



THE RADIOSENSITIZER EFFECT OF 2,6-BIS-(2,6 DICHLOROBENZYLIDENE) CYCLOHEXANONE AS A SELECTIVE THIOL ALKYLATOR ON HT29 CELL LINE

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ABSTRACT

The radiosensitizer compounds are used in order to increase the efficiency of radiotherapy. Different agents have been evaluated for radiosensitizer effects. 2,6-bis-(2,6 dichlorobenzylidene) cyclohexanone is a α , β unsaturated ketone derivatives. Conjugated unsaturated ketones can be readily reacted with thiol groups which are not found in nucleic acid. Theoretically, the genotoxicity side effect should not be observed from these agents. The suggested conjugated unsaturated ketone was synthesized from the reaction of cyclohexanone and 2, 6 dichlorobenzaldehyde in the presence of catalytic amount of NaOH. The ¹HNMR analysis demonstrated that the desired compound could be successfully prepared with the appropriate yield. The cytotoxicity of prepared compound and 2 Gy ionizing irradiation was investigated on HT 29 cell line which was resistant to 2 Gy radiation due to MTT assay. The outcome of this approach indicated that the prepared selective thiol alkylator with radiation could significantly increase the mortality of treated cells versus to the cells were only treated with the prepared compound. Therefore, the conjugated unsaturated ketone can open the new field for the preparation of new radiosensitizer agents.

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INTRODUCTION

In spite of progress in radio therapy (RT) over the years, approximately one-third of patients with solid tumors receiving curative treatment will suffer local recurrence due to residual tumor (Beck-Bornholdt *et al.*, 1997). One of the critical step is how to deliver effective cytotoxic doses of ionizing irradiation to tumor cells and meanwhile to minimize the side effects of irradiation to normal and healthy cells. Theoretically, the solution of dilemma is to deliver the ionizing irradiation beam to tumor cells as targeting. Therefore, conformal three-dimensional and intensity modulated radio therapy are used in order to provide closer conformation of irradiation fields to tumor region to decrease ionizing irradiation of healthy cells as non-targeting tissues (Han *et al.*, 2016), (Isa, 2013) and (Zabihzadeh *et al.*, 2016). Despite these advances in conformal radio therapy, treatment with irradiation is still to remain cause of morbidity among patients with cancer. It can be leaded to limit the delivery of curative irradiation doses to tumor cells. The other attempt is the combination of radiotherapy and anticancer agents. Chemotherapeutics have the potential to enhance the

sensitization of tumor cells to cytotoxic effect of ionizing irradiation are defined as radiosensitizer and the process is commonly called as radiosensitization (Candelaria *et al.*, 2006), (Kimura *et al.*, 2012), (Lawrence *et al.*, 1996) and (Nair *et al.*, 2008). A variety range of anticancer agents have been examined as radiosensitizer in preclinical stage (Herchenhorn *et al.*, 2010), (LI *et al.*, 2010) and (Weigel *et al.*, 2010). Different of conjugated unsaturated ketones such as 2,6-bis-(2,6 dichlorobenzylidene) cyclohexanone were designed as antineoplastic agents (Dimmock *et al.*, 1994). These compounds have a preferentially or exclusively affinity for thiols and not amino or hydroxyl groups which are found in nucleic acids (Das *et al.*, 2008) and (Das *et al.*, 2010). Hence, these compounds must lack of interactions with nucleic acids, thereby eliminating genotoxicity side effects. Colorectal cancer is the third most frequent cancer form in the world (Favoriti *et al.*, 2016). The primary treatment is surgery but radio therapy with or without anticemoterapeutic agents are used preoperatively to decrease tumor burden or postoperatively in order to reduce recurrence risk (Kye and Cho, 2014). According to the literature, HT29 colon cancer cell line has been reported to be resistant to cytotoxic effect of ionizing irradiation (Khalaj *et al.*, 2006) and (Lambin *et al.*, 1996). For this reason, HT29 cell line is recommended as a

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reliable cell for investigation the radiosensitizer effect of anyantineoplastic agents. This study was conducted to investigate the radiosensitizer effect of 2,6-bis-(2,6 dichlorobenzylidene) cyclohexanone as a α , β unsaturated ketone derivatives on HT29 cell line.

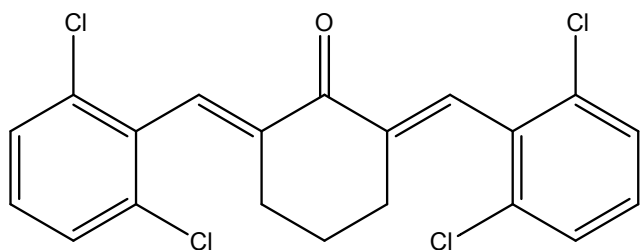


Fig 1 The structure of 2,6-bis-(2,6 dichlorobenzylidene) cyclohexanone

MATERIALS AND METHODS

All chemical compounds have been purchased from Merck and Sigma. The chemical and solvents were of the highest purity and analytical grade and used without further purification. HT29 cell line has been supplied by institute of Pasteur, Tehran, Iran.

Preparation of 2, 6-bis-(2,6) dichlorobenzylidene cyclohexanone

We modified the synthesis of desired compound that it was reported to the literature (Dimmock *et al.*, 1994). Therefore, the 2,6-bis-(2,6 dichlorobenzylidene) cyclohexanone was prepared as follow: 0.5 mole cyclohexanone and 0.5 mole 2,6 dichlorobenzaldehyde were added to 25 ml of distilled water. Then 0.025 mole NaOH in 1 ml H₂O was added to the above mixture due to the drop funnel during 15 min. The mixture was refluxed for 10 h and followed stirred for overnight at room temperature. The reaction progression was monitored by TLC. The residue was separated and recrystallized by methanol. The yellow powder crystal with melting point (mp) 163.5 -165°C (mp_{Ref}164-166 °C) was obtained. The ¹H NMR spectrometer was used to confirm the identity of the compound synthesized. The ¹H NMR spectrum of the prepared compound was as followed: ¹HNMR(D₂O): 1.77(m, 2H), 2.74(m,4H), 7.27(s,2H), 7.46(t,2H), 8.82(d,4H).

Cell Culture

The HT29 cells were seeded 10⁵ cells/T25 flasks and cultured in medium Dulbecco's Modified Eagle's Medium (DMEM), 10 % fetal bovine serum (FBS), 100U/ml penicillin and 100 μ /ml streptomycin at 37°C with 5% CO₂, 95% air and complete humidity. Stock culture was grown in 25 cm² culture flasks and all experiments were done in 96 well plates. When the stock cells were approximately 90 % confluency, they were detached by using 0.05 % trypsin / EDTA and counted by means of tryptan blue and hemocytometer. The freshly stock solutions of 2,6- bis-(2,6 dichlorobenzylidene) cyclohexanone were prepared in every experiment. The cells were seeded in 100 μ l complete medium per well of 96-multiwell plates at the density of 10⁴cells/well. The cells were incubated for 24 h at 37 °C with 5 % CO₂ ,95 % air in order to adhere on the surface of plate. When the monolayer of HT29 cell line was formed in each well, the supernatant removed and 100 μ l of different doses of 2,6 bis 2,6 dichlorobenzylidene cyclohexanone added in microtitre plates and kept for incubation at an incubator. The 96-well plates were randomly divided into two main groups. One group of

plates was irradiated with 2 Gy ionization irradiation 2 h after drug treatment and the other group without the ionizing irradiation. Then all plates were placed in the incubator at 37°C 5 % CO₂ and 95 % air for 24 h. All cells were periodically checked for shrinkage, swelling and granularity. Then the plates were removed from incubator and supernatant solution in each well decanted and 50 μ l of 3-(4,5 dimethyl thiazol-2 yl)-2,5 diphenyl tetrazolium bromide (MTT) at a final concentration of 5 mg/ml added to each well. The plates were gently shaken and incubated for 1 h in the incubator. The supernatant was removed, 125 μ l of DMSO added and gently shaken by shaker in order to solubilize the formed formazan for 10 min. The plates were read by ELISA reader (BIO-Tek Instruments, Inc, USA) using a wavelength 570nm, by using DMSO as a blank with reference reading at 690 nm. The control HT cell line was undertaken the above mentioned procedure but without any drug treatment. Eight wells were allocated for each treatment and control cells. All experiments were carried out five times. The percentages of cell growth were expressed by the ratio of the absorbance of treated to control cells multiple 100. Finally, the percentages of mortality were obtained from the subtraction of the percentages of cell growth from 100.

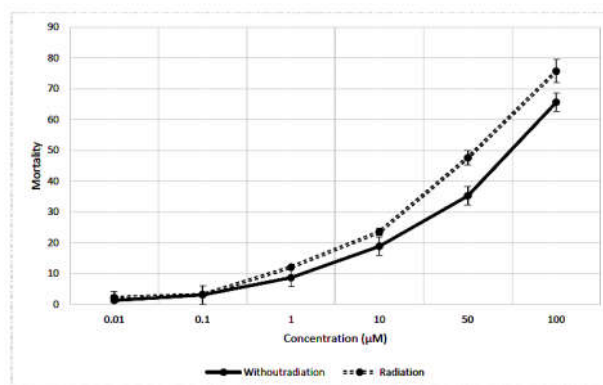


Fig 2 The percent of mortality of HT29 cell were exposed to different doses of 2,6-bis-(2,6 dichlorobenzylidene) cyclohexanone with or without 2 Gy ionizing irradiation.

Ionization irradiation

The cells were irradiated for 2 Gy ionization irradiation at room temperature by using linear accelerator (Primus, Simens, Germany) 6 MV photon beams.

Statistical analysis

Statistical analysis was performed using SPSS 11.0 for window (SPSS Inc, Chicago IL, USA) and descriptive statistics are shown as arithmetic mean \pm standard deviation. Independent samples t-test was used to investigate the differences between irradiated and non-irradiated cells and p value smaller than 0.05 was considered statistically significant.

RESULTS

The ¹H NMR spectrum indicated that 2,6-bis-(2,6 dichlorobenzylidene) cyclohexanone compound could be synthesized successfully. The desired compound was obtained with the yield above 78 %. The MTT colorimetric test is widely used in order to measure the number of alive and active metabolic cells. The basis of MTT investigation is depends on

the activity of succinate dehydrogenase enzyme which is present in the mitochondria of cells. The yellow water soluble substrate 3-(4,5 dimethyl thiazol-2 yl)-2,5 diphenyl tetrazolium bromide can be readily reduced into an insoluble colored formazon product which is measured by spectrophotometer ELIZA reader. The above mentioned reduction reaction can be occurred by the enzyme when the HT29 cells are alive. The reduction of MTT substrate can only happen in metabolically active cells. The production of resultant formazon appears to be proportional to the level of energy metabolism in the cells. For this reason, it is provided to measure the metabolically activated cells even in the absence of cell proliferation. Absorbance values that are lower than the control cells indicated that a reduction in the rate of cell proliferation. Conversely, a higher absorbance values revealed this matter that an increased in cell proliferation has happened. The preliminary assessment with different number of HT29 cells demonstrated that there was a linear relation between the density of cells and the measured absorbance. The radiosensitivity of HT29 cell line was investigated in order to choose the dose of ionizing irradiation that irradiation itself could not kill any cells. Therefore, 2 Gy ionizing irradiation was obtained from the above preliminary analysis. When 2 Gy irradiation was applied no toxicity observed on HT29 cell line. The effect of different doses of 2,6-bis-(2,6 dichlorobenzylidene) cyclohexanone on the mortality of HT29 cell line with or without 2 Gy ionizing irradiation has been assessed. As it has been shown in Fig 2, the prepared compound at the concentration of 50 μ M could increase the mortality of cell approximately to 35 % versus the control cells without 2 Gy irradiation. This mortality effect could be enhanced near about to 50 % in comparison to control cells with the combination of 2 Gy irradiation. The statistical significant increased mortality of HT29 cells could not be observed between the irradiation or non-irradiation intervention of the cells when the concentrations of the synthesized compound were below 50 μ M. The cytotoxicity of the prepared compound has been considerably increased when the dose of compound increased from 50 μ M to 100 μ M. The combination of ionizing irradiation and the prepared compound could not increase further cytotoxicity. The remaining cells might be part of subpopulation which by some mechanisms was extremely resistant to treatment or the compound could not enter to these cells and subsequently 2 Gy irradiation could not enhance the cytotoxicity of the prepared compound. The IC₅₀ of prepared compound (n=5) was 85.5 \pm 0.28 μ M. This value with the combination of 2 Gy ionizing irradiation (n=5) was 51.81 \pm 0.42 μ M. The outcome of our investigation demonstrated that the prepared compound has showed the radiosensitizer effect at the concentration 50 μ M without significant cytotoxicity effect on HT29 cell line.

DISCUSSION

Radiotherapy is an effective intervention in cancer treatment in order to enhance mortality rate in tumor cells. The cancer cells proliferate and grow very rapidly in comparison to the normal cells. The majority of solid tumors are accompanying by abnormal vascular blood supply. When the increased rate of tumor growth cannot be sustained by tumor angiogenesis, it can lead to limit oxygen supply to the tumor cells that they are distant from the blood vessels (Vaupel, 2004). This condition is known as hypoxia; tumor hypoxia is the situation where the

tumor cells have been deprived from oxygen. Hypoxic tumor cells exhibit increased aggressiveness, metastasis and are resistant to radiation and chemotherapy (Plavetic *et al.*, 2014). To overcome this dilemma, the high dose of ionizing irradiation must be delivered to tumor cells. This modality cannot be feasible in clinical practice, because the normal tissues around the tumor cells are well perfused vascularized and oxygenated. Therefore, the normal cells are prone to affect by cytotoxicity effects of ionizing irradiation. This problem can be minimized by development of selective radiosensitizer agents which would be able to increase the cytotoxicity of ionizing irradiation to tumor cells without increasing the cytotoxicity to normal cells. Therefore, a variety range of compounds are examined as radiosensitizer in preclinical stage (Khalaj *et al.*, 2006) and (Sahlberg *et al.*, 2012). It is necessary to do researching on identification of molecular targets responsible for radioresistant of tumor cells in order to develop an appropriate radiosensitizer agent. Ionization irradiation is clinically administered either by an external source such as gamma irradiation or high energy photons created by linear accelerator directed toward the tumor cells (Pollom *et al.*, 2015) or an internal source, radioactive decay from the affected area by cancer cells (Villard *et al.*, 2012). The following mechanisms have been proposed for the interaction of ionizing irradiation with biological matter. These mechanisms are the photoelectric effect, the Compton effect and the pair production effect. In addition to the above mentioned effects, the photodisintegration has the pivotal role to damage the biomolecules directly. The Compton effect is widely considered as the mode of interaction most relevant for the range of energies used in clinical ionizing radiation therapy. The observed biological effect results from photons creating multiple ionizations by ejection of electrons from the target biomolecule by the Compton effect. The extent of biological responses in the target cells after exposure to ionization radiation is largely due to oxygen with the subsequent production of free radicals. Therefore, different kinds of reactive oxygen species (ROS) are created due to ionizing irradiation. These free radicals are responsible to break chemical bonds present in target cell structures and biomolecules, basically cellular DNA. DNA is by the most critical target for the biological effects of ionization radiation. Cell death is associated with the extent of DNA damage (Nunez *et al.*, 1996). Therefore, a free radical is any atom or molecule that has one or more unpaired electrons and for this reason is highly reactive, seeking to acquire electrons from other substances. Free radical scavenger molecules can inhibit or quench free radical reactions and delay or inhibit cellular damage (Nimse and Pal, 2015) and (Young and Woodside, 2001). Glutathione (GSH) is a non-protein free thiol present in high concentrations in living organisms. It is a crucial role in a number of biochemical reactions. GSH has potential the antioxidant, radical scavenging effects by its electron donating ability (Ahmadpoor *et al.*, 2009), (Pastore *et al.*, 2003) and (Pereira *et al.*, 2013). GSH is a tripeptide (γ glutamyl cysteinyl glycine) found in most tissues. Therefore, GSH can neutralize free radicals, particularly ROS like the superoxide, hydroperoxy and hydroxyl radicals are electron deficit to accept electrons. According to the literature, GSH could be readily reacted to α,β unsaturated ketone compounds (Monks and Lau, 1988). The nucleophilic addition of GSH to electron deficient carbon-carbon double bonds

occurs mainly with a α,β unsaturated double bonds. The α, β unsaturated systems undergo Michael addition reaction with GSH to produce corresponding GSH adduct. Hence, 2,6-bis-(2,6dichlorobenzylidene) cyclohexanone belongs to α, β unsaturated ketone derivatives. Therefore, it could be preferentially reacted with GSH nucleophile and the concentration of GSH reduced in the cells. For this reason, the cells were rendered more susceptible to the cytotoxicity effects of ROS that created by ionizing irradiation. The results from this investigation demonstrated that the response of HT29 cells to cytotoxicity effects of 2 Gy irradiation was dependent on the concentrations of prepared compound. When the dose of compound was low (1 μ M), it could not show any cytotoxic effect alone or with the combination of 2 Gy irradiation. The cytotoxicity could be observed, when the dose of compound was increased to 50 μ M. The dose of compound alone was not sufficient to induce significant mortality of HT29 cells but it caused to sensitize the cells to cytotoxic effects of ionizing irradiation. The combination of 2 Gy ionizing irradiation with 50 μ M of the compound could be demonstrated the synergistic effect on the radioresistant HT29 cells. When the dose of compound was enhanced above 50 μ M, the substance could react with the other components of the cell due to probably direct reaction the carbonyl group with the nucleophile groups such as amine, were present in the cells. The selectivity of reaction to thiol group might be lost and the dose of substance was sufficient to kill the cells. Therefore, the genotoxicity side effects of compound would be appeared by the reaction with amine or hydroxyl groups which are found in nucleic acid.

CONCLUSION

The 2,6-bis-(2,6 dichlorobenzylidene) cyclohexanone as α, β unsaturated ketone has potential to react with thiol groups which are not found in nucleic acid. It could sensitize the HT29 cells to the cytotoxic effects of ionizing irradiation. If the results of our study prove to be true by in-vivo investigations, it could be confirmed this theory that the pretreatment with low dose of selective thiol alkylator agents, may be optimized the balance between local tumor control and damage effect to normal tissue in modern radiotherapy.

Abbreviations

Gy: Gray, IC: Inhibitory Concentration, MTT: 3-(4, 5 dimethyl thiazol-2 yl)-2,5 diphenyl tetrazolium bromide, ROS: Reactive Oxygen Species, μ M: Micro Molar

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