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Oridonin Enhances Radiotherapy Efficacy by Inducing Late-Stage Cell Death and G2/M Arrest in U87MG Glioma Cells

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Abstract

Background: Glioblastoma multiforme is a deadly brain tumor with limited treatment options. The aim of this study was to investigate whether oridonin (OR) could enhance the radiosensitivity of U87MG glioma cancer cells, and compare this effect with that of temozolomide (TMZ).

Method: In this experimental study, we investigated the effects of OR on U87MG glioma cells, alone and in combination with radiotherapy and TMZ cell viability assay, cell cycle analysis, and apoptosis assays were performed to assess the impact of these treatments. The differences between groups were assessed using nonparametric tests including Wilcoxon and chi-square using GraphPad Prism software.

Results: OR showed dose-dependent cytotoxicity in U87MG glioma cells. Radiation further enhanced the anti-proliferative effect of OR. TMZ, a standard treatment, also showed significant cytotoxicity, especially with radiation. Both treatments induced G2/M cell cycle arrest, particularly when combined with radiation. Notably, OR and radiation caused a more pronounced G2-phase arrest (55.6%) than TMZ (39.9%) ($P < 0.001$). While neither treatment significantly affected early apoptosis, both increased late-stage cell death. Importantly, OR and radiation significantly increased late-stage cell death (60.07 ± 1.4%) compared with TMZ and radiation alone (48.04 ± 2.9%) ($P = 0.02$).

Conclusion: These findings suggest the potential of OR as a radiosensitizer for Glioblastoma multiforme treatment. Further research is needed to understand the mechanisms and validate these

results in living organisms. This paves the way for exploring OR as a radiosensitizer for Glioblastoma multiforme treatment in the future.

Keywords: Glioma, X-rays, Temozolomide, Cells, Apoptosis

Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive primary brain tumor in adults. It is characterized by a poor prognosis due to its infiltrative nature, resistance to standard therapies, and high recurrence rate.[1] Despite surgical resection being the first-line treatment, complete removal is often difficult due to tumor infiltration into surrounding healthy brain tissue.[2] The current standard treatment combines surgery with postoperative radiotherapy and Temozolomide (TMZ) chemotherapy, resulting in a median survival of only 15 months.[3, 4] Limited treatment options and GBM's resistance to RT pose significant challenges for improving patient outcomes.[5, 6] Therefore, the radioresistance and limited alternatives in therapeutic strategies create the greatest challenges for an oncologist to improve the survival of glioma cancer patients.

Radiation sensitizers are drugs that enhance the effectiveness of RT, potentially overcoming treatment resistance and improving survival for GBM patients.[7, 8] These agents ideally increase tumor cell sensitivity to radiation while allowing for reduced radiation doses, minimizing damage to healthy brain tissue [5]. However, currently available sensitizers often have limitations including side effects, toxicity to normal tissues, and poor solubility.[3, 9]

Natural products offer a promising source of novel cancer therapeutics with potentially lower toxicity profiles.[10, 11] Oridonin (OR) derived from the Chinese herb Rabdosia Rubescens, has been extensively studied for its broad-spectrum antitumor activity. Research has shown that OR exhibits promising antitumor effects in various types of cancer, including lung cancer,[12] breast cancer,[13] nasopharyngeal cancer,[14] and GBM.[15] While some studies suggest OR may enhance the radiosensitivity of lung cancer cells,[12, 16] its impact on glioma cells remains unclear. To investigate the potential of natural compounds as radiosensitizers, we hypothesized that OR would enhance the radiosensitivity of U87MG glioma cells by inducing cell cycle arrest and apoptosis. The primary objective of this study was to assess the impact of OR on U87MG glioma cells through evaluating its cytotoxic effects, determining its ability to enhance the effectiveness of radiation therapy, and comparing its efficacy with the standard-of-care chemotherapeutic agent, TMZ.

Material and Methods

In this experimental study Cell viability, cell cycle analysis, and apoptosis assays were used to assess the impact of OR treatment, alone and in combination with radiation. All experiments were performed in triplicate.

Cell culture

The U87MG Glioma cell line was grown in minimum essential medium supplemented with Earl's balanced salt solution (EBS) and 10% heat-inactivated fetal bovine serum,1% non-essential amino acids, 2 mM l-Glutamine, 100 µg/ml streptomycin, and 100 U/ml penicillin. The cells were incubated at 37° C in 5% CO2 in the air.

Cell viability assay

The well-known assay to assess the viability of living cells in many biological samples is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). For this method, 15×10^3 cells were seeded in each well of a 96-well plate and 200 µl of medium was added. Then, exposed to varying

concentrations of OR (Adooq, USA) (10, 20, 30, 40, 50, 60, and 80 μM/L) or of TMZ (Sigma, Germany) (1000, 2000, 3000, 4000, 6000 and 8000 μM/L^6) for 24 and 48 hours, respectively; a concentration of 0.1% Dimethyl sulfoxide (DMSO) was used as a control. The tests were performed in the existence or absence of radiation. Subsequently, after washing each well in Phosphate-buffered saline (PBS), 100 μl of culture medium and 50 μL of MTT (Sigma, Germany) were added and incubated for 4 hours. After solving the formazan crystals in DMSO solution the absorption of the solution was measured by an ELISA – a reader device at 570 nm wavelengths. The 50% inhibitory concentration (IC50) is the concentration of the drug at which 50% of the cells are killed. A Wilcoxon test was employed to compare cell viability after 24 and 48 hours of OR or TMZ treatment.

Cell cycle analysis

All wells of the tissue plate were filled with a 2-ml cell suspension containing 5×10^5 cells. The cells were divided into six groups: Control group (no treatment), radiation (no other treatment), TMZ (no radiation), OR (no radiation), *TMZ*/radiation, and OR/ radiation groups.

Following a 24-hour incubation to allow cell attachment, the culture medium was replaced, and treatments were administered according to the designated groups. For TMZ/radiation and OR/radiation groups, cells were treated with their respective IC50 doses of TMZ or OR for the initial 24 hours. After this initial incubation, these groups were then exposed to a 2 Gy dose of radiation. All groups were incubated for an additional 24 hours (48 hours total for control, TMZ-only, and OR-only groups) before fixation. Cells were then fixed with cold 70% ethanol overnight at -20°C. Subsequently, the cells were added 50 μL of a 100 μg/ml RNase-free DNase solution and incubated for 30 minutes at room temperature. Finally, 20 μL of 1 mg/ml PI solution was added, and the cells were incubated for an additional 30 minutes in the dark to allow PI staining of cellular DNA. The cell cycle distribution was then analyzed using a FACScan Flow Cytometer (or Attune NxT) according to the manufacturer's instructions.

Apoptosis assay

A total of 5 x 10^5 cells were seeded in each well of a tissue culture plate. The cells were then divided into six experimental groups as mentioned in the previous section. Following a 24-hour incubation period to allow cell attachment, cells were trypsinized and washed twice with cold PBS to remove any residual media or attached factors. Cells were then resuspended in a binding buffer and stained with 5 μL each of Annexin V-FITC and propidium iodide (PI) according to the manufacturer's instructions for the Annexin V-FITC Apoptosis Detection Kit (MabTag). Specifying the volume of both stains improves clarity. The stained cells were incubated for 20 minutes in a dark place to allow for proper binding of the dyes. After incubation, the percentage of apoptotic cells was quantified using a FACScan Flow Cytometer (or Attune NxT) according to the manufacturer's instructions. To assess for significant differences between the treatment groups and a control group in the presence and absence of radiation, chi-square tests were employed.

Irradiation conditions

All irradiations were treated with a 2 Gy dose using a 6 MV photon beam from a clinical linear accelerator (Artist, Siemens Company, Germany). The field size was 20 x 20 cm^2 and the source-to-surface distance was 100 cm. To ensure accurate dose delivery within a 2% tolerance, radiation dose measurements were conducted using a diode detector provided by PTW, a German company.

Ethical considerations

Ethical approval was obtained from the Institutional Review Board and the Ethics Committee of Dezful University of Medical Science (IR.DUMS.REC.1399.011). The cell line was purchased from the Iranian Biological Resource Center with ID number C10982 and the researcher does not have access to the donor information. All experiments and methods were performed under relevant guidelines and regulations.

Statistical analysis

The results are presented as mean ± SD from three independent experiments. The IC50 of treated samples, representing the concentration required to inhibit 50% of cancer cell growth, was calculated using GraphPad Prism 5. The differences between groups were assessed using nonparametric tests such as the Wilcoxon and chi-square tests. A *P*-value of less than 0.05 was considered to indicate statistical significance.

Results

Cell viability assay

The effects of OR and TMZ on the proliferation of U87MG glioblastoma cells, with or without prior radiation exposure, were evaluated using the MTT assay. Figure 1a (without radiation) and Figure 1b (with radiation) present the results of the viability test, where U87MG cells were treated with increasing concentrations of OR extract (10, 20, 30, 40, 50, 60, and 80 μM/L) for 24 and 48 hours. OR treatment alone resulted in a dose-dependent decrease in cell viability. At the highest concentration (80 μM/L), OR reduced cell viability by 26.6% and 10.4% after 24 and 48 hours, respectively. Co-treatment with radiation appeared to attenuate the effectiveness of OR, with reductions of 13.5% and 6.2% observed at 24 and 48 hours, respectively.

Figure 2 illustrates the dose-dependent cytotoxicity of TMZ on U87MG cells, with or without prior radiation exposure. The cells were treated with increasing concentrations of TMZ (1000, 2000, 3000, 4000, 6000, and 8000 μM/L) for 24 and 48 hours (Figure 2a: without radiation; Figure 2b: with radiation). A comparison of the panels reveals that co-treatment with radiation significantly enhances the cytotoxicity of TMZ on U8MG cells.

Table 1 summarizes the IC50 values (concentration inhibiting 50% of cell growth) for OR and TMZ in U87MG cells after 24 and 48 hours of treatment, both in the absence and presence of radiation. The table reveals that for both OR and TMZ, the IC50 values decrease when radiation is added to the treatment regimen.

Cell cycle analysis

The radiosensitivity of cells is known to vary depending on the cell cycle phase. Understanding the cell cycle distribution of glioma cells is crucial for comprehending their response to radiation. To this end, we investigated the cell cycle distribution in U87MG glioma cells following treatment with IC50 concentrations of TMZ or OR, both in the presence (Figure 3) and absence (Figure 4) of radiation. As shown in the histograms (Figure 3a) and the corresponding quantitative analysis (Figure 3b), minimal changes were observed in the G2/M phase cell population upon treatment with TMZ or OR when cells were not irradiated.

Flow cytometry analysis of PI-stained cells revealed a G2/M phase arrest in both the TMZ/radiation and OR/radiation groups compared with the control and radiation-alone groups (Figure 4a, 4b). Notably, the combination of radiation and OR (55.6%) induced a remarkably higher G2/M cell population compared with TMZ with radiation (39.9%). Interestingly, while the G1 phase fraction decreased in the radiation and OR group, this change was not as pronounced as compared with the control and radiation-alone groups.

Apoptosis assay

Flow cytometry analysis using the Annexin V/PI apoptosis detection kit was employed to investigate the effects of TMZ and OR on U87MG cell apoptosis, with or without radiation exposure (Fig 5a-b). Cells were categorized into four populations based on staining: live (Annexin V-/PI-), early apoptotic (Annexin V+/PI-), late apoptotic/necrotic (Annexin V+/PI+), and necrotic (Annexin V-/PI+).

The analysis revealed no significant changes in the percentage of early apoptotic cells upon treatment with TMZ or OR compared with the control group, in both the absence and presence of radiation (P =0.09). This suggests minimal impact of these drugs on the early stages of apoptosis. However, a statistically significant increase (P = 0.001) was observed in the percentage of cells undergoing late apoptotic/necrotic cell death (Annexin V+/PI+) in both TMZ and OR-treated groups compared with the control.

Interestingly, within the radiation-treated groups, the combination of OR and radiation-induced a significantly higher percentage of cells undergoing late apoptosis/necrosis (60.07 ± 1.4%) compared with the TMZ and radiation group (48.04 ± 2.9%) (P = 0.02).

Discussion

This study investigated the combined effects of OR and TMZ with radiation therapy on U87MG glioma cells. Our results demonstrate that OR and TMZ, particularly in combination with radiation, significantly reduced cell viability and induced cell cycle arrest in the G2/M phase. Furthermore, both agents, especially when combined with radiation, led to increased late-stage apoptosis and necrosis in U87MG cells.

The results confirmed the dose-dependent cytotoxicity of OR against U87MG glioma cells. This aligns with previous studies suggesting its anti-cancer properties.[16-18] Interestingly, co-treatment with radiation appeared to increase the effectiveness of OR. Our study determined the concentration of OR required for a near 50% reduction in U87MG cell viability. This concentration was 50 μM/L and 30 μM/L for 24 and 48 hours, respectively. However, Zhang et al.[15] reported a lower IC50 (concentration inhibiting 50% of cell growth) for OR in these cells, at 20 μM/L and 10 μM/L, respectively. This discrepancy might be attributable to differences in the experimental conditions.

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TMZ displayed a significant cytotoxic effect, and this effect was notably enhanced when combined with radiation. This finding is consistent with the established clinical use of TMZ for glioma treatment, where it acts by inducing DNA damage that is potentiated by radiation therapy.[2, 19]

The radiosensitivity of glioma cells is heavily influenced by their cell cycle phase. To gain insights into this relationship, we investigated the cell cycle distribution of glioma cells following treatment with the IC50 concentrations of TMZ or OR, both in the presence and absence of radiation. Our analysis revealed that neither TMZ nor OR significantly impacted the proportion of cells in the G2 phase when administered alone (without radiation exposure). This observation was confirmed by both the cell cycle distribution histograms and the corresponding quantitative analysis. However, when combined with radiation, both the TMZ/radiation and OR/radiation

groups displayed a notable increase in the G2 phase cell population. Interestingly, the OR/radiation group exhibited a significantly higher G2 phase population compared with the control and radiation-alone groups. This finding suggests a potentially more effective strategy by targeting a broader cell population undergoing division, potentially leading to a more pronounced antitumor effect.

Our findings regarding TMZ-induced cell cycle arrest align with the observations reported by Li et al.[20] This consistency strengthens the generalizability of our results, and suggests a potential shared mechanism for TMZ-mediated G2/M arrest across different glioma cell lines.

Our findings regarding the effects of OR on cell cycle progression align with previous studies conducted on other cancer cell lines.[21, 22] These studies have demonstrated the ability of OR to induce G2/M phase arrest. This concordance suggests that OR's cell cycle arrest properties may be broadly applicable to various cancer types. However, Zhang et al.[15] reported a different effect, with OR increasing the proportion of cells in the S phase and decreasing the population in the G2/M phase.

Interestingly, neither OR nor TMZ treatment significantly impacted the early stages of apoptosis. However, both drugs induced a significant increase in the percentage of cells undergoing late apoptosis/necrosis. These findings suggest that OR and TMZ might trigger cell death through alternative pathways compared with classical apoptosis inducers. Some studies showed that OR can induce apoptosis by activating the intrinsic apoptotic pathway, involving the formation of the apoptosome complex. This complex consists of cytochrome c, Apaf-1, and pro-caspase-9. Additionally, OR can modulate the expression of Bcl-2 family proteins, upregulating pro-apoptotic Bax and Bid while downregulating anti-apoptotic Bcl-2 and Bcl-XL.[23, 24]

The combination of OR and radiation led to a significantly higher percentage of cells undergoing late apoptosis/necrosis compared with TMZ and radiation. This finding highlights the potential of OR to enhance the efficacy of radiotherapy by promoting a more pronounced cell death response. The underlying mechanisms of this synergy need further exploration. It is possible that OR might sensitize glioma cells to radiation-induced damage or potentiate the apoptotic signaling cascades triggered by radiation. While the exploration of anticancer properties of OR in combination with radiation remains a relatively understudied area, our findings find support from existing research. Park et al.[16] reported that the combined treatment of 5 μM OR and radiation significantly enhanced apoptotic cell death in H460 non-small cell lung cancer cells. This observation aligns with our own results, suggesting potential broader applicability of radiosensitizing effects of OR across different cancer types.

This study was conducted in vitro using a single glioma cell line (U87MG). While this model system provides valuable insights into the mechanisms of action of OR and TMZ, it may not fully capture the complexity of the tumor microenvironment and patient-specific variations. Further studies using patient-derived xenografts or primary cell lines from a broader range of glioma subtypes are necessary to assess the generalizability of our findings. Additionally, in vitro studies inherently lack the physiological complexity of in vivo conditions, limiting our ability to fully evaluate the systemic effects and potential side-effects of the treatments.

Future directions for this study include conducting in vivo studies to evaluate the efficacy and safety of OR and TMZ in animal models. Additionally, clinical trials can explore the potential clinical application of

OR in combination with standard treatments. Further mechanistic studies can delve deeper into the molecular mechanisms underlying the effects of these drugs, particularly in combination with radiation. Investigating potential synergistic effects with other therapies and exploring strategies to overcome drug resistance are also crucial areas for future research.

Conclusion

This study provides compelling evidence for the potential of OR as a promising therapeutic agent for glioma, particularly in combination with radiation therapy. The observed synergistic effects of OR and radiation suggest a promising strategy for improving patient outcomes. However, further research is needed to fully elucidate the mechanisms of action and to translate these findings into clinical applications. By addressing the limitations and pursuing the suggested future directions, we can advance our understanding of glioma biology and develop more effective therapeutic approaches.

Data availability

The datasets used and analyzed during the present study are available from the corresponding author upon reasonable request.

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Authors' Contribution

Conception and design: M. Khorramizadeh, Z. Arab-bafrani; Administrative support: M. Ghasemi, N. Banazadeh; Provision of study materials or patients: M. Khorramizadeh; Collection and assembly of data: All authors; Data analysis and interpretation: All authors; Manuscript writing: All authors. All authors read and approved the final manuscript version and agreed with all parts of the work in ensuring that any queries about the accuracy or integrity of any component of the work are appropriately investigated and handled.

Conflict of Interest

None declared.

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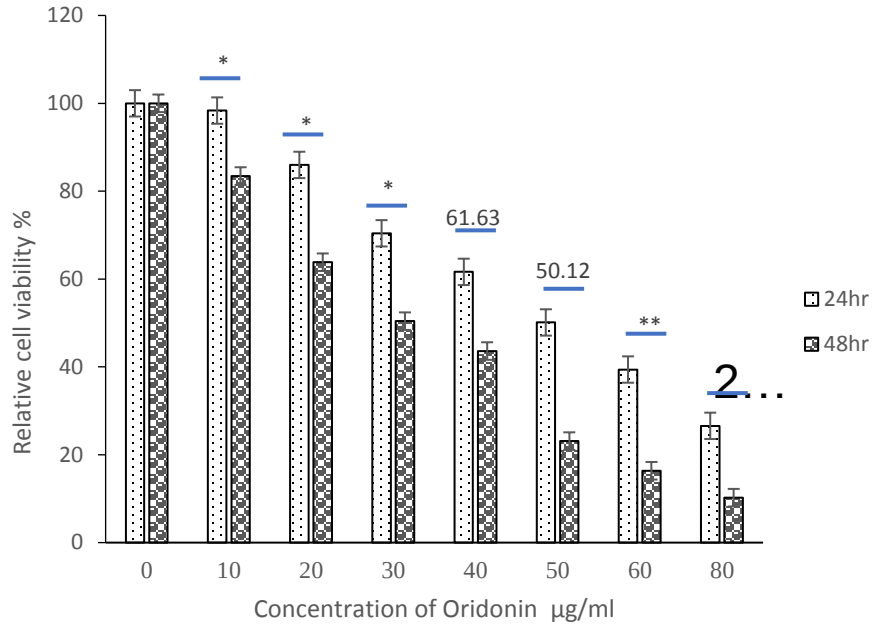
Table 1. The IC50 dose (μM/L) of Oridonin and TMZ on U87MG cells at 24 and 48 hours with or without radiation

	Without radiation		**With radiation**	
Time (Hour)	24	48	24	48
Oridonin (μM/L)	50.07	30.82	30.20	20.12
TMZ (μM/L)	4000.07	3000.30	2000.11	1000.31

TMZ: Temozolomide



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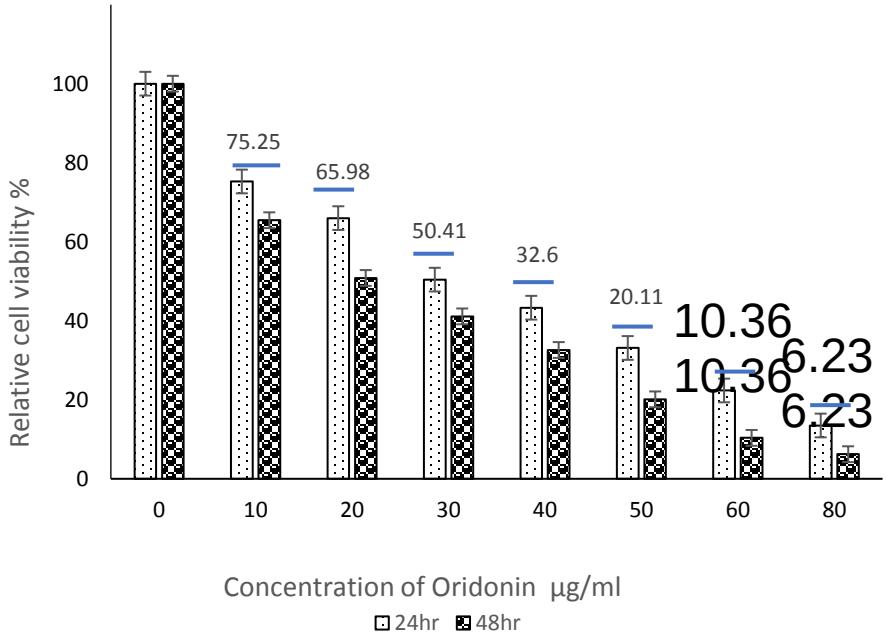

B

Figure 1. A and 1B illustrate the antiproliferative effects of Oridonin on U87MG cells at 24 and 48 hours, respectively. Figure 1A shows the effects of Oridonin with radiation, while 1B shows the effects without radiation.

*$P < 0.05$, **$P < 0.01$ were regarded as significant compared with the untreated control group.

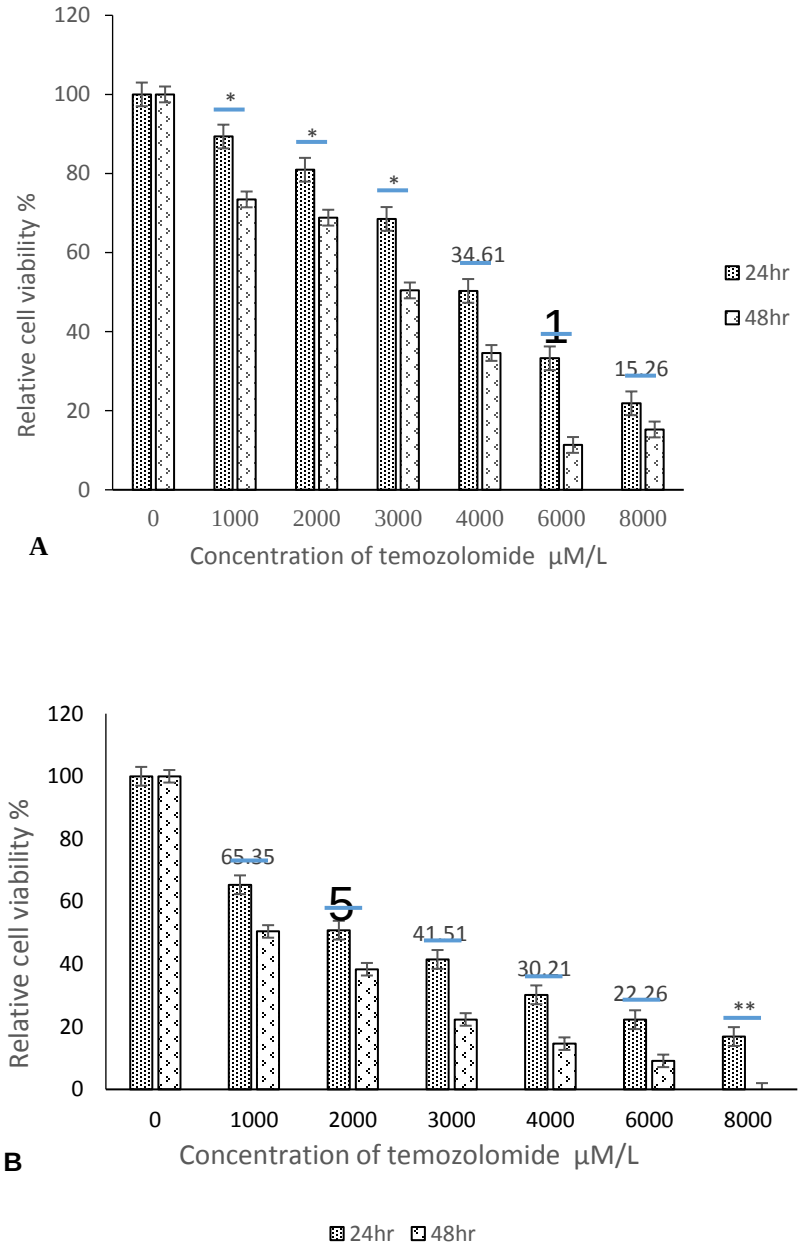

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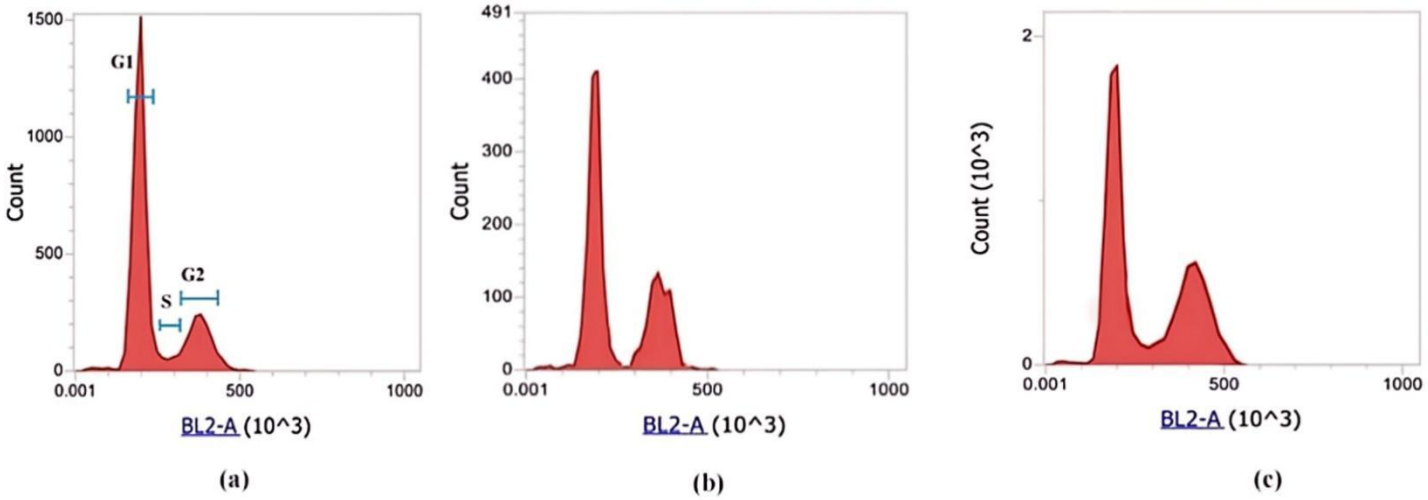
Figure 2. (A) Figure 2A shows the antiproliferative effects of temozolomide on U87MG cells at 24 and 48 hours. (B) Figure 2B illustrates the combined effect of Temozolomide and radiation on U87MG cell proliferation at 24 and 48 hours. A synergistic effect was observed, with a further significant reduction in cell viability compared to the temozolomide-only treatment (*$P < 0.05$, ** $P < 0.01$).



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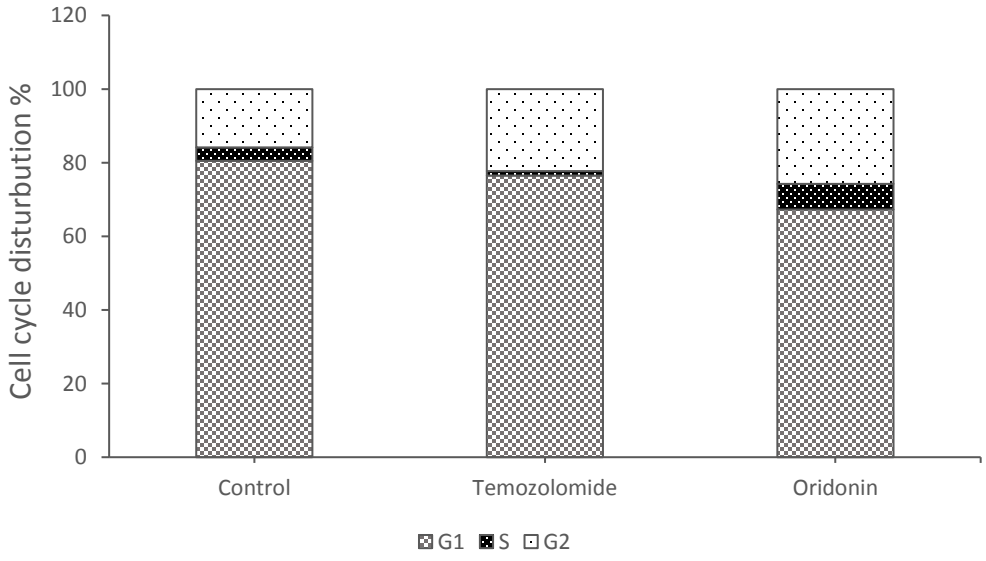

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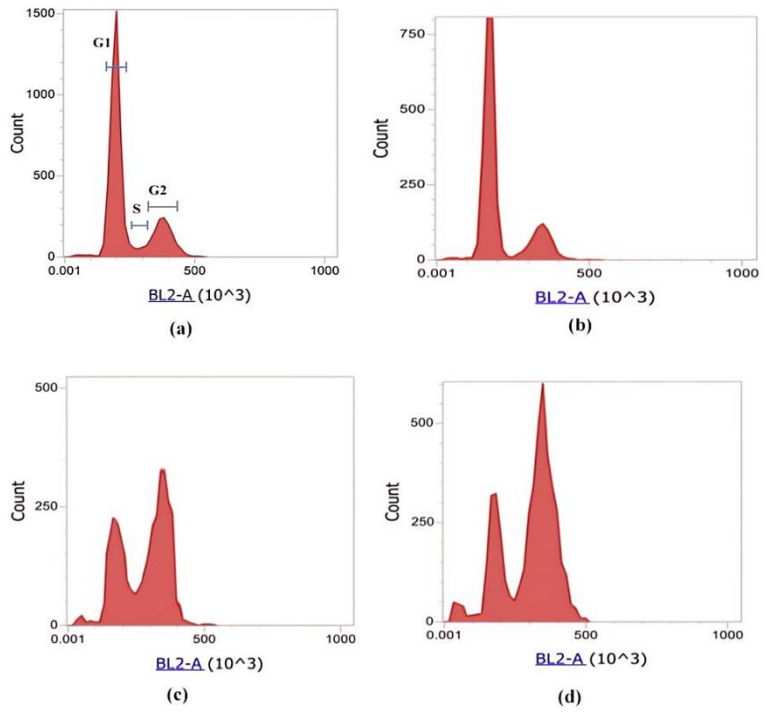
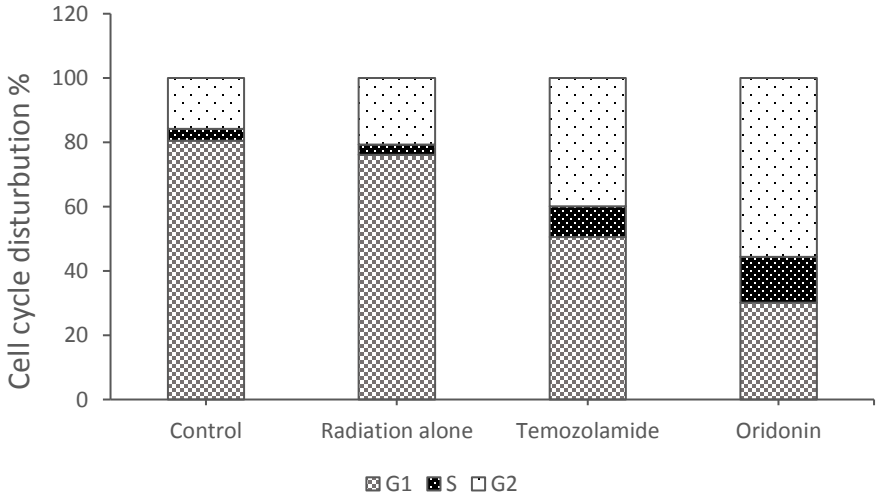
Figure 3. Cell cycle analysis of U87MG cells. (A) Flow cytometry histograms showing the cell cycle distribution determined by PI staining. The panels represent the following conditions: (a) Control, (b) Temozolomide treatment, and (c) Oridonin treatment. (B) Quantification of the proportion of cells in different cell cycle phases (G0/G1, S, and G2/M) under the different treatment conditions.

PI: Propidium Iodide



A



B

Figure 4. (A) Flow cytometry histograms illustrating the cell cycle distribution of U87MG cells treated with different agents. PI staining was used to assess cell cycle phases. (a) Control, (b) Radiation, (c) Temozolomide, (d) Oridonin. (B) Quantification of the proportion of cells in different cell cycle phases based on the flow cytometry data shown in panel (A).
PI: Propidium Iodide

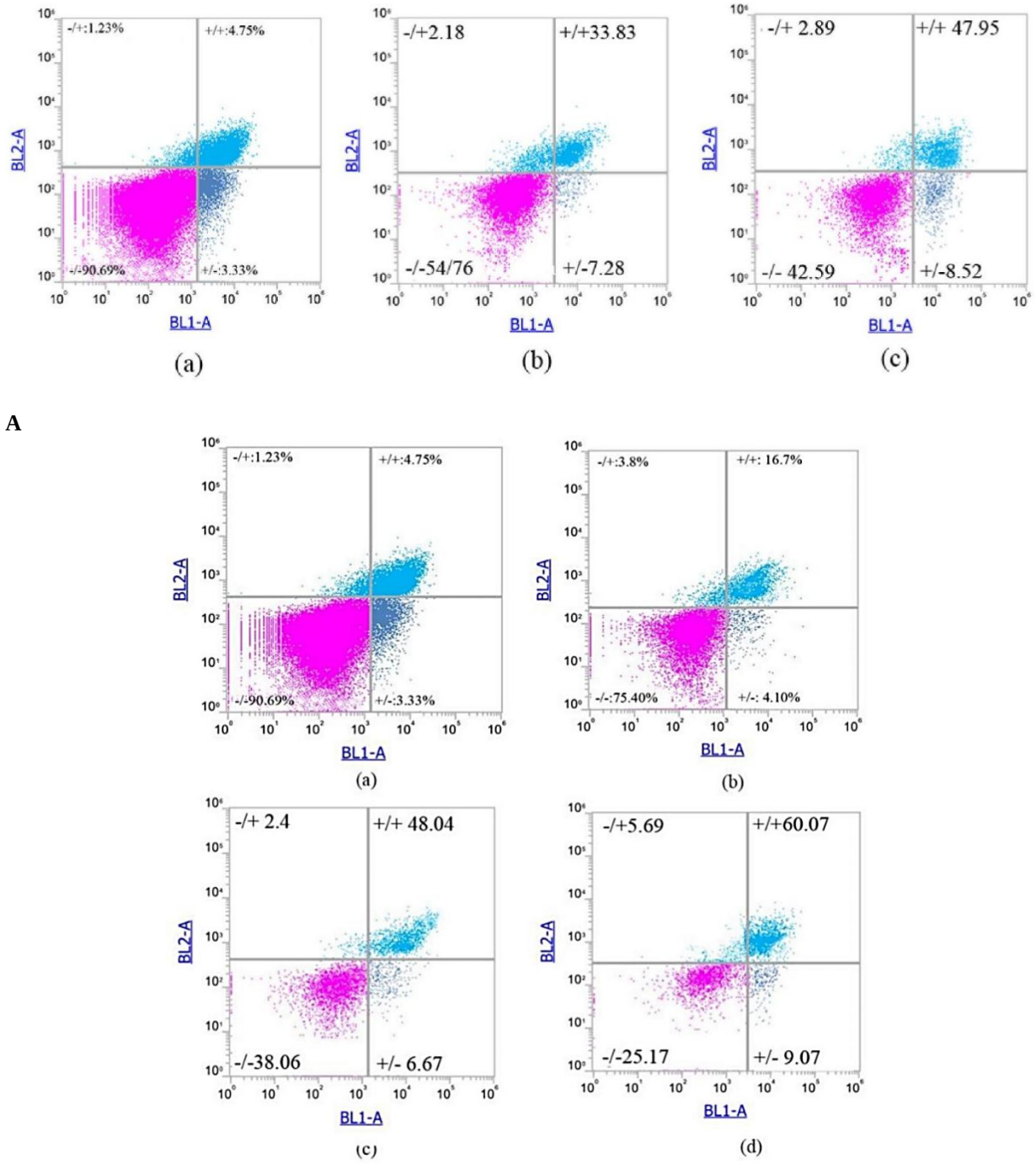


Figure 5. Flow cytometry analysis of cell apoptosis in U87MG cells. (A) Cells without radiation: (a) Control, (b) Temozolomide, (c) Oridonin. (B) Cells with radiation: (a) Control, (b) Radiation, (c) Temozolomide + Radiation, (d) Oridonin + Radiation. The lower left quadrant represents live cells (double negative), the lower right quadrant represents early apoptotic cells (annexin V+ / Propidium Iodide-), the upper right quadrant represents late apoptotic or necrotic cells (double-positive), and the upper left quadrant represents necrotic cells (annexin V- / Propidium Iodide+).