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Synergistic Effect of some Chemotherapeutic Drugs with Gamma Radiation on the Proliferation of Myeloma Cells

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LENALIDOMIDE is an immunomodulatory drug that has antiangiogenic, and anti-inflammatory properties. It has shown an efficient anti-myeloma activity. Dexamethasone is a corticosteroid used to treat multiple myeloma (MM). This study aims to evaluate *in-vitro* and *in-vivo* the proliferation of myeloma cells after treatment with Lenalidomide, Dexamethasone, γ- irradiation and their combination. *In-vitro* studies: the treatment effect on the growth of myeloma cells with each of Lenalidomide (0.5-10 µM/ml) or Dexamethasone (10-100 nM/ml) or γ-irradiation (0.5-10 Gy), double combination of the two drugs (Lenalidomide + Dexamethasone) with different doses and triple combination of the two drugs with γ-irradiation doses (0.5-8 Gy) were carried out during 6 days. *In-vivo* studies: mice bearing ascites were treated with each of Lenalidomide, Dexamethasone or γ-irradiation, double combination of the two drugs and triple combination of the two drugs with γ-irradiation for 3 weeks. Different biochemical parameters were estimated before and after treatment to evaluate the antitumor activities such as β_2-microglobulin, cell cycle analysis (flow cytometry), and caspase enzymes.

In-vitro, treatment of myeloma cells with the triple combination (3 µM/ml Lenalidomide +40 nM/ml Dexamethasone + 3 Gy γ-irradiation) has inhibited the growth of myeloma cells within 4 days.

In-vivo, in mice bearing ascites, the triple combination inhibited the growth and decreased the viability % of tumor cells to zero% within 2 weeks. The results obtained from the study of the effective doses showed a reduction in the concentrations of β2-Microglobulins and activation of the levels of caspase 8 and caspase 9 causing a control of the cell cycle.

Treatment of myeloma cells with dual therapy (Lenalidomide-Dexamethasone as a chemotherapy with γ-irradiation as a radiotherapy) resulted in a synergistic effect for treatment of multiple myeloma. This effect increases the growth inhibition, apoptosis, and regression of tumors compared to either treatment agent alone.

Keywords: Dexamethasone, Lenalidomide, Myeloma, γ-irradiation.

Introduction

Multiple myeloma (MM) is a cancer that forms in a type of white blood cells in the immune system called plasma cells that accumulate in the bone marrow and crowd healthy blood cells out (Mitsiades et al., 2004; Siegel et al., 2015). Plasma cells fight infections by producing antibodies that recognize and attack germs, whereas myeloma cells produce irregular proteins that can cause complications such as skeletal destruction (bones fractures), renal failure, anemia and hyper viscosity (Kyle & Rajkumar, 2004; Slovak, 2011). Myeloma cells accumulate in the bone marrow and crowd out healthy white blood cells and red blood cells, resulting in fatigue and an inability to fight infections. MM is the second most common hematologic malignancy with non-Hodgkin's lymphoma (Bianchi et al., 2015).

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Lenalidomide is a synthetic immunomodulatory drug produced via modification of thalidomide's chemical structure to enhance its effectiveness and reduce its side effects. It is a thalidomide 4-amino-glutamyl analog that has emerged as a drug with activity against various hematological and solid tumors. Lenalidomide prevents the adhesion of MM cells to bone marrow stromal cells and thus can resolve cell adhesion mediated drug resistance, inhibit the activity of the vascular endothelial growth factor and the essential fibroblast growth factor secreted by MM cells and stromal cells of the bone marrow(Lu et al., 2008). Lenalidomide inhibits the bioactivity and secretion of cytokines in MM cells and bone marrow stromal cells such as interleukin and tumor necrosis factor which increases MM cell development, survival, drug resistance, migration and adhesion molecules expression(Corral et al., 1999; Muller et al., 1999). Lenalidomide, also, interacts against MM by increasing the activation of cytotoxic T-cells and natural killer cells (Quach et al., 2010) associated with interleukin-2 and interferon secretion (Davies et al., 2001; LeBlanc et al., 2004; Hayashi et al., 2005).

Dexamethasone is a corticosteroid which prevents inflammatory compounds from discharging into the bloodstream. Dexamethasone is used to treat MM and inflammatory diseases, such as, skin conditions, ulcerative colitis, arthritis, lupus, eczema or allergic, pulmonary disorders (Hideshima et al., 2003; Wang et al., 2008).

Radiotherapy (RT) is ionizing radiation therapy that is normally provided by a linear accelerator, which is commonly used as part of cancer treatment to control or suppress malignant cells. RT may be curative in a number of cancer types, if it is concentrated in one region of the body. This may also be used as a treatment to prevent tumor recurrence after a primary malignant tumor has been removed. Radiation therapy is usually used in treatment of the cancerous tumor due to its ability to inhibit cell growth. Ionizing radiation works by damaging the DNA of cancerous tissue leading to cellular death (Miralbell et al., 2004).

The present study aims at researching the *in-vitro* and *in-vivo* treatment of myeloma cells using Lenalidomide or Dexamethasone drugs and their combination with gamma irradiation. Several biochemical parameters were assessed before and after treatment such as, caspases and β2-microglobulin, and cell cycle analysis to evaluate the antitumor activity.

Materials:

Drugs

Lenalidomide and Dexamethasone were provided by Celgene Corporation (San Diego, CA), Sigma.

Chemicals

Chemicals were obtained from Sigma-Aldrich Co.: RPMI-1640 media, Foetal bovine serum, Antibiotic anti-mycotic mixture (10,000 Unit penicillin, 10 mg streptomycin and 25 µg amphotericin B/1 ml 0.9% Na Cl), L-glutamine (200 mM solution), Pristine (2, 6, 10, 14, Tetramethyl penta-decane).

Myeloma cell line

The myeloma cell line SP2/OR was supplied through the IAEA project EGY/2/007, 1998.

Mice

Female Balb/C mice with body weight ranged from 20-25 gm and age of 12 weeks old were used as experimental animals. During the experimental phase, Balb/C mice were kept under constant environmental and nutritional conditions and kept at room temperature (22±2 ^{o}C) with a 12-hour on / off light schedule. Through the experiments standard, food and water were provided to mice.

Methods

Irradiation Source and Technique

The source used for irradiation was 137Cs gamma cell 40, installed at the Egyptian Atomic Energy Authority (EAEA), National Center for Radiation Research and Technology (NCRRT), Cairo, Egypt. During the irradiation cycle, cell culture flasks were put in the gamma cell irradiation chamber at variable doses of 0.5, 1.5, 3, 6, 8 and 10 Gy and mice were irradiated at a dose of 6 Gy at a dose rate 0.175 Gy/min.

Myeloma cell growth

SP2/OR myeloma cells were grown in the RPMI-1640 medium, supplemented with 10% bovine fetal serum, 1% antibiotic anti-mycotic and 1% L-glutamine, respectively. Cells have been plated in 25 cm^2 culture flasks and incubated at 37 °C in humidified air with 5% CO_2 before

cell growth reaches log phase with a viability of more than 95%. Before adjusting the cell count to 2.5 – 5 cells, the cells were counted daily with a viability check. The total number and viability of myeloma cells were determined by adding 100 µl of cell suspension with 100 µl of trypan blue dye and counted by hemocytometer.

In-vitro study

In this study the myeloma cells (1×10^6 /ml) were treated with Lenalidomide, Dexamethasone and γ-irradiation as a follows:

Treatment of myeloma cells with Lenalidomide

Lenalidomide was applied to myeloma cells at variable doses (0.5, 1.5, 3, 5, 7 and 10 µM/ml) and incubated at 37 °C for 6 days with 5% CO_2. Each dose the total count and viability percentage of myeloma cells were calculated daily.

Treatment of myeloma cells with Dexamethasone

Dexamethasone was added to myeloma cells at variable doses (10, 20, 40, 60, 80 and 100 nM/ml) and incubated at 37 °C for 6 days with 5% CO_2. Each dose the total count and viability of myeloma cells were calculated daily.

Treatment of myeloma cells with γ-irradiation

Myeloma cells were treated with γ-irradiation at variable doses of 0.5, 1.5, 3, 6, 8 and 10 Gy. Myeloma cells were incubated at 37 °C for 6 days with 5% CO_2 after exposure to γ-irradiation. The total number and viability of myeloma cells for each dose was calculated daily.

Treatment of myeloma cells with combination of Lenalidomide and Dexamethasone

Myeloma cells were treated with variable doses of the combination between Lenalidomide and Dexamethasone as follows: Lenalidomide from 0.5, 1.5, 3, 5, 7 and 10 µM/ml and Dexamethasone from 10, 20, 40, 60, 80 and 100 nM/ml respectively. The treated myeloma cells were incubated for 6 days at 37 °C with 5% CO_2. The total count and viability of myeloma cells were calculated daily.

Treatment of myeloma cells with triple combination of Lenalidomide, Dexamethasone and γ-irradiation

Myeloma cells were treated with a triple combination using variable combination doses as follows: Lenalidomide from 0.5, 1.5, 3, 5, 7 and 10 µM/ml, Dexamethasone from 10, 20, 40, 60, 80 and 100 nM/ml respectively, and each of the different combined doses of drugs being exposed to various doses (0.5, 1.5, 3, 6 and 8 Gy) of γ-irradiation source. The treated myeloma cells were incubated for 6 days at 37 °C with 5% CO_2. The total count and viability of myeloma cells were calculated every day.

In-vivo study

The studies were performed in compliance with the guidelines issued by the Egyptian Atomic Energy Authority and approved by the Animal Ethics Committee, Labeled Compounds Department. The research protocol was authorized by the Faculty of Pharmacy's Research Ethics Committee, Cairo University (REC-FOPCU), Egypt. The effect of the treatment with Lenalidomide, Dexamethasone and γ-irradiation in mice bearing ascites were carried out as a follows:

Induction of ascites tumor in mice

Myeloma cells(SP2/OR) were used in the induction of ascites tumor in mice as follows: about 0.5ml pristine (2,6,10,14-tertramethyl-decanoicnacid) was injected in each mouse intraperitoneally. After 20 days of injection with pristine the myeloma cells 250 µl (1×10^6 /ml) were injected to each mouse. The injection was repeated every week for about 4 weeks. Allmice were maintained until the tumor development appeared (Cristina et al., 2008) and each mouse was weighed daily to evaluate and follow up the ascites formation. Treatment started after the development of ascites tumor.

Effect of Lenalidomide, Dexamethasone and γ-irradiation in mice bearing ascites

Mice bearing ascites were divided into seven groups, each group comprises five ascites bearing mice. The 1st group was normal control group without ascites, the 2nd group was ascites bearing mice without treatment. Mice groups bearing ascites were treated as follows: the 3rd group was treated with Lenalidomide (5 mg / kg) twice a week for 3 weeks, the 4th group was treated with Dexamethasone (1.25 mg /kg) twice a week for 3 weeks and the 5th group was treated with γ-irradiation once at exposure dose (6 Gy), the 6th group was treated with combination of two drugs (5 mg /kg Lenalidomide +1.25 mg/kg Dexamethasone)twice a week for 3 weeks and the 7th group was treated with triple combination of mixture of two drugs (5 mg/kg Lenalidomide

+1.25 mg /kg Dexamethasone) twice a week for 3 weeks in combination with γ-irradiation exposure (6 Gy) for one time at the beginning of the first week. Ascetic fluid was withdrawn using 5 cm plastic syringe and myeloma cells were counted before and after treatment. For all groups, the total count and viability percentage of the myeloma cells were determined for each mouse.

Biological and biochemical analysis

Some biological and biochemical parameters were determined for the untreated and treated myeloma cells with either Lenalidomide or Dexamethasone, mixture of the two drugs, γ-irradiation, or triple combination between two drugs mixture with γ-irradiation. The parameters include β2-microglobulin, caspases and cell cycle analysis which were performed before and after treatment of myeloma cells (1×10^6) to evaluate the antitumor activity.

β2 -Microglobulins assay

β2 -Microglobulins were determined using Abbott AxSYM system assay.

Caspase assay

Both caspase 8 and 9 enzymatic activities were measured using caspase colorimetric assay kits (Cristina et al., 2008; Brentnall et al., 2013).

Cell cycle analysis

After 48 h from the different treatment and without treatment (as control), cell cycle analysis was carried out according to the method of Murata et al. (2006). All myeloma cells were analyzed using flow cytometer (Becton-Dickinson, CA, USA) (Mitsiades et al., 2002; Kyle & Rajkumar, 2008).

Results

In-vitro study

Myeloma cells SP2/OR (1×10^6/ml) were treated with Lenalidomide at various concentrations (0.5-10 μM/ml), the total number of myeloma cells and their viability were determined daily for 6 days. Lenalidomide caused both time and dose-dependent decrease in myeloma cell survival, as shown in Fig. 1. The results obtained showed that the viability % of myeloma cells treated with Lenalidomide (0.5 - 10 μM/ml) decreased from 99% to 57% at 0.5 μM/ml, from 99% to 49% at 1.5 μM/ml, from 99% to 43% at 3 μM/ml, from 99% to 29% at 5 μM/ml, from 99% to 19% at 7 μM/ml and from 99% to 11% at 10 μM/ml, at6 days. These results showed that variable doses of lenalidomide had an effect on myeloma cells' survival and that the viability percentage of myeloma cells decreased significantly by increasing the Lenalidomide concentration and the optimum dose was up to 5 μM/ml.

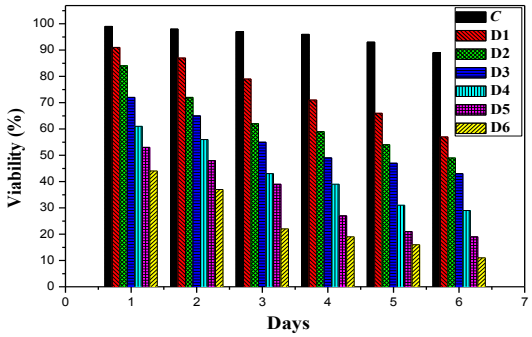

Fig. 1. Effect of variable doses of Lenalidomide on the viability % of myeloma cells (C= Control, D1= 0.5 μM/ml, D2= 1.5 μM/ml, D3= 3 μM/ml, D4= 5 μM/ml, D5= μM/ml and D6= 10μM/ml)

Myeloma cells SP2/OR (1×10^6 /ml) were treated with Dexamethasone during six days at different doses (10– 100 nM/ml), the total count and viability were calculated daily. Dexamethasone induced both time and dose-dependent reduction in myeloma cell survival, as shown in Fig. 2. The viability percentage decreased from 99% to 58 % at 10 nM/ml, from 99% to 28% at 20 nM/ml, from 99% to 21% at 40 nM/ml, from 99% to 19% at 60 nM/ml, from 99% to 11% at 80 nM/ml, and from 99% to 8% at 100 nM/ml, at 6 days. These results indicated that Dexamethasone at variable concentrations had an effect on the survival of myeloma cells and that the viability percentage of myeloma cells is significantly reduced by increasing the Dexamethasone concentration. It can be concluded that the optimal dose is 80 nM/ml. The optimum dose 80 nM/ml of Dexamethasone was selected because this dose inhibits the growth and viability % of myeloma cells during 6 days lifespan and induced apoptotic cell death pathways similar to higher dose 100 nM/ml of Dexamethasone. Therefore, must be selected the lowest dose to avoid side effects.

The effect of variable doses of γ-irradiation (0.5, 1.5, 3, 6, 8 and 10 Gy) on myeloma cell growth during the lifetime of 6 days compared to the control group is illustrated by Fig 3. The data obtained indicated that for each dose (0.5, 1.5, 3

and 6 Gy), the viability percentage of myeloma cells decreased from 99% to 48%, 34%, 21% and 15%, respectively at 6th day. The viability percentage also decreased from 99% to zero% on the 4thday with 8 Gy and from 99% to zero% on the 2nd day with 10 Gy. It can be concluded from the above results that the effective dose of γ-irradiation for the treatment of myeloma cells was 8 Gy where at this dose the viability % of myeloma cells reached 0% at the 4th day of exposure to γ-irradiation.

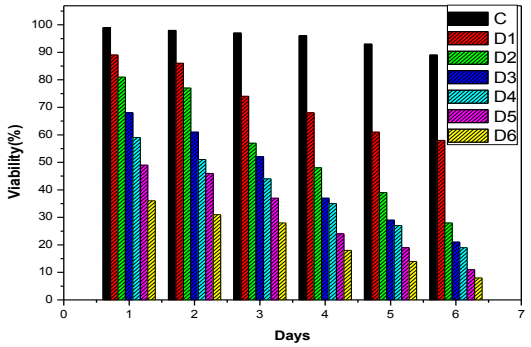

Fig. 2. Effect of variable doses of Dexamethasone on the viability % of myeloma cells (C= Control, D1=10 nM/ml, D2=20 nM/ml, D3= 40 nM/ml, D4= 60 nM/ml, D5= 80 nM/ml and D6= 100 nM/ml)

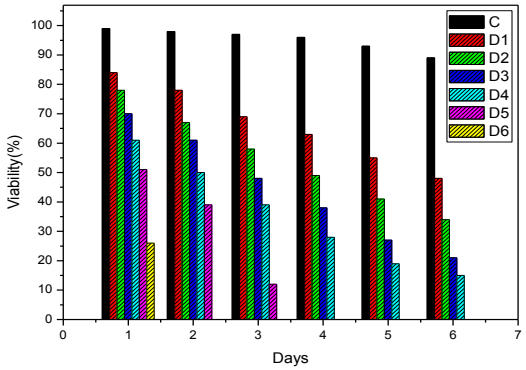

Fig. 3. Effect of variable doses of γ-irradiation (Gray) on the viability % of myeloma cells (C= Control, Gy= Gray, D1= 0.5 Gy, D2= 1.5 Gy, D3= 3 Gy, D4= 6 Gy, D5= 8 Gy and D6= 10 Gy)

Myeloma cells SP2/OR (1×10^6/ ml) were treated with a combination of the two drugs at various concentrations of Lenalidomide(0.5, 1.5, 3, 5, 7 and 10 μM/ml) and Dexamethasone (10, 20, 40, 60, 80 and 100 nM/ml), respectively, through 6 days. The total count and percentage of viability were calculated daily for the treated myeloma cells. The mixture of the two drugs induced both time and dose-dependent decrease in myeloma cell survival as shown in Fig. 4. The viability percentage decreased from 99% to 62% in the 1st dose (0.5 μM/ml + 10 nM/ml), from 99% to 49% in the 2nd dose (1.5 μM/ml + 20 nM/ml), from 99% to 0% in 3rd dose (3 μM/ml + 40 nM/ml) in 6 days of treatment, from 99% to 0% in 4th dose (5 μM/ml + 60 nM/ml) in 3 days. In addition, the viability percentage for the 5th dose (7 μM/ml + 80 nM/ml) and the 6th dose (10 μM/ml + 100 nM/ml) was 0% in one day. The reported results indicated that low doses of the drug mixture Lenalidomide and Dexamethasone had a slight effect on myeloma cell survival while the percentage of cell viability was significantly inhibited with the 4th dose (5 μM/ml Lenalidomide + 60 nM/ml Dexamethasone) after 3 days. From the obtained results, it can be concluded that by increasing the concentration of combination of the two drugs the viability % of myeloma cells decreased, this is because the combined drugs increased inhibition the growth of cells and the optimum dose of the combined two drugs was the 4th dose (5 μM/ml+ 60 nM/ml) after 3 days.

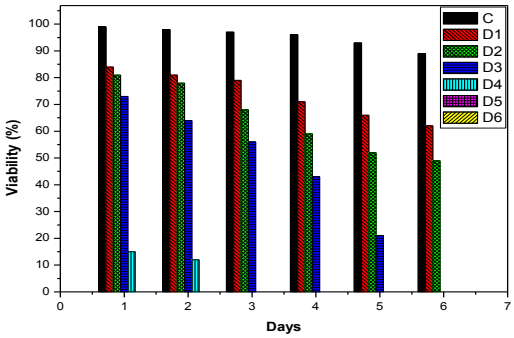

Fig. 4 . Effect of variable doses of combined treatment of Lenalidomide and Dexamethasone on the viability % of myeloma cells (C= Control, D1= 1st dose, D2= 2nd dose, D3= 3rd dose, D4= 4th dose, D5= 5th dose and D6= 6th dose)

Myeloma cells were treated with a combination of the two drugs (Lenalidomide 0.5, 1.5, 3, 5, 7 and 10 μM/ml) and (Dexamethasone 10, 20, 40, 60, 80 and 100 nM/ml), respectively with exposure to γ-irradiation at doses: 0.5, 1.5, 3, 6 and 8 Gy for each dose of combined drugs. The total number and viability percentage of treated myeloma cells were estimated for 6 days. Combined drugs with γ-irradiation triggered both time and dose-dependent reduction in myeloma cell survival, as shown in Figs. 5-9.

As shown in Fig. 5, the effect of 0.5 Gy of γ-irradiation with different doses of combination

of two drugs revealed that the viability percentage decreased from 99% to 38% at the 1st dose (0.5 µM/ml +10 nM/ml), from 99% to 46% at 2nd dose (1.5 µM/ml+ 20 nM/ml), from 99% to 0% at the 3rd dose (3 µM/ml + 40 nM/ml) during 6 days, from 99% to 0% at the 4th dose (5 µM/ml + 60 nM/ml) at 2 days. Also, the viability percentage reached 0% for each of the 5th dose (7 µM/ml + 80 nM/ml) and 6th dose (10 µM/ml + 100 nM/ml) after one day. The reported results indicated that low doses of combination of two drugs with 0.5 Gy of γ-irradiation had a slight effect on the survival of myeloma cells while the percentage of cell viability was significantly inhibited at the 4th dose (5 µM/ml Lenalidomide + 60 nM/ml Dexamethasone) at 2 days.

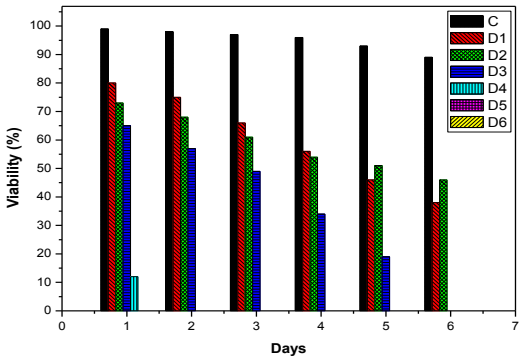

Fig. 5. Effect of variable doses of the combined treatment Lenalidomide + Dexamethasone and 0.5 Gy of γ-irradiation on the viability % of myeloma cells (C= control, D1= 1st dose, D2= 2nd dose, D3= 3rd dose, D4= 4th dose, D5= 5th dose and D6= 6th dose)

The results, as shown in Fig. 6, the effect of 1.5 Gy of γ-irradiation with various doses of the combined tow drugs indicates a decrease in the cell viability percentage from 99% to 29% at the 1st dose (0.5 µM/ml +10 nM/ml), from 99% to 25% at the 2nd dose (1.5 µM/ml+ 20 nM/ml) during 6 days, from 99% to 0% at the 3rd dose (3 µM/ml + 40 nM/ml) at 5 days. However, the viability percentage reached 0% for each of the 4th dose (5 µM/ml + 60 nM/ml), the 5th dose (7 µM/ml + 80 nM/ml) and the 6th dose (10 µM/ml + 100 nM/ml) at the first day. According to the data obtained, the successful dose reduces the development of myeloma cells and decreases the viability percentage was 1.5 Gy of γ-irradiation with combined drugs at the 3rd dose (3 µM/ml Lenalidomide+ 40 nM/ml Dexamethasone) at 5 days.

The data in Fig. 7 indicate the effect of 3 Gy of γ-irradiation with combined two drugs at different doses, inducing a decrease in the percentage of cell viability from 99 % to 17 % at the 1st dose (0.5 µM/ml +10 nM/ml), from 99 % to 11 % at the 2nd dose (1.5 µM/ml+ 20 nM/ml) during 6 days, from 99 % to 0 % at the 3rd dose (3 µM/ml + 40 nM/ml) at 4 days. However, the viability percentage reached to 0 % for each of the 4th dose (5 µM/ml + 60 nM/ml), the 5th dose (7 µM/ml + 80 nM/ml) and the 6th dose (10 µM/ml + 100 nM/ml) at the first day. The data indicated that the effective dose that decreases the viability percentage of myeloma cells was 3 Gy of γ-irradiation with the3rd dose of the combined drugs (3 µM/ml Lenalidomide+ 40 nM/ml Dexamethasone) at 4 days.

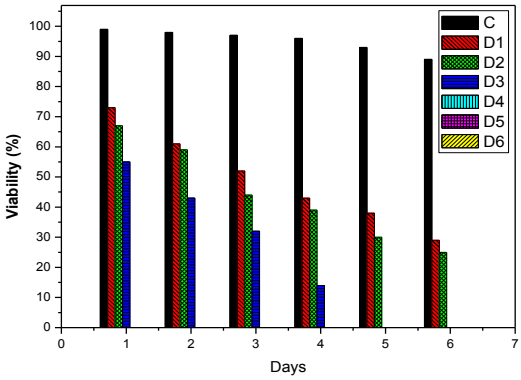

Fig. 6. Effect of variable doses of the combined treatmentLenalidomide + Dexamethasone and 1.5Gy of γ-irradiation on the viability % of myeloma cells (C= control, D1= 1st dose, D2= 2nd dose, D3= 3rd dose, D4= 4th dose, D5= 5th dose and D6= 6th dose)

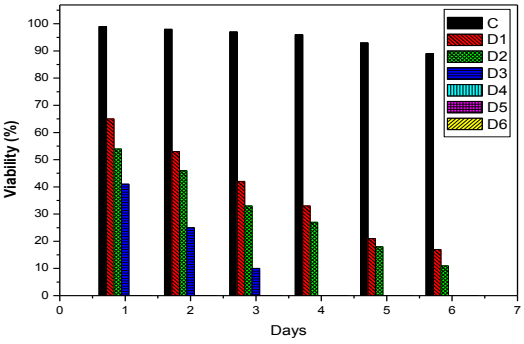

Fig. 7. Effect of variable doses of the combined treatment with Lenalidomide + Dexamethasone + 3 Gy of γ-irradiation on the viability % of myeloma cells (C= control, D1= 1st dose, D2= 2nd dose, D3= 3rd dose, D4= 4th dose, D5= 5th dose and D6= 6th dose)

The results in Fig.8 showed that the effect of 6 Gy of γ-irradiation with different concentrations of the combined tow drugs indicate a decrease

of the percentage of cell viability from 99% to 10% at the 1st dose (0.5 µM/ml +10 nM/ml) at 6 days, from 99% to 0% at the 2nd dose (1.5 µM/ml+ 20 nM/ml) at 6 day, from 99% to 0% at the 3rd dose (3 µM/ml + 40 nM/ml) at the 2nd day. however, the viability percentage reached 0% for each of the 4th dose (5 µM/ml + 60 nM/ml), the 5th dose (7 µM/ml + 80 nM/ml) and the 6th dose (10 µM/ml + 100 nM/ml) at the first day. The data indicated that the effective dose which decreases the viability percentage of myeloma cells was 6 Gy of γ-irradiation with the combined drugs at the 3rd dose (3 µM/ml Lenalidomide+ 40 nM/ml Dexamethasone) at the second day.

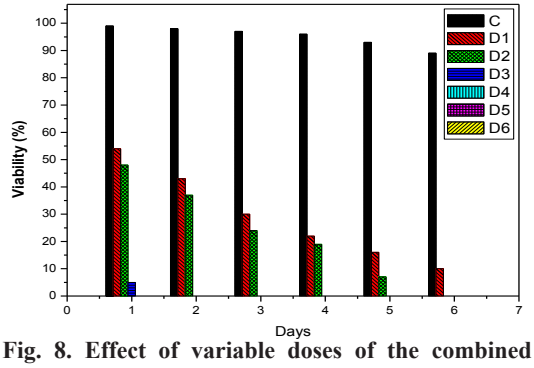

Fig. 8. Effect of variable doses of the combined treatment Lenalidomide + Dexamethasone + 6 Gy of γ-irradiation on the viability % of myeloma cells (C= control, D1= 1st dose, D2= 2nd dose, D3= 3rd dose, D4= 4th dose, D5= 5th dose and D6= 6th dose)

The results as observed in Fig. 9, showed that 8 Gy of γ-irradiation with different doses of the combined tow drugs indicate that the percentage of cell viability decreased from 99% to 0% at the 1st dose (0.5 µM/ml +10 nM/ml) at 3days. Otherwise, the viability percentage reached 0% for each of the 2nd dose (1.5 µM/ml+20 nM/ml), the 3rd dose (3 µM/ml + 40 nM/ml), the 4th dose (5 µM/ml + 60 nM/ml), the 5th dose (7 µM/ml + 80 nM/ml) and the 6th dose (10 µM/ml + 100 nM/ml) at the first day. The data indicated that the effective dose which decreases the viability percentage of myeloma cells was 8 Gy of γ-irradiation with the combined drugs at the 1st dose (0.5 µM/ml +10 nM/ml), at the third day.

From the above obtained results, it can be concluded that by increasing the concentration of the combined two drugs at the 3rd dose with 3 Gy of γ-irradiation, the viability percentage of myeloma was decreased because the combination of the two drugs with γ-irradiation increased inhibition the growth of cells and the optimum dose was the 3rd dose (3 µM/ml + 40 nM/ml + 3 Gy- γ-irradiation) at 4 days.

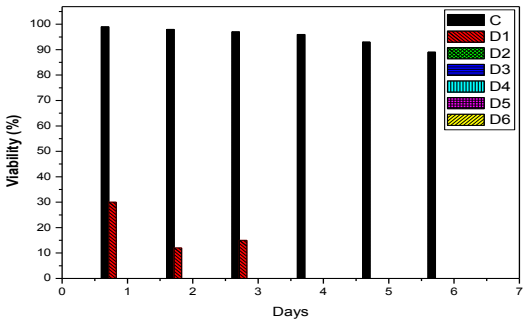

Fig. 9. Effect of variable doses of the combined treatment Lenalidomide + Dexamethasone + 8 Gy of γ-irradiation on the viability % of myeloma cells (C= control, D1= 1st dose, D2= 2nd dose, D3= 3rd dose, D4= 4th dose, D5= 5th dose and D6= 6th dose)

In-vivo treatment

The results of the groups of ascites bearing mice untreated and treated with Lenalidomide, Dexamethasone, γ-irradiation, mixture of Lenalidomide+ Dexamethasone and Lenalidomide+Dexamethasone + γ-irradiation combination, are illustrated in Table 1.

The obtained data of the animals treated with each of both of Lenalidomide, Dexamethasone alone and Lenalidomide + Dexamethasone drugs mixture showed a decreased viability percentage of tumor cells from ~ 100% to 16%, 21% at the 3rd week and 0% at the 2nd week, respectively. Also, the viability percentage of the tumor cells treated with γ-irradiation was decreased to 11% at the 3rd week while treatment with triple combination of Lenalidomide + Dexamethasone+ γ-irradiation decreased the viability percentage of the tumor cells to zero % at 2 weeks.

Determination of β2-Microglobulins

β2-microglobulins were measured for tumor cells before and after treatment. The data presented in Table 2 showed that the concentrations of β2-microglobulins for the untreated myeloma cells were 2.6± 0.4 mg/dl while after treatment with each of Lenalidomide, Dexamethasone and γ-radiation alone, Lenalidomide+ Dexamethasone drugs mixture, or triple combination of Lenalidomide + Dexamethasone+ γ-irradiation, they were decreased to 1.6±0.13, 1.8±0.2, 1.4±0.15, 1.1±0.18 and 0.6±0.1 mg/dl, respectively. These results showed that the treatment of tumor myeloma cells

reduced the concentration of β2-Microglobulins and indicated that the triple combination is more effective in myeloma cell growth inhibition.

TABLE 1.Total count and viability % of myeloma cells of untreated and treated ascites bearing mice

Time weeks	No. of injection	Parameters	Mice groups: Mean ± SD (n=5)					
			2nd Group Control without treatment)	3rd Group Treated with Lenalidomide (5 mg/kg)***	4th Group Treated with Dexamethasone (1.25mg/kg)***	5th Group Treated with γ-irradiation (6 Gray)****	6th Group Treated with Lenalidomide +Dexamethasone (5 mg/kg + 1.25 mg/kg) ***	7th Group Treated with Lenalidomide +Dexamethasone (5mg/kg+ 1.25 mg/kg) *** +γ-irradiation (6 Gray) ****
1	1	*T.C/ml	$17\times10^4\pm 0.4\times10^4$	$15\times10^4\pm 0.3\times10^4$	$13\times10^4\pm 0.4\times10^4$	$8\times10^4\pm 0.2\times10^4$	$6.5\times10^4 \pm 0.8\times10^4$	$5\times10^4\pm 1.5\times10^4$
		**V (%)	84±2.0	77±1.3	72±0.3	67±0.7	39±0.7	23±0.4
	2	T.C/ml	$35\times10^4\pm 2.4\times10^4$	$26\times10^4\pm 0.7\times10^4$	$17\times10^4\pm 0.3\times10^4$	$11\times10^4\pm 0.6\times10^4$	$4\times10^4\pm 0.4\times10^4$	$3\times10^4\pm 0.4\times10^4$
		V. (%)	88±0.7	79±1.6	60±0.7	51±0.9	10±0.7	7±0.9
2	3	T.C ml	$49\times10^4\pm 1.7\times10^4$	$31\times10^4\pm 0.7\times10^4$	$22\times10^4\pm 0.4\times10^4$	$14\times10^4\pm 0.8\times10^4$	$3\times10^4\pm 0.3\times10^4$	$2\times10^4\pm 0.3\times10^4$
		V. (%)	90±1.2	66±0.7	53±0.7	42±0.4	4±0.5	0
	4	T.C/ml	$62\times10^4\pm 4.8\times10^4$	$40\times10^4\pm 0.7\times10^4$	$37\times10^4\pm 0.5\times10^4$	$17\times10^4\pm 0.7\times10^4$	$1\times10^4\pm 0.7\times10^4$	0
		V. (%)	92±1.5	41±0.8	45±0.7	33±0.7	0	0
3	5	T.C/ml	$93\times10^4\pm 4.2\times10^4$	$49\times10^4\pm 0.3\times10^4$	$43\times10^4\pm 0.8\times10^4$	$21\times10^4\pm 0.5\times10^4$	0	0
		V. (%)	95±1.1	28±0.7	32±0.7	19±0.7	0	0
	6	T.C ml	$114\times10^4\pm 6.5\times10^4$	$51\times10^4\pm 0.7\times10^4$	$58\times10^4\pm 0.4\times10^4$	$15\times10^4\pm 0.4\times10^4$	0	0
		V. (%)	97±1.3	16±0.4	21±0.7	11±0.7	0	0

*T.C: Total count of myeloma cells.

**V: Viability of myeloma cells.

*** Drugs treatment: 2 doses/ week/ 3 weeks.

**** γ-irradiation: 1 dose for one time at the beginning of the first week.

TABLE 2.The level of β2-microglobulins(mg/dl) in Myeloma cells treated with drugs and γ-irradiation

Groups	β2-microglobulin(mg/dl)	
	Range	Mean ±SD
Control	1.9 - 3.1	2.6±0.4
Lenalidomide	1.3-1.8	1.6±0.13
Dexamethasone	1.4-2.2	1.8±0.2
Lenalidomide + Dexamethasone	0.8-1.2	1.1±0.18
γ-irradiation	0.9-1.3	1.4±0.15
Lenalidomide+ Dexamethasone+ γ-irradiation	0.4 - 0.9	0.6±0.1

Caspase activity assays

The results, in Fig. 10, demonstrated enzyme activity levels of caspase-8 and 9 in single-treated myeloma cells (Lenalidomide or Dexamethasone or γ-irradiation) or double-treatment combination (Lenalidomide + Dexamethasone), or triple-treatment combination (Lenalidomide + Dexamethasone+ γ-irradiation) compared to the control group. Lenalidomide treatment increased caspase-8 enzyme activity and Dexamethasone treatment increased caspase-9 enzyme activity. The treatment with each of γ-irradiation, double treatment combination (Lenalidomide + Dexamethasone) drugs and triple treatment combination of Lenalidomide + Dexamethasone + γ-irradiation increased the enzyme activities of both of caspase-8 and caspase-9. The obtained results showed that the triple treatment with combination of Lenalidomide + Dexamethasone + γ-irradiation was higher in increasing the levels of the enzyme activities of both caspase-8 and caspase-9 than γ-irradiation and double treatment combination (Lenalidomide + Dexamethasone) drugs.

Cell cycle analysis

The results in Table 3 showed that treatment of ascites-bearing mice of myeloma cells with Lenalidomide increased the percentage of cells in phase G0/G1 (48.76%) and decreased the percentage of phase S cells (30.27%) and G2/M phase change (19.88%), while Dexamethasone therapy increased the percentage of cells in phase G0/G1 (51.96%), the percentage of cells in the S phase and G2/M phase decreased (29.18% and 18.86%respectively). The consequence of treatment with a combination of drugs (Lenalidomide + Dexamethasone) increased the percentage of cells in phase G0/G1 (78.96%), reduced the percentage of cells in phase S (15.18%) and phase G2/M (5.86%).Treatment with γ-irradiation decreased the percentage of cells in G0/G1 phase (29.57%), decreasing the percentage of cells in S phase (58.79%) and reducing phase G2/M (12.87%) while treatment with triple combination (Lenalidomide + Dexamethasone + γ-irradiation) increased the percentage of cells in phase G0/G1 (47.28%), decreased the percentage of cells in phase S (38.98%) and phase G2/M (11.74%). The results revealed that the triple-combination treatment induced phase S and phase G2/M arrest and apoptosis induction.

Discussion

The research strategy for this work was intended to study the effect of in-vitro and in-vivo treatment of myeloma cells using each of Lenalidomide, Dexamethasone alone as a medication, as well as using a combination of the two drugs as chemotherapy. Also, the effect of γ-irradiation was studied as radiotherapy. The effect of triple combination of two drugs mixture with γ-irradiation was studied as a combination between a chemotherapeutic drugs and radiotherapeutics.

In this study, the treated myeloma cells with Lenalidomide drug, the optimum dose was 5 μM/ml during 6 days lifespan because these doses decreased the growth and percentage viability of the myeloma cells. When the myeloma cells are treated with Dexamethasone drug, the optimum dose was 80 nM/ml, this dose inhibits the growth and viability percentage of myeloma cells during 6 days lifespan. The results revealed that Lenalidomide and Dexamethasone are effective drugs in myeloma therapy and induced apoptotic cell death pathways (Frankfurt & Rosen, 2004; Greenstein et al., 2002).

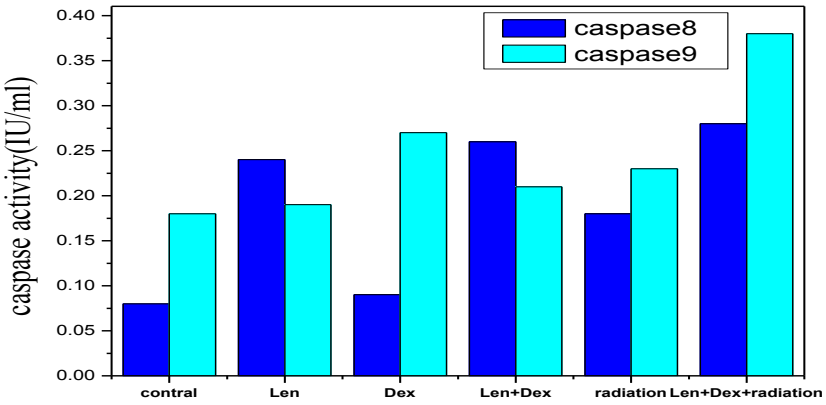

Fig. 10. Levels of caspase-8 and caspase-9 activities in myeloma cells

TABLE 3. Effects of Lenalidomide, Dexamethasone, Lenalidomide + Dexamethasone mixture, γ-irradiation, Lenalidomide + Dexamethasone + γ- irradiation combination on Cell Cycle Phase Distribution

Conditions	% of Cells in Cell Cycle Phases		
	S	G0/G1	G2/M
Control	51.52	37.54	9.93
Lenalidomide	30.27	48.76	19.88
Dexamethasone	29.18	51.96	18.86
Lenalidomideand Dexamethasone	15.18	78.96	5.86
γ-irradiation	58.79	29.57	12.87
Lenalidomide+ Dexamethasone+ γ-irradiation	38.98	47.28	11.74

G0/ Quiescence, G1/ GAP1 phase, S/ Synthetic phase, G2/ GAP2 phase, M/ mitosis phase

Treatment of the myeloma cells with a mixture of Lenalidomide+ Dexamethasone (5 μM/ml +60 nM/ml) increased the inhibition of the growth and decreased the viability percentage of myeloma cells after 3 days. Lenalidomide combined with Dexamethasone is one of the most effective treatment options for multiple myeloma. This induces a direct cytotoxicity in myeloma cells (Armoiry et al., 2008; Hideshima et al., 2000).

The treatment of the myeloma cells with γ-irradiation showed that the effective dose was 8 Gy which decreased the percentage viability of the myeloma cells to zero % after 4 days from the exposure to γ-irradiation. Radiation therapy works by damaging the DNA of cancerous cells, bysingle-strand DNA damage and double-stranded DNA damage.

Single-strand DNA damage is then passed by cell division; damage to the DNA of the cancer cells accumulates, causing them to die or grow more slowly, and this may be due to the direct effect of γ-irradiation on the DNA causing damage to chromosomal abnormalities, genetic deletions and double-stranded breaks, which increases the likelihood of myeloma cell apoptosis (Lomax et al., 2013; Harrison et al.,2002). So this study revealed that radiotherapy is a powerful treatment modality for multiple myeloma.

The data obtained revealed that the treatment of myeloma cells with the triple combinations(Lenalidomide+ Dexamethasone + γ-irradiation; 3 μM/ml + 40 nM/ml + 3 Gy, respectively) increased the inhibition of the growth of myeloma cells after 4 days. Moreover, the results indicate that the triple combination of the two drugs (Lenalidomide + Dexamethasone) with γ-irradiation have a superior activity on the inhibition of myeloma cell growth when compared to each drug alone as well as the combination of the two drugs. Accordingly, radiation therapy must be used as part of adjuvant therapy to reduce and avoid tumor recurrence, and to eliminate a primary malignant tumor so that, radiation therapy is synergistic with chemotherapy (Mill, 1975; Bosch & Frias, 1988).

In-vivo treatment of tumor cells with triple combination (Lenalidomide + Dexamethasone + γ-irradiation) is more effective inhibition of cell growth leading to zero viability percentage through 2 weeks.

β2-Microglobulins are specific tumor marker protein for multiple myeloma and normally found on the surface of the cells (Jing & Qing, 2011). They are secreted by B-cells that correlate with the myeloma cell mass. The value of β2-microglobulins was reduced to (0.6±0.1) with triple combination treatment. These results indicated that the treatment was more efficient on the inhibition of myeloma cells growth.

A family of aspartate-specific cysteine proteases is caspase which plays an important role in apoptosis and a variety of physiological and pathological processes (Chauhan et al., 2001; McComb et al., 2019). Previous results revealed that Lenalidomide treatment activates caspase-8 dependent apoptosis, while Dexamethasone triggers caspase-9 dependent apoptosis activation. The triple combination (Lenalidomide + Dexamethasone + γ-irradiation) led to more increase in the level of caspase-8 and caspase-9 enzyme activity than treatment with γ-irradiation or / double combination (Lenalidomide + Dexamethasone) drug mixture. Therefore, treatment with triple combinations triggers dual apoptotic signaling cascades.

The results showed that treatment with each Lenalidomide or Dexamethasone had a strong effect on the progression of the cell cycle by increasing the percentage of cells arrested in the phase G0/G1 and decreasing the percentage of cells arrested in the phase S and G2/M in myeloma cells compared to the control.

Treatment with Lenalidomide + Dexamethasone combination increased the number of apoptotic cells and induced to greater level of G0/G1 growth arrest. Treatment with γ-irradiation inhibited myeloma cells proliferation by inducing cell cycle arrested at S phase and led to apoptosis. Triple combination of Lenalidomide + Dexamethasone + γ-irradiationwas more effective in cell cycle progression control and apoptosis (Mitsiades et al., 2002; Hideshima et al., 2000). The results indicated that the triple combination led to arrest S phase and G2/M phase and induced apoptosis.

The triple combinations of Lenalidomide, Dexamethasone and γ-irradiation induced a greater level of growth inhibition and enhanced growth inhibition and apoptosis compared to the double combinations (Lenalidomide and Dexamethasone) or either agent alone in myeloma cell line.

Conclusion

In conclusion, the effect of dual therapy (chemotherapy-radiotherapy) for MM treatment showed that the combination of the two drugs lenalidomide and dexamethasone with γ-radiation causes tumor cells apoptosis due to the activation of caspase cascade and apoptosis may be attributed to decreasing of multiple myeloma cells proliferation by induction of S phase and G2/M phase arrest. This study introduced the synergistic effect of γ-irradiation with chemotherapy.

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