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LOMUSTINE AND 6 MV X-RAY COMBINATION EFFECT ON U87-MG CANCER CELLS

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ABSTRACT

Glioblastoma multiforma (GBM), the most malignant brain cancer, is currently treated with surgery followed by radiotherapy and concurrent chemotherapy. In spite of much efforts made to improve the treatment, outcome is still unsatisfactory. Lomustine is an alkylating agent which is used for GBM treatment. Combination of different doses of lomustine with radiation may result in different cytotoxicity against cancer cells. Optimization of radiation dose and lomustine concentration to generate synergy can help to resolve some problems with GBM treatment. In this study, in order to examine the combination effect on U87-MG cancer cells concentrations of 25, 50 and 100 ug/ml lomustine were used in combination with doses of 1, 2, 4 and 6 Gy 6 MV X rays' radiation. MTT was performed to evaluate the cytotoxicity of either lomustine or radiation separately as well as in combination. For examination of survival fraction of U87-MG cells treated with lomustine and irradiated to radiation colony assay was performed. Combination index calculations were used for investigation of combination effect. Results showed that U87-MG cells viability decreased by increasing lomustine concentration and radiation dose ($p< 0.05$). For combination of 1 Gy radiation dose with lomustine at concentrations of 25, 50 and 100 ug/ml CI was close to 1 representing additive effect and for other situations, the effect was antagonism (CI < 1). It is promising for patients with GBM to be treated by radiotherapy and concurrent chemotherapy if possible to increase lomustine uptake by cancer cells to the above levels through targeted drug delivery or by other mechanisms.

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Introduction

The most malignant brain cancer is now glioblastoma multiforma (GBM) which has a very poor prognosis [1-3]. Standard method for treatment of GBM is currently surgery followed by radiotherapy and concurrent chemotherapy [4]. Remaining of cancer cells in the tumor site after surgery, resistance of the tumor cells to radiation and drugs, insufficient uptake of chemotherapy agents by the cancer cells and some difficulties associated with dose delivery techniques in the radiotherapy are several limiting factor for the standard treatment [5-7]. Lomustine is an alkylating agent which is used for GBM treatment. It is nonspecific for cancer cells and also affects rapid dividing normal cells [8, 9]. Reducing cytotoxicity and in turn side effects of chemotherapy agents require lowering drug dose but cancer cells have to receive enough lethal dose to be killed [10]. The alkylating agents react through an electrophilic alkyl group or a substituted alkyl group to covalently bind to cellular nucleophilic sites [11]. Synergy that the effect of two agents given together is more effective than would be

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predicted based on their individual activity in turn can enhance the radiotherapy effect and reduce the side effects of lomustine [11]. Combination of different doses of lomustine with radiation may result in different cytotoxicity effects. Optimization of prescribed dose of lomustine in order to create synergy while combining with radiation seems to be a requirement for treatment of GBM. One way to examine the combination effect is median effect principle [12]. The purpose of this study was to investigate the combination effect for lomustine and 6 MV X ray on U87-MG cancer cells by median effect principle.

Experimental

Materials

Lomustine was purchased from EXIR Company of Austria. For cell culture High glucose Dulbecco's Modified Eagle's Medium (DMEM) (BioIdia), Phosphate buffer saline (PBS) (BioIdia) and penicillin – streptomycin (BioIdia) along with Fetal Bovine Serum (FBS) (INCOLON) were used. For cytotoxicity evaluation 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (sigma Aldrich) was used and dimethyl sulphoxide (DMSO) molecular grad was bought from Pars Tuse. Four colony assay formaldehyde (37%) were purchased from Merk and crystal violet from Sigma Aldrich. U87-MG cell line was obtained from Pasteur institute of Iran.

Biological tests

Cell culture

U87-MG Cells were cultured in T-75 flasks containing high glucose DMEM with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and grown in incubator (BINDER) under 5% CO2 pressure at 37^0C temperature. When cells well grown and reached to a suitable confluence, they were separated from flask floor by 0.25 % trypsin/EDTA solution and were counted for biological tests.

MTT assay

Cytotoxicity of lomustine and 6 MV X ray on U87-MG cells were examined by MTT test. Cells were cultured in 12-well plates at a concentration of 20000 cells per well and incubated for 24 h to attach to the floor of the wells. Three groups were chosen to examine the cytotoxicity of lomustine based on concentration including 25, 50 and 100 ug/ml and one group left as control without drug. For each concentration, three wells were cultured. Lomustine contained medium was filtered through 0.2 µm filter before addition to wells. After incubation, the culture medium was removed and 200 µl fresh medium containing 5mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliµm bromide (MTT) was added to each well and remained in incubator for 4 h at 37^0C. After that, supernatant was carefully removed and 440 µl DMSO was added to each well and remained for 2 h. Cells viability was examined by measuring absorbance of the solution using microplate reader (BIO-RAD Model 680) at 570nm. The cells viability was calculated as percentage of optical density reading of cells treated with lomustine divided by the same for untreated cells. Measurements were repeated three times.

For evaluation of cytotoxicity of 6 MV X ray on U87-MG cancer cells, they were irradiated using an Anchor accelerator. Cells were cultured in the 24 wells plate at density of 20000 cells per well and were exposed to doses of 1, 2, 4 and 6 Gy in a 20×20 cm^2 field at percentage depth dose of 97.8. Dose calculation was performed using PDD table of the accelerator and 5 cm thickness backscatter Plexiglas acrylic sheets were placed under plates to ensure that the dose is uniform through the irradiated field. In order to ensure that correct radiation dose is delivering to the cells, dose verification was performed for doses of 2 and 4 Gy using thermoluminescent dosimeter (TLD-100). Two TLDs were embedded in water resistance bags and placed in water filled wells such as shown in Figure 1.

Figure 1: Dose verification using TLD-100 dosimeters

For evaluation of cytotoxicity of lomustine in combination with 6 MV X ray, cells were first cultured in the 12 wells plate. After removing cultured medium and washing with PBS, fresh medium containing 25, 50 and 100 µg/ml lomustine were added to them and for each concentration, three wells were cultured. Plates were then irradiated to doses of 1, 2, 4 and 6 Gy by 6 MV X ray by the same way for untreated cells. Kruskal-Wallis test was used for statistical analysis of the MTT test results.

Colony assay

To prevent cancer cells from recurrent, killing of those cells which can form a colony is very important [13]. Colony assay as a gold standard method is usually performed to investigate survival of irradiated or treated cancer cells [14-16]. Cells were first cultured in the 12 wells plate at density of 50000 cells per each well. Medium containing lomustine at concentrations of 25, 50 and 100µg/ml was added to them after removing the cultured medium. Cells were then exposed to doses of 1, 2, 4 and 6 Gy at room temperature. X ray irradiation was performed by 6 MV X ray with output of 1cGy/MU. Irradiated cells then were carefully counted and numbers of either 1000 or 2000 cells were cultured in 60 mm petri dishes in triplicate. After irradiation, cells were left in incubator for 14 days. Culture medium was then removed and petri dishes were washed twice by PBS. Colonies were fixed by formaldehyde and stained with 5% crystal violet in PBS. Colonies which had more than 50 cells were counted and survival fraction was determined for each group by the equation 1[15].

$$SF = \frac{\text{no.of colonies formed after treatment}}{\text{no.of cells seeded} \times \text{PE}} \qquad (1)$$

Which PE is the ratio of the number of colonies to the number of cells seeded (equation 2):

$$PE = \frac{\text{no.of colonies formed}}{\text{no.of cells seeded}} \times 100 \qquad (2)$$

To determine combination effect between radiation and lomustine, Chou analysis was performed using compusyn software[17]. The combination index (CI) was used to determine if the effect is synergy, additive or antagonist. CI less than 1, greater than 1 and equal to 1 indicate antagonism, synergy and additive respectively.

Results

Maximum absorption intensity of dissolved lomustine in the ethanol is located at wavelength 228 nm on UV-Vis spectra (Figure 2). This is in a good accordance with standard spectrum of lomustine on which the peak is located at 230nm.

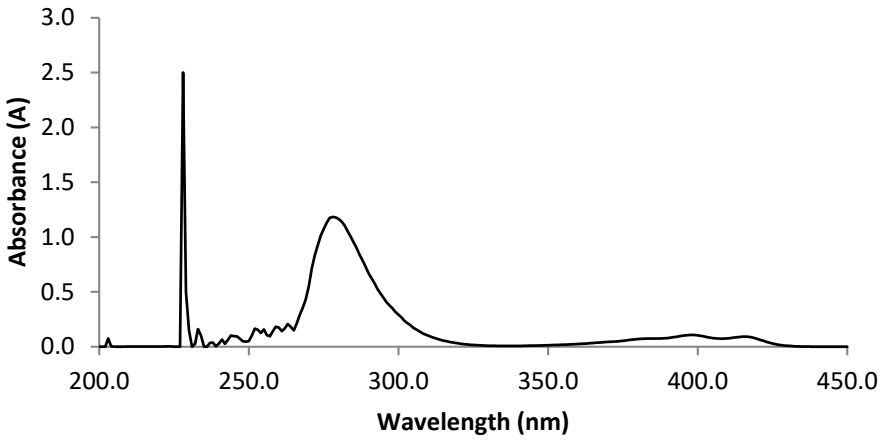

Figure 2: Maximum absorption intensity of dissolved lomustine in the ethanol is located at wavelength 228 nm on UV-Vis spectra.

Figure 3 shows the image of U87-MG cells after well growing and reaching to a suitable confluence.

Figure 3: image of U87-MG cells after well growing and reaching to confluence

U87-MG cells viability which were irradiated to doses of 1, 2, 4 and 6 Gy from 6 MV X rays decreased significantly ($P < 0.001$) with increasing radiation dose (Figure 4).

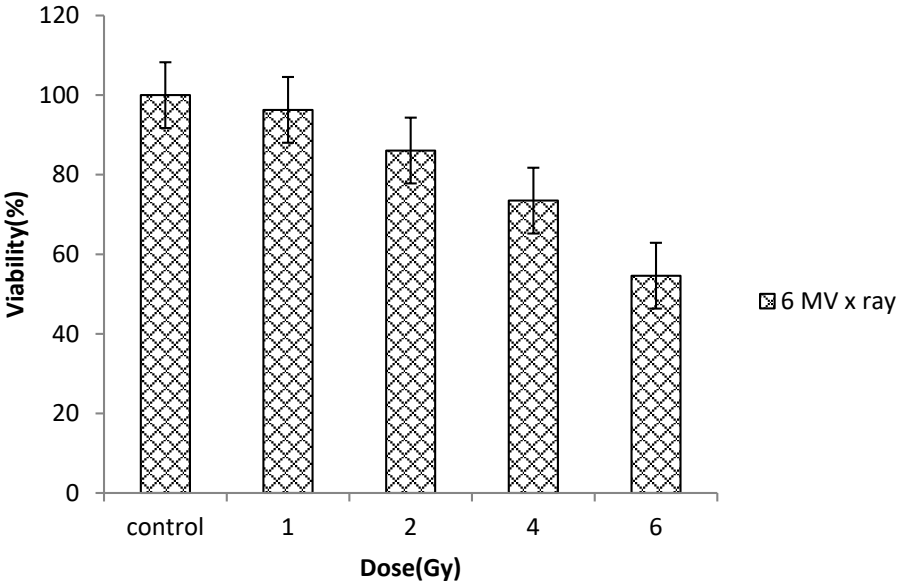

Figure 4: Viability of U87-MG cancer cells exposed to doses of 1, 2, 4 and 6 Gy from 6 MV X rays.

Dose verification results by TLD for doses of 2 and 4 Gy to cancer cells were 2±0/19 and 4±0/12 Gy respectively.
Figure 5 shows viability percent of U87-MG cells treated with lomustine. Viability decreased ($P<0.05$) with increasing concentration of lomustine.

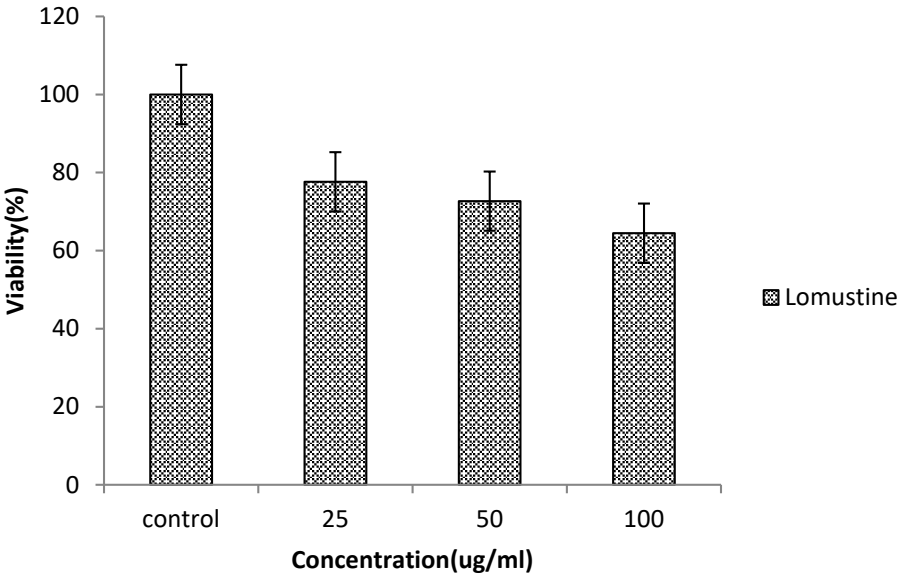

Figure 5: Viability of U87-MG cancer cells treated with lomustine

MTT results for combination of lomustine with 6 MV X ray are shown in Figure 6. For a specific dose of radiation viability decreased significantly ($P<0.05$) with increasing lomustine concentration except for dose of 4 Gy ($P=0.55$). For a specific concentration of lomustine viability decreased significantly ($P<0.05$) with increasing radiation dose.

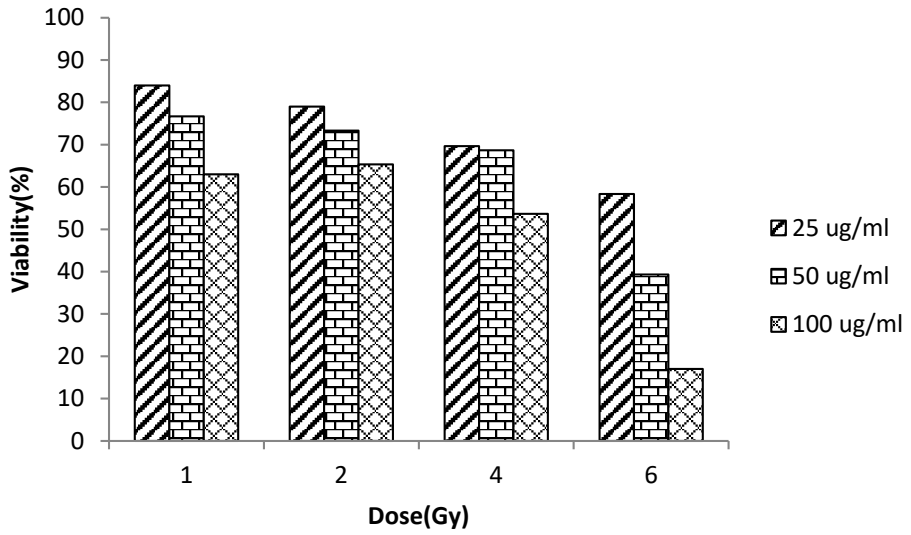

Figure 6: Viability of U87-MG cancer cells which were treated with concentrations of 25, 50 and 100µg/ml lomustine and irradiated to doses of 1, 2, 4 and 6 Gy from 6 MV X ray

Colony assay results are usually shown by dose response curves in which a vertical axis with a logarithmic scale and a horizontal axis represent survival fraction and radiation doses respectively. Survival curve for combination of 6 MV X rays and lomustine is shown in Figure 7. Survival fraction decreased significantly ($P<0.05$) by dose increasing for a specific concentration of lomustine. It also decreased with increasing lomustine concentration at a specific radiation dose ($P<0.05$).

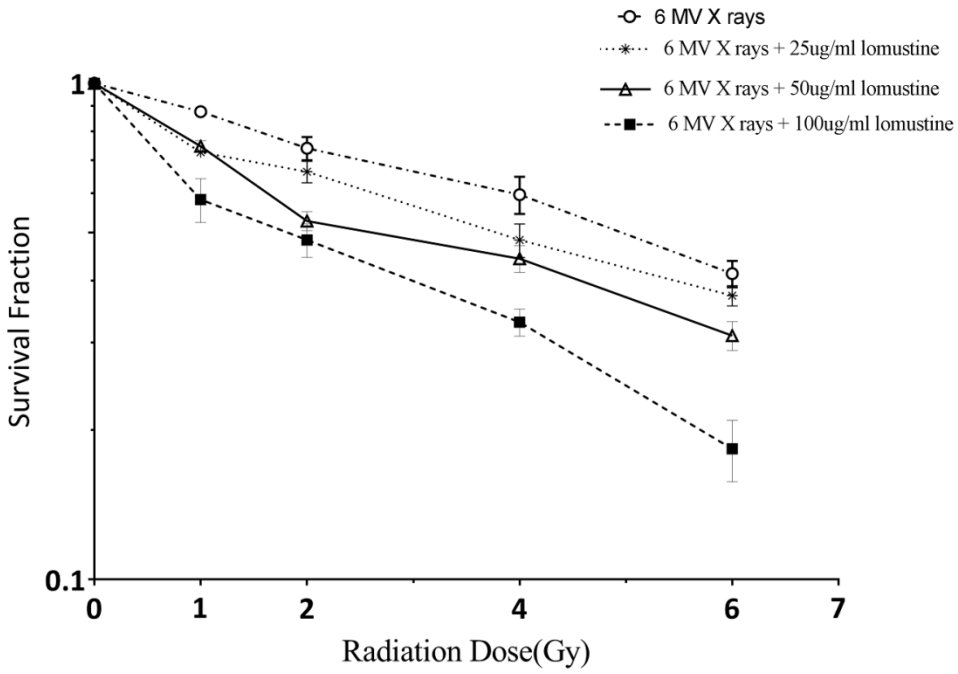

Figure 7: Survival curves of U87-MG cancer cells treated with combination of 6 MV X rays and lomustine at different doses

Combination index (CI) calculations results performed by compusyn software are shown in Figure 7. CI nearly equals to 1 for the combination of radiation and lomustine in such a way: 1 Gy - 25ug/ml, 1Gy - 50ug/ml and 1Gy - 100ug/ml. Based on these CI values, the effect can be interpreted as additive for the above situations. CI is less than 1 for other situations of combination and the effect is antagonism. No synergy was observed in this study.

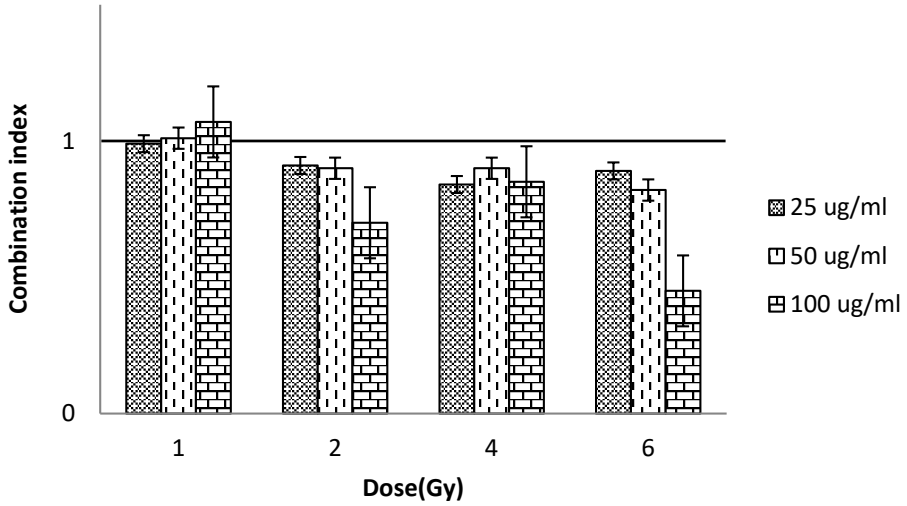

Figure 7: Combination index (CI) for the combination of radiation and lomustine

Discussion

In this study, we examined the combination effect of lomustine and 6 MV X ray on U87-MG cancer cells. The results showed that U87-MG cancer cells viability decreased with increasing concentration of lomustine. It is an alkylating agent which reacts through an electrophilic alkyl group or a substituted alkyl group to covalently bind to cellular nucleophilic sites [11]. This led to covalent linkages by alkylation with bases in DNA which in turn is responsible for cytotoxicity. Radiation interaction with cancer cells can damage DNA directly or produce free radicals by radiolysis of water molecules that in turn is destructive and killer of cells [18, 19]. Reactive oxygen spices (ROS) are generated as a result of reactions of free radicals with biological molecules which cause oxidation of lipids, protein and DNA that induce apoptotic and necrotic cell death due to mitochondrial dysfunction [20]. Different mechanisms of cytotoxicity for lomustine and 6 MV X ray may be a justification to explain why the combination effect is either additive or antagonism.

Conclusion

Our study revealed that combination of low radiation doses which are usually used in daily fractionation radiotherapy with those concentrations of lomustine used in this study can result in additive effect. It is promising for treatment of patients with GBM if possible to increase lomustine uptake by cancer cells to the above levels through targeted drug delivery or other mechanisms.

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