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Dihydroartemisinin induces apoptosis in skin cancer cell line A-431 via ROS pathway

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Abstract—Dihydroartemisinin (DHA) is a derivative of artemisinin that has been shown to inhibit cell growth and induce apoptosis in the several cancer cells via reactive oxygen species (ROS) formation. The present study was designed to understand the mechanism underlying Dihydroartemisinin-induced apoptosis in the A-431 human skin cancer cell line.MTT viability assay was used to study the cell proliferation effect of Dihydroartemisinin. Annexin V-FITC and reactive oxygen species (ROS) formation were assessed to detect apoptosis. Dihydroartemisinin significantly inhibited cell proliferation in a concentration-dependent manner in A-431 cell line. Dihydroartemisinin significantly induced apoptosis, which was determined by Annexin V-FITC staining. Moreover, increase of ROS formation was observed in response to Dihydroartemisinin treatment. The results of this study suggest that Dihydroartemisinin inhibited cell proletarian and induced apoptosis in skin cancer cells via ROS pathways. These data might suggest that Dihydroartemisinin could be used as an agent for the treatment of skin cancer.

Keywords—Skin cancer, Dihydroartemisinin, apoptosis, ROS.

I. INTRODUCTION

Skin cancer is one of the major causes of cancer death worldwide .Non-melanoma skin cancer (NMSC) is one of the most commonly diagnosed malignancies in the world [1, 2]. The standard treatments for this disease are surgery and chemotherapy using 5% fluorouracil. While there is a high rate of response to this therapy, most treated patients relapse with tumor [3]. Therefore, effective skin cancer therapy will require novel strategies to target and eliminate skine cancer cells. The new therapies have been directed towards identifying agents that block proliferation and induce apoptosis of skin cancer cells. Artemisinin, a natural product isolated from the plant Artemesia annua L [4, 5]. It has been used to treat malaria and is still widely used as an effective antimalaria drug [5]. Recently, studies have suggested that artemisinin and its derivatives have anticancer properties, such as in breast, oral squamous cell carcinomas, pancreas, prostate, colon and neuroblastoma [6-12]. Although many studies have been performed about the underlying mechanisms of Artemisinin, the exact mechanism of this compound is still highly controversial [13, 14]. It has a defined mechanism of action, with artemisinin and its derivatives effecting heme-mediated decomposition of the endoperoxide bridge in artemisinin to produce free radicals [15]. This heme-catalyzed excess of reactive oxygen species (ROS) induces apoptosis through a mitochondrial mediated pathway or blocking cell cycle kinetics [16-18]. However, reactive oxygen species production is not enough to explain anticancer activities of artemisinin [19]. Previous study demonstrated that Dihydroartemisinin (DHA) can activate p38 MAPK pathway independently of ROS [19, 20]. Recent studies have illustrated that several cellular process and pathways including cell cycle, apoptosis contribute to the anticancer activities of artemisinin [21, 22], the ROS-independent mechanisms of artemisinin and its derivatives remain to be elucidated. Taking into account what said above and the fact that Dihydroartemisinin can induce cell apoptosis; we have investigated on the apoptotic induction as a possible tool in the therapeutic protocols for the cure of skin cancer. In the present study we used A-431 as Non-melanoma skin cancer cell lines to examine the effect of Dihydroartemisinin on the cell apoptosis. Furthermore, the effect of Dihydroartemisinin on the ROS production was also detected.

II. MATERIAL & METHODS

1. Chemical reagents

RPMI 1640, trypsin/EDTA, phosphate-buffered saline (PBS), penicillin and streptomycin were purchased from Gibco (Rockville, USA). Dihydroartemisinin, Annexin-V-FITC apoptosis detection kit, MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) and DMSO (dimethyl sulfoxide) were obtained from Sigma-Aldrich (Munich, Germany). Cell culture plastic ware was obtained from Nunc Co. (Roskilde, Denmark). Fluorescent reactive oxygen species (ROS) Detection Kit was purchased from Marker Gene TM Live Cell Co. ((St. Louis, MO, USA).

2. Cell culture

The A-431 human skin cancer cell line was purchased from National Cell Bank of Iran (NCBI). The cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 µg/ml of streptomycin and maintained at 37°C in a humidified incubator with 5% CO2. Cells were harvested at 70–100% confluence with trypsin/EDTA and either used fresh or were frozen on liquid nitrogen and stored at -70°C.

3. Cell viability assay with MTT reduction

Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as described previously [23, 24]. The A-431 cells were seeded at 5×10^3 cells/well in 5% CO2 at 37°C in RPMI medium (containing 10% FBS, 100 units⁄ml penicillin and 100 µg⁄ml streptomycin) in 96-well plates. After incubation overnight to allow for cell attachment, the RPMI medium in each well was replaced with media containing various concentrations of Dihydroartemisinin (0.01-20 µM) and incubated for 48 h. Afterwards, 20 µl of MTT (5 mg/ml in PBS) was added to each well and the cells were incubated for another 4 h at 37°C. The supernatants were then aspirated carefully and 200 µl of dimethyl sulfoxide (DMSO) was added to each well. The plates were shaken for an additional 10 min and the absorbance values were read by the microplate reader (Bio-Rad, Hercules, CA, USA) at 570 nm. Data were collected from several experiments and the percentage of cell growth inhibition was determined by comparison to untreated control cells**.**

4. Assay for apoptosis using Annexin V/ PI staining

Detection of apoptosis was conducted using the annexin V-FITC/PI apoptosis detection kit according to manufacturer's protocol. Briefly, the treated and untreated cells, control cells, were harvested after 24h and washed twice with cold PBS. The cell pellets were re-suspended in 500 µl of 1x binding buffer at a concentration of 1×10^6 cells/ml. 5 µl of annexin V-FITC and 5 µl of PI were added into the cell suspension, followed by gentle vortexing. The staining samples were incubated for 10 minutes at room temperature in darkness. Samples were analyzed by a FACScalibur flow cytometer (BD Biosciences,San Jose, USA) using the software supplied in the instrument.

5. Measurement of Reactive Oxygen Species (ROS)

Formation of reactive oxygen species (ROS) was evaluated using the Marker Gene TM Live Cell Fluorescent ROS Detection Kit according to the manufacturer's instructions. Briefly, cells plated to a density of 25×10^3 per well in 96-well plate and incubated with different concentration of Dihydroartemisinin in the presence and absence of NAC (1 mM) for 48 hr. after drug treatment, cells were loaded with 2',7'-Dichlorofluorescin diacetate (20 µM) in HBSS at 37 ◦C for 30 min in the dark. The cells were then washed with HBSS and Fluorescence caused by DCF in each well was measured and recorded at 485 nm (excitation) and 528 nm (emission) by using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT).

6. Statical analysis

Non parametric one-way analysis of variance (ANOVA) was performed with the Dunnett's test, using software Graphpad Prism. Each experiment was carried out in triplicate and repeated three to four times independently. $P < 0.05$ was considered significant. All data are expressed as means ± SD.

III. RESULTS

The effects of Dihydroartemisinin on the cell growth

To evaluate the effects of Dihydroartemisinin on the viability of A-431 skin cancer cells, MTT assay was carried out. MTT assay measures the activity of mitochondrial dehydrogenase enzyme based on its ability of cleaving tetrazolium ring to produce formazan; thus, the assay can be used as an index of cell viability. Treatment of A-431 cell lines with the Dihydroartemisinin for 48 h resulted in a dose-dependent reduction in the cell viability when compared with that of control (Figure 1). In the A-431 cells, as indicated in Figure 1, a significant inhibitory effect was observed at 1µM ($P < 0.05$ versus control group) which reached to the maximum at 20 µM ($P < 0.01$ versus control group). The IC50s (The effective dose that inhibits 50% growth) for treatment of A-431 cell by Dihydroartemisinin was 5.5µM.

Figure1:

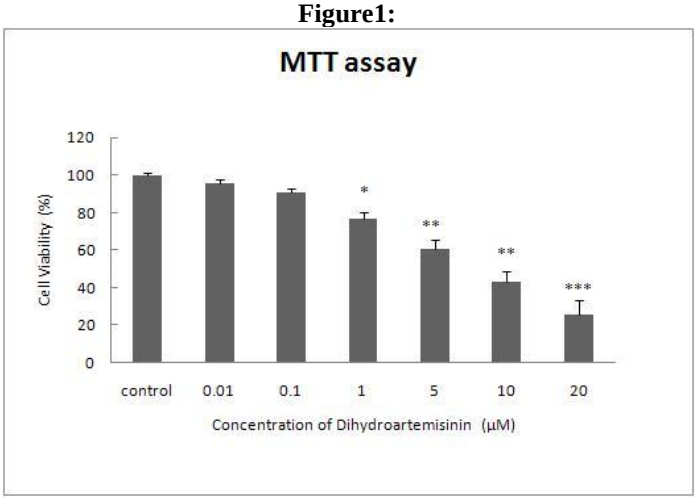

Figure1: The effect of Dihydroartemisinin on the cell viability in A-431 skin cancer cells. Cells were treated with various concentrations (0.01μM- 20 μM) of Dihydroartemisinin for 48h, and viability was assessed by MTT assay. Dihydroartemisinin reduced cell viability in A-431 cells in a dose-dependent manner. Results (mean ± SD) were calculated as percent of corresponding control values. *P<0.05, **P<0.01 and ***P<0.001 are significant. Statistical analysis was performed by ANOVA. Each point represents 4 repeats, each triplicate.

Detection of apoptosis by flow cytometry

To determine whether growth-inhibitory effect of Dihydroartemisinin in A-431 cells was associated with the induction of apoptotic cell death, FITC-conjugated annexin V (FL1-H) and PI (FL2-H) staining (detected by flowcytometry) was used as a criterion to distinguish apoptotic cells (Figure 2). Therefore, A-431 cells treated with various concentration of Dihydroartemisinin were evaluated by flowcytometry. All data were analyzed by specific software Partec FloMax. Control cells were negative for both annexin V-FITC and PI. Annexin V-FITC-positive, PI-negative cells were considered to be in early apoptotic stage, while annexin V-FITC-positive, PI-positive cells were considered to be late apoptotic or necrotic and cells were positive for PI were considered mostly necrotic cells. As shown in Figure 2, a significant increase in the percentage of both early (annexin V positive, PI negative) and late (annexin V positive, PI positive) apoptosis were detected after treatment with Dihydroartemisinin in a concentration-dependent manner ($P < 0.01$).

Figure2:

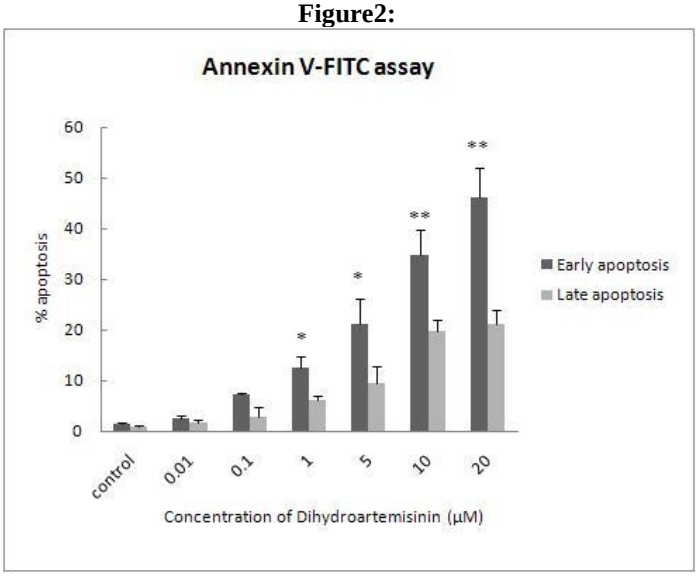

Figure 2: Detection of apoptosis using flow cytometry. Flow cytometric analysis of A-431 apoptotic cells after annexin V-FITC/propidium iodide (PI) staining. Cells were treated with various concentration of Dihydroartemisinin for 48h. The results shown are mean ± SD of three independent experiments. * $P < 0.05$; ** $P < 0.01$ compared with control group.

Role o Reactive Oxygen Species (ROS) in Dihydroartemisinin-induced cell apoptosis

To examine whether Dihydroartemisinin exert their apoptotic effects in A-431 cells by inducing oxidative stress, we evaluated the levels of ROS after 48 hr treatment with Dihydroartemisinin, with or without NAC. The results indicated that Dihydroartemisinin promoted ROS production in these cells in a dose-dependent manner (Fig. 3).

Figure3:

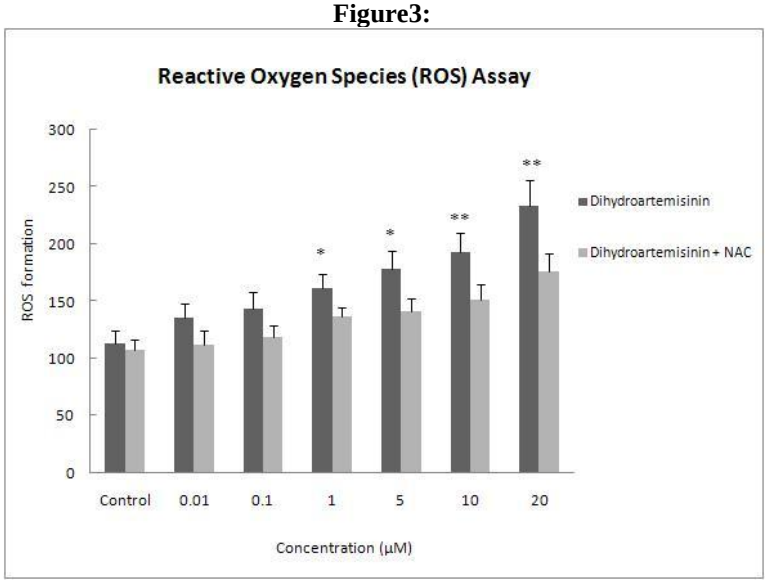

Figure3: Effects of Dihydroartemisinin on reactive oxygen species (ROS) generation in A-431 cell line. After treatment with different doses (0.01-20μM) of Dihydroartemisinin in the presence and absence of NAC (1 mM) for 48 hr, cells were loaded with dichlorofluorescin diacetate and fluorescence was measured by Microplate Reader. The results shown are mean ± SD of three independent experiments. * $P < 0.05$; ** $P < 0.01$ compared with control group.

IV. DISCUSSION

In this study, we have investigated the effect of Dihydroartemisinin (DHA) as novel anticancer drug for skin cancer. Our results demonstrated that DHA inhibits cell proliferation and induces apoptosis in concentration-dependent manner. In addition, our results also indicate that DHA efficiently triggers cell apoptosis via ROS signaling pathways.

Artemisinin and its derivatives such as Dihydroartemisinin (DHA) have been reported to induce apoptosis in pancreas, prostate, colon and neuroblastoma [8-11]. Dihydroartemisinin has been found as an anti-proliferative agent in vitro and in vivo [25]. In particular, Dihydroartemisinin was considered as a potential antitumor agent in several cancer cell lines [6–12]. Based on these findings, we explored to determine the growth inhibitory effect of Dihydroartemisinin on the human skin cancer cells and the possible mechanism of its action was investigated. Here we demonstrated that Dihydroartemisinin inhibited skin cancer cell proliferation through induction of cell apoptosis.

Cell death has been shown to occur by 2 major mechanisms, necrosis and apoptosis (a programmed cell death). Classical necrotic cell death occurs due to noxious injury or trauma, while apoptosis takes place during normal cell development, regulating cellular differentiation. While necrotic cell death results in cell lysis, cellular apoptosis is characterized morphologically by cell shrinkage, nuclear pyknosis, chromatin condensation, and blebbing of the plasma membrane. It seems that all known anticancer drugs kill cancer cells predominantly through apoptosis [26, 27].

In addition, induction of apoptosis by the treatment of the cells with the different concentrations (0.01-20 μM) of Dihydroartemisinin was observed as externalization of phosphatidylserine, and ROS formation. Lu et al. demonstrated that Dihydroartemisinin induced apoptosis in human lung adenocarcinoma cells by up-regulation of Bax protein family [17].ROS are involved at several different points in the apoptotic pathway, including loss of mitochondrial membrane integrity with the attendant release of mitochondrial apoptogenic factors, intracellular caspase activation, and DNA damage [26, 27]. The role of ROS in the apoptosis induced by Dihydroartemisinin investigated in cancer cells [18]. Lu et.al demonstrated that Dihydroartemisinin induced apoptosis via ROS formation in ASTC-a-1 cells [18]. In this study, it has been shown that Dihydroartemisinin

induced ROS formation in A-431 human skin cancer cells. Moreover, we demonstrated that NAC is capable to protect cells from cytotoxic effect of Dihydroartemisinin.

V. CONCLUSION

The present study shows that Dihydroartemisinin can inhibit A-431 skin cancer cells proliferation via induction of apoptosis. Apoptosis induced by Dihydroartemisinin was determined by externalization of phosphatidylserine, and ROS formation. Furthermore, this study introduces a possible mechanism for Dihydroartemisinin on the control of skin cancer cell growth, i.e. involvement of ROS pathway. In this study, the primary anticancer effect of Dihydroartemisinin *in vitro* is evaluated, and to determine the effect of Dihydroartemisinin *in vivo* further investigation are required.

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