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# Effects of Radiotherapy in Combination With Irinotecan and 17-AAG on Bcl-2 and Caspase 3 Gene Expression in Colorectal Cancer Cells

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## Abstract

**Introduction:** In this study, the cytotoxic and anti-cancer effects of Irinotecan as a conventional chemotherapeutic agent compared to 17-(allyl amino)-17-demethoxygeldanamycin (17-AAG) as possible radiosensitizers in the HCT-116 cell line were investigated.

**Methods:** HCT-116 cells were treated with various concentrations of irinotecan and 17-AAG and also irradiated with a 2-Gy of X-ray radiation. Then, the cell viability was examined by a water-soluble tetrazolium-1 assay after 24 hours. For single therapies and double and triple combination cases, $IC_{50}$, $0.5 \times IC_{50}$ and $0.25 \times IC_{50}$ concentrations of each drug were selected respectively for a terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay and other tests. In treated and untreated cells, the caspase 3 and Bcl-2 gene expression ratios were evaluated by the real-time PCR method. Likewise, caspase 3 activity was detected with a colorimetric assay.

**Results:** In all combined treatments, including 17-AAG- radiation, irinotecan - radiation, irinotecan -17-AAG, and irinotecan-17-AAG-radiation, decreased cellular viability and increased TUNEL positive cells were presented versus the control group ($P<0.05$). There were increased TUNEL positive cells in the triple combination, in concentrations of $0.25 \times IC_{50}$ of each drug, in comparison with single and double agent treatments. Moreover, in triple combination, the caspase 3 mRNA level and caspase 3 activity increased versus related single treatments. Likewise, in the irinotecan-17-AAG-radiation combined treatment and the 17-AAG-radiation double treatment, the Bcl-2 gene expression level decreased in comparison with single therapies.

**Conclusion:** It can be indicated that the combination of chemo-radiotherapy versus single treatments has significant anti-cancer effects.

**Keywords:** Irinotecan; 17-AAG; Radiotherapy; HCT-116 cell; Apoptosis.

## Introduction

Colorectal cancer (CRC) is considered as one of the prevalent lethal cancers worldwide. A large number of new cases of CRC and its related mortality have been reported.[1-5]

Various treatment options, including chemotherapy in combination with novel molecular targeted drugs, led to survival benefits in choosing patients as first and second-line treatment of CRC.[6]

Developments in multimodal treatment approaches, including major oncologic disciplines, have been related to a better prognosis for patients.[7]

In this regard, the rapid progress of laser irradiation and photosensitizer systems has led to photodynamic therapy progressively becoming a novel method in the treatment of numerous tumor types.[8] Near infrared ray laser energy is one of the commonly utilized methods because of deeper tissue penetration and low absorption by human tissue. A nanomaterial combination of chemotherapy and photothermal therapy has been commonly confirmed to increase the anti-cancer effect. Consequently, it is imperative to investigate multi-functional nanoparticles (NPs) that actively combine with targeted chemotherapy and photo-thermal therapy in the treatment of colon cancer.[9]

There was no difference in the disease-free survival between the radiation therapy and trans-oral laser microsurgery treatment groups.[10]

Despite the progress in conventional treatments, radiotherapy-related side effects and local recurrence which are the problems in CRC treatment bring about a high rate of mortality.[11]

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The application of anticancer polychemotherapy or combined chemotherapy has been characterized as an effective treatment of cancers since it attains higher therapeutic efficiency than single chemotherapeutic drugs.[12]

Some main treatment strategies are chemotherapy with fluorouracil, oxaliplatin and irinotecan.[13-15] Irinotecan is a camptothecin derivative. Camptothecin derivatives are the DNA topoisomerase I enzyme inhibitors and specifically interfere with replication of DNA.[16,17] Recent study have indicated that irinotecan induces apoptosis.[17] Indeed, the use of chemotherapeutics drugs has not been completely successful in the treatment of CRC.[18]

In order to reduce the resistance to some chemotherapeutic drugs, researchers have investigated new therapeutic compounds along with conventional treatments.[15,16,19,20]

The common treatments for CRC due to resistance to cytotoxic compound may do not provide effective results. Therefore, newer therapeutic methods can alter the current treatment options.[21] (17 allyl amino)-17-demethoxygeldanamycin (17-AAG) is a heat shock protein 90 (HSP90) inhibitor, which exerts anti-tumor effects and induces apoptosis in cancer cells.[21,22] In addition, in preclinical models, it exerts anticancer activity in different cancer types.[22-24]

The combination of chemotherapy and radiation therapy in other studies increased the treatment efficiency.[22,25] Chemoradiotherapy prevents tumor proliferation. Indeed, the aim of these therapies are cancer growth inhibition.[26,27] One of the most effective methods in efficient radiation therapy is combining it with chemotherapy, to improve the cytotoxicity against cancer cells. In addition, when cancerous cells are radiation-resistant, the combination of chemotherapy with this method can increase treatment response.[22,27] Therefore, radiotherapy with a new chemotherapy drug is expected to reduce complications of chemotherapy and increase the treatment outcome.

It has been revealed that irinotecan acts as an effective radiosensitizer (in vitro). These effects are related to ATM/Chk/CDC25C/CDC2 signaling, leading to G2/M phase arrest and the apoptosis of CRC cells.[28,29] Moreover, the combinatorial treatment of conventional photon radiation and Hsp90 inhibitor displayed tumor growth inhibitory effects in comparison with radiation alone.[30] 17-AAG exhibits anticancer activity when combined with X-ray radiation. Indeed, 17-AAG improves radiation efficiency by apoptosis induction and increases the major mediators of oxidative and nitrosative stress.[22] The 17-AAG binding affinity to HSP90 protein in cancerous cells is higher than in normal cells.[31]

With the purpose of overcoming resistance in CRC therapies, the study of new treatments options is essential to recognize effective treatments including combinatorial treatments.[22,27] Consequently, in this study, the anti-cancer effects of irinotecan as the conventional chemotherapeutic agent, in comparison with 17-AAG as possible radiosensitizers, in the CRC cell line were investigated.

Radiotherapy induced damages through oxidative stress could activate the apoptosis intrinsic pathway.[32] Indeed, cancer cells death induction (after radiotherapy) is one of the main goals of this study.

Nevertheless, of the stimuli that can induce apoptosis cascade, mitochondrial permeability, through releasing the pro-apoptotic factors, will occur. This pathway is related to the Bcl-2 family proteins. Anti-apoptotic (Bcl-2) and apoptotic proteins (Bax family) are two major proteins belonging to the Bcl-2 family, which is engaged in nuclear damage and apoptosis induction.[32]

Therefore, we evaluated the Bcl-2 and caspase 3 gene expression levels as the possible mechanisms of radiotherapy effects in combination with irinotecan and 17-AAG in CRC cells.

Indeed, we examined the effects of radiation therapy in combination with a common chemotherapy drug (irinotecan) and a newer drug (17AAG) on inducing apoptosis-related genes to target their possible molecular mechanisms in HCT-116 cells.

## Materials and Methods

### *Cell Culture, Treatments and Cell Viability Assay*

HCT-116 cell line was purchased from (Pasteur Institute, Iran) and cultured in Dulbecco's Modified Eagles Medium (Biowest, France) supplemented with 10% fetal bovine serum (Biowest, France) and 1% penicillin/streptomycin (Biowest, France). Irinotecan and 17-AAG were obtained from LC Company (USA) and Sigma Aldrich (St. Louis, MO, USA) respectively.

The cells were treated in eight separate groups: (irinotecan), (17-AAG), (X-ray radiation), (17-AAG-radiation), (irinotecan-radiation), (17-AAG-irinotecan), (17-AAG-radiation- irinotecan), and the control group.

HCT-116 cells were treated with various concentrations of irinotecan (1, 2, 4 and 8 μM) and 17-AAG (10, 25, 50 and 100 nM) for 24 hours. Then cell viability was evaluated by the WST-1 method. After that, the $IC_{50}$ was measured by CompuSyn software (CombuSyn, Inc., Paramus, USA).

After treatments with irinotecan and 17-AAG, microplates were coated with 1 cm of a Plexiglass sheet (water equivalent) and irradiated with a clinical linear accelerator machine with single dose irradiation (2 Gy) at room temperature (dose rate of 200 cGy/min - field size of 10 cm × 15 cm - source-to-surface distance (SSD) = 100 cm). To ensure the output of the accelerator, the physicists of the radiotherapy unit performed calibration before each irradiation dosimetry. After 24 hours of incubation and exposure to drugs ($0.25 \times IC_{50}$ and $0.5 \times IC50$, $1 \times IC_{50}$ and $2 \times IC_{50}$ concentrations for both agents in triple and double combinations) and 2 Gy of radiation, the cell

viability was assessed. Each single drug treatments were in IC50 concentration for all tests.

In following $0.25 \times IC_{50}$ for triple and $0.5 \times IC_{50}$ for double treatments of irinotecan and 17-AAG were used in all tests including a TUNEL assay, real-time PCR and caspase 3 activity assay tests.

Cell viability was determined by the colorimetric water-soluble tetrazolium salts (WST-1) test. In brief, $1 \times 10^4$ cells were cultured in each well and treated with different concentrations of both drugs and 2-Gy X-ray radiation (as mentioned above), and the cell viability was measured using the WST-1 kit instruction (Takara, Japan).

### *Evaluation of Cell Apoptosis Rate by a TUNEL Assay*

Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay was conducted by in situ cell death detection Kit (Roche, Germany) for detection of apoptotic cells according to kit instruction,[33,34] in total number of treatment wells and control as described above. The slides were observed through a light microscope.

### *Real-time PCR*

In order to evaluate the gene expression levels of Bcl-2 and caspase 3 (after 24 hours), the treated and untreated cells were trypsinized and total RNA was isolated based on the kit's protocol (GeneAll, South Korea). The purity of the extracted RNA was assessed by calculating the optical density ratio at 260 nm to that at 280 nm.

Subsequently, cDNA was synthesized by cDNA synthesis kit (GeneAll, South Korea) and real-time PCR was carried out (in the total volume of 25 µL) by Real Q Plus 2x Master Mix Green (Ampliqon) according to the kit protocol.

Real-time PCR was performed using specific primers of β-actin, Bcl-2 and caspase 3, at 30 cycles of denaturation at 95°C, 30 seconds at annealing temperature ( at 59°C for caspase 3, 58°C for Bcl-2 and β-actin), and extension at 72°C for 30 seconds. The primer sequences are presented in Table 1. The relative mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

### *Caspase 3 Enzyme Activity Assay*

In order to detect the caspase 3 enzyme activity, a colorimetric assay kit from Abnova Company (Taiwan) was used. Briefly, after 24 hours, the treated and untreated cells were trypsinized and collected by centrifugation. Then, based on kit instructions, a cell lysis buffer was added and HCT-116 cells were kept on ice. In the next step, protein concentration was assessed according to the Bradford method. The lysis buffer was added to Proteins (50 µg) and then 50 µL 2×reaction buffer and 5 µL DEVD-*p*NA 4 mM substrate were added. After 2-hour incubation at 37°C, the plates were read at 405 nm. The caspase 3 activities were evaluated by comparing the obtained values with the level of the uninduced control.

**Table 1**. Primer Sequences Used for Real-Time PCR (Bcl-2 and β-Actin,[35] Caspase 3[36])

| Gene | Primer Sequence |
|---|---|
| Bcl-2 | Forward: 5′-CATCAGGAAGGCTAGAGTTACC-3′ |
| | Reverse: 5′-CAGACATTCGGAGACCACAC-3′ |
| Caspase 3 | Forward: 5′-TGGTTCATCCAGTCGCTTTG-3′ |
| | Reverse: 5′-CATTCTGTTGCCACCTTTCG-3′ |
| β-Actin | Forward: 5′-TGCCCATCTACGAGGGGTATG-3′ |
| | Reverse: 5′-CTCCTTAATGTCACGCACGATTTC-3′ |

### *Statistical Analyses*

The data were expressed as the mean ± standard deviation. $IC_{50}$ values were determined by CompuSyn software based on the data from a cell viability assay. Significant differences between groups were evaluated through one-way ANOVA, followed by the Tukey test, using GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, California) and SPSS software, version 10.0 (SPSS Inc, Chicago, Illinois). All tests were performed in three replicates. A $P$ value $< 0.05$ was considered as the significant level.

## Results

The growth inhibitory effects of both drugs were tested after 24 hours of exposure in the HCT-116 cell line and results were calculated as the percentage of the viable cells relative to the controls.

On the basis of the results presented in the dose-response curve (Figure 1A-B), cell viabilities decreased in the dose-dependent patterns in various concentrations of irinotecan and 17-AAG drugs. The growth inhibitory effects of 17-AAG and irinotecan are presented in Figure 1A-B.

According to the dose-response curve and CompuSyn software (CombuSyn, Inc., Paramus, USA), the IC50 amounts were 15 ± 4.04 (nM) and 1.20 ± 0.30 (µM) for 17-AAG and irinotecan respectively.

In single radiation treatment, cell viability decreased in comparison with the control group ($P < 0.05$) (Figure 2).

The growth inhibitory effect of radiation, irinotecan and 17-AAG (in $IC_{50}$ concentrations of both drugs) in HCT-116 cells after 24h were shown in Figure 2.

According to results of WST-1 assay in double combinations ( $0.5 \times IC_{50}$ concentration), there was significant difference in cell viability rate of 17-AAG-radiation and irinotecan- radiation and 17-AAG-irinotecan treatments in comparison with related single treated cases ($P < 0.05$). Similarly, in comparing double combinations at the concentration of $0.5 \times IC_{50}$ of each drug, there was significant decrease in the cell viability rate in 17-AAG-radiation versus 17-AAG-irinotecan treatment, indicating the higher cytotoxic effect of 17-AAG-radiation versus other double drug treatment. Low concentrations of drugs ($0.5 \times IC_{50}$ in double and

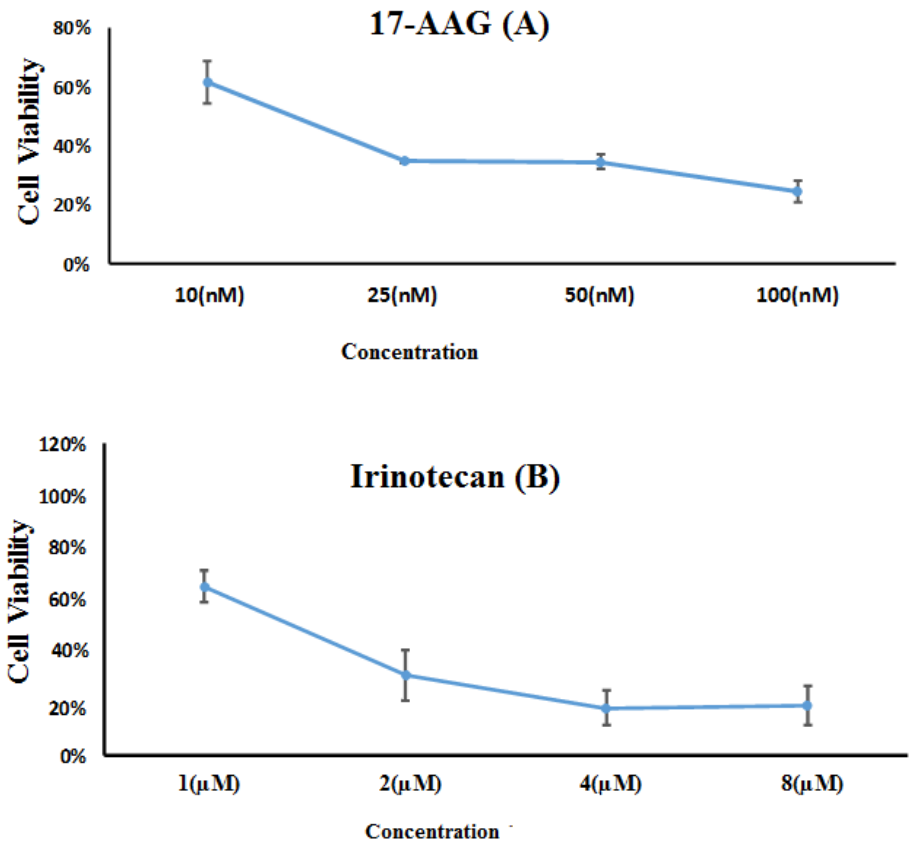


**Figure 1**. Cell Growth Inhibitory Effects (Dose-Response Curves) of 17-AAG and Irinotecan in Various Concentrations. Cells were treated with increasing concentrations of both drugs and after 24 h, cell viability assessed by WST-1 method. Data shown as mean ± standard deviation.

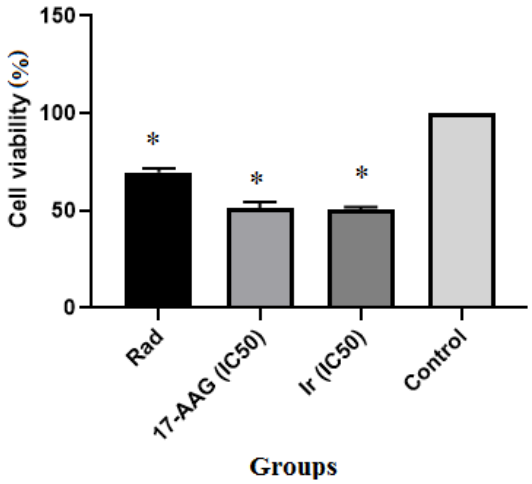


**Figure 2.** Results of MTT Cell Viability Assay in Single Treatments of Radiation, 17-AAG and Irinotecan. Cell growth inhibitory effects were assessed in single treatments of both drug ($IC_{50}$ concentration) and 2Gy of X-ray radiation after 24 h. Data displayed as % of controls (mean ± standard deviation). *Significant difference versus control. Ir, irinotecan; Rad, radiation.

$0.25 \times IC_{50}$ in triple combinations) in combination with radiation decreased cellular viability versus single agent treated cells ($P < 0.05$). Insignificant decreased cellular viability in the 17-AAG-radiation treated case was observed in comparison with irinotecan-radiation treatment at the concentration of $0.5 \times IC_{50}$ (Figure 3).

In the triple treatment, in $0.25 \times IC_{50}$ concentration, the cell viability was significantly reduced compared to double treatment (at the concentration of 0. $5 \times IC_{50}$) including irinotecan-radiation. The cellular viability in triple treatment in $0.25 \times IC_{50}$ concentration significantly decreased in comparison with the single treatment in $IC_{50}$ concentration of both drugs as well as 2-Gy of radiation ($P < 0.05$).

In order to evaluate the percentage of apoptotic cells in single-drug treatments, the $IC_{50}$, $0.5 \times IC_{50}$ and $0.25 \times IC_{50}$ concentrations of both drugs were selected for single, double and triple combinations respectively. In all treatments, an X-ray was used at 2 Gy. The results of the TUNEL test are presented in Figure 4.

The percentage of TUNEL positive cells counted toward the total cells. In single treatments of radiation, irinotecan and 17-AAG, there were significant increase in TUNEL positive cells in comparison with control cells ($P < 0.05$). Moreover, in all double and triple combinations, including 17-AAG- radiation ($P = 0.008$), irinotecan-radiation ($P = 0.007$), irinotecan-17-AAG ($P = 0.003$), and irinotecan-17-AAG- radiation ($P < 0.05$), the significantly higher numbers of TUNEL positive cells were presented versus the control sample.

In addition, the percentage of TUNEL positive cells in triple treatments (irinotecan-17-AAG-radiation) was

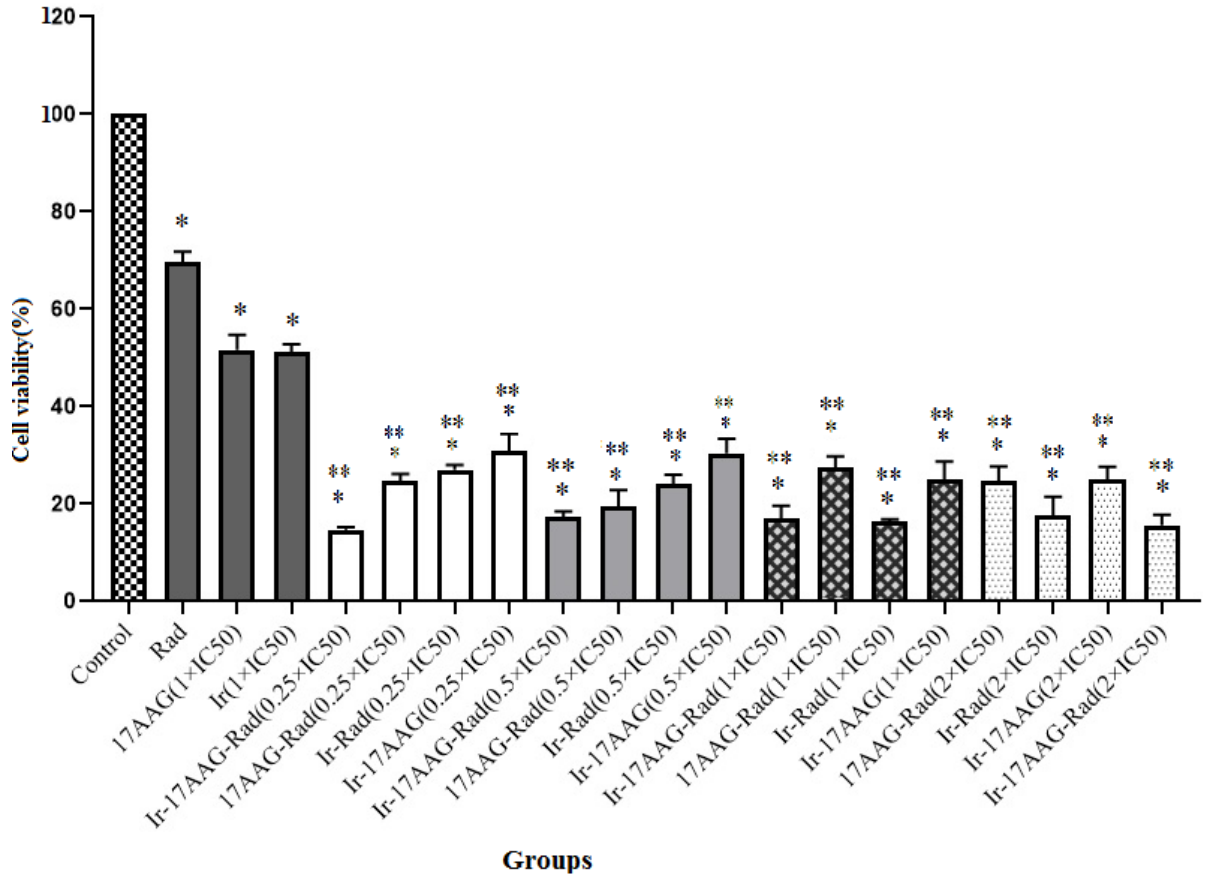


**Figure 3.** Cytotoxic Effects of Double and Triple Treatments of Radiation, 17-AAG and Irinotecan. Cell viability were evaluated in 0.25×IC50, 0.5×IC50, 1×IC50 and 2×IC50 concentrations of both drugs and 2 Gy of X-ray Rad after 24 h. Data showed the WST-1 assay, in combined cases in HCT-116 cells (mean±standard deviation). *Significant difference versus control. **Significant difference versus related single treatments. Ir, irinotecan; Rad, radiation.

significantly higher than double treatments (17-AAG-radiation ($P=0.003$), irinotecan-radiation ($P=0.004$), irinotecan-17-AAG ($P=0.008$)).

The results indicated that the triple treatment (irinotecan-17-AAG-radiation) at the concentration of $0.25\times IC_{50}$ had the highest effect on the induction of cell apoptosis versus other treated cases.

### *The Bcl-2 and Caspase 3 Gene Expression Levels*

Based on Real-time PCR analysis (Figure 5), there was a significant increase in caspase 3 mRNA expression in all treatments (except radiation single treatment) versus the untreated controls. Likewise, in double combinations including 17-AAG-radiation, irinotecan-radiation, and irinotecan-17-AAG, increased caspase 3 gene expression levels were presented in comparison with related single treatments. Further analysis showed a significant increase in caspase 3 mRNA in triple combination versus single and double agent treatments ($P<0.05$).

According to our results, the Bcl2 gene expression assay showed insignificant decrease in Bcl-2 mRNA in single treatments including radiation ($P=0.147$) and irinotecan ($P=0.105$) versus the untreated controls. Nevertheless, a significant decrease in the Bcl-2 gene expression ratio was detected in triple and double combinations (17-AAG-radiation ($P=0.001$), irinotecan- radiation ($P=0.008$), irinotecan-17-AAG (P=0.017), and irinotecan-17-AAG-radiation ($P<0.05$) in compared to the controls. In the double combination of 17-AAG-radiation, there was a significant decrease in the Bcl-2 expression ratio in comparison with single radiation treatment ($P=0.042$). Moreover, in the triple combination of irinotecan-17-AAG-radiation, there was a significant decline in the Bcl-2 expression level versus the single treatment.

### *Caspase3 Enzyme Activity Assay*

As presented in Figure 6, there was a significant increase in caspase 3 enzyme activity in triple and double treatments compared to the untreated control cells ($P<0.05$). In addition, increased caspase 3 activity was detected in single treatments including irinotecan and 17-AAG versus uninduced cells ($P<0.05$). Likewise, higher caspase 3 activity was detected in double and triple combinations in comparison with single treatments ($P<0.05$).

In the triple combination of irinotecan-17-AAG-radiation, there was significantly elevated caspase 3 activity versus double combinations [17-AAG- irinotecan and radiation- irinotecan ($P<0.05$)]. In addition, there was a significant increase in caspase 3 enzyme activity in the triple combination versus 17-AAG- radiation treated

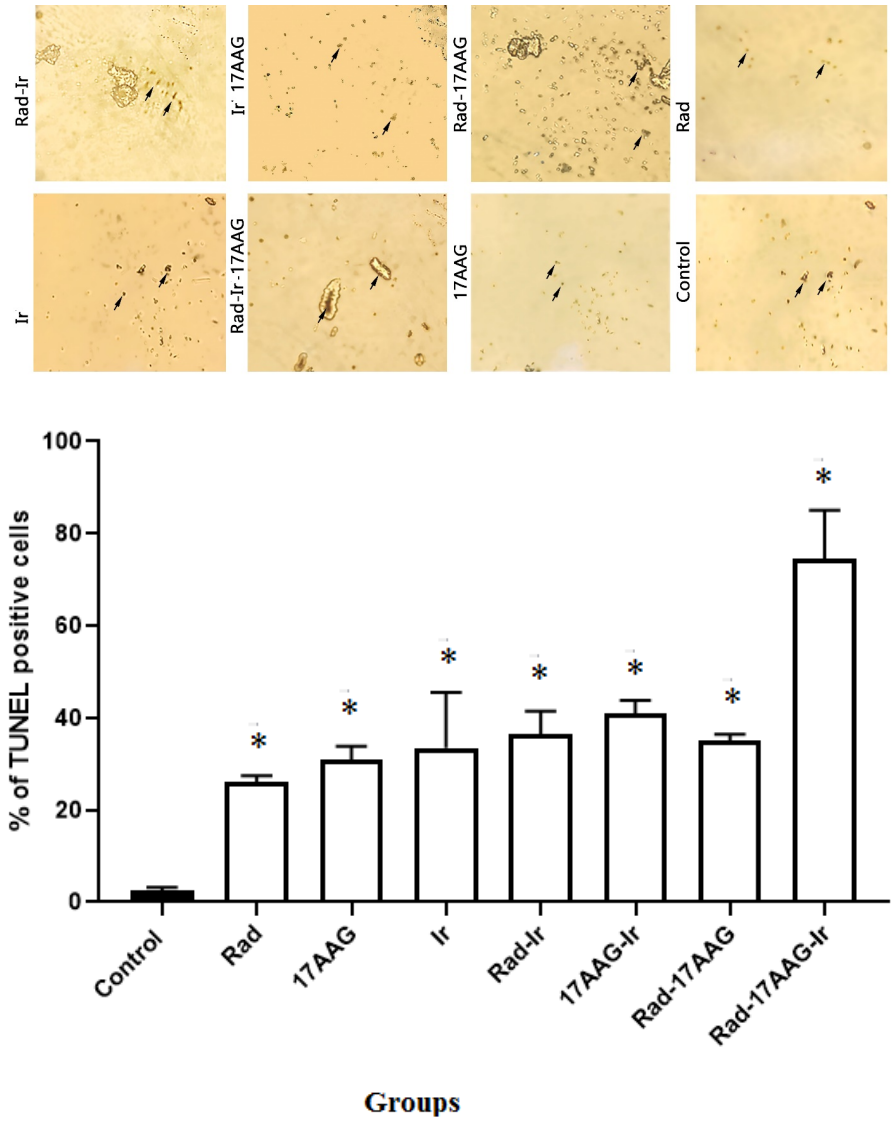


**Figure 4.** Results of TUNEL assay, in single, double and triple treatments of Radiation, 17-AAG and Irinotecan. Percentage of apoptotic cells (TUNEL positive cells) were assessed (0.25 × IC50, 0.5 × IC50 concentration of each drug in triple and double combinations respectively and 2Gy of X-ray Rad after 24 h). Data shown as mean ± standard deviation. *Significant differences in compared to control. Ir, irinotecan; Rad, radiation.

cells ($P < 0.05$).

## Discussion

New therapeutic options along with conventional methods may lead to the successful treatment of CRC. In this regard, chemotherapy alone or in combination with other methods such as radiation therapy could be effective.[37-42] Therefore, in this study, we evaluated the anti-cancer efficiency of irinotecan when combined with 17-AAG and radiotherapy to achieve the better treatment outcome in CRC. The results of the present study indicated the efficiency of combination therapy with radiation, 17-AAG, and irinotecan in very low concentrations of both drugs (lower than $IC_{50}$). The combination therapies induced anti-cancer effects by regulating apoptosis mediators. These results might suggest the new treatment option for CRC. More precisely, this triple combination exhibited an effective cytotoxic effect on the HCT-116 cell line, proposing the potential efficiency of chemo-radiotherapy.

It has been shown that chemotherapy in combination with radiotherapy improved the treatment response.[42] In order to reduce the complications of chemotherapy, improvement of treatment efficacy, reduction in the resistance to usual methods, the combination of radiotherapy with new chemotherapy drugs can be an appropriate solution to CRC treatment.[27,42] Numerous anti-cancer agents prompt their impacts by apoptosis induction.[43]

In this regard, HSP-90 inhibitor and 17-AAG has been applied in some previous studies for different types of cancers to overcome resistance to cancer therapy and also as effective treatment.[44-48] This cytotoxic agent (17-AAG) could exert cell growth inhibition and induce cancer cell

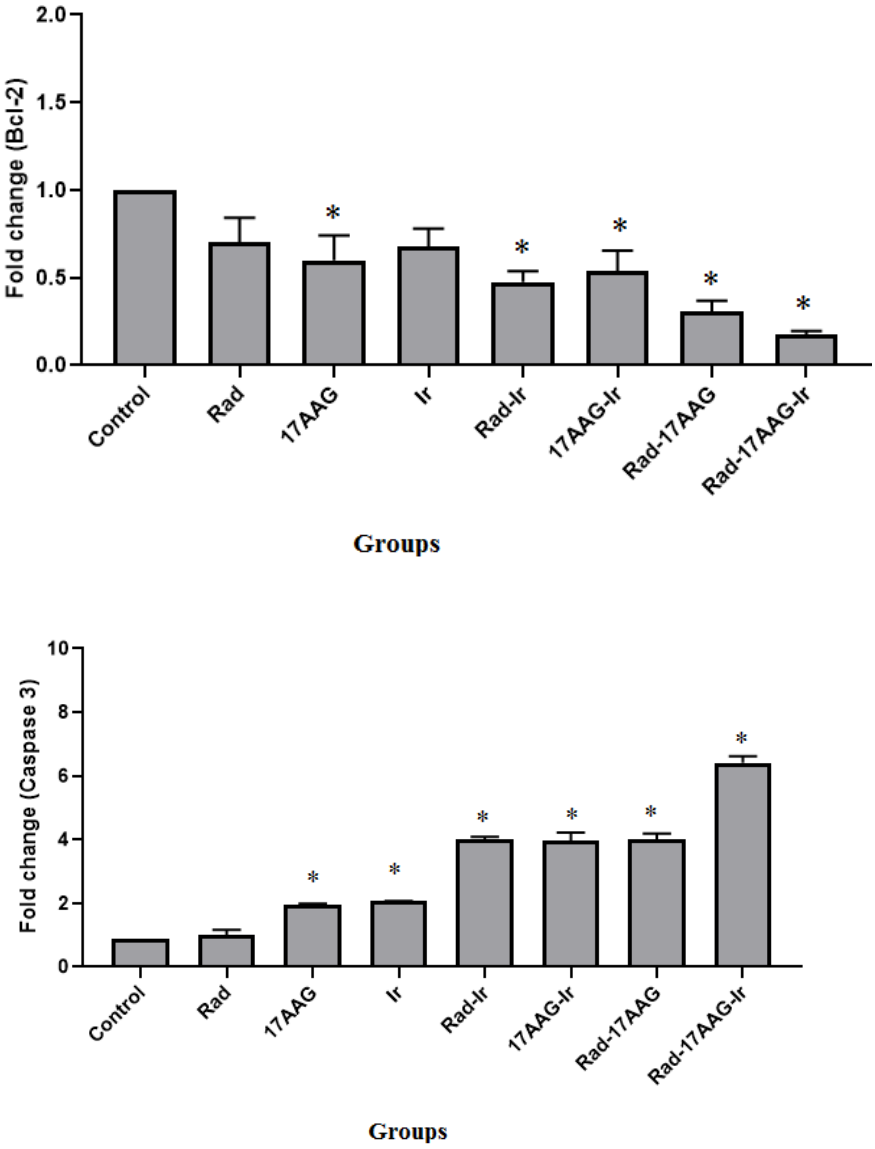


**Figure 5**. Evaluation of Bcl-2 and Caspase 3 Gene Expression Ratios by Real-Time PCR Method. The mRNA expression ratios were measured in untreated and treated HCT-116 cells (in $0.25 \times IC_{50}$, $0.5 \times IC_{50}$ concentration of each drug in triple and double combinations respectively and 2Gy of X-ray radiation after 24 h). Data were presented as mean fold change ± SD. * Significant differences in compared to control.

death.[48]

Based on the results, in single-drug treatments, the cell viability with increasing the drug dosage decreased. Besides, various drug concentrations in double treatments decreased the cellular viability (compared to single-drug treatment).

In a similar survey, the cytotoxicity of 17-AAG in combination with oxaliplatin and other agent was investigated.[48] In this study, the cellular viability in double treatments at low concentrations were decreased in comparison with single treatments even at higher doses. Similarly, our results showed that double treatments in lower doses reduced cellular viability compared with higher doses in single treatments.

According to our results, in double treatments, significant differences in cellular viability and apoptosis rate were presented in compared with single treatments. Also, in double and triple combinations, caspase 3 mRNA and caspase 3 activity (as apoptosis major mediator) increased versus single treatments in higher concentrations of both drugs. These data showed the potential anti-cancer efficiency of each drug, especially 17-AAG as a possible radiosensitizer agent in combination with 2 Gy radiation, because in combination cases, the low concentrations of both drugs were used ($0.5 \times IC_{50}$ for double and $0.25 \times IC_{50}$ for triple combinations). Moreover, in the double combination of 17-AAG-radiation and the triple combination of irinotecan-17-AAG- radiation, there were significant decline in the Bcl-2 mRNA expression ratio (as oncogenic gene) in comparison with the single treated cases. It could be indicated that this drug increased the efficiency of radiotherapy.

In a similar study, the cellular responses to chemo-radiotherapy in HCT-116 and HT-29 cancer cells were

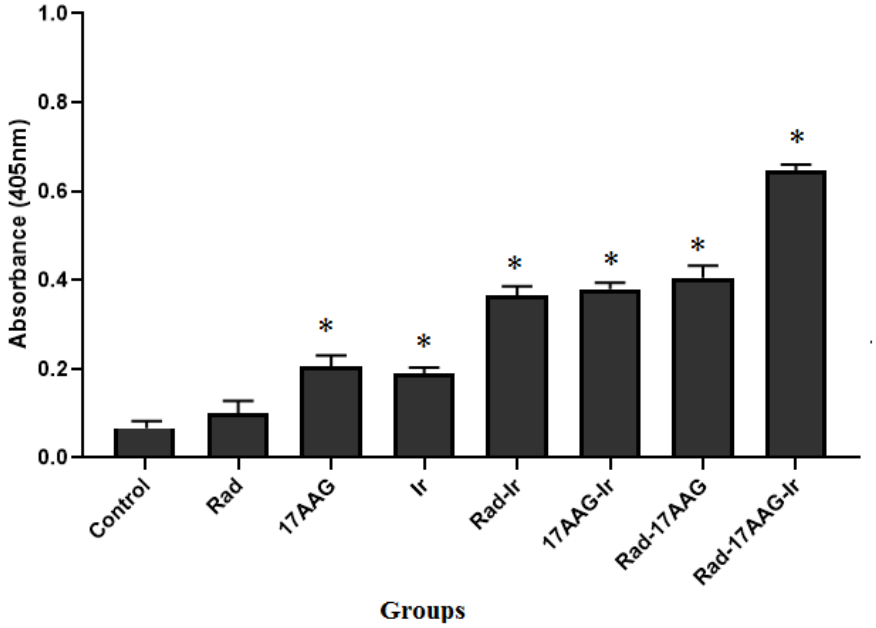


**Figure 6.** Caspase 3 Activity Assay in Uninduced and Treated Cells (in $0.25 \times IC_{50}$, $0.5 \times IC_{50}$ Concentration of Each Drug in Triple and Double Combinations Respectively and 2 Gy of X-ray Radiation After 24 h). Each value displayed the mean ± standard deviation. * Symbol indicates a significant difference in compared with untreated control. Ir, irinotecan; Rad, radiation.

evaluated, and 5-fluorouracil was used as the radio-sensitizer agent. Their results showed the DNA damage and induction of cell death after treatments,[27] which is consistent with the results of our study

Likewise, in a comparable report, CRC cell lines including HT-29, SW48, SW707 and SW480, were exposed to single-dose radiation (2 Gy), and apoptosis were evaluated. Results showed that there was an association between survival fractions with a 2-Gy dose of irradiation. Furthermore, according to this study, radio sensitivity predicted by apoptosis as detected via surviving fraction at 2 Gy.[49]

Similarly, the effects of SN-38 in combination with 17-AAG on colorectal cancer cells have been studied. In p53-defective cells treated with 17-AAG, a downregulation of Chk1 has occurred.[50]

It could be indicated that 17-AAG affects the multiple oncogenic proteins, which are targets for HSP 90. In fact, the Hsp90 inhibition attenuates the invasive properties of colon cancer cells and, also, improves the function of some other chemotherapy drugs.[51,52]

On the other hand, 17-AAG inhibits the G1 proteins in colon cancer cells.[53] In another survey, the effects of radiation induced by the 17-AAG drug in the oral squamous cell carcinoma cell line. This drug inhibits cell proliferation and increases the effects of radiation. In fact, the effect of 17-AAG depends on the status of p53, and treatment with an HSP 90 inhibitor with radiation therapy seems to improve the therapeutic outcomes of conventional treatments in patients with wild-type p53,[54] which may be related to our results about 17-AAG-radiation treatment efficacy in HCT-116 cells.

Also, other surveys report that the administration of 17-AAG increases the sensitivity of esophageal cancer cells to gamma photon radiation because it affects growth factor-mediated signal transduction.[55] It has been indicated that decreased expression of Bcl-2 in treatment with 17-AAG was presented.[56]

Another parallel study showed the significant therapeutic effects, induced by a low dose of an Hsp90 inhibitor in combination with radiotherapy. In this study, monotherapy in a low dose was not effective; nevertheless, once combined with radiotherapy, it led to significant tumor growth inhibition,[57] which was similar to our findings.

Indeed, the efficiency of novel treatments should be evaluated in vitro before utilizing in vivo. In this regard, another study by Minaei et al[58] was conducted to present the Z-scan technique as an accurate, in vitro procedure to investigate the cytotoxicity of different treatments. In this way, OLN-93 and C6 cells were treated with radiofrequency hyperthermia (HT), Temozolomide (TMZ), and chemo-hyperthermia (HT + TMZ). The cytotoxic effects of treatments were studied by the Z-scan method and colony formation assay. The results of Z-scan method were in accordance with the data of clonogenic assay. [58]

Moreover, in another study, researchers demonstrated that the Z-scan technique can be considered as a reliable method by the potential to detect cell changes throughout treatments with HT.[59]

In addition, a previous report confirmed the advantage of chemo-photothermal (PTT) effect to understand the release of DOX from hairpin DNA. The researcher

of that study also verified that AS1411 based NPs showed significant targeted binding towards SW480 cells. Amazingly, AS1411 based NPs showed significant cytotoxicity and inhibited cancer cell proliferation under laser exposure versus NPs with chemotherapy or PTT .[9]

In addition, combined treatment with irinotecan and radiation led to G2/M phase arrest and apoptosis occurrence. The western blot analysis in the combined group showed the greatest increase in the expression levels of proteins attributed to the cell cycle (Tyr15p-Cdc2 and cyclin B1) and DNA damage response system. Certainly, irinotecan probably radiosensitized the p53-mutant HT29 cells by the ATM/Chk/Cdc25C/Cdc2 pathway.[28]

It has been reported that 17-AAG when combined with radiation, increased the mediators of oxidative and nitrosative stress. In addition, this treatment increases the Bax and decreases the Bcl-2 gene expression ratios that are associated with apoptosis induction.[22] Similarly, our results showed downregulation of Bcl-2 mRNA in double and triple combination of 17-AAG with irinotecan and radiation treatments which promoted apoptosis occurrence.

17-AAG as an HSP90 inhibitor with 2-Gy radiation in breast cancer cells increased the hydrogen peroxide and nitric oxide levels.[22]

The radiosensitizing efficacy of an Hsp90 inhibitor may be affected by the modulation of proteins attributed to radioresistance, involving ErbB2, Akt and Raf-1, resulting in decreased NF-κB activity, altered DNA repair ability, and apoptosis.[60]

The cancer cells could be resistant to multiple therapeutic options. Thus, evaluating effective treatments and related molecular mechanisms are important.[22,48,61-63] In this study, increased efficacy of radiotherapy with 17-AAG and irinotecan may be related to apoptosis induction and accelerate radiation sensitivity. In fact, the induction of apoptosis and Bcl-2 downregulation are engaged in possible mechanisms of 17-AAG in combination cases with anti-cancer agent and radiation.

The results of this work still need to be confirmed with in vivo animal studies and also only two apoptosis-related genes were analyzed; consequently, future evaluations should be conducted to examine other related pathways influenced by 17-AAG, radiation and irinotecan combined treatments.

## Conclusion

Numerous studies have shown several advantages of chemoradiation treatment (combined chemotherapy and radiotherapy) versus chemotherapy or radiotherapy alone.[64] Therefore, in this study, the potential anti-cancer efficiency of 17-AAG and irinotecan in combination with X-ray radiation was confirmed in concentrations lower than $IC_{50}$ of both drugs. Since the growth inhibition of cancer cells after treatment is essential, we confirmed that low concentrations of two chemotherapeutics increased the efficiency of radiation therapy. Indeed, these combinations regulated apoptosis by affecting related genes including caspase 3 and Bcl-2. It can be concluded that the combination of chemo-radiotherapy has better effects than multi-drug chemotherapy.

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### Conflict of Interests

The authors declare no potential conflicts of interest.

### Ethical Consideration

The ethical committee of Urmia University of Medical Sciences, Urmia, Iran (Ethics Code; IR.UMSU.REC.1396.155), approved the study protocol.

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