The Dopamine D₂ Receptor Contributes to the Spheroid Formation Behavior of U87 Glioblastoma Cells

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Keywords

Dopamine · Glioblastoma · Spheroids · Stem cell · Neurotransmitters

Abstract

Background: Glioblastoma multiforme (GBM) is a common and lethal cancer of the central nervous system. This cancer is difficult to treat because most anticancer therapeutics do not readily penetrate into the brain due to the tight control at the cerebrovascular barrier. Numerous studies have suggested that dopamine D₂ receptor (D₂R) antagonists, such as first generation antipsychotics, may have anticancer efficacy in vivo and in vitro. The role of the D₂R itself in the anticancer effects is unclear, but there is evidence suggesting that D₂R activation promotes stem-like and spheroid forming behaviors in GBM. **Objectives:** We aimed to observe the role of the dopamine D₂R and its modulators (at selective concentrations) in spheroid formation and stemness of GBM cell line, U87MG, to clarify the validity of the D₂R as a therapeutic target for cancer therapy. **Methods:** Spheroid formation assays and Western blotting of the glioblastoma cell line, U87MG, were used to observe responses to treatment with the D₂R agonists

sumanirole, ropinirole, and 4-propyl-9-hydroxynaphthoxazine (PHNO); and the D₂R antagonists thioridazine, pimozide, haloperidol, and remoxipride. Extreme limiting dilution analysis was done to determine the impact of sumanirole and remoxipride treatment on sphere-forming cell frequency. Proliferation was also measured by crystal violet staining. Stable lentiviral transduction of *DRD2* or *shDRD2* was used to validate the role of the D₂R in assay behaviors. **Results:** D₂R antagonists thioridazine, pimozide, haloperidol, and remoxipride decrease spheroid formation behaviors at a selective 100 nmol/L concentration, while D₂R agonists PHNO, sumanirole, and ropinirole increase the formation of spheroids. Similarly, 100 nmol/L remoxipride decreased sphere-forming cell frequency. These results were recapitulated with genetic overexpression and knockdown of the D₂R, and combination experiments indicate that the D₂R is required for the effects of the pharmacological modulators. Furthermore, spheroid proliferation and invasive capacity increased under treatment with 100 nmol/L sumanirole and decreased under treatment with 100 nmol/L thioridazine. Expression levels of the stemness markers Nestin and Sox2, as well as those of differentiation marker glial fibrillary acidic protein, were not altered by 100 nmol/L thioridazine or sumanirole for 72 h or

continuous treatment with these compounds for 7 days during a spheroid formation assay. **Conclusions:** Signaling activity of the dopamine D₂R may be involved in the spheroid formation phenotype in the context of the U87MG cell line. However, this modulation may not be due to alterations in stemness marker expression, but due to other factors that may contribute to spheroid formation, such as cell-cell adhesion or EGFR signaling.

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Introduction

A common central nervous system cancer, glioblastoma multiforme (GBM), has a remarkably poor prognosis [1]. Less than 10% of patients will survive for 5 years after diagnosis [2]. Even among patients who engage in clinical trials, only about a quarter of patients will survive for 2 years [2]. Additionally, while responding to treatment, patients often suffer from debilitating symptoms such as headaches, memory loss, seizures, and mood swings depending on tumor location [3]. There are many complications of GBM treatment, including limitations on resection (due to tumor location or diffuseness) and exclusion of potential therapeutics from the tumor by the cerebrovascular or blood-brain barrier (BBB) [2, 3]. Thus, current surgical options, chemotherapeutics, and radiotherapy can only marginally improve patient survival and not cure GBM [1–3]. It is clear that new options are needed for GBM patients.

One potential treatment modality for GBM, as well as for other treatment-resistant cancers, is to target cancer stem cells (CSCs), a purported group of slow-cycling, undifferentiated cells capable of continual self-renewal [4]. CSCs are linked to increased tumor metastasis and chemoresistance [5, 6]. GBM cells grown in spheroid-inducing, serum-free media are enriched in stemness markers and are thought to provide a better model for in vivo behaviors of GBM as they mimic the highly proliferative, undifferentiated state of most GBMs [7]. Spheroid formation and spheroid culture in this type of system have become a common method of studying GBM due to enhanced behavioral similarities to in vivo tumors [7]. Treatments that are able to cross the BBB and are selective for CSCs over nonmalignant cells may offer a high-efficacy, low-adverse-effect option for patients with GBM that could potentially extend survival or improve quality of life.

Interestingly, dopamine D₂-like receptor (D₂R)-targeting compounds were identified as potential selective

CSC-targeting therapeutics in acute myeloid leukemia, lung cancer, and GBM through pharmacological screens [8–10], and dopamine itself contributes to GBM stem-like self-renewal characteristics [11, 12]. These receptors are G_{ai/o}-coupled GPCRs that are found throughout the nervous system, though they are also present elsewhere in the body [13]. High expression of D₂R has been tied to poor prognosis in numerous cancers [14–18], and D₂R knockdown impairs the growth of the GBM cell line, U87MG [19]. Critically, D₂R antagonists such as those used in the above studies can cross the BBB and readily access CNS tumors. Many of these compounds are also FDA-approved treatments for conditions such as schizophrenia with well-characterized adverse effect profiles, pharmacokinetics, and pharmacodynamic profiles [13, 20]. If the D₂R is involved in stemness, treatment with already-available, relatively safe compounds could extend survival for GBM patients. Here, we use sphere formation assays to study the role of this receptor, and its activity, in GBM.

Although the D₂R and its antagonists have been identified in numerous screens, no study has yet clearly shown a direct relationship between D₂R antagonist cytotoxicity and D₂R receptor activity in order to elucidate the role of the receptor in cancer cell growth. Moreover, these studies were carried out with relatively nonselective concentrations (typically micromolar, when D₂R receptor affinities are typically in the low nanomolar range for these compounds) of D₂R antagonists, a concentration at which binding to other receptors and off target enzymes is likely. In this work, we further characterized the role of D₂R in GBM spheroid formation and stem-like molecular signatures in the context of the commonly used GBM cell line, U87MG. To that end, we employ a combination of genetic modulation and the application of varying chemotypes of D₂R modulators at concentrations selective for D₂R binding to reduce the likelihood of off target effects interfering in the study.

Materials and Methods

Reagents and Cell Culture

U87MG cells were obtained from ATCC (Manassas, VA, USA), and all experiments were performed within 20 passages of receipt. U87MG-shDRD2 and U87MG-OE-DRD2 were generated according to manufacturer's protocols with lentiviruses, respectively, DRD2 GIPZ-shRNA viral particles (Dharmacon, Lafayette, CO, USA) or myc-DDK tagged DRD2 lentiORF particles (Origene Technologies, Rockville, MD, USA). For monolayer cultures, cells were maintained in MEM (Gibco, Waltham, MA, USA) + 10% FBS

(Hyclone, Logan, UT, USA). Spheroids were generated and maintained in spheroid media [DMEM/F12 (1:1) + 20 ng/mL epidermal growth factor (Sigma Aldrich, St. Louis, MO, USA), 5 µg/mL insulin (Sigma Aldrich), 10 ng/mL basic fibroblast growth factor (Sigma Aldrich), and 0.4% bovine serum albumin (Research Products International, Mount Prospect, IL, USA). Media was mixed fresh for each experiment, and 1× B-27 (Gibco) was added to spheroid media immediately before use. All cultures were incubated at 37°C and 5% CO₂. D₂R modulators were the generous gift of Dr. Richard B. Mailman (Penn State College of Medicine, Hershey, PA, USA) aside from sumanirole, which was obtained from Sigma Aldrich (St. Louis, MO, USA).

Spheroid Forming Assays

Spheroid forming assays were carried out as previously described [21]. Briefly, cells were plated at 200 cells/well in given concentrations of compounds in the spheroid media in low adhesion 96 well plates (Corning Costar, Corning, NY, USA) and incubated for 7 days before quantification. PBS was used in all outside wells to reduce evaporation. For counting, spheroids were defined as rounded aggregates of cells with a smooth surface and poor cell-to-cell definition. Loose cellular aggregates of well-defined cells were not included in spheroid counts.

Immunoblotting

Western blotting was performed as previously described [22] using the ScanLater (Molecular Devices, San Jose, CA, USA) visualization system, and 4–12% bis/tris gels (Life Technologies, Carlsbad, CA, USA) were loaded with even amounts of protein (8–12 µg/well) as determined by Pierce BCA assay (Thermo Fisher Scientific, Waltham, MA, USA) and run at 175 V for 60–90 min in MOPS buffer (Life Technologies). Transfer was carried out on ice at 20 V for 120 min. Primary antibodies (Cell Signaling Technology, Danvers, MA, USA) included nestin, glial fibrillary acidic protein (GFAP), Sox2, and vinculin (at dilutions of 1:1,000, 1:500, 1:500, and 1:1,000 respectively, all from Cell Signaling Technology, Danvers, MA, USA). ScanLater Eu-conjugated secondary antibodies (Molecular Devices) were used for visualization with a SpectraMax i3x multimode plate reader (Molecular Devices). Images were quantified with ImageStudio Lite software (Licor, Lincoln, NE, USA).

Invasion Assays

Invasion assays were carried out with Corning BioCoat Matrigel Invasion Chamber 8 µm 24 well plates (Corning, NY, USA) according to manufacturer's instructions. U87 spheroid cells were plated in transwell inserts at 15 × 10⁴ cells/well in DMEM/F12 not supplemented with growth factors, and the complete spheroid media was used as a chemoattractant. Invasion assays were carried out for 48 h before insert removal and cell counting. Inserts were washed and stained with DiffQuik (Siemens, Munich, Germany). Photos were taken of the inserts and quantified with ImageJ.

MTT Assays

MTT assays were carried out as previously described [22], with the following exception. Due to the suspension nature of these cells, spheroid cells were plated at 35,000 cells/well in 50 µL of complete spheroid media and then treated with 50 µL of 2× treatment media as noted before 48 h incubation.

Crystal Violet

Crystal violet was used to observe cell proliferation due to concerns with the potential for limited bromodeoxyuridine delivery to the center of spheroids. For this assay, 20,000 spheroid cells/well in 1 mL media were plated in 24 well plates in complete spheroid media with the conditions noted. At 48, 96, and 168 h, cells were harvested by centrifugation and fixed with methanol for 15 min at room temperature before 3 PBS rinses. These cells were then stained with crystal violet solution (0.5% in 20% methanol, 80% water) for 30 min before 3 PBS rinses and centrifugations (until the PBS had no traces of crystal violet). The crystal violet was then solubilized in methanol and the absorbance at 590 nm was observed for technical triplicates for each independent sample. These values were then normalized to the reading at time zero and reported as fold change.

Extreme Limiting Dilution Assays

Extreme limiting dilution assays were done based on protocols previously described [23, 24]. U87MG cells were plated at 100, 20, 10, 5, and 1 cell(s)/well in 200 µL spheroid media in low adhesion 96 well plates (Corning Costar, Corning, NY, USA) with 100 nmol/L sumanirole, remoxipride, or vehicle. For this assay, plates were incubated for 9 days before quantification rather than 7 days as done for the spheroid forming assays described above to account for the lower plating densities used. At this time point, there was maximum spheroid formation without significant cell death. PBS was used in all outside wells to reduce evaporation. For counting, spheroids were defined as above. Analysis was done using the online extreme limiting dilution analysis (ELDA) webtool [25].

Statistics

All data shown are representative of 2–3 independent experiments. Spheroid counts were carried out in quadruplicate or more for each experiment, whereas cell counts and invasion assays were carried out in triplicate. Time course data was analyzed with two-way ANOVA followed by a Dunnett's post hoc, whereas spheroid count data and immunoblot signals were analyzed with one-way ANOVA plus Dunnett's posthoc. For spheroid forming assays, at least 8 replicate wells were counted; results are presented as box plots, with the whiskers representing minimum and maximum values and the box representing quartiles. For extreme limiting dilution assays, 240 replicate wells were counted for cells plated at 100 cells/well, 480 replicate wells were counted for cells plated at 20, 10, and 5 cells/well and a minimum of 70 replicate wells were counted that were verified to contain a single cell at plating. Analysis was done with the ELDA webtool that uses maximum likelihood estimations in generalized linear models to determine the spheroid forming cell frequency followed by chi-square tests for significance. ELDA results are presented as 95% CIs. All other data points shown are presented as mean ± SEM.

Results

Selective Concentrations of D₂R Modulators Alter Sphere Formation of U87MG

First, we clarified the function of D₂R modulators in a common model of stemness by measuring spheroid form-

Fig. 1. Spheroid formation is altered by selective concentrations of D₂ modulators. **a** 100 nmol/L of D₂R antagonists thioridazine, pimozide, haloperidol, and remoxipride significantly reduce spheroid formation of U87MG cells in a 7 day assay. 100 nmol/L of D₂R agonists PHNO, sumanirole, and ropinirole significantly increased the number of spheroids formed. **b** 100 nmol/L concentrations of D₂ antagonists thioridazine, haloperidol, pimozide, and remoxipride are nontoxic to monolayer cultures of U87MG. **c** 100 nmol/L concentrations

of D₂ antagonists are nontoxic to U87MG cells cultured as spheroids. Spheroid formation assays were analyzed by one-way ANOVA with Dunnett's posthoc. **d** 100 nmol/L remoxipride significantly reduced the spheroid forming cell frequency of U87MG cells in a 9 day assay. 100 nmol/L sumanirole did not significantly reduce the spheroid forming cell frequency. Upper and lower bars represent the 95% CI. Analysis was done with the ELDA webtool. ** $p < 0.01$; **** $p < 0.0001$. PHNO, 4-propyl-9-hydroxynaphthoxazine.

ing capability of the U87MG cell line. For this assay, we used a standard 100 nmol/L concentration, which is closer to expected receptor affinity and thus should provide higher selectivity for D₂R than concentrations of these compounds that have historically been used [14, 26–31]. Over the course of 7 days, 100 nmol/L of D₂ antagonists thioridazine, pimozide, haloperidol, and remoxipride reduced spheroid formation, whereas 100 nmol/L of agonists sumanirole, ropinirole, and 4-propyl-9-hydroxynaphthoxazine increased the number of spheroids formed (Fig. 1a). At these concentrations, there is no significant effect on MTT measurements of overall cell metabolic activity in either monolayer or spheroid cultures

(Fig. 1b, c). Thus, at non-cytotoxic concentrations, multiple chemotypes of D₂ antagonists reduce the number of spheres formed while multiple chemotypes of agonists increase it in the context of U87MG. To determine if this effect is mirrored by alterations in the frequency of spheroid forming cells, ELDA was done. For this assay, cells were plated at decreasing cell densities, and the quantity of sphere-positive wells for each density was used to calculate the frequency of spheroid forming cells in the U87MG population. Over the course of 9 days, 100 nmol/L of remoxipride significantly reduced spheroid forming cell frequency, whereas 100 nmol/L of sumanirole did not significantly increase the frequency of spheroid forming

Fig. 2. Genetic modulation of D₂R alters spheroid formation capacity. **a** Stable transduction with lentiviral vectors produced significant alterations in D₂R protein expression level. Transduction with *shDRD2* reduced protein expression by half (KD on blot), whereas stable overexpression of *DRD2* increased protein expression by ~3.5 fold (OE on blot). **b** Overexpression of *DRD2* slightly increase cell proliferation rates as measured by cell counts, while knockdown slightly decreased it over the course of 4 days. **c** Ex-

pression levels of D₂R correlate with spheroid formation capacity. Knockdown reduced spheroid formation by half, while overexpression more than doubled it. Spheroid formation assays and Western blot data were analyzed by one-way ANOVA with Dunnett's posthoc, while cell counts were analyzed with two-way ANOVA with Dunnett's posthoc. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. KD, knockdown; OE, overexpression; D₂R, D₂ receptor.

cells (Fig. 1d). Thus, while the D₂ agonist sumanirole did increase the number of spheres formed per well it did not alter how many cells in the U87MG population could form spheres. In contrast, the D₂ antagonist remoxipride reduced both the number of spheres and the frequency of cells that could form spheres in this population.

Genetic Modulation of D₂R Alters Sphere Formation of U87MG

While such pharmacological evidence is strongly supportive of a D₂R-mediated reduction in spheroid forming capacity, we utilized genetic manipulation of the *DRD2* gene to observe the role of the D₂R in spheroid-forming phenotypes. Stable, lentivirally-transduced cell lines were used due to the 7-day time period of these assays. Stable transduction of *sh*-RNA against *DRD2* was able to reduce protein expression by about half, whereas stable overexpression of *DRD2* increased expression nearly 4-fold over that of the parental line (Fig. 2a, $p < 0.01$ by one-way ANOVA with Dunnett's post hoc). Overexpression of *DRD2* slightly increased cell proliferation, while knockdown slightly decreased it over the course of 96 h as measured by cell counts (Fig. 2b, $p < 0.01$ by two-way ANOVA with Dunnett's post hoc). However, the differences in

number of spheres formed were more pronounced. Knockdown reduced spheroid formation by nearly half, and overexpression doubled it (Fig. 2c, $p < 0.001$ by one-way ANOVA with Dunnett's post hoc). While it is possible that this effect is due to the proliferative changes in the monolayer culture, the differences in magnitude make it more likely that the presence of D₂R more greatly facilitates survival in the more restrictive environment of spheroid media.

Effects on the Sphere-Forming Phenotype Are Responsive to D₂R Expression Levels

To further control for the possibility of off target pharmacological effects, we assayed the role of the receptor further with a combination of pharmacological competition and genetic manipulation. First, we ascertained the effects of co-treatment with D₂R antagonists and agonists. In these studies, we found that co-treatment with 100 nmol/L 4-propyl-9-hydroxynaphthoxazine, sumanirole, or ropinirole was able to at least partially block the reduction in spheroid formation caused by treatment with 100 nmol/L thioridazine, pimozide, and haloperidol (Fig. 3a). We then compared the behaviors of wild type (Fig. 1a), *DRD2* overexpressed (Fig. 3b) and *DRD2* knockdown (Fig. 3c) U87MG

Fig. 3. The D₂R is involved in pharmacologically induced spheroid formation phenotypes. **a** Co-treatment with 100 nmol/L D₂R agonists PHNO, sumanirole, and ropinirole partially ablated the reduction in spheroid number induced by 100 nmol/L of D₂ antagonists thioridazine, pimozide, and haloperidol over the course of 7 day. **b**, **c** Modulation of D₂R expression impacted compound response in a

7-day spheroid formation assay. Wild-type (Fig. 1a) responses to antagonists were reduced by overexpression of D₂R (**b**), while knockdown blocked all compound responses (**c**). Spheroid formation assays were analyzed by one-way ANOVA with Dunnett's posthoc. * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$. ns, non significant; D2R, D2 receptor; PHNO, 4-propyl-9-hydroxynaphthoxazine.

cell lines under treatment with D₂R modulators. When *DRD2* was overexpressed (Fig. 3b), the ability of antagonists to reduce sphere formation was impaired ($p < 0.05$ for all 4 antagonists by one-way ANOVA with Dunnett's post hoc). Of the agonists, only 100 nmol/L sumanirole significantly increased spheroid formation ($p < 0.05$). It is possible that overexpression led to the near-saturation of signal through

the D₂R at a basal level, thus ablating any further effects from agonist treatment. When *DRD2* was knocked down, none of the compounds, either agonist or antagonist, were able to alter the spheroid formation phenotype of the cells (Fig. 3c, not significantly different from control by one-way ANOVA and Dunnett's post hoc). This suggests that the observed effect requires the D₂R, and is thus not off target.

Fig. 4. D₂R modulators alter spheroid behaviors but not stemness markers. Pharmacological modulation of D₂R activity alters malignant behaviors of spheroids, but do not change stemness marker expression levels. **a** U87MG spheroids were treated with vehicle, 100 nmol/L sumanirole, or 100 nmol/L thioridazine for 72 h. Expression of stemness markers Nestin and glial differentiation marker GFAP were not significantly altered, while expression of Sox2 was reduced in thioridazine-treated cells. **b** U87 MG spheroids were formed over the course of 7 days in the presence of vehicle, 100 nmol/L sumanirole, or 100 nmol/L thioridazine. Expression of markers was unchanged by treatment. **c** Quantification of (a, b). Densitometry values were normalized to loading control, vinculin. **d** Spheroid proliferation was measured by crystal violet staining

over 7 days under treatment with vehicle, 100 nmol/L sumanirole, or 100 nmol/L thioridazine. D₂R agonist sumanirole increased spheroid proliferation at 96 and 168 h, whereas 100 nmol/L thioridazine significantly reduced it. **e** Invasion of cells from U87MG spheroids was measured via transwell assay after 48 h under treatment with 0, 100, and 1,000 nmol/L concentrations of sumanirole, thioridazine, and remoxipride. Sumanirole significantly increased invasion at 100 and 1,000 nmol/L, while thioridazine and remoxipride inhibited it in a concentration-dependent manner. Western blots were analyzed via one-way ANOVA, while two-way ANOVA was used for cell proliferation and invasion assays. Dunnett's posthoc was used for each. ** $p < 0.01$; **** $p < 0.0001$. GFAP, glial fibrillary acidic protein.

Invasive and Proliferative Behaviors Are Affected by D₂R Modulators

The combination of results from pharmacological and genetic modulation in this assay suggested that there was a clear D₂R effect on the spheroid forming phenotype, but we wished to ascertain if this phenotype was due to increased stemness. It is possible that increased spheroid formation is related to increases in adhesion proteins or over-

all proliferation rate more than to stemness markers. We chose to study the glial differentiation factor, GFAP, and 2 common stemness markers, nestin and Sox2 (sex determining region Y-box 2) in both preformed spheres treated for 72 h (Fig. 4a, c), and spheres formed over 7 days in media containing the D₂R modulators (Fig. 4b, c). As elsewhere, we used the more selective 100 nmol/L concentration for all treatments. There was a trend in reduced ex-

pression of nestin and Sox2 for thioridazine treated cells in the preformed sphere condition, but only the reduction in Sox2 expression was significant ($p = 0.002$). In the spheroid forming condition, no significant changes were observed, as expression levels of these proteins were highly variable between replicates, suggesting the potential for significant drift over the 7-day period, perhaps due to variable compensatory mechanisms in the surviving cells. However, significant changes were observed in crystal violet staining, which was used as a surrogate marker of spheroid proliferation (Fig. 4d). Sumanitrole was able to increase total cell membrane staining at 96 and 168 h, but thioridazine significantly decreased it ($p < 0.01$ for both compounds at 96 and 168 h). Spheroids were also more able to invade in a 48 h transwell assay when treated with sumanitrole than thioridazine or remoxipride (Fig. 4e, $p < 0.001$ by two-way ANOVA with Dunnett's post hoc). Taken together, these data suggest that there is an effect on cellular proliferation and invasive behavior with D₂R modulation, but this effect is independent of the regulation of stemness markers, nestin and Sox2.

Discussion/Conclusions

In this work, we have observed a clear spheroid formation phenotype tied to D₂R activity in U87MG cells. Pharmacological and genetic manipulations are able to modulate spheroid formation in this cell line in a manner that can be ablated with the addition of antagonistic factors. It is worth noting that overexpression and knockdown of this receptor was sufficient to ablate both agonist and antagonist effects. In the case of overexpression, it is possible that the effects of agonism were overwhelmed by increased low-level constitutive signaling from the D₂R. It appears that these receptors are engaging in appreciable signaling in the absence of supplied D₂R ligand, though it is possible that the cells are producing dopamine on their own or a factor present in the growth media is providing some level of agonism. Indeed, recent reports suggest that GBM cells can secrete dopamine themselves [11]. Similarly, heightened receptor availability negated the effects of pharmacological antagonism.

These findings modify our understanding of D₂R in GBM spheroids, as we have seen clear spheroid formation effects at selective concentrations of D₂R modulators, but we were unable to observe alterations in expression of nestin, or the differentiation marker, GFAP, but Sox2 expression was significantly reduced in spheroids which were treated with thioridazine for 72 h. This suggests that

while there are changes in spheroid formation, they may not be fully attributable to increase in stemness markers. In the absence of increased stemness characteristics in the sumanitrole treated condition, it appears that cell viability and spheroid growth in the context of serum-free, suspension growth must be largely due to another factor. These could include, but are not limited to, increased cell-cell adhesion, reduced susceptibility to death signals, or an enhanced reliance on other growth signaling systems which are favored in this media, such as EGFR signaling.

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Statement of Ethics

The authors have no ethical conflicts to disclose. No human or animal research was conducted as part of these studies.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

J.S.W. conceived, designed, carried out, and analyzed all experiments with the aid of J.L.R., E.J.K., and M.V.G. J.S.W. wrote the manuscript. J.D.N., G.-L.M., and R.J.H. contributed to experimental design and analysis and edited the manuscript.

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