

Mitochondrial Targeted Peptide (KLAKLAK)₂, and its Synergistic Radiotherapy Effects on Apoptosis of Radio Resistant Human Monocytic Leukemia Cell Line

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ABSTRACT

Background: Ionizing radiation plays a significant role in cancer treatment. Despite recent advances in radiotherapy approaches, the existence of irradiation-resistant cancer cells is still a noteworthy challenge. Therefore, developing novel therapeutic approaches are still warranted in order to increase the sensitivity of tumor cells to radiation. Many types of research rely on the role of mitochondria in radiation protection.

Objective: Here, we aimed to target the mitochondria of monocytic leukemia (THP-1) radio-resistant cell line cells by a mitochondrial disrupting peptide, D (KLAKLAK)₂, and investigate the synergistic effect of Gamma-irradiation and KLA for tumor cells inhibition *in vitro*.

Material and Methods: In this experimental study, KLA was delivered into THP-1 cells using a Cell-Penetrating Peptide (CPP). The cells were then exposed to gamma-ray radiation both in the presence and absence of KLA conjugated with CPP. The impacts of KLA, ionizing radiation or combination of both were then evaluated on the cell proliferation and apoptosis of THP-1 cells using MTT assay and flow cytometry, respectively.

Results: The MTT assay indicated the anti-proliferative effects of combined D (KLAKLAK)₂ peptide with ionizing radiation on THP-1 cells. Moreover, synergetic effects of KLA and ionizing radiation reduced cell viability and consequently enhanced cell apoptosis.

Conclusion: Using KLA peptide in combination with ionizing irradiation increases the anticancer effects of radio-resistant THP-1 cells. Therefore, the combinational therapy of (KLAKLAK)₂ and radiation is a promising strategy for cancer treatment in the future.

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Keywords

Combination Therapy; Ionizing Radiation; Radio-Resistance; Mitochondria; Pro-Apoptotic Peptide; Antimicrobial Peptide; Cell survival; Flow Cytometry

Introduction

Radiotherapy is an efficient approach for cancer treatment. Despite significant technical improvements in radio therapy applications, little advances have been made in identification of optimal combinations including radiotherapy and targeted therapy in order

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to increase the efficacy of cancer treatment [1]. Another major challenge is that the efficacy of radiotherapy is limited by the radio-resistance properties of many cancer cells [2]. Therefore, innovative approaches are needed to enhance the outcome of radiotherapy treatment.

The major part of initial cell damage caused by Ionizing radiation (IR) is double-strand DNA breaks [3]. Furthermore, irradiation induces a number of indirect effects through generation of reactive oxygen species (ROS). Formation of ROS as the main point of secondary effects of radiation can indirectly cause DNA damage [4]. It has been suggested that the ROS generated by ionizing irradiation has a variety of biological functions [5-7]. One of the notable functional and structural impairments associated with IR-induced ROS is the peroxidation of membrane phospholipids and proteins [8, 9]. Thus, the plasma membrane of the cells and the membranes of cytoplasmic organelles are another major target of ROS and radiation. This oxidative stress brings on alterations in both the lipid bilayer permeability and fluidity traits [10]. In addition, the ROS burst following ionizing radiation can result in mitochondrial membrane oxidative damage. This in turn may eventually trigger a cascade of events leading to increased permeability of mitochondrial membrane, cytochrome c release, activation of caspase-3, and consequently the cell death [11].

Since, cancer cells have the ability to repair the radiation-induced damage efficiently, the radio resistance properties are developed and consequently improve the survival rate of cancer cells and their replication [12]. From a radiobiological point of view, radiation resistance may occur via many pathways. One of them is adaptive pathway which occurs as a result of mitochondrial signaling as pro-survival signaling pathways for promoting radio-adaptive resistance [13]. Moreover, recent studies suggested that following the mitochondrial DNA-damage induced by irradiation, increasing mitochondrial DNA copy number may be

a subsequent response [14]. This can cause the upregulation of mitochondrial proteins such as superoxide dismutase, the most powerful antioxidant, that might diffuse into the other mitochondria and intensify the damage signal. This in turn might propagate to the daughter cells and result in the recurrence of secondary cancer in cells long after the first radiation exposure [14-16]. Another theoretical speculation is that irradiation may lead mitochondrial DNA fragments to be inserted into the nuclear genome and could Subsequently increase cancer risk [17].

According to all of the experimental data, the targeting of mitochondria may be an appropriate approach to increase lethal efficacy of radiation therapy [14].

Anti-microbial peptides are approved to serve as anti-cancer peptides. KLA peptide, (KLAKLAK)₂, is a well-known example and the most successful one of them. It has an amphipathic alpha-helical structure which exhibits remarkable selectivity for anionic membranes of prokaryotes [18]. If KLA is internalized into eukaryotic cells via an appropriate targeting mechanism, it can destroy mitochondrial membranes and initiate the tumor cell apoptosis process [19-22].

Many studies confirmed that THP-1, a human acute monocytic leukemia cell line, can develop radio resistance properties [23-25]. Therefore, the aim of current study was to evaluate the synergistic effect of ionizing irradiation and mitochondrial damage caused by KLA peptide on THP-1 radio-resistant cells *in vitro*.

Material and Methods

Cell line

In this experimental study, THP-1 suspension cell line was purchased from the cell bank of Pasteur Institute of Iran. The cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mm L-glutamine, 0.05 mm 2-mercaptoethanol

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and 100 unit/ml penicillin-streptomycin. Cells were then cultured in tissue culture flasks and incubated at 37°C under 5% CO₂.

Gamma Irradiation of Cancer Cells

The cells were seeded in 96 well plates and subjected to uniform irradiation at room temperature using a Cobalt 60 tele therapy radiation therapy unit (Theratron 780, Best Theratronics, Canada) with a dose rate of 70.5 c/min. After the irradiation, the plates were incubated at 37°C with 5% CO₂ and harvested 24 hours after irradiation for the further analysis.

Peptide and Cell Treatments

CPP44 fused previously, a leukemia-homing cell penetrating peptide [26] to D-form of (KLAKLAK)₂ peptide with the feature of specific THP-1 cell internalization and cell proliferation inhibition (Unpublished data) was provided by Biomatik Corporation (Biomatik, Ontario, Canada).

THP-1 cells were divided into 4 groups. Group I received a concentration of 5 μM of FITC-labeled CPP44-KLA peptide alone. Group II were exposed to 10 Gy gamma irradiation. The third group of the cells were incubated with 5 μM concentrations of KLA peptide for an hour and then exposed to 10 Gray gamma-rays. Group IV consisted of THP-1 cells without any treatment received. Experiments were performed in triplicate. The cells were then incubated for 24 hours (37° C, 5% CO₂).

MTT Cell Proliferation Assay

MTT assay was performed to measure cell proliferation rates and to assess the effect of KLA peptide on THP-1 cells. Briefly, THP-1 cells were seeded on a 96-well cell culture plate in triplicate (2×10⁴ cell/well) for 12 hours. 24 hours after treatment, 20 μl of MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (5 mg/ml) was added into each well and incubated at 37°C for

4 hours. After centrifugation (5 min, 1000 g, room temperature), the supernatant was carefully removed and DMSO (100 μl/ well) was added into each well and the plate was gently shaken for 15 minutes. The absorbance of MTT at 540 nm was monitored by the microplate reader.

Analysis of Cell Apoptosis

After incubation at 37°C for 24 hours in an atmosphere of 5% CO₂, treated THP-1 cells with 5 μM CPP44-KLA peptide and/or 10 Gray of Gamma irradiation, the cells were harvested, washed twice with cold PBS and then resuspended in 1X binding buffer at a concentration of 1×10⁶ cell/ml. For the PE-Annexin V and 7AAD staining, 5 μl of each 7AAD and PE-Annexin V (BD Biosciences, San Jose, CA, USA) were added to 100 μl of the solution followed by incubation in the dark for 15 minutes at the ambient temperature. Samples were then analyzed in a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA). Fluorescence was measured in FL-2 channel (PE) and FL-3 channel (AAD). The control group received no treatment.

Statistical Analysis

All experiments were repeated at least three times. Data analysis was performed with SPSS Version.17.0 statistic software package and presented as means ± 3SD. The normality of the data was evaluated using Shapiro-Wilk normality test. The One-way Analysis of Variance (ANOVA) with Tukey post-hoc test was utilized to statistical analyses in the minimum significance at P<0.05.

Results

Penetration of Peptide into the Cells

Twenty-four hours after treatment with KLA peptide at concentration of 5 μM, the cells were harvested and washed twice in phosphate buffered saline (PBS). The green fluorescence

of fluorescein isothiocyanate, labeled to the N-terminal of CPP44-KLA peptide, was assessed by fluorescent microscopy. The FITC-labeled peptide was internalized into THP-1 cells successfully (Figure 1).

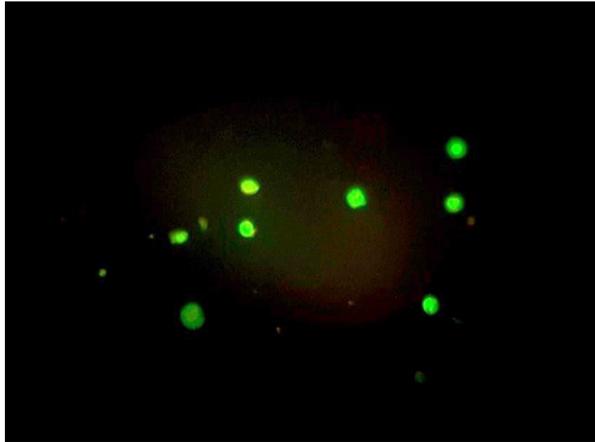


Figure 1: Cellular localization of cell penetrating peptide44 (CPP44)-KLA in THP-1 cell line. The green Fluorescein isothiocyanate (FITC) conjugated with the CPP44-KLA was assessed by fluorescent microscopy.

The Synergistic Effect of KLA Peptide combined with Irradiation against Viability of THP-1 Cells

The influence of (KLAKLAK)₂ pro-apoptotic peptide, radiotherapy and combination of both treatments on THP-1 cell viability were evaluated by MTT assay. Cell survival rates were determined at time point of 24 hours following the treatment and the results were statistically evaluated using ANOVA one-way method. THP-1 cell viability decreased in cells that had been treated with combined treatment of irradiation together with KLA peptide was applied (43.3± 6.5%) (Figure 2). However, the cell viability in groups that treated with irradiation or KLA peptide alone was 82.2±6.1% and 58.5±8%, respectively (Figure 2).

Comparing treatment groups with or without KLA peptide, a significant reduction in survival rate of the cell treated both with irradiation and incubation with KLA peptide in compare with group II that received irradiation alone was determined (P<0.001).

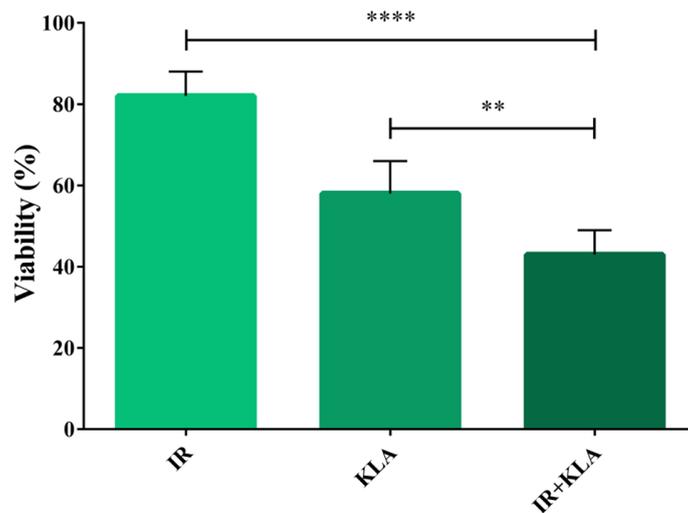


Figure 2: The inhibitory effect of cell penetrating peptide44 (CPP44)-KLA, radiotherapy (IR) and combination of CPP44-KLA peptide and IR (KLA+IR group) on thp-1 cells viability acquired through MTT proliferation assay. In addition to the IR group that the viability evaluated in the absence of CPP44-KLA under exposure of gamma-irradiation (10 Gy), thp-1 cells were also treated by CPP44-KLA peptide in absence (KLA group) and the presence of irradiation with same Dose. ****: P<0.0001 compared to IR group, **: P<0.001 compared to KLA group (Tukey was utilized as the post hoc test).

Synergistic Apoptotic Effects of KLA peptide and ionizing Irradiation on THP-1 cells

The induction of THP-1 cell death by each KLA peptide or gamma-ray radiation alone and combined therapy was determined by dual staining with Annexin V and 7AAD followed by flow cytometry analysis. Simultaneously, staining the cells with 7AAD and Annexin V demonstrated that a significant proportion of

the cells treated with both peptide and irradiation became stained with Annexin V, which inferred early-stage apoptotic cell death (****: $P < 0.0001$); some of the cells showed dual staining with both 7AAD and annexin V, and indicated late apoptotic cell death (Figure 3).

Flow cytometry analysis of stained cells indicated the overall increase in the percentage of apoptotic cells (both early and late) in different assays in comparison with control

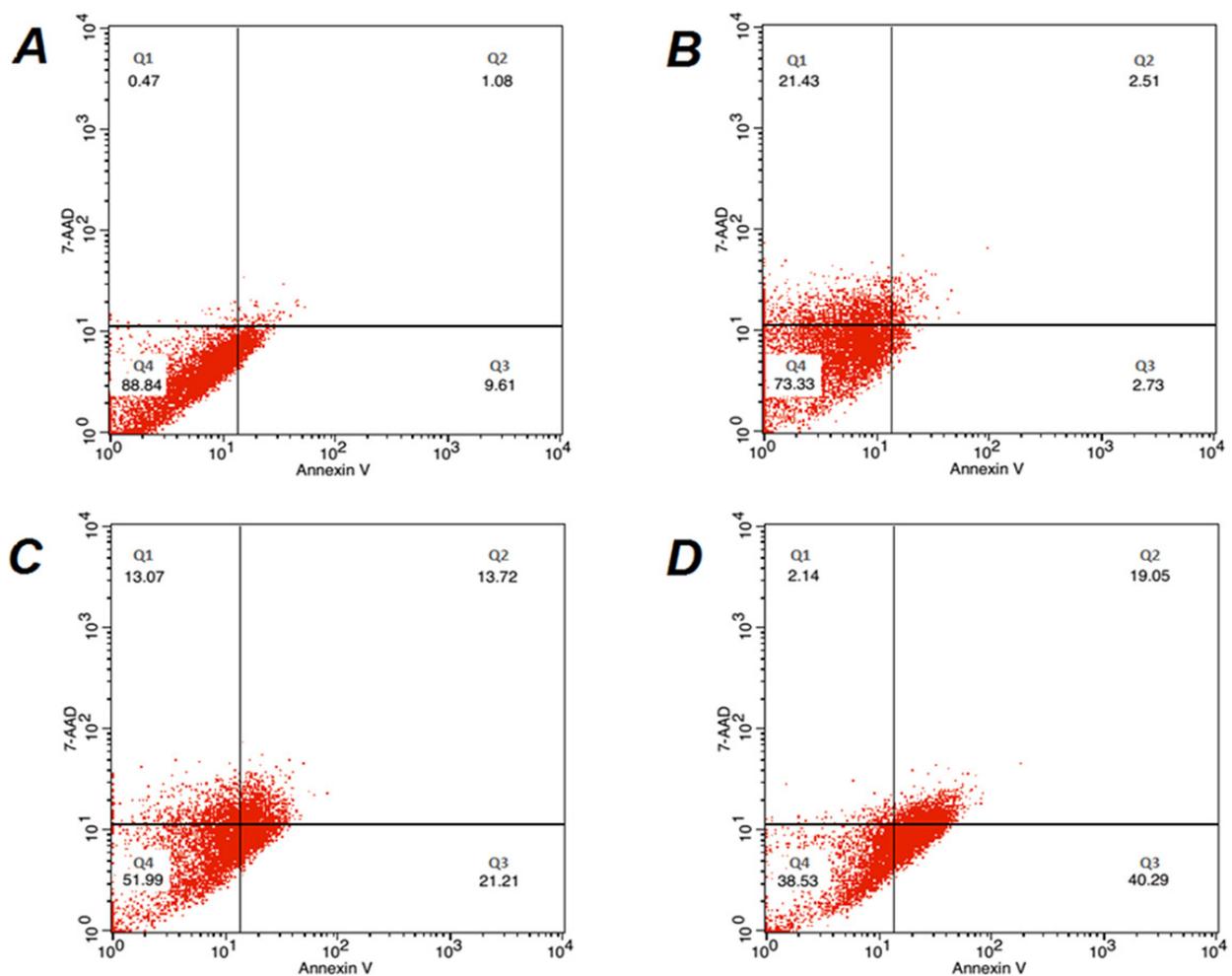


Figure 3: Flow cytometry analysis of cell apoptosis, with panels showing 7AAD/annexin V staining of the cells exposed to any treatments as control (A), irradiated cells (B), cell penetrating peptide44 (CPP44)-KLA treated cells (C) and cells exposed to combination of irradiation and peptide (D). The quadrants (Q) represent live cells (Q4), early apoptotic cells (Q3), late apoptotic cells (Q2) and necrotic cells (Q1). As illustrated in chart D, combination of KLA and irradiation synergistically could increase total cell apoptosis percentage.

group. However, higher percentage of cell apoptosis was determined when the combination of KLA peptide and gamma-ray radiation was applied to indicate the synergistic effect of (KLAKLAK)₂ peptide and radiotherapy on apoptosis of radio-resistant THP-1 cells (Figure 4).

Discussion

Nowadays, attempts to improve the efficacy of radiotherapy have focused on synergistic with chemotherapy. To optimize the biological efficacy of radiotherapy, co-administration of radiotherapy and therapeutic agents making cancerous cells susceptible to irradiation are needed. Apart from radiotherapy combined with traditional chemotherapeutic drugs, lim-

ited advances have been made to use potential synergies between radiotherapy and targeted systemic therapies. Specifically, a few novel drug-radiotherapy combinations are established [1]. Based on our knowledge, few approaches overcoming radio-resistance process have focused on the use of peptide-based combinational therapies.

Several researches declare that many signaling survival pathways are committed to cause resistance against ionizing radiation [13, 27].

Recent evidence suggests that mitochondria play a critical role in such pathways and control the cell survival and repopulation after irradiation [13, 14]. Nevertheless, most of the therapeutic agents combined with radiotherapy are designed as DNA-repair inhibitors in

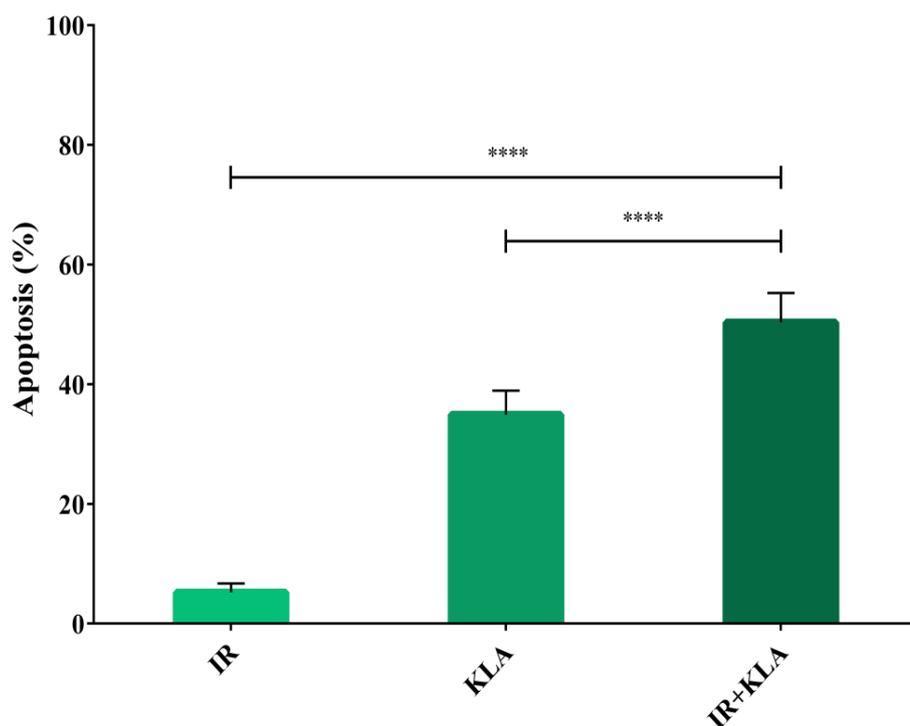


Figure 4: Total apoptosis Comparison between cells treated with 10 Gy gamma-ray Irradiation (IR), 5 μ m cell penetrating peptide44 (CPP44)-KLA peptide (KLA) or combination of both (KLA+IR). The results showed that combination of gamma-ray radiation and pro-apoptotic KLA peptide significantly induced apoptosis more than each treatment alone represented a predominant synergistic effect. ****: P<0.0001 compared to KLA and IR groups.

order to overcome the radio resistance properties [27-29].

In current study, considering the aim of radio-sensitization, we investigated the synergistic effect of ionizing radiation together with (KLAKLAK)₂, a potent cytotoxic peptide which can target and disrupt the mitochondrial membrane. KLA peptide has been demonstrated to possess anticancer properties as reported by many experimental data. KLA with submicromolar cell penetrating abilities results a dramatic release of cytochrome c into the cytosol and induces caspase-3-dependent cell apoptosis [30-33]. Furthermore, the releasing of cytochrome c induces a variety of destructive effects, including generation of free radicals and subsequent cell damage. [34]. On the other hand, potentially damaging levels of mitochondrial reactive oxygen species (ROS) makes the cells more susceptible to irradiation.

Besides, the mitochondria potentially upregulate superoxide dismutase antioxidant which can counteract the deleterious effects of radio-induced ROS as mentioned before [16]. It has been suggested that cells with more frequency of mitochondria can resist more against irradiation effects even if it eliminates a major fraction of the mitochondria. Therefore, it has been proposed that mitochondria can determine the cell radio-sensitivity [34]. Based of all these findings, targeting of this pivotal organelles would be a proper approach to develop radio-sensitization.

As demonstrated in our results, the effect of irradiation on its own was an approximately 24% reduction in the viability of radio-resistant THP-1 cells after 24 hours. This is in reasonable agreement with other reported findings at similar dose rates, 60 cGy/min [24] and 100-104 cGy/min [35].

MTT assay results revealed that the cell group only exposed to gamma-rays showed slight cytotoxicity. The results also indicated that KLA peptide reduced the viability of THP-1 cells alone. While pretreatment of

CPP44-KLA combined with of gamma-radiation significantly suppressed cell growth and decreased survival chance of THP-1 cells after 24 hours.

To determine the mechanism of this synergistic effect, the apoptotic rates were next investigated by flow cytometry. The results suggest that mitochondrial damage caused by (KLAKLAK)₂ peptide could synergistically sensitize radio-resistant THP-1 cells to ionizing irradiation and trigger rapid cell death consequently.

It is worth mentioning that the ionizing radiation could not affect the structural and functional properties of the D-form peptide, pre-administered to the cells, and KLA peptide functioned as predicted.

Conclusion

We hypothesized that developing of a novel radio-sensitization strategy, based on a mitochondrial targeting manner, can be a suitable choice to increase the efficacy of radiotherapy even in the cells with radio-resistance properties. In addition, it is supposed that combining of ionizing irradiation with KLA, conjugated to a specified targeting agent like CPP44, will augment the positive cancer treatment outcomes. However, further investigation will be necessary to determine the exact mechanisms in which KLA peptide could sensitize various cells to irradiation effects.

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Conflict of Interest

None

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