed to the writing of the manuscript:* A.M. Mikheev, Rostomily, Safe.

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