

Metformin Enhances the Sensitivity of Glioblastoma Cancer Cells to Cisplatin through DNA Damage Assessment

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Abstract

Background: Glioblastoma (GBM) stands out as the most prevalent primary brain tumor characterized by its high aggressiveness. Numerous therapeutic approaches have been employed, and the utility of combination therapies has been substantiated, particularly in GBM treatment. Cisplatin, an anticancer chemotherapeutic agent, is employed for the management of various malignancies, including GBM; however, it is associated with significant systemic toxicity. In the realm of combination therapy, metformin, a biguanide drug conventionally used as a first-line treatment for type 2 diabetes, has recently emerged as a valuable adjunct in the treatment of a diverse spectrum of tumors. This study aimed to elucidate the impact of metformin on sensitizing the human cerebral GBM cancer cell line (AMGM) to cisplatin chemotherapy by employing the comet assay as a means to assess DNA damage, thereby advocating the potential of metformin as an adjuvant for cisplatin-based therapy.

Method: In this experimental study, the AMGM cell line was cultured and subsequently treated with either single-agent cisplatin, metformin, or a combination of both drugs. Cell viability was assessed through growth inhibition calculations. The Chou–Talalay analysis was used to assess the cooperative effect of this drug combination. Furthermore, DNA fragmentation was quantified using the alkaline comet assay technique.

Results: The findings demonstrate that metformin significantly potentiates the therapeutic efficacy of cisplatin by synergistically inhibiting the growth of AMGM cells and reducing DNA damage.

Conclusion: These results underscore the potential utility of metformin as a valuable adjunct in enhancing the clinical effectiveness of chemotherapy regimens.

Keywords: Metformin, Cisplatin, Glioblastoma, Synergism effect, DNA fragmentation

Introduction

In Iraq, according to the annual Iraqi Cancer Registry 2022, the top 10 cancer incidences in both genders

per 105 population in breast, lung, leukemia, non-Hodgkin lymphoma, thyroid, and brain with other central nervous system (CNS) were 22.2,

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7.5, 6, 5.1 and 4.7 respectively.¹⁻²

While in the statistics of the United States, malignant brain and other CNS tumors account for a small proportion, about 1% of all invasive cancer cases, but are the most commonly diagnosed solid tumor in children and adolescents and represent the leading cause of cancer death among males aged <40 years and females aged <20 years. In 2021, about 83,570 individuals were diagnosed with brain and other CNS tumors in the United States (24,530 malignant tumors and 59,040 non-malignant tumors), and 18,600 people died from the disease. Glioblastoma (GBM) is recorded to be less than one-third of all brain and other CNS tumors diagnosed in the United States; however, the majority of deaths are from its incidence.³

Cisplatin (cis-dichlorodiammineplatinum, CDDP) represents one alkylating agent as one of the well-known platinum-based chemotherapeutic drugs, induces DNA damage, interferes with its replication and transcription, and disrupts its structure. Cisplatin exhibits a high affinity towards sulfur donors such as cysteines and methionine, forming stable (Pt-S) bonds. It competes with the affinity towards the nitrogen atom in the backbone of DNA, thus contributing to resistance against

the cytotoxic action of cisplatin.⁴⁻⁵

Although cisplatin shows a broad spectrum of anticancer activity, its utility is limited due to acquired drug resistance, side-effects, damage to non-targeted tissues, and long-term off-target effects, which represent one of the significant factors causing mortality in cancer survivors in a later stage of patients life.⁶⁻⁷

Several chemotherapeutic drugs used, like biguanides metformin, phenformin, and buformin, were initially derived from the herb *Galega officinalis* (French lilac) and were developed for the treatment of individuals with hyperglycemia and type 2 diabetes diseases; in addition, was evaluated as anticancer agent.⁸⁻⁹

Metformin (1,1-dimethyl biguanide hydrochloride) was associated with decreased cancer incidence and mortality in diabetic patients, and the insulin-lowering effects of metformin may be integral to its anticancer properties.¹⁰⁻¹²

In review,¹³ they used metformin to enhance the activity of standard glioma therapies. In studies conducted by Adeberg et al. on a cohort of 276 patients with primary GBM, longer progression-free survival was demonstrated in diabetic patients treated with metformin.¹²⁻¹⁴

Another previous study on high-grade glioma

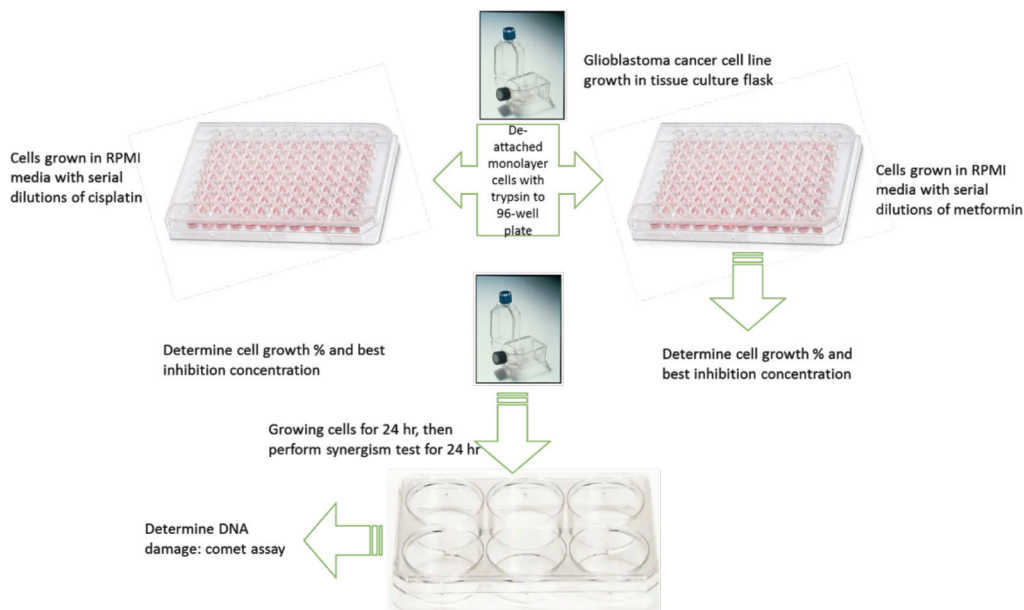


Figure 1. This figure illustrates the flowchart depicting the sequence of current consequence experiments. A Glioblastoma cancer cell line was cultured and tested for viability through a cytotoxicity assay and DNA damage assessment using a comet assay.

(HGG) patients included 1,093 patients with HGG; metformin was found to give a better overall and progression-free survival of patients with World Health Organisation (WHO) grade III, suggesting the mechanisms of isocitrate dehydrogenase (IDH) mutations which might sensitize to the metabolic drug metformin.¹⁵

In a recent study by Liu et al. published in October 2022 working on the association of high glucose levels in GBM patients, their findings were the lack of intrinsic differences among glioma patients, and the importance of decreasing glucose levels and glioma clinical trials could incorporate molecular subclasses by reproducible and widely adopted method such as DNA methylation. Suggested that the absence of methylation phenotype differences between tumors in different glucose levels leads to differences in how tumor cells utilize glucose.¹⁶

This study investigated metformin's potential effects in sensitizing the human cerebral glioblastoma-multiforme cancer cell line (AMGM) GBM cancer cell line to cisplatin chemotherapy using comet assay to determine DNA damage. The use of metformin as an adjuvant for cisplatin-based therapy was suggested.

Materials and Methods

This study represents an experimental study, and the flowchart experiments are displayed in figure 1. The study was approved according to the "Application for Biomedical Research Ethics Review" from the Research Ethics Committee of Mustansiriyah University (BCSMU code/2022).

Chemotherapeutic agent

Cisplatin (Celon laboratories, India), the anticancer drug, was gifted from the Radiation and Atomic Medicine Hospital (Baghdad, Iraq) with a concentration (50mg/50ml: IP). This drug was diluted with a medium without calf bovine serum just before use for in vitro studies.

Metformin, a drug used for diabetic patients, was purchased from Sigma Chemical Co. and dissolved in a culture medium without calf bovine serum before being used in vitro studies.

Cell line maintenance

Brain GBM cancer cell line (AMGM) was established by Al-Shammari et al.,¹⁷ cells were maintained and cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C and 5% CO₂. The cells were a kind gift from the experimental therapy department, the Iraqi Center for Cancer Medical Genetics Research, and Mustansiriyah University.

Cytotoxicity assays

In order to assess the cytotoxic impact of metformin and cisplatin, the AMGM GBM cancer cell line was cultivated in two distinct 96-well plates until the formation of confluent monolayers. In the first plate, cells were subjected to metformin treatment alone, with serial dilutions ranging from 1000, 100, 80, 60, 40, 20, and 10 to 1 mg/ml, respectively. In the second plate, cells were exposed to the chemotherapeutic agent cisplatin, again with serial dilutions of 1000, 100, 10, 8, 6, 4, 2, and 1 mg/ml. Following a 24-hour incubation period, the manufacturer's protocol conducted a crystal violet assay utilizing a 96-well plate.

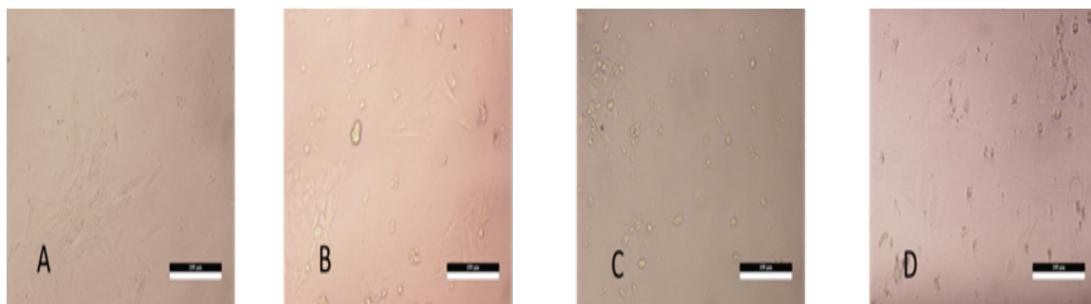


Figure 2. This figure depicts the growth of AMGM cells in identical exposure conditions: (a) control (untreated cells), (b) cisplatin alone, (c) metformin alone, and (d) a combination of both cisplatin and metformin.

AMGM: Human cerebral glioblastoma-multiforme cancer cell line

Subsequently, the culture media were aspirated, and the plates were washed with PBS before being stained with crystal violet for 20 minutes. Once the plates were thoroughly dried, absorbance measurements were obtained utilizing an ELISA reader spectrophotometer set at 570 nm (EnSpire Multiplate reader, Perkin Elmer, Boston, USA). Cell viability was represented as a percentage of viable cells relative to the untreated control cells. This assay was carried out in triplicate, and the inhibition rate of cell growth, expressed as the percentage of cytotoxicity, was calculated using the following equation:

$$\text{Cell inhibition \%} = ((A-B)/A) \times 100\%$$

, where A denotes the mean optical density of the untreated wells, and B signifies the optical density of the treated wells.

Combination assay according to Chou-Talalay (1984)

To evaluate the effect of the combination of metformin and cisplatin, the cells were cultured in 96-well plates until confluent monolayer formation. Cells were treated with a combination of the two (metformin + cisplatin) in serial dilutions for 24 hours. After an incubation duration, crystal violet stained the plates, and the absorbance was measured using an ELISA reader spectrophotometer at 570 nm (EnSpire Multiplate reader, Perkin Elmer, Boston, USA). To analyze the result of combination drugs, CompuSyn software (ComboSyn Inc., Paramus, NJ, USA)

was utilized to compute the Chou–Talalay assay combination indices (CIs) and variable ratios of metformin and cisplatin. If a CI value less than 1 indicates synergism, greater than 1 indicates antagonism, and equal to 1 indicates additivity.¹⁸

Genotoxicity assay (comet assay)

For the detection of DNA fragmentation associated with apoptosis, the alkaline comet assay method pH=13, as described by Collins, was used:¹⁹

The AMGM cells were cultured in replicates of 6-well plates until a monolayer had formed. Once the monolayer had formed, the cells were incubated with cisplatin, metformin, and a combination of both. After a 24-hour incubation period, the cells were detached using a scraper, centrifuged, and the supernatant was removed. From the resulting precipitate, 10 µl was extracted and mixed with low-temperature melting agarose at a ratio of 1:10 (v/v). This mixture was then spread onto a previously prepared glass slide coated with agarose gel. Subsequently, the slides were immersed in pre-cooled lysis buffer (comprising 2.5 M NaCl, 100 mM EDTA, pH 10, 10 mM Tris base, and 1% Triton X-100) and kept at 4°C for 90 minutes. Following lysis and thorough rinsing, the slides were equilibrated in a TBE solution (composed of 40 mM Tris/boric acid and 2 mM EDTA, pH 8.3), electrophoresed at 1.0 V/cm² for 20 minutes, and then subjected

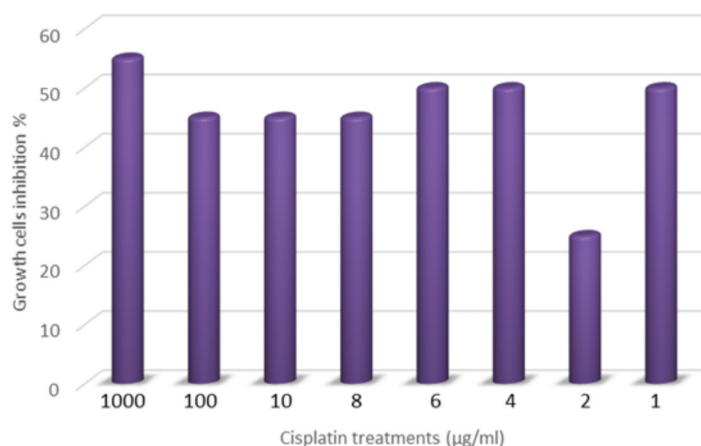


Figure 3. The cytotoxicity activity of cisplatin, at varying concentrations, is compared to untreated AMGM cells over 24 hours of incubation. The data represent the mean of three replicate experiments.

AMGM: Human cerebral glioblastoma-multiforme cancer cell line

to ethidium bromide staining for 5 minutes.

For comet pattern evaluation, 50 nuclei were counted from each slide. The scoring of apoptotic comets was conducted using the method devised by Collins. Images of the comets were captured under a fluorescence microscope (Leica) at $\times 100$ magnification. For each sample, a minimum of 50 comets were analyzed, and the olive tail moment, calculated as $[\text{tail DNA} (\%) \times (\text{tail mean} - \text{head mean})]$, was quantified using the Comet Assay Software Project (CASP) Lab version 1.2.3b1, developed by the Free Software Foundation Inc. in Boston, MA, USA.

Statistical analysis

Each experiment was conducted thrice using independent cell passages. Statistical analysis was carried out using GraphPad Prism version 8.1.0. The data are represented as the mean \pm standard deviation (SD). Differences between the means of treated and untreated samples were assessed through a one-way analysis of variance (one-way ANOVA). $P < 0.05$ was deemed statistically significant.

Results

Cytotoxicity of drugs on the human GBM AMGM cancer cell line

Assessing the antiproliferative activity of metformin and cisplatin on AMGM, various

concentrations of both substances were employed to determine cell growth inhibition (GI) values. Depictions of cells before and after treatment can be seen in figure 2. Cytotoxicity results for cisplatin and metformin against the AMGM cell line are presented in figure 3 and 4 after a 24-hour incubation period, compared to untreated control cells.

Employing different dilutions of each substance to investigate their impact on GBM cancer cell line proliferation (AMGM), the optimal concentration that inhibited 50% of cancer cell growth after 24 hours of incubation with cisplatin was observed at a high concentration of 1000 mg/ml, while the lowest concentrations were 6 and 4 mg/ml, as shown in figure 3. Meanwhile, treatment with metformin during the same incubation period demonstrated that the highest concentration of 1000 mg/ml inhibited 60% of cancer cell growth, with the lowest concentration of 100 mg/ml inhibiting 55% of cell growth when compared with untreated AMGM cells, as depicted in figure 4.

Combination treatment of cisplatin and metformin on AMGM cell line growth

The combination treatment yielded a 50% GI, whereas cisplatin alone exhibited a 40.5% GI, and metformin treatment in isolation also demonstrated a 45.5% GI. No significant

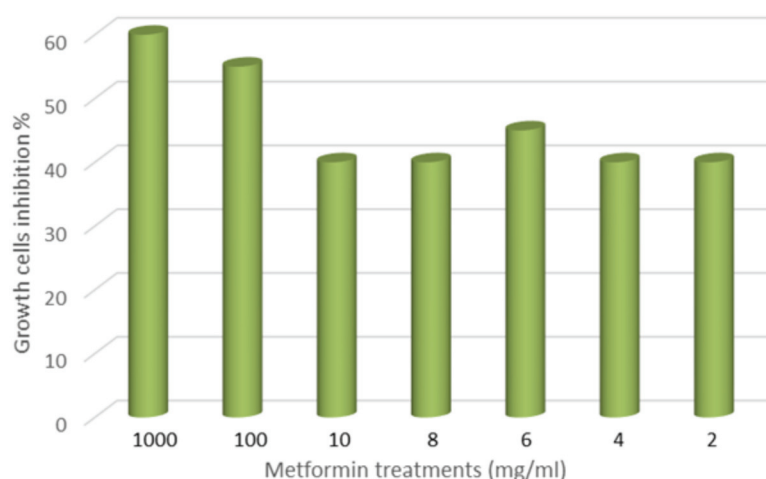


Figure 4. The cytotoxicity activity of metformin at different concentrations is compared to that of untreated AMGM cells during a 24-hour incubation period. The data represent the mean of three replicate experiments.

AMGM: Human cerebral glioblastoma-multiforme cancer cell line

differences were observed between the combination therapy and the individual treatments, as depicted in figure 5.

Chou-Talalay equations were employed to investigate potential interactions between cisplatin and the chemotherapeutic drug cisplatin. A CI less than 0.9 indicates a favorable interaction, a CI between 0.9 and 1.1 suggests an additive effect and a CI greater than 1.1 indicates an antagonistic interaction. Employing the dose-oriented isobologram technique, it was determined that the AMGM cell line demonstrated a favorable interaction between metformin and cisplatin at 50% GI doses, as illustrated in figure 6. This highlights the positive combined effects observed at points 2 (CI: 0.077), 3 (CI: 0.181), 4 (CI: 0.421), 5 (CI: 0.292), and 8 (CI: 0.069), respectively.

Induction of DNA damage by combination treatment of AMGM cancer cell line

The comet assay method assessed the DNA damage induced by metformin, cisplatin, and their combination on the AMGM cell line. The DNA tail moment and tail migration were analyzed as indicators of DNA damage. After a 24-hour incubation period, it was observed that metformin alone induced less DNA damage in AMGM cells, while cisplatin exhibited significantly higher toxicity to AMGM cells compared with untreated control cells. Figure 7 presents the combined effect of metformin, which minimized DNA damage on AMGM cells when used with cisplatin.

Discussion

In this study, the combination of two therapies used in the GBM cancer cell line, cisplatin and metformin, as well as determining the cytotoxic activity of combination therapies in inhibiting the growth of the GBM cell line, then determining the effect on DNA damage of this combination therapy on cancer cell line using the comet assay.

The treatment of cancer and GBM especially faces several main obstacles: blood-brain barriers, drug chemotherapy resistance, and cancer recurrence. Researchers and studies focused on developing better strategies to be adapted for

increasing the efficacy of treating GBM patients.

The cytotoxicity of both treatments using different doses show inhibition to the GBM cell line at 50% after 24 hours of treatment; the combination therapy showed several synergism effects using different concentrations of metformin and cisplatin on the GBM cancer cell line. The comet assay DNA damage of the GBM cell line after being treated with combination therapy was higher than in treated cells with cisplatin alone. Hence, combination therapy using metformin and cisplatin against the GBM cell line was recommended according to the results of the current scenario.

In a study aimed at addressing resistance in two distinct glioma cancer cell lines, namely TMZ-resistant (T-98) and drug-sensitive (U-87) glioma cell lines, researchers investigated the efficacy of three novel drug combinations (TMZ with AC2, AC7, and AC26). The study findings showed a significant cooperative effect and high selectivity with minimal toxicity when using different doses. These promising results suggest the potential future use of these three novel drugs in treating drug-resistant glioma, offering hope for the management of GBM.²⁰

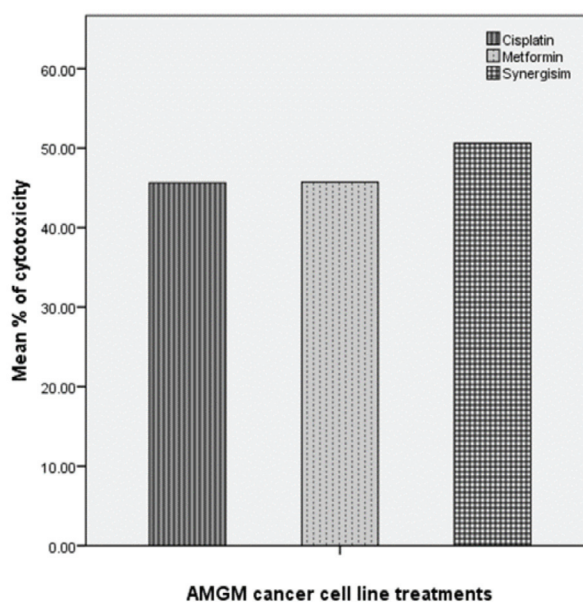


Figure 5. The mean cytotoxicity activity resulting from the combination of metformin and cisplatin is compared to untreated AMGM cells over 24 hours of incubation based on data from three replicate experiments.

AMGM: Human cerebral glioblastoma-multiforme cancer cell line

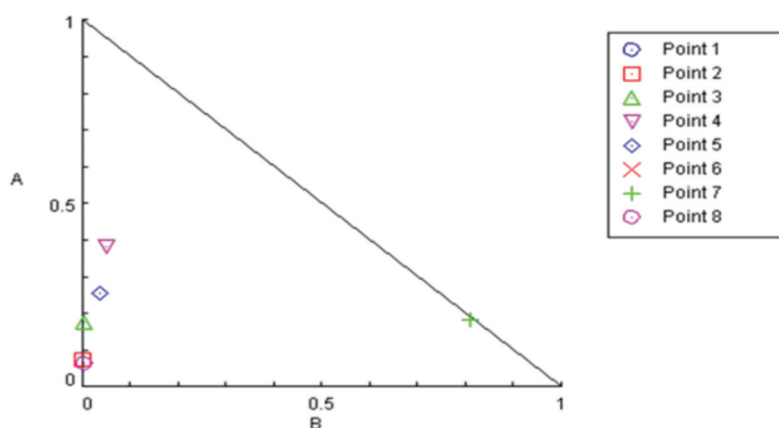
A preclinical phase II study proved that a dose of 2250 mg/day of metformin in combination with temozolomide in patients with newly diagnosed GBM appeared to be well tolerated with acceptable toxicity. Suggesting that cancer stem cells were resistant to existing radiotherapy or chemotherapy and targeting glioma-initiating cells using metformin as a novel therapeutic regimen that could improve the outcome of GBM.²¹

A recent study developed nanoparticles that efficiently co-load TMZ and CDDP and transport these across the blood-brain barriers to target glioma cells precisely. While using mice bearing U87MG or drug-resistant U251R GBM tumor and treated with this developed nanoparticle and TMZ+CDDP showed a potent anti-GBM effect, greatly extending survival time relative to mice receiving single-drug loaded nanoparticles or equivalent doses of free drugs without any side-

effects in histological analyses or blood routine studies. Suggesting that this new nanoparticle formulation overcomes several obstacles that limit the efficacy of combined TMZ and CDDP drug therapy and could be a promising strategy for GBM combinatorial chemotherapy treatment.²²

Another study suggested the efficiency of using metformin in treating SF268 glioma cancer cells, showing that metformin decreases the survival of glioma cells, inhibiting 2D cell motility and cell invasion and increasing cellular adhesion. Finally, this study recommended the anti-invasive anti-metastatic potential of metformin and its mechanism of action in GBM cells.²³

Determination of response to chemotherapy is a significant requirement of personalized medicine single-cell gel electrophoresis, known as a comet assay, used to detect DNA damage in cells. The current study used comet assays to determine the response to chemotherapeutic drugs



Points	Dose met	Dose Cis	Effect	CI	Interaction
1	1000.0	1000.0	0.55	2.58812	
2	100.0	100.0	0.6	0.07747	Synergism
3	80.0	10.0	0.55	0.18101	Synergism
4	60.0	8.0	0.5	0.43187	Synergism
5	40.0	6.0	0.5	0.29201	Synergism
6	20.0	4.0	0.4	8.19547	
7	10.0	2.0	0.45	0.99646	Synergism
8		1.0	0.5	0.06993	Synergism

Figure 6. Isobologram analysis reveals the synergistic effect of cisplatin and metformin after combination treatment on AMGM cells in vitro. The accompanying table presents each concentration's CI data, calculated using CompuSyn software.

CI: Combination indices; Cis: Cisplatin; met: Metformin; AMGM: GBM cancer cell line

widely used in the GBM cell line. The comet assay technique could allow authentic and quick results with the minor items and could be administrated to various drugs, human breast, and colon cancer cell lines treated with chemotherapies. The study results showed that drug activities varied even in the same cancer types, suggesting the heterogeneity of different cancer types.²⁴

The limitation of using single chemotherapy in treating glioblastoma cells is that these chemotherapies do not kill all the cancer cells, and some cells survive, leading to the appearance of new cells resistant to the treatment and needing other therapies that sensitize the chemotherapy. The second limitation in the treatments could be the heterogeneity of glioblastoma cells. These

could be overcome by combination therapy, drugs safe for normal cells, reduced cancer risk, and increased selectivity, specificity, and sensitivity of treatments.

Conclusion

The drug combinations involving cisplatin and metformin proposed in this study provide novel insights for treating drug-resistant GBM cell lines. As suggested in our study, the effective dosages of each combination have been determined through assessments of cytotoxic activity and DNA damage. The combination developed herein underscores the effectiveness of these dosage combinations in inhibiting glioma cell growth across a wide range of dosages, which would be impractical to screen experimentally. This

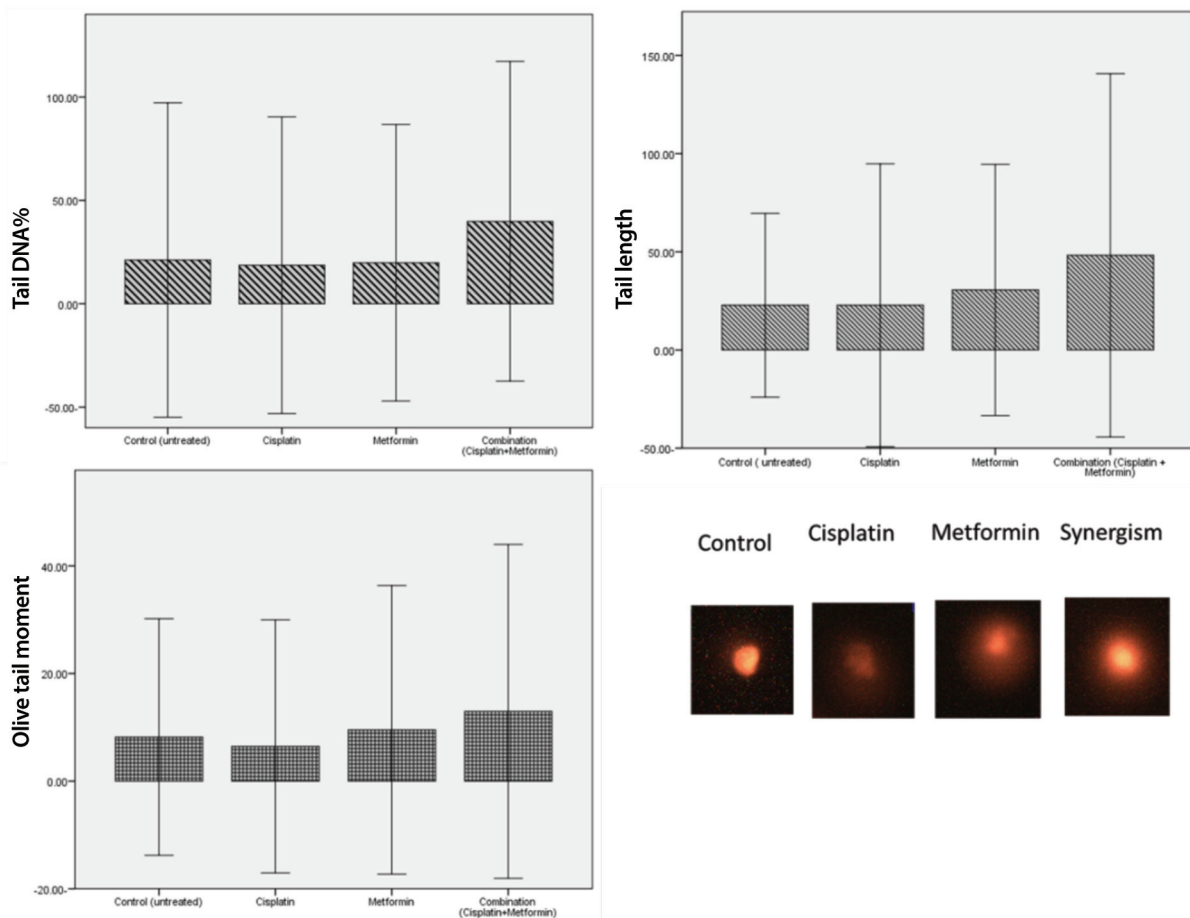


Figure 7. Alkaline comet assay results for the AMGM cancer cell line following treatments with metformin and cisplatin, either alone or in combination, after 24 hours of incubation. The represented results from the comet assay aim to detect DNA damage (original magnification, $\times 100$). Olive tail moments were measured using CASP software, and values are presented as means \pm SEM.

Con: Control; Met: Metformin; Cis: Cisplatin; SEM: Standard error of the mean; Synergism: Cisplatin combined with metformin; AMGM: Human cerebral glioblastoma multifrome cancer cell line; Correlation is significant at the 0.01 level (2-tailed). Cis/ con $P < 0.0001^{**}$.

represents a crucial step in estimating the synergistic effects of the drug pairs.

This study elucidates the potential of metformin in conjunction with cisplatin for treating GBM cancer cell lines in an in vitro model. Furthermore, it employs the comet assay technique to demonstrate DNA damage, showcasing its reliability, speed, and value in assessing GBM treatment. Lastly, it suggests that metformin holds promise as a therapeutic candidate for GBM treatment. Further investigations are necessary to evaluate the efficiency of metformin in treating GBMs in vivo.

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Conflict of Interest

None declared.

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