Synthesis, in vitro evaluation of cytotoxicity and radiosensitizer activity of novel 2-(2, 4-dinitrobenzylidene) cyclohexanone derivatives against the radioresistant HT29 cell line under aerobic condition

Original Article

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ABSTRACT

Introduction: Bifunctional radiosensitizer agents in which nitroaromatic moieties are attached through a linker to antineoplastic moieties have demonstrated higher cytotoxicity and radiosensitizer effects than the corresponding counterparts. This study was conducted to investigate the cytotoxicity and radiosensitizer activities of 2, 4-dinitrobenzene as a radiosensitizer moiety which connected to α, β unsaturated aryl ketones against the radioresistant human HT29 colon cancer cells.

Methods: A series of bifunctional radiosensitizer derivatives that are composed of electron-affinic 2, 4-dinitrophenyl moiety and thiol reactive unsaturated conjugated ketones were prepared. The designed compounds were synthesized by the reaction of the corresponding 2, 4-dinitrobenzaldehyde, cyclohexanone and different aryl aldehydes. The cytotoxicity and radiosensitizer activity of the tested compounds were examined against HT29 colon cancer cells under aerobic condition. The IC_{50} value of the tested compounds and percent of survival cells were analyzed by the MTT assay. The clonogenic assay was used to assess the cell viability following treatment with the tested compounds with or without the combination of radiation.

Results: This approach demonstrated that the tested compounds at the concentrations utilized have little or no cytotoxicity towards the radioresistant HT29 cell line but have great cytotoxicity and radiosensitizer activity when combined with irradiation.

Conclusion: The novel bifunctional unsaturated conjugated aryl ketones which are thiol alkylators found to exhibit radiosensitivity activity. Consequently, these new developed compounds should be evaluated further to assess their potential efficacy with radiotherapy to combat malignancies in a pre-clinical animal model.

Key words: Glutathione; HT29 cell line; Radiosensitizer; Thiol alkylator; Unsaturated conjugated ketone

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INTRODUCTION

Radiotherapy has a pivotal role in the treatment of a variety of malignancies in clinical practice. The results of radiotherapy are related on the sensitivity of tumor cells in comparison to the normal cells to ionizing irradiation. Considering the remarkable progress made in the treatment of cancer through radiotherapy in recent years, the patients with solid tumors who are used radiotherapy to treat cancer, they will potentially suffer from relapsing disorder due to the residual cancer cells [1]. The cancerous cells usually grow very rapidly in contrast to their vascular blood supply particularly in the middle of solid tumor. This factor can lead to a hypoxic region and the cells present in these areas are resistant to radiotherapy and chemotherapy [2, 3]. Higher doses of ionizing irradiation must be delivered to these cells in order to overcome this dilemma. This effort is practically not feasible, because the normal tissues surrounding the tumor cells are well perfused and vascularized. Therefore, the normal tissues in comparison to the tumor cells remain oxygenated and are prone to ionizing irradiation damage [4-6]. Radiosensitizer compounds are used in order to enhance the radiation susceptibility of tumor cells versus normal cells in the absence of significant drug-induced cytotoxicity [7-9]. Therefore, different antineoplastic agents have been examined for their radiosensitizer effects in preclinical and clinical phases [10-12]. The use of hyperbaric oxygen was the most primitive effort to overcome the resistance of tumor cells to ionizing irradiation [13]. A more promising strategy is the combination of a radiosensitizer agent and conventional dose of ionizing irradiation that exclusively sensitize tumor cells versus normal cells without inducing considerable cytotoxicity to the normal tissues. Adams et al., reported that compounds with potential electron affinic capability could sensitize hypoxic cells to an oxygen dose [14]. They concluded that there is a close relationship between electron affinity and potency of a compound as a radiosensitizer. A variety different of nitroaromatic and nitroheterocyclic agents have been examined as electron affinic compounds. These compounds increase the sensitivity of the cancer cells to irradiation by creation radical anion species [15]. The new class of radiosensitizer compounds were prepared by the attachment of nitroaromatic compounds to the cytotoxic moieties through linker. The combination of these compounds with ionizing radiation were more potent than the components toward the tumor cells [16, 17]. These compounds were known as bifunctional radiosensitizer agents. According to the literature, it has been demonstrated that the paramount radiosensitizer effect could be observed when a 2, 4-dinitrophenyl moiety was attached to 5-fluorouracil or hydroxyurea through a three- carbon chain on the HT29 cell line [18, 19]. Different unsaturated ketone derivatives were prepared as antineoplastic agents possessing thiol alkylating properties. These compounds have a preferentially or exclusively affinity for thiols and not amino or hydroxyl groups which are found in nucleic acids [20, 21]. Hence, these compounds have little or no capacity to interact with nucleic acids, thereby preventing genotoxicity. The HT29 colon cancer cells were resistant to ionization radiation and dose of radiation could induce cytotoxicity on other cell lines did not demonstrate any damage against the HT29 cells [22-24]. Therefore, the HT29 cells are a suitable model for investigating of radiosensitizer effect of the synthesized compounds in preclinical phase. The main aim of the study is to evaluate the cytotoxic and aerobic radiosensitizer effects of the 2, 4-dinitrophenyl moiety to act as radiosensitizer which was tethered to the different benzylidene cyclohexanones for evaluation against human HT29 colon cancer cells (Figure 1).

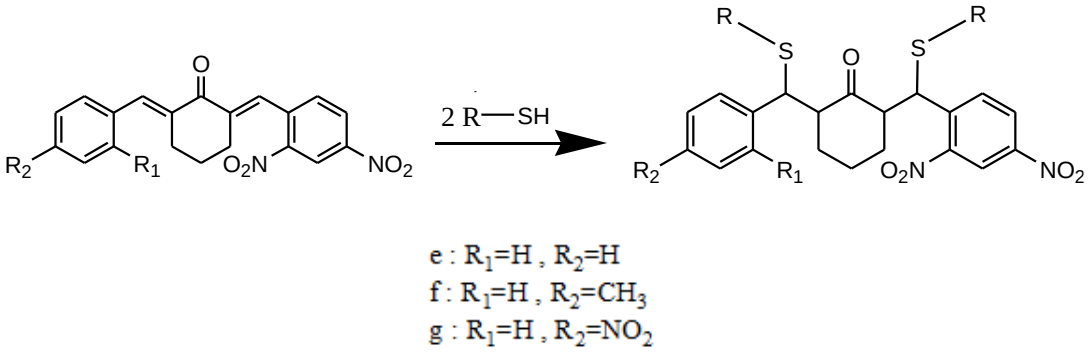

Fig 1. Graphical abstract scheme.

METHODS

All chemical and solvents were purchased from Merck and Sigma-Aldrich companies. The chemical and solvents were of the highest purity and analytical grade and used without further purification. Thin layer chromatography was carried out using silica gel (Kieselgel 60,230-400 mesh, Merck) to check the progress of reactions. Melting points were measured on an Electerothermal IA 9200 model and are uncorrected. ^{1} H NMR spectra were recorded on 500 MHz Bruker, using D2O or CDCl3 as solvents. Chemical shifts (δ) are reported in ppm relative to TMS as the internal standard. Mass spectra were obtained on a Varian instrument. Infrared spectra were recorded on a Bruker, Tensor 27 (Germany). All cell culture experiments were carried out on the HT29 cell line, originally derived from a human colorectal carcinoma and were provided by the Pasteur Institute of Iran. HT29 cells were grown as an attached monolayer in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (50 U/ml) and streptomycin (50µg/ml). Cells were grown in tissue culture flasks and kept in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Chemistry

The schematic representations of designed synthesized compounds are depicted in Figure 2.

Chemistry preparation of compounds a and b

The compounds (a) and (b) were synthesized according to the protocol that it was reported to the literature [25]. Briefly, the mixture of cyclohexanone (0.2 mol), benzaldehyde or para methyl benzaldehyde (0.1mol) were added to 100 ml distilled water. Then NaOH (0.1 mol) in 2 ml water was added the mixture by during 15 min. The mixture was refluxed for 6 to 8 h. The reaction progress was check by TLC. After heating, the reaction mixture was stirred at room temperature for 12 h. The cooled reaction mixture was extracted three times with chloroform, and the organic extracts dried with $MgSO_4$. The solvent was evaporated under reduced pressure and residue obtained. The residue was distilled under reduced pressure for 6 h. The distillate solidified on standing at room temperature for 24 h. Finally, the desired compounds were recrystallized from methanol. 2-benzylidene cyclohexanone mp=50-52°C (mp $_{Ref}$=50-52°C) in 54% yield.^{1}HNMR (D_2O) δ: 1.71(m,2H), 1.93(m,2H), 2.54(t,2H), 2.81(t,2H),7.39(t,1H, Ar-H), 7.42(t,2H, Ar-H), 7.49(d,2H, Ar-H), 7.59(s,1H, =CH). IR (KBr, cm^{-1}): 1673 (C=O), 1594(C=C).

2-(4-methyl benzylidene) cyclohexanone: mp=72-74°C (mp $_{Ref}$=71-72°C) in 45% yield. ^{1}HNMR (D_2O) δ: 1.78(m,2H), 1.92(m,2H), 2.39(s,3H), 2.57(t,2H), 2.82(t,2H),7.21(d,2H, Ar-H),7.38(d,2H, Ar-H), 7.71(s,1H, =CHs). IR (KBr, cm^{-1}): 1673(C=O), 1584(C=C).

Chemistry preparation of compounds c and d

These compounds were prepared by literature procedure [26] as follow: A solution of NaOH (0.015 mol) in 5 ml of distilled water was added over a period of 15 min to a mixture of 4-nitrobenzaldehyde (0.041 mol) or 2, 4-dinitrobenzaldehyde (0.041 mol) and cyclohexanone (0.06 mol) in water (50 ml). The mixture was stirred at room temperature 6h for compound d and 24 h for compound c, after which time the precipitate was collected and triturated with diethyl ether (200 ml) for 30 min at room temperature. The solid was collected by filtration and dried. The residue was dissolved in the mixture of 100 ml ethanol and 2 ml of HCl 37% w/v and refluxed at 40 °C. The reaction progress was carefully monitored by TLC. After heating, the cooled mixture was evaporated under reduced pressure. The residue was triturated with distilled water for 20 to 30 min. The solid was collected and dried under reduced pressure to produce the compound c or d. 2-(4-nitrobenzylidene cyclohexanone): mp=118-120°C (mp $_{Ref}$ =119-120°C) in 64% yield. ^{1}HNMR (D_2O) δ: 1.8(m, 2H), 1.96(m, 2H), 2.57(t, 2H), 2.82(t, 2H), 7.45(s, 1H, =CH), 7.53(d, 2H, Ar-H), 8.24(d, 2H, Ar-H). IR (KBr, cm^{-1}): 1675(C= O), 1506 and 1333(NO_2). 2- (2, 4-dinitrobenzaldehyde) cyclohexanone: mp=86-88 °C in 45% yield. ^{1}H NMR (D_2O) δ: 1.75(m, 2H), 1.87(m, 2H), 2.47(t, 2H), 2.76(t, 2H), 7.27(s, 1H, =CH), 7.58(d, 1H, Ar-H), 8.47(d, 1H, Ar-H), 8.96(s, 1H, Ar-H). ^{13}C NMR δ: 23.8, 26.5, 27.8, 42.5, 54.6, 66.8, 76.5, 77.5, 120.5, 127.1, 141,146,200.6.

MS (m/z, %): 277.1 (M^{+}, 57), 231.1(32), 203.1(15), 185.12(25), 164.1(58), 115.1(41), 95.1(53), 77.1(36), 55.2(90). IR (KBr, cm^{-1}): 1675(C= O), 1506 and 1333(NO_2).

Chemistry preparation of compounds e, f and g

A solution of compound of a, b or c (0.002 mol) and 2,4-dinitrobenzaldehyde (0.002 mol) in 10 ml of diethyl ether and 0.5 ml of methanol was stirred at room temperature for 10 min. Dried hydrogen chloride was passed into solution for 30 min at room temperature. The reaction was continued 2h at room temperature for compound g and 6 h for compound e or f. The solid was collected by filtration and triturated in diethyl ether for 30 min at room temperature. Finally, the desired compound was collected by filtration and dried under reduced pressure. 2-benzylidene-6-(2,4-dinitrobenzylidene)-cyclohexanone: mp= 161- 163°C in 65% yield.

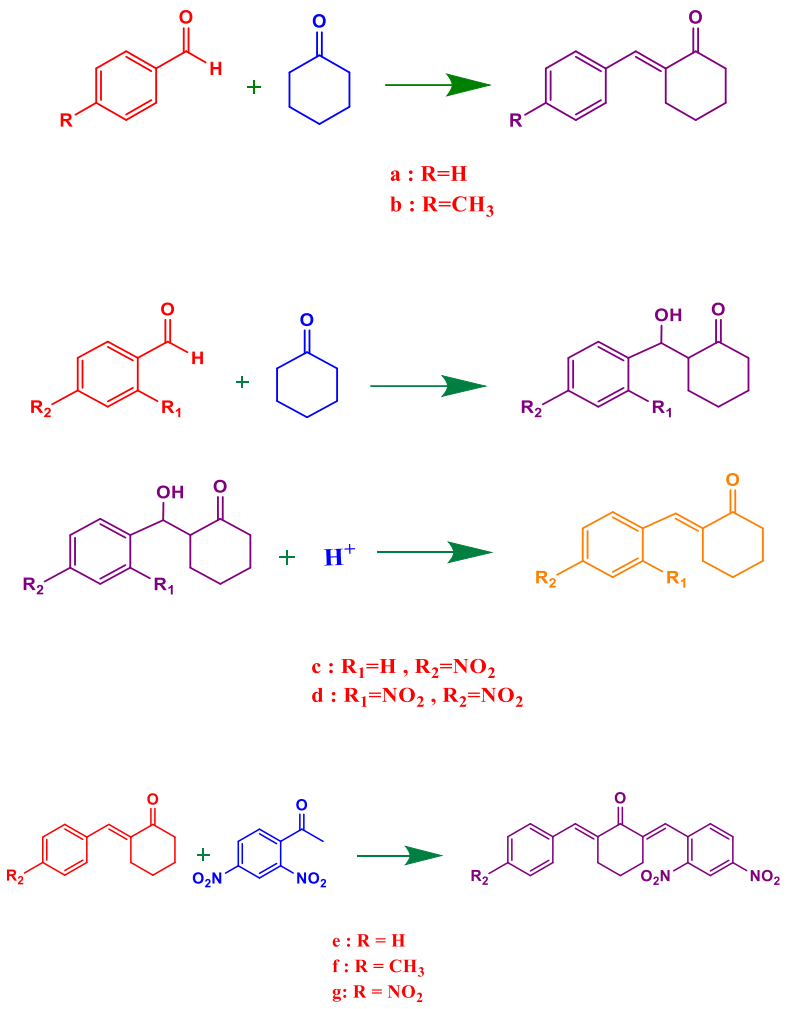

Fig 2. The scheme of synthesized unsaturated conjugated ketone derivatives.

^{1}H NMR ($CDCl_3$) δ: 1.82(m, 2H), 2.64(t, 2H), 2.96(t, 2H), 7.39(t, 1H, Ar-H), 7.41(t, 2H, Ar-H), 7.49(d, 2H, Ar-H), 7.61(s,1H, = CH), 7.88(d,1H Ar-H), 7.95(s, 1H, = CH), 8.5(d, 1H, Ar-H), 9.01(s, 1H, Ar-H). ^{13}C NMR ($CDCl_3$) δ: 22.8, 28.1, 28.5, 47.7, 120.5, 127.1, 128.5, 129.1, 130.3, 130.4, 130.5, 130.6, 132.7, 135.1, 135.5, 138.5, 139.1, 188.7.

MS (m/z, %): 363.1 (M^+, 47), 347.2(78), 330.2(32), 318.2(51), 301(51), 289.1(15), 272.1(35). IR (KBr, cm^{-1}): 1693 (C= O), 1354 and 1531(NO_2).

2-(2, 4-dinitrobenzylidene)-6-(4- methyl benzylidene)-cyclohexanone: mp= 169.5-171°C, in 64% yield. ^{1}H NMR ($CDCl_3$) δ: 1.79(m, 2H), 2.39(s, 3H), 2.60(t,2H), 2.95(t,2H), 7.25(d, 2H, Ar-H), 7.40(d, 2H, Ar-H), 7.61(d, 1H, Ar-H), 7.83(s, 1H, = CH), 7.92(s, 1H, = CH), 8.49 (d,1H, Ar-H), 8.97(s,1H, Ar-H). ^{13}C NMR ($CDCl_3$) δ: 13.5, 13.7, 21.4, 22.8, 23.4, 28.1, 28.6, 33.6, 85.5, 120.5, 127.1, 129.3, 130.1, 130.7, 132.2, 132.7, 139.3, 164.6, 196.6. MS (m/z, %): 377.2(M^+, 74), 361.2(94), 315.2(30), 270.1(14), 213.1(38), 171.1(42), 115.1(68), 77.1(28). IR (KBr, cm^{-1}):1663(C= O), 1343 and 1529 (NO_2).

2-(2, 4-dinitrobenzylidene)-6-(4-nitrobenzylidene) cyclohexanone: mp= 160-162°C in 65% yield. ^{1}H NMR ($CDCl_3$) δ: 1.54(m,2H), 2.63(t,2H), 2.93(t, 2H), 7.39 (d, 2H, Ar-H), 7.61(d,2H, Ar-H), 7.83(s, 1H, = CH), 7.94(s, 1H, = CH), 8.28 (d, 1H, Ar-H), 8.51(d, 1H, Ar-H), 8.98 (s, 1H, Ar-H). ^{13}C NMR ($CDCl_3$) δ: 22.5, 27.9, 28.4, 109.6, 117.2, 120.6, 123.7, 127.2, 130.9, 131.4, 132.6, 134.9, 135.8, 138.1, 139.3, 142, 147.5, 188.3. MS (m/z, %): 408.7(M^+,38), 363.2(65), 317.2(41), 239.1(48), 200.1(91), 152.1(46), 115.1 (87), 77.1(47). IR (KBr, cm^{-1}):1662 (C= O), 1344 and 1521(NO_2).

Compounds treatment

The stock solution of each synthesized compounds was prepared in the culture media with minimum amount of Dimethyl sulfoxide (DMSO) (0.1- 0.5%). The solutions were stored at -20°C and kept away from light. The desired concentrations of each tested

compounds were obtained by dilution of its stock solution with the culture media on experiment day. The final concentration of DMSO was the same in control and test compounds.

Cell culture

The cell culture was undertaken on the basis of procedure that reported to the literature [18, 19, 23, 24]. Briefly, the HT29 cell line was seeded with the density of 10^5 cells in T25 flasks and cultures in DMEM culture media and enriched with 10% FBS, 100U/ml penicillin and 100 µg/ml. The flasks were incubated at 37 °C in a humid incubator in 5% CO_2, 95% air. The cells were grown in single layer in each flask. When the cells reached to 90% confluency, the growing cells were detached using trypsin 0.05% and EDTA 0.05%. Then, the 10^4 cells in 100 µl culture media were seeded in each well of 96-well plates and incubated for 24 h in order to adhere to the surface of plates. The 96-well plates were randomly divided into two groups. One group of 96-well plates was considered for treatment of the tested compounds and control cells. Other group was treated with the combination of 2 Gy radiation and the synthesized compounds and control cells. The control cells were tested like other cells except they were not exposed to the synthesized compounds. Ionizing irradiation was undertaken 2 h post drug treatment in all studies. When the cells were covered the surface of each well of 96-well plates, the supernatant solution decanted and different doses of each compound in 100 µl of culture media added to each well of 96-well plates. The plates were incubated at 37°C, 5% CO_2 and 95% air for overnight. Then the plates were removed from the incubator and the supernatant solution in each well decanted. The 50 µl of 3-(4, 5 dimethyl thiazol-2 yl) 2, 5 diphenyl tetrazolium bromide (MTT) at a concentration of 5 mg/ml was added to each well. The plates were gently shaken for 10 min and incubated at 37°C, 5% CO_2 and 95% air for 1 h. The supernatant solution of each well was removed after incubation and 125 µl of DMSO added to each well of 96-well plates and shaken for 10 min in order to solubilize the produced formazon. The absorbance of each well of 96-well plates was recorded using an ELIZA reader (Bio-Tek instrument, Inc, USA) at wavelength 570 nm with DMSO as a blank with reference reading at 690 nm. All experiments were repeated three times and percent of cell survival calculated by dividing the absorbance of treated cells to control cells multiple 100. The survival curve was plotted for each synthesized compound. These curves were obtained by plotting the percentage of survival cells versus the concentrations of the prepared compounds in the presence or absence of 2 Gy ionizing radiation. The corresponding IC_{50} value of tested compounds was calculated [27]. The cell viability of the examined cells with or without the combination of 2 Gy radiation was evaluated by clonogenic analysis under aerobic condition. The clonogenic assay was undertaken as follow: the culture media of the cell cultures after exposing to the tested compounds were separated in order to remove the tested compounds and washed with phosphate buffer solution (PBS). The cells with density of 5×10^3 were seeded in 6-well plates and incubated at 37°C for 14 days. Then the plates were removed from the incubator and the cells fixed in methanol and finally the colonies were stained with crystal violet and those colonies containing50 cells or more than 50 cells were scored and recorded. The number of colonies of treated and untreated with 2 Gy radiation with the tested compounds were investigated. The overall clonogenic curves were derived from the percentage of surviving cell versus the different doses of the prepared compounds.

Radiosensitizer enhancement ratio (RER)

The radiosensitizer effect of each prepared compound was evaluated by measurement of the cell growth inhibition using clonogenic analysis. The results were stated as radiosensitizer enhancement ratio (RER). These values were obtained by dividing the percentage of cells killed in the presence and absence of ionizing irradiation for each dose of the tested compounds. Each experiment was repeated three times independently. The data are shown in Table 1.

Ionizing irradiation

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

Statistical analysis

Statistical investigation was performed using SPSS 11.0 for window (SPSS Inc, Chicago IL, USA) and descriptive statistics are shown as mean± standard deviation. Independent samples t-test was used to assess the differences between irradiated and non-irradiated cells treated with the test compounds. P value smaller than 0.05 was considered statistical significant.

RESULTS

The MTT assay was used in order to determine the cytotoxicity of the synthesized compounds against the radioresistant HT29 cell line under aerobic condition. The results obtained from this assay indicate the IC_{50} values of the tested compounds as well as the concentration of the tested compounds to demonstrate the radiosensitizer effect toward these cells. The reduction of tetrazolium salts is widely used as a reliable method to evaluate the proliferation of cells.

Table 1: Radiosensitizer enhancement ratio (RER) of different doses of tested compounds against HT29 cell line under aerobic condition. Each experiment was repeated three times independently and the standard deviations expressed for each point.

Compound	Concentration(μM)	RER
a	0.01	1.002±0.04
	0.1	1.04±0.03
	1	1.93±0.07
	10	1.41±0.04
	50	1.15±0.11
	100	1.08±0.17
b	0.01	1.001±0.02
	0.1	1.5±0.07
	1	1.47±0.12
	10	1.29±0.9
	50	1.19±0.17
	100	1.10±0.15
c	0.01	1.04±0.02
	0.1	1.05±0.07
	1	2.92±0.24
	10	2.34±0.14
	50	1.14±0.17
	100	1.11±0.19
d	0.01	1.81±0.07
	0.1	1.071±0.04
	1	1.91±0.08
	10	2.11±0.21
	50	1.33±0.17
	100	1.13±0.13
e	0.01	1.25±0.05
	0.1	1.73±0.19
	1	3.79±0.67
	10	1.5±0.18
	50	1.17±0.69
	100	1.08±0.14
f	0.01	1.26±0.01
	0.1	1.72±0.04
	1	2.44±0.13
	10	1.89±0.11
	50	1.17±0.18
	100	1.03±0.28
g	0.01	2.6±0.13
	0.1	3.92±0.16
	1	3.8±0.13
	10	1.5±0.15
	50	1.12±0.12
	100	1.01±0.09

MTT is reduced by live and metabolically active cells. The reductive reaction is carried out by the action of dehydrogenase enzymes which present in the mitochondria of cells and generate intracellular purple formazon. The produced formazon can be readily solubilized and quantified by spectrophotometric assay. When metabolic events lead to apoptosis or necrosis, the reduction of cell viability happens. The linear relationship between cell number and signal produced must be established, thus allowing an accurate quantification changes in the rate of cell proliferation by MTT assay. Therefore, the sensitivity of MTT assay to cell number was performed. The result of this preliminary investigation indicated that there was a direct relationship between the absorption intensity and the cell number in this approach. The susceptibility of HT29 cell line to different doses of radiation was evaluated. The result revealed this matter that no cytotoxicity has been observed when 2 Gy ionizing irradiation applied. Therefore, 2 Gy ionizing irradiation was used for investigation of radiosensitizer effect of tested compounds in all studies. Cell cultures were exposed to a range of concentrations 0.01, 0.1, 0.5, 1, 10, 50 and 100 μM of each compound which were lower than their corresponding IC_{50} values. As it was stated in Figure 3, all synthesized compounds demonstrated cytotoxicity effect on HT29 cell line at the concentrations up to 100 μM. The IC_{50} value for compound (a) was 82.3± 3.1 μM without irradiation (Table 2).

Table 2: The IC_{50} and p values for tested compounds determined by MTT assay on HT29 cell line. The data have been obtained from three independent tests and the standard deviations given for each point.

Product	IC_{50} (μM) without irradiation	IC_{50} (μM) with irradiation
a	82.3±3.1	80.16±0.69
b	68.7±0.75	66.9±1.49
c†	31.15±1.75	26.87±2.45
d††	41.8±1.5	28.3±0.33
e††	17.97±0.27	10.42±1.65
f†	14.68±2.5	9.3±0.97
g††	12.14±3.7	3.37±0.54

(Mean± SD), (P < 0.05 = †, P < 0.01 =† †)

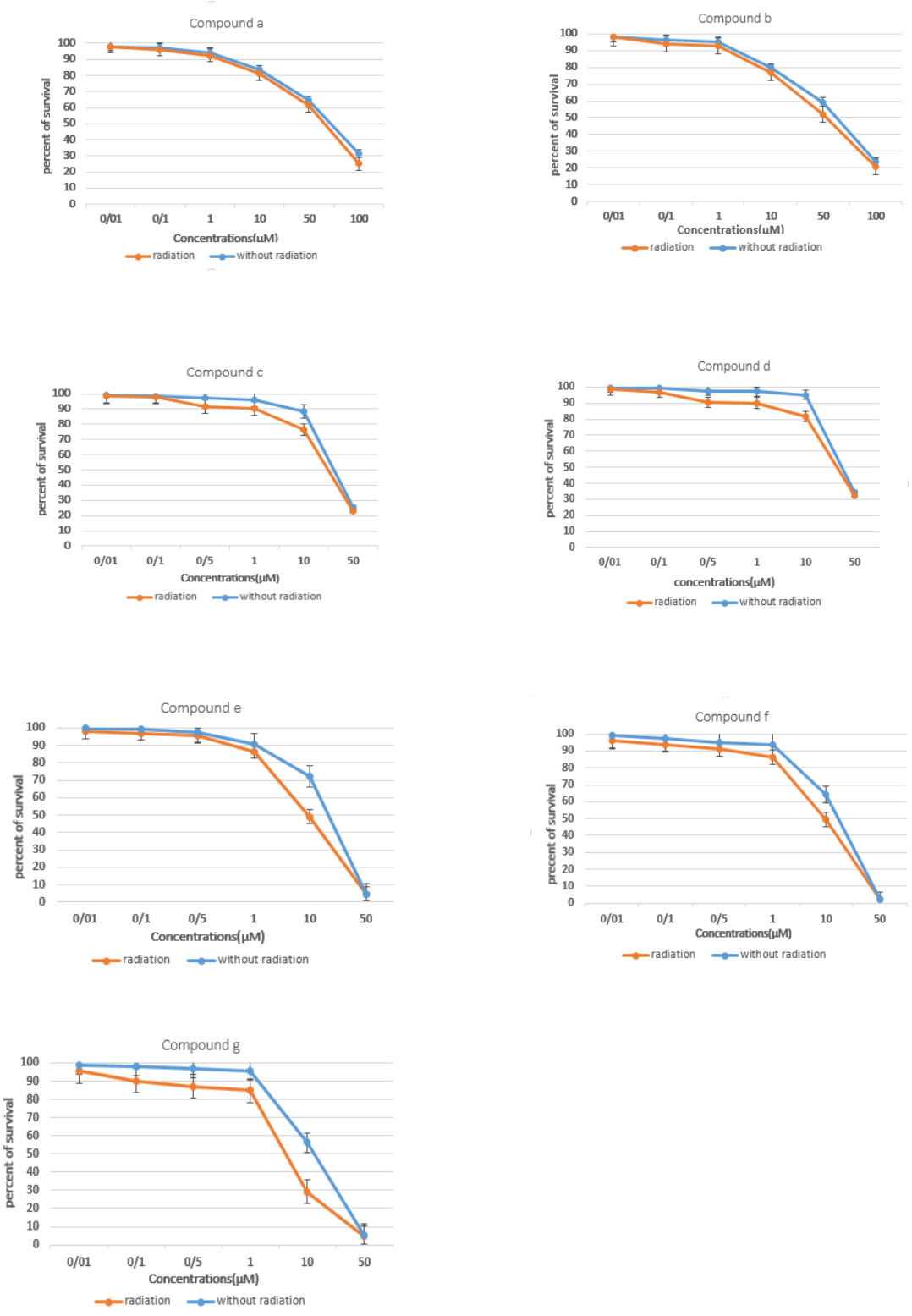

Fig 3. The percent of survival of HT29 cells have been obtained after exposure to the different concentrations of test compounds alone or with the combination of 2 Gy ionizing irradiation.

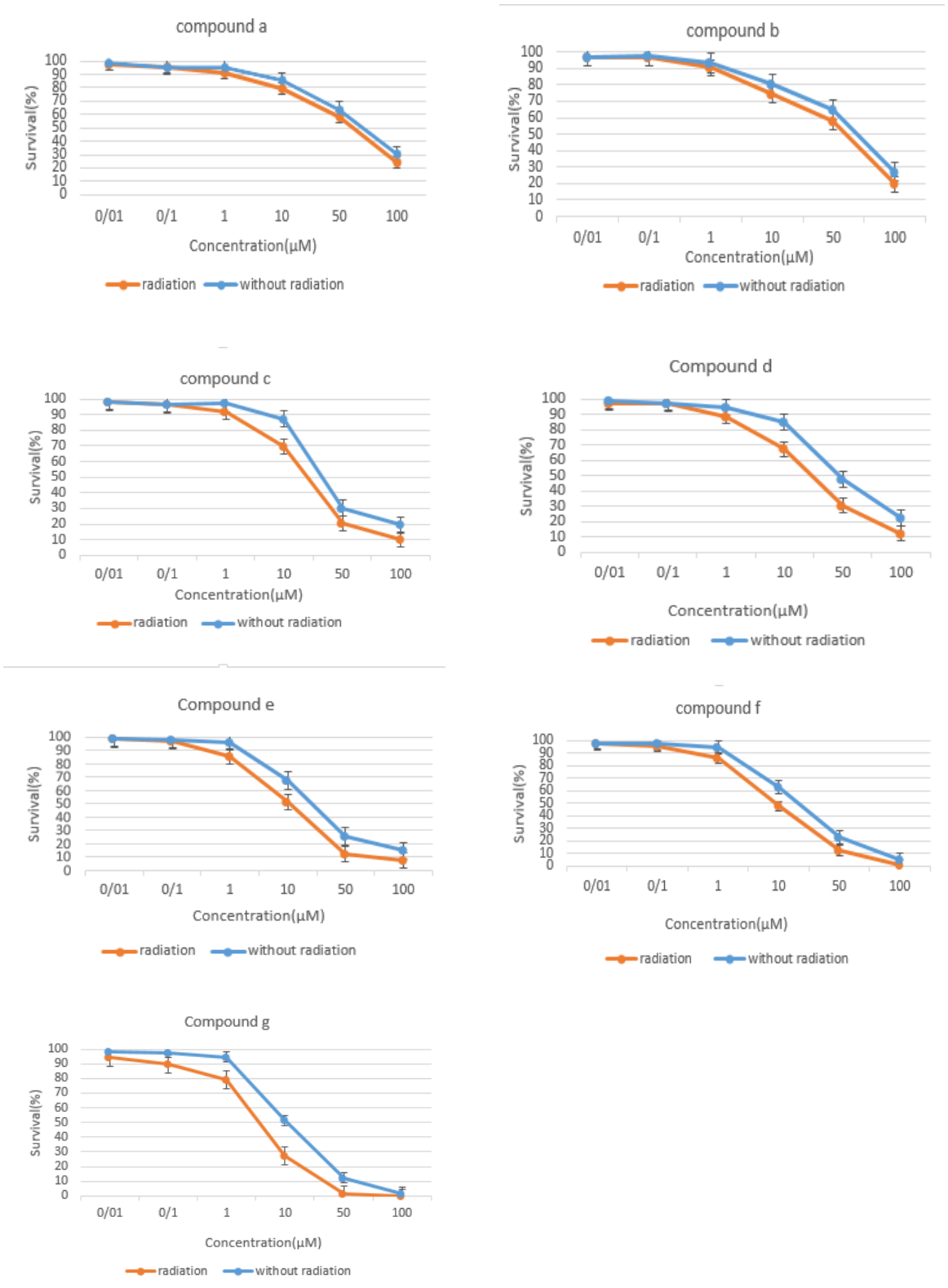

Fig 4. Clonogenic survival HT29 cells with or without the combination of 2 Gy ionizing irradiation after exposure to the different doses of tested compounds. The data were obtained from three independent tests.

This value was reduced to 80.16± 0.69 µM when 2Gy irradiation was used. It revealed that the radiosensitizer effect was not observed with the combination of compound (a) and 2Gy radiation. The IC_{50} value was 68.7± 0.75 µM for compound b. It indicated that compound b was more potent than compound a. Compound b was more lipophilic than compound (a) for the substitution of para hydrogen of benzene ring by methyl group. Hence, the compound b could enter to the cells more readily than compound a. As it is shown in Figure 3, the radiosensitization could not be shown by compound b. The IC_{50} value was 31.15± 1.75 µM for compound c. It indicated that this compound was more potent than compounds (a) and b. This matter was related to the substitution para hydrogen of benzene ring by nitro group. The nitro is electron attractive group and thereby, the electron density of benzene ring could be effectively reduced by resonance effect which the α, β unsaturated ketone was rendered more susceptible than compounds a and b toward nucleophilic addition of thiol group to produce thiol adduct. Therefore, the presence of electron releasing group like methyl could increase electron density of benzene ring and finally the α, β unsaturated ketone group was rendered less reactive than to Michael addition reaction by thiol group. As it was shown in Figure 3, compound c shows radiosensitization effect on the HT29 cell line. Compound d was prepared by the introduction another nitro group at the ortho position of benzene ring. The IC_{50} for compound d was 41.8± 1.5 µM when was used alone. This value was reduced to 28.3± 0.33 µM when the cells were affected by the combination of compound d and 2 Gy ionizing irradiation. Compound d was less potent than compound c without the combination of 2 Gy irradiation. It might be related to steric hindrance for the presence of nitro group at the ortho position. For this reason, the benzene ring is not co-planar with the α, β unsaturated ketone group in comparison compound c. Therefore, the electron attractive effect of nitro groups on benzene ring could not be effectively felt by α, β unsaturated ketone group thereby, compound d was less reactive than versus compound c toward Michael addition reaction. But the radiosensitizer effect of compound d was greater than compound c. It indicated that the more reactive oxygen species (ROS) were created by reduction of nitro group due to exposure of ionizing irradiation. Compounds e, f and g were synthesized by attachment of effector moiety a, b and c to 2, 4 dinitrobenzene as radiosensitizer portion due to cyclohexanone linker respectively. The IC_{50} values of these compounds were 17.97± 0.27, 14.68± 2.5 and 12.14± 3.7 µM when they were applied alone on HT29 cell line. Therefore, these compounds were more cytotoxic than the above-mentioned compounds. It indicated that the further nucleophilic addition site was created by the presence of another unsaturated conjugated ketone group in these molecules. The IC_{50} values of compounds e, f and g were 10.42± 1.65, 9.3± 0.97 and 3.37± 0.54 µM respectively when these compounds were used with the combination of 2 Gy ionizing irradiation on the HT29 cell line. These compounds successfully demonstrate radiosensitization effect on the HT29 cell line. The radiosensitizer effect has been observed at the doses of 0.01 to 1 µM of these compounds in which concentrations could not induce any cytotoxicity effect on the HT29 cell line. The cytotoxicity of compounds e, f and g has been significantly enhanced when the dose of them increased from 1to 50 µM. The combination of these compounds and ionizing irradiation could not increase further cytotoxicity. The following assumptions might be suggested for the resistance of remaining cells to treatment. First the tested compounds could not enter to these cells and consequently the cytotoxicity could not be enhanced by 2 Gy radiation. Second these cells belonged to a subpopulation of cells that they were extremely resistant to treatment by some unknown mechanisms.

As it is shown in Figure 4, the clonogenic assay indicated that the viability of HT29 cells in the presence of the tested compounds with the combination of 2 Gy ionizing irradiation has been decreased. This reduction was dose dependent to the concentrations of tested compounds. The RER factor demonstrated that the radiosensitization activity was considerable for the presence of the compounds d, e, f and g under aerobic condition. As it is stated in Table 1, the maximum radiosensitizer effect was observed for compound g at the concentrations between 1 to 10 µM. This achievement was completely consistent with the result obtained from the MTT assay. The outcome of this approach showed that the bifunctional bioreductive unsaturated conjugated ketones have the radiosensitizer effect at the doses in which have little or no cytotoxic effect on the radioresistant the HT29 cell line.

DISCUSSION

Extensive efforts have been investigated in the area of cancer treatment with radiotherapy to develop synthetic radiosensitizer compounds with minimal toxicity in normal tissues. A variety of compounds have been examined for radiosensitizer effects. Although many of the screened compounds demonstrated effective radiosensitization in vitro and in vivo system, the majority of them failed to show promising outcomes in clinical trials for human application. The most important limitation is inability to discriminate between cancerous cells and normal cells. This above-mentioned factor causes to induce severe toxic effects during treatment. Therefore, any

modalities that increase the radiation therapeutic efficiency without dose modification of ionizing irradiation can be considered for clinical practice. Successful radiotherapy strongly depends on to the sensitivity of tumor cells versus normal cells. Ionizing irradiation has been demonstrated to increase the production of ROS in cells [28]. It has been suggested that exposure to the compounds which can enhance oxidative stress may be eligible to sensitize tumor cells to the cytotoxic effects of ionizing irradiation. For this reason, it is highly desirable to identify the molecular targets responsible for radioresistance of tumor cells in order to develop an appropriate radiosensitizer compound. Ionizing irradiation is clinically administered either by an external source such as gamma irradiation or high energy photons created by linear accelerator toward the tumor cells [29], or an internal source, radioactive decay from the affected region by tumor cells [30,31]. Ionizing irradiation can remove tightly bound electrons from their atomic orbitals. Ionization can cause the atom to become charged or ionized. The ionized atom can readily react with neighboring atoms and forming new chemical bonds. Biological damage happens due to chemical changes caused by ionizing irradiation at the cellular level. These effects are a consequence of the ionization of atoms of biomolecules, which might cause chemical changes and alter or eradicate its functions. Energy transmitted by ionizing irradiation can act directly causing ionization of the biological molecule or can act indirectly through the free radicals resulting from the ionization of the water molecules that surround the cells. Direct ionization is predominant with high linear energy transfer (LET) radiation like alpha heavy charge particle. Indirect ionization is predominant with low LET radiation like gamma or x-rays. Low LET radiation must first undergo interactions to generate free radicals, which can then ionize the atom of biomolecules in the cell. Protein molecules can lose the functionality of its amino groups and modify its behavior. Unsaturated Lipids may suffer from peroxidation process and carbohydrates may suffer dissociate reaction. DNA is the primary target for ionizing irradiation because it contains genes that hold information for cell functioning and reproduction that are highly critical to cell survival. Direct ionization of the DNA molecule, which may result in genetic damage. Radiation ionizes water molecules, which causes to produce free radical species in indirect action. Free radical species attack DNA molecules which this manner is more common than direct action. The four following possibilities can be considered when ionizing irradiation enters a cell. First, the radiation may pass through without creation of any damage to the cell. Second, the radiation may damage the cell, but the cell can effectively repair the damage. Third, the radiation may damage the cell and the damage is not efficiently repaired and the cell replicates itself in the damage form. Forth, if the repair of DNA is incomplete, signaling pathways leading to cell death through terminal differentiation and apoptosis. The processes of energy absorption and induced ionization, as well as biomedical events triggered by the living organism response could occur within fraction of seconds. Repair of cellular damage, particularly DNA repair, may undertake from minutes to hours after exposure to ionizing irradiation and depending on the nature of damage that is induced in the cell. Single-strand breaks are the most damage to DNA molecule that can be repaired with no long-term effects in the cell. Double-strand breaks are not readily repaired and more potential for long-term damage to the cell versus single-strand breaks. Scavenging of free radical species is the most important mechanism of radiation protection in the cell. Free radical scavenging is mediated by several enzymes present in cells which effectively reduce the oxidative stress. These enzymes such as catalase, glutathione transferase, glutathione peroxidase and superoxide dismutase can counteract the oxidative stress-induced by ionizing irradiation [32]. Glutathione (GSH) is a tripeptide (γ glutamyl, cysteinyl and glycine) found in most tissues. GSH is a non-protein free thiol present in high concentration in living organisms. It is a pivotal role in a number of biochemical reactions in the cells. GSH has potential the antioxidant and radical scavenging effects by its electron donating ability [33]. Therefore, GSH can readily neutralize free radicals, especially ROS like the superoxide, hydroperoxy and hydroxyl radicals are electron deficit to accept electrons. GSH could be reacted to α, β unsaturated ketone compounds [34, 35]. The nucleophilic addition of GSH toward to electron deficient carbon-carbon double bonds occurs mainly with the conjugated unsaturated double bonds. The α, β unsaturated ketone undergoes Michael addition reaction with GSH to produce correspond GSH adduct. Hence, any factors can effectively decrease the concentration of GSH in the cell, has the potential characteristic to sensitize the cells to the cytotoxic effect of ionizing irradiation. A number of aryl and heteroaryl nitro compounds sensitize cells to ionizing irradiation. Various alkylating agents have similar biological effects as ionizing irradiation such as inducing chromosome breaks, killing dividing cells and interacting with the genetic material in the cell. Different of conjugated ketones have been examined as antineoplastic agents. These compounds are thiol alkylator having little or no capacity to interact with amino or hydroxyl groups [36, 37] since these later groups, but not thiols are found in nucleic acids. Theoretically, enones may be devoid of the genotoxic side effects displayed by a number of antineoplastic agents [38, 39]. In the present approach, the compounds were designed to alkylate thiol constituents in order to sensitize the cells to cytotoxic

effect of ionizing irradiation. Compounds (a) and (b) were thiol alkylator and they did not have the functional group to produce ROS in the cell after exposure to ionizing irradiation. These tested compounds at different concentrations reduced the cell viability significantly, and these compounds were cytotoxic at concentrations up to 100µM and the cell viability could be decreased to less than 20% (Figure 3). Radiosensitization experiment was carried out under conditions identical to the cytotoxicity measurement by incubating cells with compounds under aerobic condition. The cytotoxicity of compounds (a) and (b) against HT 29 cell line at all concentrations were not different significantly and these tested compounds showed no radiosensitizer activity in this study. The introduction of nitro group in compounds c, d, e, f and g were potential capable to produce reactive intermediates by electron reduction of nitro group after exposure to ionizing irradiation. The presence of nitro group in these tested compounds, they were more susceptible than compounds (a) and (b) to nucleophilic addition toward α, β carbon-carbon double bond in order to produce thiol adducts. These tested compounds in addition to thiol alkylator capability, they had to generate ROS in the cells after interacting by ionizing irradiation. These compounds are bioreductive bifunctional agents. As it was stated in Figure 3, the compounds d and g were perfectly demonstrated radiosensitizer effect on the HT29 cell line. Compound d at the concentration up to 10 µM did not show any considerable cytotoxicity on the cell, but the viability of cells with the combination of 2 Gy ionizing irradiation was reduced to 80%. Cytotoxicity was not observed after compound g treatment up to 1 µM concentration but the viability was decreased to 80 % after exposure to radiation. The high cytotoxicity of compound g could be related to the presence of nitro groups in these compounds. Radiosensitizer effects of tested compounds d and g enhanced by increasing in their concentrations higher than 50 µM. Therefore, the sensitization to ionizing irradiation could be sequentially occurred by initial thiol alkylation at the olefinic carbon atom, followed by reduction of the aryl nitro groups and finally consecutive oxidative stress processes in the cells. The present investigation demonstrated that thiol alkylator agents with additional functional group to produce reactive intermediates after exposure to ionizing irradiation are toxic and have radiosensitizer activities on the radioresistant HT29 cell line. The use of these agents may be advantageous in adjuvant cancer treatment by radiotherapy.

CONCLUSION

2,4-Dinitro benzylidene cyclohexanone derivatives are new class of bioreductive radiosensitizer agents. These compounds have potential not only to react with thiol groups which are not found in nucleic acid but also to induce oxidative stress after counteracting with ionizing irradiation. If our finding is verified by in-vivo and preclinical investigations, the low dose of new developed radiosensitizer compounds can be recommended along with radiotherapy in the treatment of cancer patients in order to increase the sensitivity of cancer cells to radiation and reduce the side effects of radiotherapy in clinical practice.

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