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Article

A Pyrimidine-Based Tubulin Inhibitor Shows Potent Anti-Glioblastoma Activity In Vitro and In Vivo

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Abstract

Background: Glioblastoma (GBM) presents as an aggressive and treatment-resistant brain tumor with few effective therapies. We targeted the colchicine-binding site on tubulin—a validated but underutilized site—to develop novel small-molecule inhibitors as alternatives to temozolomide (TMZ), the current standard treatment. **Methods:** We synthesized a focused library of pyrimidine- and dihydroquinoxalinone-based analogs and tested nine compounds for cytotoxicity in GBM cell lines using the Sulforhodamine B (SRB) assay. We identified compound 4 as the most promising compound and evaluated its effects on cell growth using live-cell imaging, assessed apoptosis via cell death ELISA, and tested its anticancer activity in GBM xenografts in vivo. **Results:** Several compounds demonstrated nanomolar IC₅₀ values and outperformed TMZ. We identified compound 4—a pyrimidine analog with a secondary amine—as the lead candidate. Compound 4 reduced cell viability in a dose-dependent manner and induced complete growth arrest within 48 hours at concentrations as low as 3–10 nM in LN229 and MT330 GBM cell lines. ELISA confirmed that compound 4 triggered dose-dependent apoptosis, whereas TMZ failed to induce apoptosis even at micromolar concentrations. In vivo, compound 4 significantly inhibited GBM xenograft growth in immunocompromised mice by 66%. **Conclusions:** Compound 4 acts as a potent tubulin inhibitor with strong anti-GBM activity and merits further preclinical development.

Keywords: pyrimidine dihydroquinoxalinone; microtubule-targeting agents (MTAs); glioblastoma (GBM); cancer

1. Introduction

Glioblastoma (GBM) is the most common and aggressive type of primary brain tumor, with approximately 12,000 new cases diagnosed annually in the United States [1]. Despite advances in surgical resection, radiotherapy, and chemotherapy, GBM remains highly lethal due to its rapid progression and resistance to conventional treatments. The median survival time for patients with GBM remains poor, typically ranging from 12 to 18 months [2]. Temozolomide (TMZ), an oral DNA-alkylating agent, which can cross the blood-brain barrier (BBB), is the standard chemotherapeutic used in GBM treatment [2]. By interfering with DNA replication in cancer cells, TMZ induces cell death and helps slow tumor growth. However, its clinical effectiveness is often limited by the development of resistance mechanisms within tumors. As a result, TMZ rarely extends patient survival beyond 15 months [2]. The emergence of TMZ resistance poses a major challenge in GBM therapy, highlighting the urgent need for novel, potent small molecules capable of effectively

targeting GBM cells. Developing such agents to overcome the limitations of current treatments, particularly TMZ resistance, is a critical focus in GBM research.

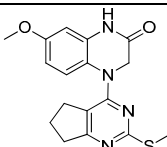
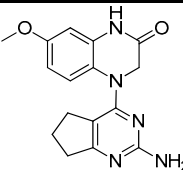
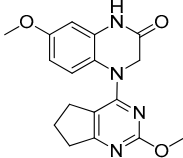
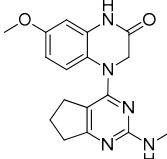
Microtubule-targeting agents (MTAs) have become a significant area of focus in the development of anticancer drugs. Microtubules are dynamic heteropolymers composed of α - and β -tubulin subunits [3-5]. These structures play a pivotal role in various cellular processes, including maintaining cell shape, enabling intracellular transport, and facilitating cell division [6]. MTAs disrupt polymer dynamics, triggering cell cycle arrest that leads to cell death. Thus, microtubules are a good target for anticancer therapy. Three major binding sites have been identified in tubulins: the taxane [7], vinca alkaloid [8], and colchicine sites [9]. A broad variety of small molecule tubulin-binding agents have been developed as therapeutics for a variety of other cancers. Hence, developing novel compounds that can induce cell death without resistance is crucial for improving outcomes in GBM therapy. In this study, we have selected a series of MTAs that were developed in our laboratory for melanoma, breast, pancreatic, and prostate cancers and we selected nine of the most potent microtubule inhibiting molecules to test against two glioblastoma cell lines.

2. Results

2.1. MTAs reduce GBM cell viability in vitro.

Over the past decade, our laboratory has developed a series of small-molecule tubulin inhibitors targeting the colchicine binding site, potentially offering therapeutic advantages over other MTAs [10–13]. To advance these compounds as pharmacological agents against glioblastoma (GBM), we focused on our most potent derivatives containing pyrimidine and dihydroquinoxalinone moieties with diverse functional groups. From this library, nine analogs (compounds 1–9, Table 1) were selected and tested for their effects on the viability of two GBM cell lines (U251 and LN229) using the Sulforhodamine B (SRB) assay, a standard method to measure cytotoxicity in cell-based assays. TMZ, the current anti-glioma drug, was included as an internal control.

Table 1. Cytotoxic Activities of MTAs on U251 and LN229 GBM Cell Lines.

S.No	Structure	IC ₅₀ (nM)	
		GBM	cell lines
		U251	LN229
1		2.49	1.88
2		>1000	>1000
3		80.1	15.6
4		2.36	2.09

5		3.1	3.15
6		0.81	5.82
7		6.56	8.36
8		5.71	5.66
9		2.09	2.36
10	Temozolomide	>1000	>1000

Based on the structures of previously characterized compounds such as Verubulin, BPR0L075, and BNC105 (Figure 1), which demonstrated preclinical efficacy, we hypothesized that the methoxy (OMe) group on the aromatic ring is critical for biological potency. We selected compound 3, which contains this group, and found it exhibited moderate cytotoxic activity with IC_{50} values of 15.6 and 80.1 nM in the two glioma cell lines, respectively. Modification of the OMe group in compound 3 to a thioether (SMe, compound 1) markedly increased potency, with IC_{50} values of 1.88 and 2.49 nM in the respective cell lines.

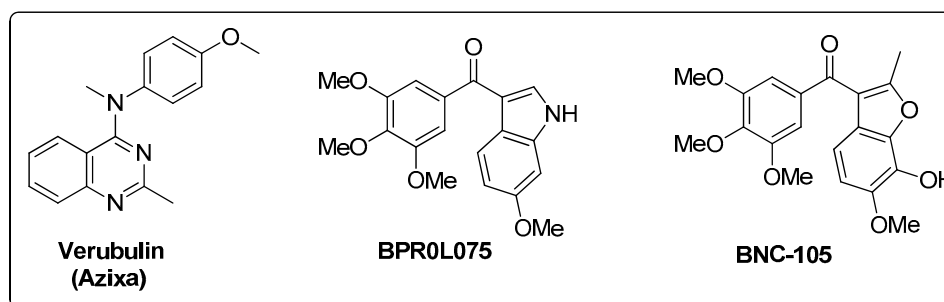


Figure 1. Previous Tubulin Binding Agents with pre-clinical efficacy in GBM.

Encouraged by these results, we then examined various pyrimidine analogs with primary (1°) and secondary (2°) amine substitutions at the 2-position of the pyrimidine ring. Interestingly, secondary amine-bearing pyrimidine analogs showed strong potency. Compound 4 exhibited IC_{50} values of 2.09 and 2.36 nM, compound 5 had 3.1 and 3.15 nM, and compound 6 showed 0.81 and 5.82 nM in the two glioma cell lines, respectively. In contrast, the primary amine analog (compound 2) was biologically inactive ($IC_{50} > 1000$ nM). Incorporation of nitrogen at the 2-position as part of a heterocyclic ring improved potency, as demonstrated by compounds 4 and 5. Additionally,

derivatives containing isothiocyanate and methyl groups (compounds 8 and 9) displayed significant cytotoxic activity (IC_{50} values ranging from 2.09 to 5.71 nM). The IC_{50} values for all compounds are summarized in Table 1.

For reference, TMZ, the standard DNA-alkylating agent used to treat GBM, required concentrations at least 1000-fold higher to induce cytotoxicity (IC_{50} of 20–400 μ M). This study revealed that most of the tested compounds induced cytotoxicity in GBM cells with nanomolar potency. Based on these promising results, we selected compound 4 for further investigation of its anticancer activity. The complete dose-response curve for compound 4 in the SRB assay is shown in Figure 2.

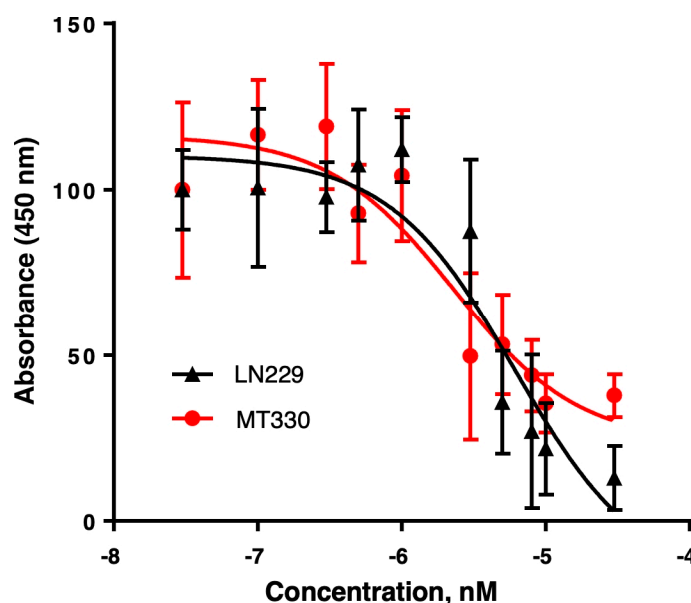


Figure 2. Compound 4 reduces GBM cell viability in a dose-dependent manner. LN229 (black triangles) and MT330 (red circles) GBM cells were treated with increasing concentrations of compound 4 for 5 days, followed by assessment of cell viability using the sulforhodamine B (SRB) assay. Absorbance at 540 nm (A_{540}) was measured and normalized to vehicle (DMSO)-treated controls. Both cell lines exhibited a dose-dependent decrease in viability, with MT330 cells showing slightly greater sensitivity to compound 4. Data are presented as mean \pm SEM from three independent experiments.

2.2. Compound 4 reduces the proliferation of GBM cells *in vitro*.

We next employed the IncuCyte Live-Cell Imaging System to monitor GBM cell proliferation and morphology in real time over a 5-day period. LN229 and MT330 GBM cells were seeded into 96-well plates and treated the following day with compound 4 at concentrations ranging from 1 to 10 nM. MT330 cells were also examined because they are a clinically relevant *in vivo* model of GBM tumorigenesis [14]. As shown in Figure 3, vehicle-treated LN229 and MT330 cells rapidly entered a rapid exponential growth phase. However, treatment with 1 nM compound 4 significantly reduced cell proliferation compared to controls. At 3 and 10 nM, both cell lines exhibited complete growth arrest within 48 hours of treatment initiation, with a more pronounced response observed in MT330 cells. Photomicrographs taken during this time revealed morphological changes in both cell lines treated with compound 4 suggestive of apoptosis.

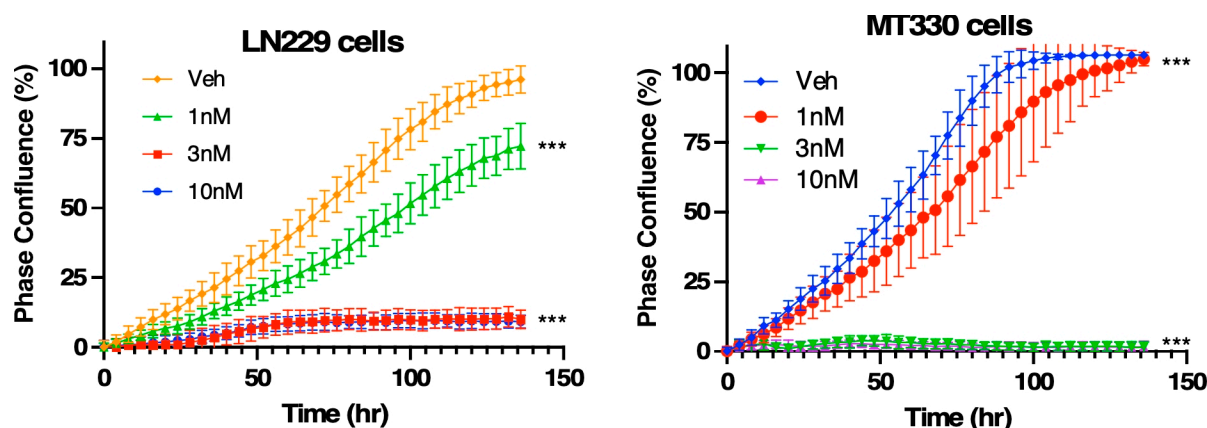


Figure 3. Compound 4 inhibits proliferation of GBM cells in a dose-dependent manner. LN229 (left) and MT330 (right) GBM cells were treated with vehicle (Veh) or compound 4 at concentrations of 1, 3, or 10 nM. Cell proliferation was monitored in real time using the IncuCyte Live-Cell Imaging System, and phase confluence (%) was measured every 4 hours over a 5-day period as an indicator of cell growth. Vehicle-treated cells entered exponential growth, while compound 4 induced a dose-dependent reduction in proliferation in both cell lines. At 3 and 10 nM, compound 4 caused complete growth arrest within 48 hours, with a more pronounced effect in MT330 cells. Data are presented as mean \pm SEM from three independent experiments. Statistical significance was determined at the final time point using one-way ANOVA with post hoc analysis.

2.3. Compound 4 induces the apoptosis of GBM cells in vitro.

We next investigated whether the reduction in GBM cell viability following compound 4 treatment was attributable in part to the induction of apoptosis. LN229 and MT330 GBM cells were treated with compound 4 for 48 hours, after which apoptosis was assessed using a highly sensitive, cell death ELISA that detects nucleosome release from apoptotic cells, as previously described [14]. As shown in Figure 4, compound 4 induced apoptosis in LN229 GBM cells in a dose-dependent manner, with significant activity observed at concentrations between 1 and 3 nM, which is consistent with its IC_{50} values from SRB assays. In addition to LN229 cells, we also examined MT330 GBM cells. Similar to LN229, MT330 cells exhibited a robust, dose-dependent apoptotic response to compound 4. However, TMZ, which is the standard-of-care chemotherapy for GBM, does not induce apoptosis in either LN229 or MT330 GBM cells even at micromolar concentrations [15], underscoring the distinct and potent pro-apoptotic activity of compound 4.

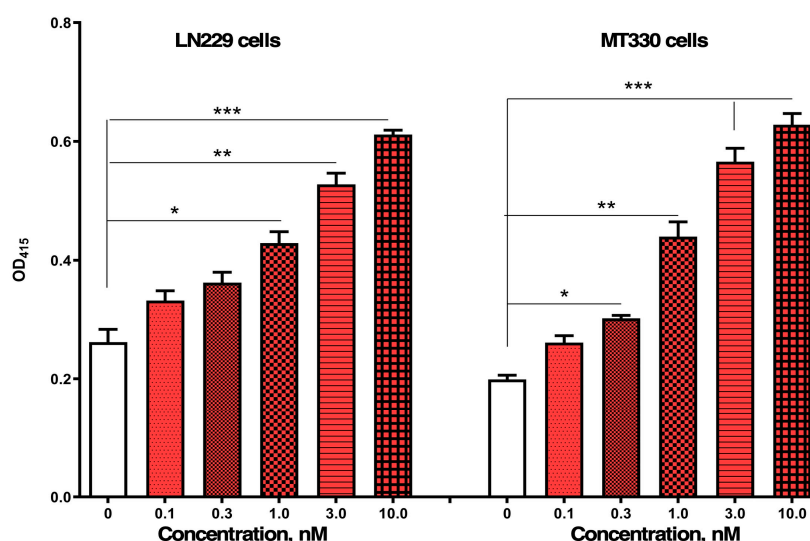


Figure 4. Compound 4 induces dose-dependent apoptosis in GBM cells. LN229 and MT330 GBM cells were treated with increasing concentrations of compound 4 for 48 hours as indicated. Apoptosis was quantified using a

nucleosome-based cell death ELISA, which detects cytoplasmic histone-associated DNA fragments released during apoptotic cell death. Data are presented as mean \pm SEM from three independent experiments. A significant, dose-dependent increase in apoptosis was observed in both cell lines, with maximal apoptotic activity detected between 1 and 3 nM. Statistical significance was determined using one-way ANOVA with post hoc analysis. *p* values are indicated as follows: < 0.05 (*), 0.01 (**), and 0.001 (***).

2.4. Compound 4 exhibits potent anticancer activity against GBM tumors *in vivo*

We have demonstrated that compound 4 significantly reduces proliferation and induces apoptosis in GBM cells *in vitro*, suggesting its potential as an anticancer agent. To further evaluate its efficacy, we tested compound 4 in a GBM xenograft model. MT330 GBM cells were injected subcutaneously into the flanks of immunocompromised mice. Eleven days post-engraftment, mice were randomized into two groups: (a) vehicle control (PEG-saline mixture), and (b) compound 4-treated (5 mg/kg, administered intraperitoneally twice weekly). Treatment continued until a humane endpoint was reached in either group.

Tumor volumes were measured two times a week using calipers. As shown in Figure 5A, throughout the 25-day time course of treatment with compound 4 there was a significant reduction in mean tumor volume compared to controls. Individual tumor volumes throughout the time course of drug treatment are depicted in Figure 5B, which further confirmed markedly reduced growth in the compound 4-treated group.

At day 36 post-injection, several mice in the control group reached the humane endpoint (tumor volume equal to or greater than 1000 mm³), whereas none of the compound 4-treated mice did. At this time point, all mice were euthanized, and tumors were excised, measured, and weighed. Figure 6A shows the dot plots of the tumor volumes in individual mice showing the trend of smaller tumors in compound-4 treated mice. The average tumor size in the compound 4-treated group being 441.3 mm³, compared to 1333.6 mm³ in the control group—a 66% reduction. Figure 6B visually illustrates the difference in tumor size with those in compound 4-treated mice being substantially smaller than those from control mice. Furthermore, as shown in Fig 6C the weight of the tumors was markedly lower in compound 4-treated mice as compared to control (0.50 g versus 1.12g)

Taken together, the results in Figure 5 and 6 clearly demonstrate that compound 4 exhibits potent anticancer activity against GBM tumors *in vivo*.

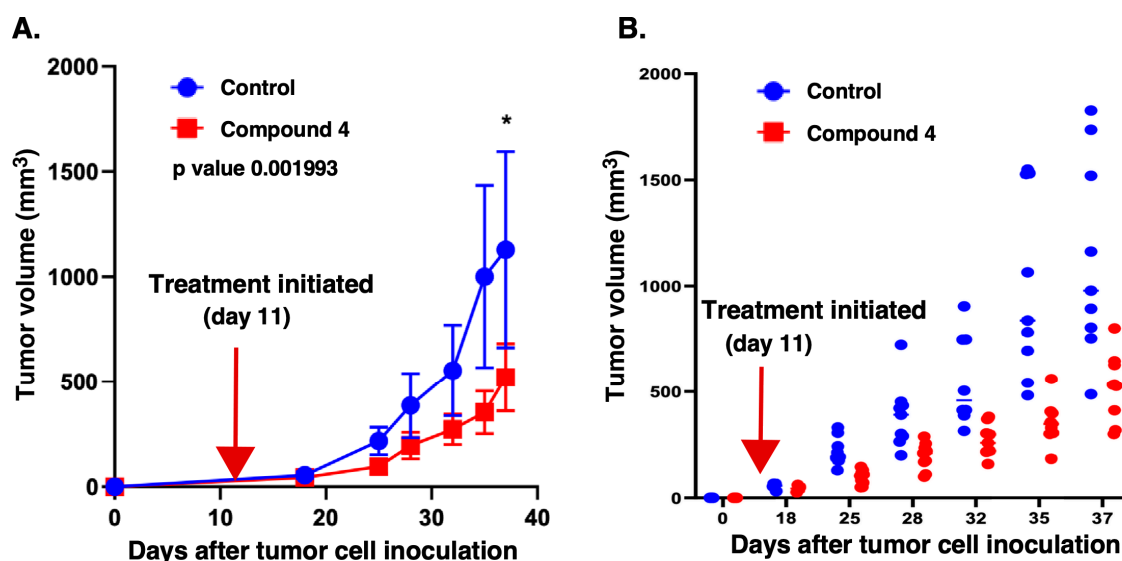


Figure 5. Compound 4 has potent anticancer activity on GBM xenografts. MT330 cells (5×10^5) were injected into the flanks of 20 female NSG (NOD.Cg-Prkd^{scid} Il2rg^{tm1Wjl}/SzJ) immunocompromised mice. At 11 days after tumor injection, palpable tumor masses could be readily discerned. The mice were then randomized into two groups (N=6 to 9): a vehicle control group (PEG-saline) and a group of mice treated with compound 4 at 5 mg/Kg twice weekly. Tumor volumes were measured with calipers on a weekly basis. (A) Linear graph of tumor volumes

plotted against the number of days after tumor inoculation. (B) Dot plot of tumor volumes in individual mice plotted against the number days after tumor inoculation. A significant inhibition in tumor growth was observed throughout the course of treatment with compound 4. Statistical significance was determined using one-way ANOVA with post hoc analysis. *p* values are indicated as follows: < 0.05 (*), 0.01 (**) and 0.001 (***).

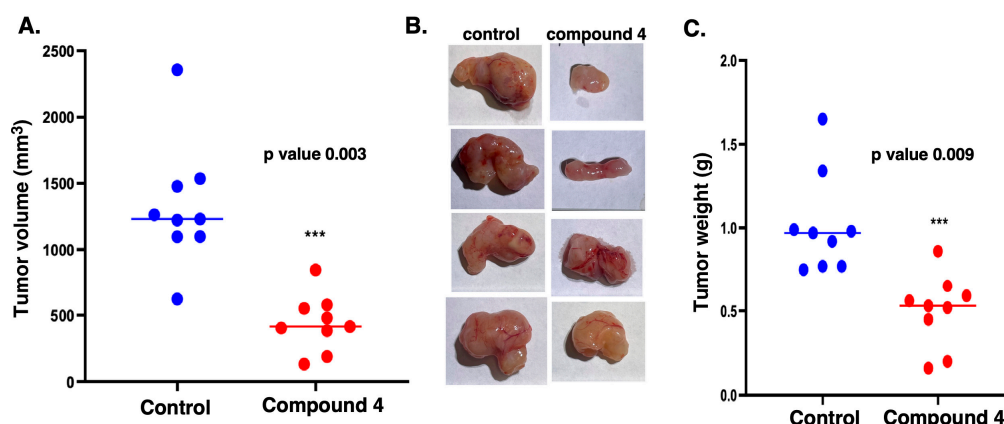


Figure 6. Compound 4 has potent effect on GBM xenograft size and weight. Mice were inoculated with MT330 GBM cells and treated with Compound 4 or vehicle as described to the legend in Figure 5. At 26 days after treatment (37 days after tumor injection), the mice were euthanized and the tumors removed. A significant inhibition in tumor growth was observed at two weeks after treatment with compound 4. (A) A dot plot of tumor volume, (B) photograph of three tumors from each group, and (C) dot plot of tumor weights. Statistical significance was determined using one-way ANOVA with post hoc analysis. *p* values are indicated as follows: < 0.05 (*), 0.01 (**) and 0.001 (***).

3. Discussion

Gliomas—particularly high-grade variants such as GBM—are among the most aggressive and treatment-resistant tumors of the central nervous system. Despite advances in surgical resection, radiotherapy, and chemotherapy, including the use of temozolomide (TMZ), the prognosis for GBM patients remains poor, with median survival rarely exceeding 15 months [10]. A major challenge in treating gliomas is their highly proliferative, invasive, and heterogeneous nature, compounded by the presence of the blood-brain barrier (BBB), which restricts effective drug delivery to tumor sites [11].

In recent years, microtubule-targeting agents (MTAs) have emerged as a promising class of chemotherapeutics capable of disrupting cancer cell division, migration, and intracellular transport. These agents act by binding to tubulin—the structural component of microtubules—thereby interfering with microtubule dynamics essential for mitotic spindle formation and cellular integrity [11]. Given the pivotal role of microtubules in glioma cell proliferation and migration, tubulin inhibitors offer potential to address critical therapeutic gaps in glioma treatment. However, translating the success of tubulin inhibitors from other cancer types to glioma therapy has proven difficult. Challenges such as limited BBB penetration, neurotoxicity, and the molecular heterogeneity of glioma cells have hindered clinical efficacy. Several tubulin inhibitors have demonstrated anticancer activity. For instance, Verubulin (MPC-6827, Azixa), a pyrimidine-derived small molecule, inhibits microtubule formation, crosses the BBB, and induces mitotic arrest and apoptosis in various cancer cell lines. Nevertheless, Verubulin failed in phase II clinical trials for recurrent GBM due to insufficient efficacy and unacceptable toxicity [12]. Similarly, BPR0L075 (SCB01A) and BNC105 showed preclinical efficacy in multiple cancer models, including GBM, but did not achieve success in clinical trials [13–15].

Recent studies have continued to explore colchicine-binding site inhibitors (CBSIs) as viable alternatives for GBM therapy. Liu et al. reported that 2-aryl-4-amide-quinoline derivatives exhibited potent antiproliferative activity and favorable pharmacokinetics in breast cancer models, suggesting

broad applicability of strategies [10]. Weng et al. emphasized the structural flexibility and strong cytotoxicity of CBSIs, positioning them as a mainstream approach for overcoming multidrug resistance [11]. In GBM-specific research, Manzoor et al. identified pyrimidine-based CDK6 inhibitors with high binding affinity and stability, reinforcing the therapeutic relevance of pyrimidine scaffolds [12]. Byrne et al. developed pyrazolopyrimidinone with selective cytotoxicity against GBM cells in vitro, further validating nitrogen-containing heterocycles as promising platforms for drug development [13].

In this study, we targeted the colchicine-binding site on tubulin with a focused library of pyrimidine- and dihydroquinoxalinone-based analogs. Compound 4, a pyrimidine analog with a secondary amine, emerged as the lead candidate, demonstrating nanomolar IC₅₀ values and superior efficacy compared to TMZ. Structural modifications—particularly secondary amine substitutions and heterocyclic enhancements—were key contributors to its cytotoxic potency.

Live-cell imaging revealed that compound 4 rapidly inhibited GBM cell proliferation, while apoptosis assays confirmed a robust, dose-dependent induction of cell death at concentrations far lower than those required for TMZ. Importantly, in vivo studies using a GBM xenograft model showed that compound 4 reduced tumor volume by 66% and tumor weight by over 50%, without apparent toxicity.

Taken together, these findings validate colchicine-site tubulin inhibition as a compelling strategy for GBM treatment and support the continued preclinical development of compound 4. Its potent activity, favorable pharmacodynamics, and ability to overcome TMZ resistance position it as a strong candidate for future translational and clinical studies.

4. Experimental methods

4.1. Biological reagents and cell cultures

U251 and LN229 GBM cell lines (ATCC, Manassas, VA), along with the MT330 cell line (Department of Neurosurgery, UTHSC), were cultured in DMEM supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 100 IU/mL penicillin, and 100 µg/mL streptomycin. Cells were maintained at 37 °C in a humidified incubator with 5% CO₂. All cell lines were authenticated by short tandem repeat (STR) analysis.

4.2. SRB assays

LN229 and U251 GBM cells were plated at a density of 5,000 to 10,000 cells per well in a 96-well plate and treated with various concentrations of MTAs. After 4 to 5 days of incubation, cells were fixed with trichloroacetic acid, and the plates were washed thoroughly. Sulforhodamine B (SRB) dye was then added to stain the cellular proteins. Following extensive washing to remove unbound dye, the bound SRB was solubilized, and absorbance was measured at 540 nm on a plate spectrophotometer. IC₅₀ values were determined using GraphPad Prism software.

4.3. Cell proliferation assays

For cell proliferation assays, LN229 and MT330 GBM cells were seeded at 2×10^3 cells per well in 96-well plates. After 24 hours, plates were treated with or without compound 4 and placed in the Incucyte Live-Cell Analysis System (Essen Bioscience, Ann Arbor, MI). Cells were incubated at 37°C, and proliferation was monitored over time by cell confluency using the manufacturer's software tools.

4.4. Cell death assays

For cell death assays, LN229 and MT330 GBM cells were seeded at 1×10^4 cells per well in 48-well plates. Following 2 days of drug treatment, apoptosis in the adherent cell population was assessed using the Cell Death Detection ELISAPLUS kit (Roche), according to the manufacturer's

instructions. This assay quantifies cytoplasmic histone-associated DNA fragments, a hallmark of apoptotic cell death [14].

4.5. Mouse xenograft experiments

All animal experiments were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at UTHSC (Protocol #17-098). Xenograft tumors were established in five-week-old female NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ immunocompromised mice (Jackson Laboratory). For xenograft studies, MT330 cells (5×10^5) were injected subcutaneously into the flanks of mice, as previously described [15]. Once tumors became palpable (usually within 10 to 14 days), mice were randomly assigned to the two treatment groups (9 mice per group) and received intraperitoneal injections of compound 4 (5 mg/kg body weight) or vehicle (DMSO) twice per week. Tumor volumes were measured weekly using calipers (calculated as $\text{width}^2 \times \text{length} \div 2$).

Author Contributions: Conceptualization, D.D.M., L.M.P., and W.L.; methodology, S.P., D.H.; validation, M.D., J.I.J., A.S., H.R.K., D.N.P.; formal analysis, M.D., J.I.J., A.S., H.R.K., C.H.Y., D.N.P.; resources, S.P., D.D.M.; writing—original draft preparation, S.P., L.M.P. and D.D.M.; writing—review and editing, S.P., D.H., W.L.; D.D.M. and L.M.P.; supervision, D.D.M., W.L. and L.M.P.; project administration, D.D.M., W.L. and L.M.P.; funding acquisition, D.D.M., W.L. and L.M.P. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by the Institutional Review Board of the University of Tennessee Health Science Center (protocol code 23-0472, 10/19/2024) for studies involving animals.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article.

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript.

Abbreviations

GBM	Glioblastoma
TMZ	Temozolomide
MTAs	Microtubule-targeting agents
IC ₅₀	half-maximal inhibitory concentration
SRB	Sulforhodamine B
NSG	NOD.Cg-Prkdc ^{scid} Il2rg ^{tm1Wjl} /SzJ6+

The

following abbreviations are used in this manuscript:

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