Original Article

Extracellular Caspase-8 Dependent Apoptosis on HeLa Cancer Cells and MRC-5 Normal Cells by ICD-85 (Venom Derived Peptides)

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Abstract

Background: Our previous studies revealed an inhibitory effect of ICD-85 (venom derived peptides) on MDA-MB231 and HL-60 cell lines, through induction of apoptosis. The purpose of this study was to investigate apoptosis-induced mechanism on HeLa and MRC-5 cells by ICD-85 through activation of caspase-8.

Methods: Cell viability, cytosolic enzyme Lactate Dehydrogenase (LDH) and cell morphology were assessed under unexposed and ICD-85 exposed conditions.Caspase-8 activity was assayed by caspase-8 colorimetric assay Kit.

Results: The results show that Inhibitory Concentration 50% (IC_{50}) value of ICD-85 for HeLa cells at 24 h was estimated and found to be 25.32±2.15 µg/mL. Furthermore, treatment of HeLa cells with ICD-85 at concentrations of 1.6×10 and 2.6×10 µg/mL did not significantly increase LDH release. Morphological changes in HeLa cells on treatment with ICD-85 compared with untreated HeLa cells consistent with an apoptotic mechanism of cell death, such as cell shrinkage which finally results in the generation of apoptotic bodies. However, when MRC-5 cells were exposed to ICD-85, no significant changes in cell morphology and LDH were observed at concentrations below 2.6×10µg/ml. Also, the apoptosis-induction mechanism by ICD-85 on HeLa cells was found through activation of caspase-8 and the activity of caspase-8 in HeLa cells was 1.5 folds more than its activity on MRC-5 cells.

Conclusion: Therefore, the apoptosis-induced mechanisms by ICD-85 are through activation of caspase-8 and concerning the least cytotoxic effect on MRC-5 cells, ICD-85 may be used as anticancer compound to inhibit growth of cancer cells.

Keywords: Caspase-8; Apoptosis; Extrinsic pathway; HeLa cells

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Introduction

Apoptosis is generally mediated by caspase cascades that lead to cleavage or activation of molecules that are important for cell death [1-3]. Different types of apoptotic stimuli can trigger cell death by different mechanisms. Bind to cell surface receptors and in turn induce the activation and cleavage of the initiator caspases, such as caspase-8 and caspase-10 [4]. Once activated, caspase-8 can activate two different apoptotic pathways [5]. First, it can directly cleave and activate effecter caspases, such as caspases-3 and 7 which mediating the apoptotic response [2, 3]. Second, caspase-8 can activate a mitochondrial pathway which is mediated by the caspase-8 substrate causing cytochrome C release leading to apoptosis [5-10]. Some reports revealed the existence of enzymes in venom causing cellular caspases activation including caspase-8 [11-14]. A haemorrhagic metalloproteinase purified from *Bothrops asper* snake venom was reported to activate caspase-8 [11]. Our previous studies on ICD-85 revealed the growth inhibition of cancer cell lines including MDA-MB 231[15, 16] and HL-60 [17]. On the other hand DNA laddering and cell morphological studies clearly showed that the inhibition of cancer cell lines by ICD-85 is through induction of apoptosis [17]. Hence, the present investigation was undertaken to answer the questions that whether the induction of apoptosis by ICD-85 is through external stimulation of cell killer receptors or internal receptors? Also, is the apoptotic inducing activity of ICD-85 on cancer cells differs from normal cells?

Materials and Methods

Chemicals

The Dulbecco's Modified Eagle Medium (DMEM) as cell culture medium, Fetal Bovine Serum (FBS), penicillin and streptomycin were purchased from Gibco BRL (Life Technologies, Paisley, Scotland). Human fibroblast cell line (MRC-5) and cervical adenocarcinoma cell line (HeLa) were obtained from cell bank (Razi Vaccine and Serum Research Institute, Karaj, Iran). The 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Roche Diagnostics GmbH (Germany). Lactate Dehydrogenase (LDH) assay Kit was purchased from Sigma-Aldrich(USA). FLICE/Caspase-8 colorimetric assay Kit was obtained from BioVision (USA).

ICD-85 (venom derived peptides)

The active fraction of ICD-85 is a combination of three peptides, ranging from 10,000 to 30,000 Da, derived from the venoms of an Iranian brown snake (*Agkistrodon halys*) and a yellow scorpion (*Hemiscorpius lepturus*). This fraction was formulated and provided by the corresponding author. The ICD-85 peptides were selected based on a study of crude venom cytotoxicity. The crude venom showed antigrowth activity on the MBA-MD231 cell line. Then, the venoms were fractionated; the active peptides were isolated and, sub sequentially, tested on the same cell line. Enzymatic characterization of the peptides was not performed [15, 16].

Cell Culture

Cell lines were cultured in the DMEM medium with addition of FBS (10%, v/v), streptomycin (100 µg/mL) and penicillin (100 U/mL). The cells were grown in CO_2 incubator (memmert, Germany) at 37˚C with 5% CO_2 and 90% humidity [18, 19].

Cell Proliferation Assay

Mitochondrial function and cell viability were measured by the MTT assay. The cells were plated into a 96 well plate at a density of 2×10^4 cells/well. The cells were grown overnight in the full medium and then exposed to serial concentrations of ICD-85(1×10^{-3} to 6×10 µg/mL) for 24 h. Following the treatment, the cells were incubated with MTT (5 mg/mL) for 4 h. The medium was then removed and 100 µL of Dimethyl Sulfoxide (DMSO) were added into each well to dissolve formazan crystals, the metabolite of MTT. After thoroughly mixing, the plate was read using ELISA plate reader at 570 nm for optical density that is directly correlated with cell quantity. Survival rate was calculated from the relative absorbance at 570 nm and expressed as the percentage of control [20-23].

The percentage of inhibition and viability were calculated using the following formula: [24, 25].

$$\text{Inhibition (\%)} = [1 - (\text{treated}/\text{control})] \times 100$$

$$\text{Viability (\%)} = 100 - \text{Inhibition (\%)}$$

Lactate Dehydrogenase (LDH) Release Assay

The LDH is a cytoplasmic enzyme retained by viable cells with intact plasma membranes, but released from necrotic cells with damaged membranes. The LDH catalyses the conversion of lactate to pyruvate upon reduction of NAD^+ to $NADH/H^+$; the added tetrazolium salt is then reduced to formazan. HeLa and MRC-5 cells (density = 1×10^6 cells/mL) were cultured for 24 h at 37˚C in the absence or in the presence of ICD-85 at various concentrations (1.6×10 to 6×10 µg/mL). This assay was performed according to the manufacturer's instructions (CytoTox 96® Promega, Mannheim, Germany). Absorbance values at 492nm were determined photometrically with a 96 well plate reader (Bio-Tek, USA) [26, 27].

Morphologic Analysis Using an Inverted Microscope

Morphological studies using a normal inverted microscope were carried out to observe the morphological changes of cell death in cancer and normal cells treated with ICD-85. The untreated cells served as the negative control [28].

Caspas-8 Assay

The activity of caspase-8 (also known as FLICE) was examined using colorimetric assay Kit according to the manufacturer's instructions (BioVision, USA). The assay is based on the spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labelled substrate that recognizes an optimal tetrapeptide sequence of the individual activation sites. Briefly, supernatants of cells were collected and added into 96 well plates. Final reaction buffer (50 µL) and 5 µL caspase-8 colorimetric substrate (IETD-pNA) were then added to each well. After incubation at 37˚C for 2 h, the release of pNA was measured at 405nm using a micro-plate spectrophotometer. Fold-increase in FLICE activity was determined by comparing the results of treated samples with the level of the uninduced control [26, 29].

Statistical Analysis

Values are expressed as means±SD of three repeats in each group. Data were analysed using student's t-test with statistical significance for $p<0.05$.

Results

Cytotoxicity on HeLa and MRC-5 cells

Figure 1 shows the effect of various concentrations of ICD-85 on HeLa cell line. It indicates the decreased HeLa cells viability after the treatment with ICD-85 in a dose-dependent manner. The loss of

cell viability is maximal at final concentrations of 5×10 and 6×10 µg/mL (Figure 1). The IC50 value of ICD-85 for HeLa cancer cells at 24 h was estimated and found to be 25.32±2.15 µg/mL. On the other hand ICD-85 showed no significant growth inhibitory effect on normal lung cells MRC-5 at concentrations less than 5×10 µg/mL as compared to unexposed cells. However, the inhibition effect of ICD-85 on MRC-5 cells was about 30% at concentrations 5×10 and 6×10 µg/mL (Figure 1).

Lactate Dehydrogenase (LDH) Activity

Although treatment of HeLa cells with ICD-85 at concentrations of 1.6×10 and 2.6×10 µg/mL did not significantly increase LDH release, indicating that the treatment with ICD-85 maintains the integrity of plasma membrane in HeLa cells (Figure 2), but when ICD-85 concentration increased to 3.6×10 µg/mL and above, the LDH activity in the cultured media increased significantly ($p<0.05$). However, the treatment of MRC-5 cells with various concentrations of ICD-85 also revealed the significant ($p<0.05$) increase in LDH activity at concentrations above 3.6×10 µg/mL (Figure 2).

Morphological Evaluation

HeLa and MRC-5 cells were exposed to ICD-85 for 24 h and morphological changes were examined using invert microscopy. Figure 3-A and 3-C showing the morphology of control (unexposed to ICD-85) HeLa and MRC-5 cells respectively. While significant morphological changes in HeLa cancer cells were observed after ICD-85 treatment characterising the features of apoptosis such as cell rounding and granulation (Figure 3-B), no significant morphological alterations were found in MRC-5 cells treated with same concentration of ICD-85 (Figure 3-D).

ICD-85 Induces Caspase-8-dependent Cell Death

To further characterize the apoptotic pathway induced by ICD-85, we determined the activities of caspase-8. Various concentrations of ICD-85 treatment of HeLa cells for 24 h, caused significant ($p<0.05$) increase in the activity of caspase-8 at IC_{50} concentration by 1.5 folds and at maximum concentration (6×10 µg/mL) by 2 folds (Figure 4). The rise in caspase-8 activity of HeLa cells exposed to ICD-85 was dose-dependent manner. However, when MRC-5 cells were exposed to ICD-85 at various concentrations, the negligible rise in the activity of caspase-8 was not significant as compared to unexposed MRC-5 cells (Figure 4). However comparing the activity of caspase-8 in

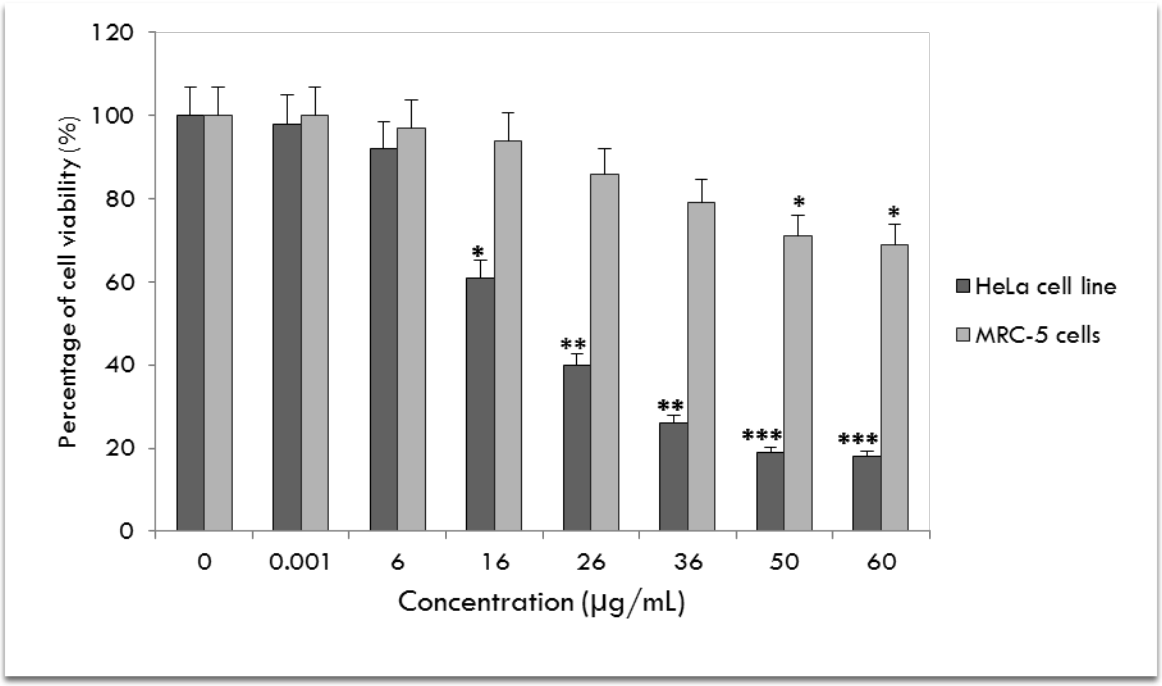


Figure 1. Cytotoxic effect of ICD-85 on HeLa and MRC-5 cells. Cell viability of HeLa and MRC-5 cells exposed to ICD-85 in concentrations ranging from 1×10^{-3} to 6×10 µg/mL at 24 h and measured by MTT assay. Data are mean ± SD from three independent determinations in triplicate. *$p<0.05$, **$p<0.005$ and ***$p<0.001$ were considered to be statistically significant, compared with values from cells incubated in the absence of ICD-85 (controls).

HeLa and MRC-5 cells after exposure to ICD-85 revealed the significant ($p<0.05$) 1.5 folds greater activity of caspase-8 in HeLa as cancer cells to MRC-5 as normal cells at IC_{50} concentration.

Discussion

Apoptosis or programmed cell death is a fundamental event that plays an important role in the homeostasis and development of an organism [30-32]. One of the major defects in cancer is the lack of cells to be driven into the apoptotic mode, due to malfunction of molecules like c-myc [33], ras [34], p53, Bcl-2, caspases and telomerase [35- 38]. Hence, we thought that targeting such molecules in cancer might provide a new therapeutic strategy. With this background we tried to evaluate the anti-proliferative effect of ICD-85 and mechanism of induction of apoptosis in cultured HeLa cells. ICD-85 used in the present study is combination of 3 peptides isolated partially from two different venoms. Both the venoms have strong cytotoxic activity on cancer cells and hence they are selected based on their anti-proliferative activity on cancer cell lines. The combination of these peptides was used because they work together synergistically having anti-proliferative activity along with anti-angiogenic activity while the toxicity in normal cells remains unchanged (unpublished data).

In this study HeLa as cancer cell line and MRC-5 as normal cells were used. Cytotoxicity measured by MTT assay clearly indicated a dose-dependent loss of HeLa cell viability after ICD-85 treatment. The efficacy of this method (MTT assay) has been extensively demonstrated [39-42]. This method is based on the ability of mitochondrial dehydrogenases in living cells to reduce soluble tetrazolium salts to a blue formazan product whose amount is directly proportional to the number of living cells [21-23]. When IC_{50} of ICD-85 in HeLa cells was determined it was found to be about 25.32 ± 2.15 µg/mL after 24 h treatment. The IC_{50} is a useful parameter for quantification of the drug effect on the cell survival [43-45]. Furthermore, in our previous studies we showed that the ICD-85 was cytotoxic to HL-60 cell line, through induction of apoptosis [17]. However, the IC_{50} determined in HL-60 cells was comparatively lower (0.04µg/ml) than HeLa cells. This can be due to the nature and

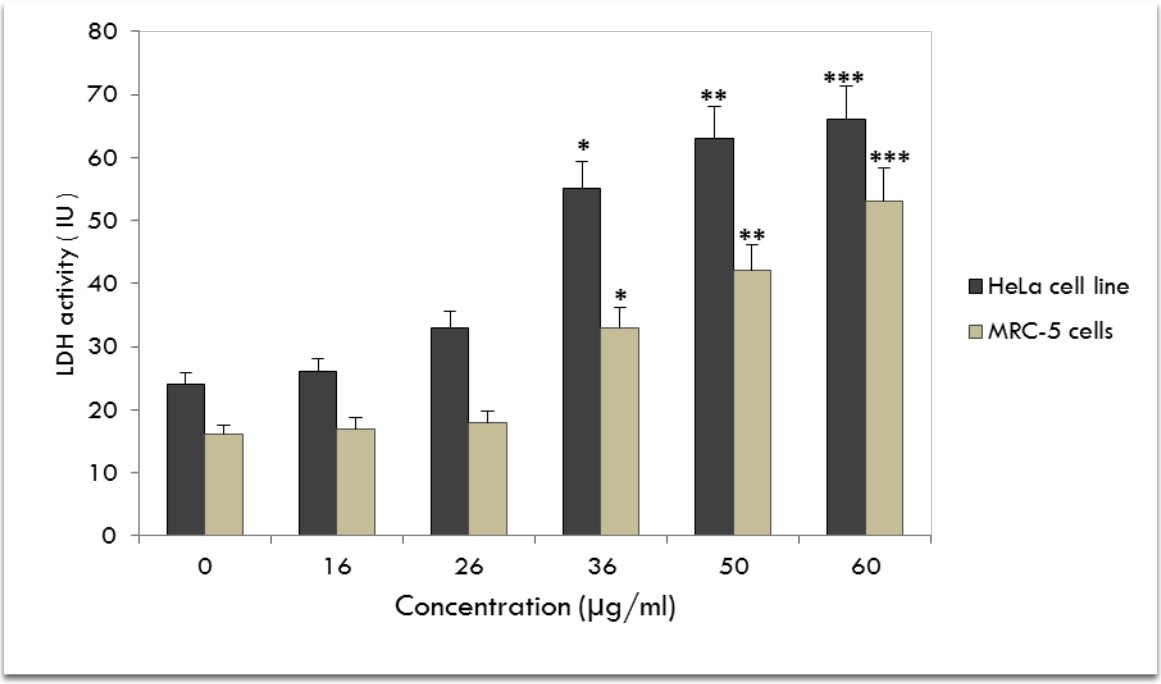
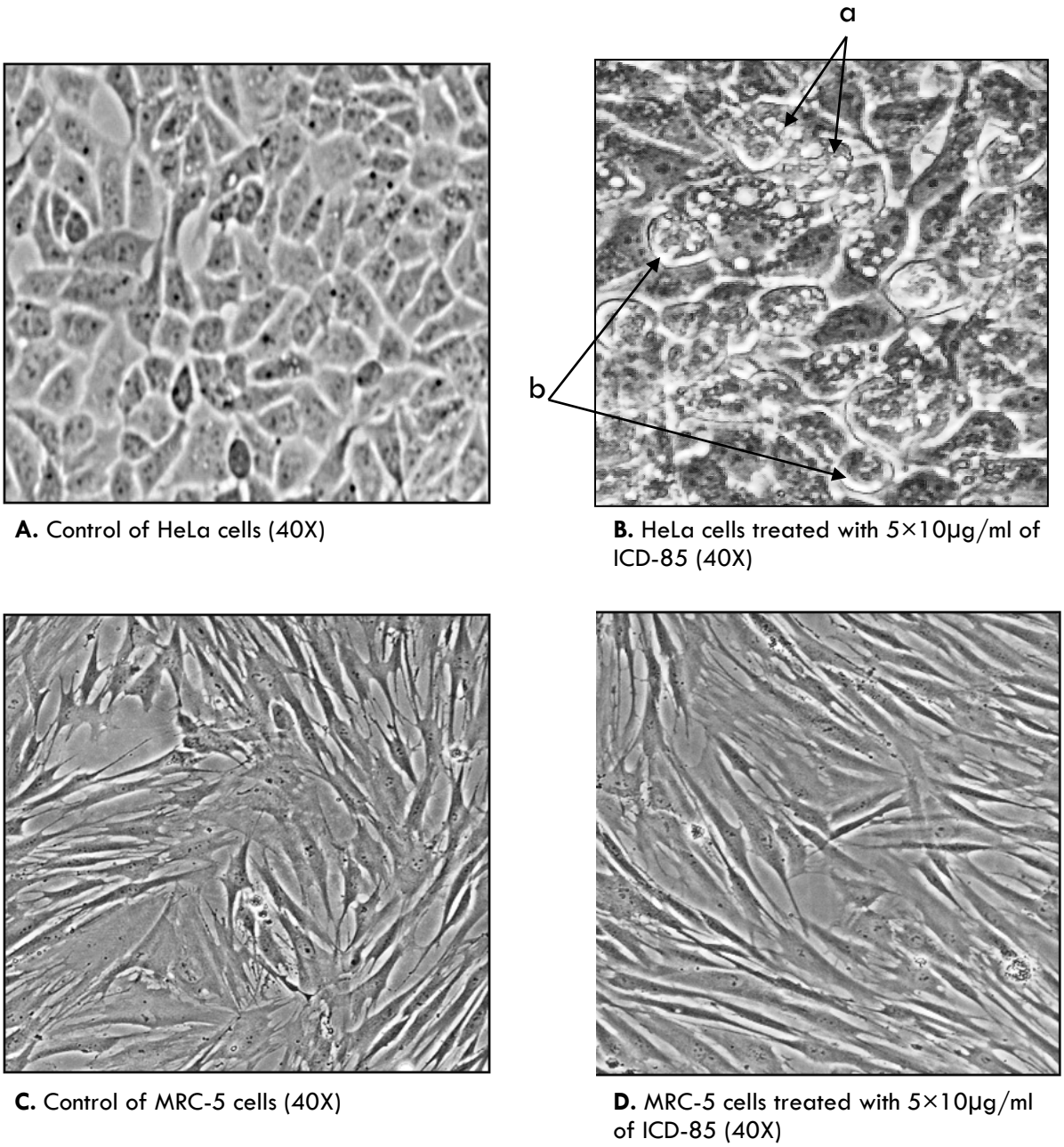


Figure 2. Lactate Dehydrogenase (LDH) activity in HeLa and MRC-5 cells treated with ICD-85. Activity of cytosolic enzyme Lactate Dehydrogenase (LDH) after treatment of HeLa and MRC-5 cells in the presence and in the absence (control) of ICD-85 at various concentrations of 1.6×10 to 6×10µg/ml. The cells were cultured in DMEM medium supplemented with 10% FBS for 24 h at 37°C. Data are mean ± SD from three independent determinations in triplicate.*P< 0.05, ** P < 0.005 and ***P < 0.001 were considered to be statistically significant, compared with values from cells incubated in the absence of ICD-85 (controls).

susceptibility of different cells.

When LDH activity of culture median which HeLa cells grown and exposed to ICD-85 was determined, no significant effects were observed in the LDH release below concentration of 2.6×10 µg/mL. However, when concentration was above 3.6×10 µg/mL, the LDH activity of ICD-85 increased significantly ($P < 0.05$). The LDH leakage assay is based on the release of the enzyme into the culture medium after cell membrane damage while the MTT



A. Control of HeLa cells (40X)

B. HeLa cells treated with 5×10µg/ml of ICD-85 (40X)

C. Control of MRC-5 cells (40X)

D. MRC-5 cells treated with 5×10µg/ml of ICD-85 (40X)

Figure 3. Effect of ICD-85 on morphology of HeLa and MRC-5 cells. HeLa and MRC-5 cells ($1×10^6$ cells/mL) were cultured in DMEM medium supplemented with 10% FBS and treated in the absence (control cells), or in the presence of ICD-85 at 5×10 µg/mL for 24 h at 37°C. Morphological changes of treated cells were observed by inverted microscopy and compared with control cells. The arrows in figure B (40X) showed cell rounding (b) and granulation (a) in HeLa cancer cells treated with ICD-85 as compared to untreated HeLa cells (A, 40X). There are no significant morphological changes in MRC-5 normal cells treated with ICD-85 (D, 40X) as compared to untreated MRC-5 cells (C, 40X).

assay is mainly based on the enzymatic conversion of MTT in the mitochondria [10, 46-48]. It has been well documented that lactate dehydrogenase levels (as a marker of necrosis) in the cell medium elevated after the cells exposed to anticancer agents [49, 50]. Kathikeyan et al. assessed the cytotoxic effect of snake venom by the release of LDH from HeLa and HEp2 cells after treating with the venom which exhibited positive results without change in LDH level [51]. No change in LDH release along with cell proliferation inhibition indicates cell recovery to normal function [51]. However, in this study, most of the HeLa cells exposed to ICD-85 showed cell shrinkage, which can be important marker of apoptosis [23]. It is possible that increase in LDH activity of HeLa cells exposed to high concentrations of ICD-85 is due to necrotic effect rather than apoptotic effect which observed at low concentrations. On the other hand MRC-5 cells exposure to ICD-85 at concentration lower than 2.6×10µg/ml did not show any significant rise in LDH levels. Previous studies by Zare Mirakabadi et al. showed that the ICD-85 effect on normal cells (MRC5), at low concentrations (5, 10 and 15 µg/mL), after 24 h incubation, has no significant cell damage and hence, it appears that ICD-85 acts selectively on cancer cells at low concentration through induction of apoptosis [15].

There are many references that point to the effect of cytotoxic agents on the cell morphology and proliferation pattern [40, 42, 52, 53]. At the present study morphological observations of the HeLa cells exposed to ICD-85 revealed a sort of granulations in the cells. Also, in this work, morphological changes consistent with an apoptotic mechanism of cell death that this phenomenon supported by obtained results of caspase-8 activity in the present study. However, these results are supported by our previous studies on MDA-MB231 [15] and HL-60 cell lines [17] exposed to ICD-85 which showed the shrinkage of cells under light and transmission electron microscopic respectively. This is in accordance with the report of Kerr *et al* too [54]. In contrast, when normal MRC-5 cells were exposed to ICD-85 at concentration similar to HeLa cells, no morphological changes observed when compared to unexposed cells. This is in accordance with the morphological studies by Ardeshiry lajimi et al. that demonstrated,

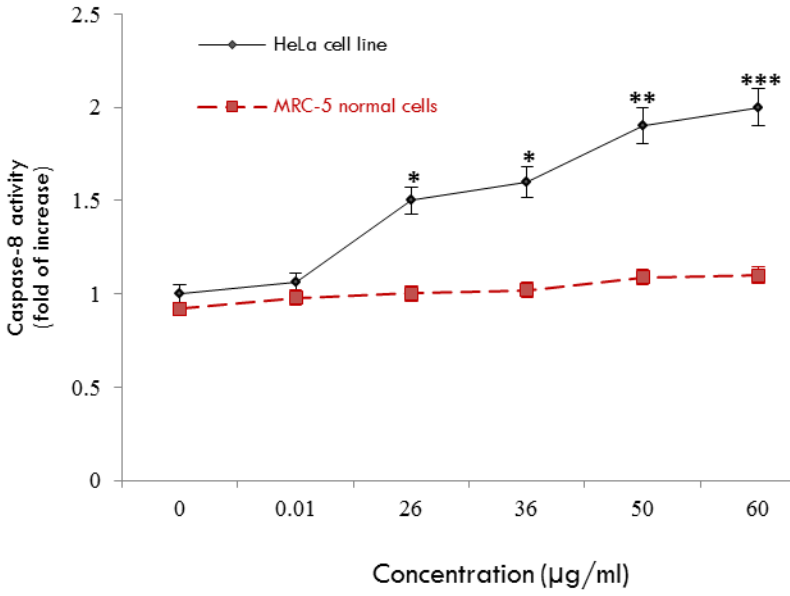


Figure 4. Determination of caspase-8 activity in HeLa and MRC-5 cells treated with ICD-85. Caspase-8 activity in HeLa and MRC-5 cells were evaluated with a colorimetric assay based on the spectrophotometric detection of the chromophore p-nitroanilide (pNA). The cells were cultured in DMEM medium supplemented with 10% FBS for 24 h at 37˚C. For determination of caspase-8 activity, HeLa and MRC-5 cells were treated in the absence and in the presence of ICD-85 at the concentrations of 1×10^{-2} to 6×10µg/ml. Data are mean ± SD from three independent determinations in triplicate. *P<0.05, **P<0.005 and ***P<0.001 were considered to be statistically significant, compared with values from cells incubated in the absence of

Scrophularia striata extract eliminates many cancer cells undergoes granulation while there is no significant effect on normal human fibroblast cell line [19].

Our investigations showed that caspase-8 activity in HeLa cells was significantly ($p<0.05$) increased in the presence of ICD-85 at concentrations of 2.6×10 to 6×10 µg/mL. However, when activity of caspase-8 in MRC-5 cells was evaluated at concentration of 2.6×10 µg/mL ICD-85 no significant increase in enzyme activity was observed. The concentration of 2.6×10 µg/mL was chosen because; it was almost the IC_{50} of ICD-85 on HeLa cells. Caspases-8 and -9 plays a critical role in anticancer drug induced apoptosis, in apoptosis resistance and anticancer drug resistance [55-60]. Since apoptosis-induced mechanism on cancer cells by ICD-85 was unknown for us, in this study we tried to measure caspase-8 activity, as activator of apoptosis through extrinsic pathway and also caspase-8 is an essential component of death receptor-mediated apoptosis [57-60]. Some reports revealed the existence of enzymes in venom causing cellular caspases activation including caspase-8 [11-14]. Many toxins and proteins have been reported to induce apoptosis in the cancer cell by activating the enzymes such as caspase-8 of the apoptotic cascade [61, 62]. In a study has been reported that lionfish venom induces apoptosis in HeLa and HEp2 cells by activating caspase-8 [63]. In the light of these observations it can be suggested that ICD-85 is able to induce caspase-8-dependent apoptosis in HeLa cells [64-66].

Conclusion

In conclusion, in the light of both our previous and current findings it is evident that function of ICD-85 is through inducing apoptosis selectively in cancer cells and one of apoptosis-induced mechanisms by ICD-85 is through activation of caspase-8.

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Conflict of Interest

The authors declare that they have no conflict of Interest.

Authors' Contribution

Ali Sarzaeem carried out the receptor study and wrote the manuscript; Abbas Zare Mirakabadi designed the research. Both authors read and approved the final manuscript.

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