Effects of resveratrol and 1,3-bis(2-chloroethyl)-1-nitrosurea combination on YKG1 glioblastoma cells

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Abstract: Glioblastoma is a primary malignant brain tumor that can be treated with 1,3-bis(2-chloroethyl)-1-nitrosurea (BiCNU/carmustine). Resveratrol is a natural phenol that can interfere with apoptosis. This study aims to investigate how the combination of BiCNU and resveratrol affects glioblastoma cells in vitro. Accordingly, YKG1 glioblastoma cells were treated with different amounts of resveratrol (50 and 100 µM) and BiCNU (10 and 20 µM) either alone or in combination. Cell viability tests and immunochemical studies were conducted on these cells. According to results, increasing the amount of resveratrol and BiCNU decreased cell viability. Additionally, when these maximal doses of resveratrol and BiCNU (100 µM resveratrol plus 20 µM BiCNU) were applied, viability decreased to the highest cytotoxicity levels. Immunohistochemical analysis also revealed the significantly upregulated H scores of beclin-1 and caspase-3 in treated groups with the highest value in maximally combined concentration. These results indicated the cumulative effects of concurrent administration of BiCNU and resveratrol on the cytotoxicity of malignant human YKG1 glioblastoma cells in vitro.

Keywords: Apoptosis, carmustine, glioblastoma, resveratrol

Özet: Glioblastoma, 1,3-bis (2-kloroetil) -1-nitrozüre (BiCNU / carmustin) ile tedavi edilebilen birincil kötü hücreler, tek başına veya kombinasyon halinde farklı miktarlarda resveratrol (50 ve 100 µM) ve BiCNU (10 ve 20 µM) ile muamele edildi. Bu hücreler üzerinde hücre canlılığı testleri ve immünokimyasal çalışmalar yapıldı. Sonuçlara göre, resveratrol ve BiCNU miktarlarının artırılması hücre canlılığını kademeli olarak azalttı. Ek olarak, maksimum resveratrol ve BiCNU dozu (100 µM resveratrol artı 20 µM BiCNU) verildiğinde, hücre canlılıkları oldukça azaldı. Immünohistokimyasal analizler, maksimum kombin kombin konsantrasyonda muamele edilen gruplarda beclin-1 ve kaspaz-3 H skorları düzeyleri önemli ölçüde arttırdığı ortaya çıktı. Bu sonuçlar, BiCNU ve resveratrolun aynı anda uygulanması, malign insan YKG1 glioblastoma hücrelerinin in vitro sitemi toksitesi üzerindeki kumulatif etkilerini göstermiştir.

Anlahtır Kelimeler: Apopozia, karmustin, glioblastoma, resveratrol


1. Introduction

The brain's primary malignant tumor, glioblastoma (GB), is considered the second most frequently encountered brain tumor following the meningioma (Omuro and De Angelis, 2013). It has been reported that GB makes up 12% to 15% of all intracranial tumors and 50% to 60% of astrocytic tumors. This tumor occurs in 3 out of 100,000 individuals per year (Williams, 2014). It has been stated that GB often starts at around 64 years of age, and incidence in males was more common than females (Tian et al., 2018).

GB presents non-specific symptoms, including headache, nausea, vomiting, seizures, changes in personality, and symptoms resembling those of a stroke initially. These symptoms might become worse rapidly and give rise to unconsciousness (Alexander and Cloughesy, 2017). The causes of GB is unknown in most cases. However, genetic disorders such as neurofibromatosis, Li Fraumeni syndrome and previous radiotherapy might pose risk factors for GB, which might originate from normal neurons or develop from an existing low-grade astrocytoma (Aliferis and Trafalis, 2015). Its diagnosis is based on computational tomography, magnetic resonance imaging, and tissue biopsy (Omuro and De Angelis, 2013; Batash et al., 2017).

Glioblastoma is a neurological pathology with high morbidity and mortality; therefore, its treatment is challenging for clinicians (Adamson et al., 2009). The first reason for this challenge is the susceptibility of brain damage from conventional treatments, and the second is the brain's limited capacity for repairing itself. The third reason is the resistance of the tumor cells to the traditional treatment methods, and the last is the inability of chemotherapeutics to cross the blood-brain barrier (Adamson et al., 2009; Batash et al., 2017).
The cure for GB refers to palliative treatment and other methods that intend to improve patients’ survival. These methods consist of surgery, chemotherapy, radiotherapy, and immunotherapy (Alikieri and Trafalis, 2015). Thus, treatment of GB usually involves surgery followed by chemotherapy and radiotherapy. It is well known that more extensive surgical removal is associated with more prolonged survival (Villà et al., 2014). Chemotherapy for GB indicates the administration of vincristine, hydroxyurea, temozolomide, 6-thioguanine, 5-fluorouracil, and 1,3-bis (2-chloroethyl)-1-nitrosurea (BiCNU) (Desai et al., 2019). BiCNU has been used both for the initial diagnosis of glioma and tumor recurrence via intravenous administration (Xiao et al., 2020). Amongst these, BiCNU which is an alkylating agent called carmustine, has been well established but may cause hepatotoxicity and pulmonary fibrosis (Desai et al., 2019). However, some studies have suggested that BiCNU provides a survival advantage for GB patients (Spiegel et al., 2007). Despite the overall treatment methods, GB usually remits, and the typical survival length following diagnosis is 12 to 15 months. Fewer than 7% of people might survive longer than five years, and without any treatment, the survival period is typically three months (Stoyanov et al., 2018; Withthayanuwat et al., 2018).

Apoptosis is a form of programmed cell death that occurs in multicellular organisms. In contrast to necrosis, which addresses traumatically cell death associated with acute cellular injury, apoptosis is an extremely controlled and regulated process that confers advantages during an organism’s life cycle. These advantages especially appear during embryogenesis (Kaczanowski, 2016). Its high rate causes atrophy, but reduced apoptosis might cause uncontrolled cell proliferation, leading to carcinogenesis (Lopez and Tait, 2015; Kaczanowski, 2016).

A natural polyphenol, resveratrol (3,5,4’-trihydroxy-trans-stilbene), is produced by grapes, blueberries, raspberries, mulberries, and peanuts naturally (Kataria and Khatak, 2019). It acts through signaling pathways related to growth factors and receptor tyrosine kinases and interferes with signal transduction by the growth factor β. Experimental studies have demonstrated that resveratrol might impact apoptosis by regulating molecules that comprise caspase-3, p53, peroxisome proliferator-activated receptor, NnkB, Bax, Bcl-2, and apoptotic protease activating factor-1 (Emsen and Turkez, 2017; Rauf et al., 2018).

Chemoprevention is defined as the administration of a pharmacological agent to prevent infection or disease. In malignancy, chemoprevention is initiated to avoid the spread of an existing condition, help differentiate molecular targets, and increase chemotherapeutics’ bioavailability (Cruz and Balkwill, 2015). The use of chemoprophylaxis is limited primarily by two factors which are biological risk and financial cost. Since all medications can cause side effects, chemoprevention should only be considered when treatment benefits outweigh the risks. The cost associated with chemoprevention may be prohibitive, mainly when the cost of treatment is high or the target disease’s incidence is low (Huang and Mellor, 2014). Resveratrol appears as an appropriate pharmacological for the chemoprevention due to its mild adverse effects and relatively low cost. Its adverse impacts are usually related to long-term use and/or ingestion of higher doses. Nausea, stomach pain, flatulence, and diarrhea are the side effects of resveratrol treatment (Vervandeur-Fasser and Latruffe, 2014). The treatment process is very limited in GB, and there is no effective treatment. Therefore, this study aims to compare the effectiveness of resveratrol as an alternative to BiCNU treatment in the YKG1 glioblastoma cell line and to determine whether it potentiates the effects of each other depending on their possible combination.

2. Materials and Method

YKG1 human glioblastoma cells were obtained commercially (RIKEN BioResource Center, Japan), whereas analytical grade BiCNU (15493-8, SigmaAldrich, USA) and trans-resveratrol (CAS 501-36-0, Santa Cruz, USA) were used for pharmacological treatment.

2.1. Cell Culture

Cells were incubated in culture media, including Dulbecco's Modified Medium with 10% Fetal Calf Serum, 1mM sodium pyruvate, and 2 mM L-glutamine in an incubator with 5% CO2 and 95% humidity at 37°C. Cells were grown in flasks of 75 cm2 at an approximate density of 4x10^5/cm2. After trypsinization, the cell suspension was transferred to sterile capped tubes and centrifuged at 400g and 25°C for 5 minutes. The cell pellet was mixed with new media that had a volume corresponding to 1/3 of the tube. Twenty µl of this suspension was mixed with 90 µl buffer solution (PBS + 0.1% FCS) and 100 µl trypan blue in an eyependorf tube. White-colored living cells were counted by a hemocytometer in microscopic examination. When the cell count became sufficient, they were passaged at a ratio of 1:4, allocated into new flasks, and allowed to replicate under convenient incubation conditions. Cell viability was assessed in samples obtained from these culture media.

2.2. Experimental Design

Malignant YKG1 human glioblastoma cells that were passaged and replicated in the laboratory were treated by different doses of BiCNU and resveratrol for 48-h. DMSO (0.1%) was the solvent for the chemicals, and all treatments are summarized in Table 1. All groups contained at least triple biological replicates.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment Description</th>
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</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>50 µM resveratrol</td>
</tr>
<tr>
<td>Group 2</td>
<td>100 µM resveratrol</td>
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<tr>
<td>Group 3</td>
<td>10 µM BiCNU</td>
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<tr>
<td>Group 4</td>
<td>20 µM BiCNU</td>
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<tr>
<td>Group 5</td>
<td>50µM resveratrol + 10 µM BiCNU</td>
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<tr>
<td>Group 6</td>
<td>50µM resveratrol + 20 µM BiCNU</td>
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<tr>
<td>Group 7</td>
<td>100 µM resveratrol + 10 µM BiCNU</td>
</tr>
<tr>
<td>Group 8</td>
<td>100 µM resveratrol + 20 µM BiCNU</td>
</tr>
<tr>
<td>Group 9</td>
<td>Control (0.1% DMSO)</td>
</tr>
</tbody>
</table>

2.3. Cell Viability Measurement

MTT (3-(4,5-methylthiazol-2-yl)-2,5-diphenyl tetrazolium -bromide) is a colorimetric agent used to access cell viability in vitro. Living cells degrades DMSO enzymatically, leading to a color change. This experiment aims to determine the percentage of cells that keep their viability with respect to the control after pharmacologic
agents' treatment. According to the method, after 48 hours of incubation with different amounts of resveratrol and BiCNU alone or in combination, the cells were treated with 20 μl MTT dye (5 mg/ml) for 2 hours. Afterward, MTT was eliminated, and 200 μl DMSO was added to each well. Following an incubation period of 10 minutes, the color change was assessed at a wavelength of 540 nm. Cell viability was accepted as 100% in the control group.

2.4. Immunocytochemistry

Human glioblastoma cells were cultured in 12-well chamber slides, and these cultures were treated by pharmacological agents. At the 48th hour of pharmacological treatment, the growing media were removed, and the cells were fixed by 4% paraformaldehyde. After washing by phosphate buffer saline (PBS), they were put on ice floating in a 0.1% Triton-X100 solution for 15 minutes, and endogenous activation of peroxidation was provided by 3% H$_2$O$_2$ for 10 minutes. The cells were washed in PBS three times in 5 minutes and then kept in a blocking solution for 10 minutes. Later, the cells were incubated with primary antibodies of Beclin-1 (ab62472, 1/200) and Caspase-3 (Sc56053, 1/200) at room temperature for 1 hour, and the suspensions were washed by PBS three times. After the cells were treated with secondary antibodies, they were incubated with biotin for 30 minutes. Then, the cells were washed by PBS three times in 5 minutes and incubated with avidin for 30 minutes. After the cells were washed by PBS three times in 5 minutes, they were colored by 3- amino-9-ethyl carbazole. Finally, they were washed with distilled water and stained with Mayer's hematoxylin. The cells were counted by a light microscopy under X20 magnification by the Image Analysis Program (NIS, Japan). The percentage of stained cells was specified semi-quantitatively depending on the following score formula.

Score = (Staining scale+1) x (Percentage of cells stained at each scale)

Staining was designated as 0 (no staining), +1 (weak staining), +2 (moderate staining), and +3 (strong staining).

2.5. Statistical Analysis

Data were analyzed by SPSS (Statistical Package for Social Sciences) software version 22.0 (IBM, Armonk, NY, USA). The Kolmogorov-Smirnov test analyzed the distribution of data. Continuous variables were expressed as mean ± standard deviation, and categorical variables were denoted as numbers or percentages where appropriate. Student t-test was used, and P-values less than 0.05 were accepted as significant.

3. Results

3.1. Viability Test Results

Figure 1 indicates the viability test results obtained after 48 hours of pharmacological treatment. Accordingly, cell cytotoxicity was significantly higher in Group 2 (cells treated with 100 μM resveratrol) than Group 1 (cells treated with 50 μM resveratrol). Similarly, cytotoxicity was significantly increased in Group 4 (cells treated with 20 μM BiCNU) than Group 3 (cells treated with 10 μM BiCNU). Compared to other groups, cytotoxicity was significantly higher in Group 8 (cells treated with 100 μM resveratrol + 20 μM BiCNU).

Figure 1. Effects of resveratrol (RSV) and 1,3-bis(2-chloroethyl)-1-nitrosurea (BiCNU) treatment on YKG1 glioblastoma cells’ viability after 48-hours of treatment.

3.2. Immunocytochemistry Results

Expression levels of Beclin-1 and Caspase-3 proteins were analysed with immunohistochemical staining, and the results are summarised in Figure 2.

Figure 2. Images of the immunostained YKG1 cells, which were treated with different amounts of resveratrol and BiCNU. The cells were stained with Beclin-1, and Caspase-3 antibodies and images were taken by a light microscopy at 20X magnification.
The images of immunostained slides were analysed semiquantitatively, and Hscore results are summarized in Figure 3.

![Figure 3. Beclin-1 and Caspase-3 HScore results obtained as a result of immunocytochemical staining.](image)

According to results, the expression of Beclin-1 was significantly higher in Group 8 (cells treated with 100 μM resveratrol + 20 μM BiCNU) as compared to Group 2, Group 4, Group 6 respectively (P < 0.0001, P = 0.001, P = 0.002). The expression of Caspase-3 was significantly higher in Group 8 as compared to Group 2, Group 4, and Group 6 (P < 0.001, P < 0.001 and P = 0.004). All groups except Group 8 showed +1 immunostaining while there was both +1 and +2 immunostaining in Group 8 (cells treated with 100 μM resveratrol + 20 μM BiCNU).

4. Discussions

Glioblastoma is the most invasive tumor of the central nervous system with the worst prognosis among the other cancer types. The typical survival duration following diagnosis is around 12 to 15 months (Omuro and DeAngelis, 2013; Alexander and Cloughesy, 2017). The treatment of GB usually involves surgery followed by chemotherapy and radiotherapy. It is well known that larger surgical removal is associated with more prolonged survival (Villà et al., 2014; Alifieris and Trafalis, 2015). However, complete excision of GB is not possible in most cases, and the role of chemotherapy is often limited in preventing recurrences (Anjum et al., 2017). Therefore, an integrated approach should be developed to prolong the patients' survival span diagnosed with this malignancy.

Nitrosoureas, including BiCNU (carmustine), was first introduced to treat gliomas and their effectiveness was modest, but doses that produced response rates up to 50% led to severe systemic side effects (Nagpal, 2012). Afterwards, polymers were developed to maintain the efficacy of BiCNU beyond the blood-brain barrier so that, subsequently, biodegradable polymers might permit more constant drug delivery. Such BiCNU polymers were used to make up carmustine wafer which is also named as BiCNU wafer or Gliadel wafer (Nagpal, 2012; Zhang et al., 2014).

The beneficial effects of carmustine wafer on patients’ survival with GB have been highlighted in various studies. A meta-analysis found that treatment with carmustine wafer and temozolomide was significantly more effective than avoiding chemotherapy in improving survival. It was also remarked that the carmustine wafer's clinical efficiency extended up to 24 months (Spiegel et al., 2007). Another systematic review also aimed to examine the potency of carmustine wafer in malignant glioma treatment and included three randomized controlled trials and one prospective cohort study. This systematic review came up with the conclusion that the patients who received the diagnosis of GB for the first time and who were treated with carmustine wafer had a significantly longer survival span than the controls (Perry et al., 2007). Based on the findings of two randomized controlled trials, a Cochrane review concluded that carmustine wafer improved GB patients’ survival without significantly contributing to the increase in adverse effects (Hart et al., 2011).

Resveratrol demonstrates many anti-carcinogenic effects on various cancer cells in vitro (Le Corre et al., 2005; Kundu and Surh, 2008; Shukla and Singh, 2011). When evidence from in vitro studies was analyzed, Jang et al. (1997) were the first showing resveratrol as a chemopreventive agent. It was found that topical application of resveratrol could inhibit tumor formation in an animal model of skin cancer. Later studies also confirmed that resveratrol’s topical application prevented tumor formation by regulating the cell cycle and endorsing apoptosis, reducing COX activity and prostaglandin production in a skin cancer mouse model (Afaq et al., 2003; Reagan-Shaw et al., 2004). The findings related to in vitro use and efficacy of resveratrol for other types of cancer that require its oral ingestion or intraperitoneal injection has been more controversial. This discrepancy has been attributed to the poor bioavailability of trans-resveratrol. Wenzel & Somoza (2005) clarify the bioavailability and metabolism of resveratrol. Accordingly, as resveratrol is consumed orally in rodents and humans, 70–80% is quickly absorbed via passive diffusion in the intestines (Kaldas et al., 2003; Walle et al., 2004). After absorption, resveratrol is conjugated into glucuronides and sulfates so that trans-resveratrol levels in peripheral circulation reach their peak 30–60 minutes after oral administration (Soleas et al., 2001; Yu et al., 2002).

In humans, circulating unmodified trans-resveratrol levels make up only about 2% of the peak serum concentration of total free resveratrol and conjugates after a single dose of 25 mg to 70 kg body weight (Goldberg et al., 2003). Another study has ended up with the conclusion that at least 70% of resveratrol is absorbed after a single 25 mg dose, and there is a peak serum concentration of 2 μM (approximately 490 ng/ml) for resveratrol and all of its metabolites (Walle et al., 2004). After administering multiple oral doses (5 g daily for 29 days), plasma concentrations of trans-resveratrol are as high as 4 μM (4.29 nmol/ml). However, it should be noted that resveratrol at this high dose was also associated with gastrointestinal side effects (Brown et al., 2010). On the contrary, in human colon tissue, resveratrol levels and its
metabolite resveratrol-3-O-glucuronide have been detected in relatively higher concentrations (674 and 86 nmol/g, respectively) when 0.5–1.0 g of resveratrol was taken orally once per day. In the study mentioned above, resveratrol supplementation decreased cellular proliferation by 5% in colorectal cancer tissue, as assessed by Ki67 staining (Patel et al., 2010). Since there are such rapid conjugation and low bioavailability of resveratrol, the in vitro use of resveratrol for cancer prevention and treatment has been a matter of debate.

As for brain cancer, Xu and coworkers were the first reporting the combination of resveratrol, and temozolomide significantly down-regulates the expression of matrix metalloproteinase-9, enhances the production of reactive oxygen species, and inhibits the anti-apoptotic protein Bcl-2. Thus, this combination has been considered to suppress cell proliferation in malignant U87MG glioma cell line. The significant pro-apoptotic effect of resveratrol has been observed through the increase in Bax expression, the decrease in the expression of Bcl-2, and the cleavage of caspase-3 (Xu et al., 2005).

Filippi-Chiela and colleagues were the first to determine that resveratrol induced autophagy formation by the up-regulation of autophagy proteins such as Atg5, Beclin-1, and LC3-II in three human glioblastoma cell lines. Correspondingly, the authors have hypothesized that resveratrol accelerates autophagy inhibition, which results in apoptosis in turn (Filippi-Chiela et al., 2011). Firouzi and coworkers found that methoxyamine and resveratrol can significantly reduce colony numbers and induce DNA damage of glioblastoma spheroid cells. This result reflected the promise of resveratrol at a 20-μM concentration in cancer-treatment therapy when used together with radiation and radiosensitizer (Firouzi et al., 2015). Other researchers demonstrated that resveratrol might reduce the expression and activity of the POK erythroid ontogenic factor (Pokemon) in glioma cells, suppress the Sp1 DNA binding activity, and enhance the recruitment of HDAC1 (Yang et al., 2016). Later, Song et al. showed that administration of 20 μM resveratrol decreases cell viability to a lesser extent and the administration of 40 μM resveratrol decreases cell viability significantly in malignant human LN18 and U87 glioblastoma cell lines (Song et al., 2019). Another study by Cilibrasi et al. found out that resveratrol at a dose of 100 μM had the highest efficiency for cytotoxicity for human glioma stem cells. In that study, cytotoxicity was observed at the minimum resveratrol dose of 50 μM but, interestingly, the resveratrol dose of 200 μM was found to have significantly lower cytotoxicity than the resveratrol dose of 100 μM (Cilibrasi et al., 2017).

In this study, cytotoxicity was significantly higher in the cells treated with 100 μM resveratrol than the cells treated with 50 μM resveratrol. Likely, toxic effects were significantly increased in the cells treated with 20 μM BiCNU than the cells treated with 10 μM BiCNU. Compared to other groups, cell viability was significantly higher in the cells treated with 100 μM resveratrol + 20 μM BiCNU. Moreover, the expression of Beclin-1 and Caspase-3 were significantly higher in cells treated with 100 μM resveratrol + 20 μM BiCNU than in the other groups. All groups except those treated with 100 μM resveratrol + 20 μM BiCNU showed +1 immunostaining when there was both +1 and +2 immunostaining in cells treated with 100 μM resveratrol + 20 μM BiCNU. The present study’s findings suggest that the concurrent administration of BiCNU and resveratrol has cumulative effects on the cytotoxicity of malignant human YKG1 glioblastoma cells in cell culture. This cytotoxicity is related to both autophagy and apoptosis as it is reflected at least enhanced expression of Beclin-1 and Caspase-3. The present study’s findings should be interpreted carefully as their power is limited by the in vitro design of the research and the administration of relatively lower doses of BiCNU and resveratrol to the cell culture. A clinical implication of these findings might be the utilization of resveratrol as a chemoprevention agent in patients receiving BiCNU to treat malignant glioma. To verify this implication, clinical studies should be undertaken to specify the efficacy and safety of concurrent BiCNU and resveratrol treatment in patients diagnosed with malignant glioma. Hence, further research is warranted to clarify the effects of BiCNU and resveratrol combination for the glioblastoma cells in vitro and the GB patients in vitro.

Conflict of interest
The authors state no conflict of interest.

Author Contribution
GP, EA, HG, BDY, and SC performed the research. EA and HG helped during the experimental study and statistical analysis. GP wrote the manuscript. GP and EA drafted the manuscript. GP and EA conceived and designed the study and critically revised the manuscript.

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