

Original Article

Cytotoxic Effect of ICD-85 (Venom-derived Peptides) on HeLa Cancer Cell Line and Normal LK Cells using MTT Assay

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Abstract

Background: Cancer is the fifth leading cause of death worldwide. There are considerable efforts to identify naturally occurring substances for use as new drugs in cancer therapy. Some components of animal venoms have been identified that possess substantial anticancer properties. In our previous studies, the cytotoxic effects of ICD-85 (venom-derived peptides) have been reported on HL-60 and MDA-MB231 cell lines. This has prompted us to investigate the comparative cytotoxic effects of ICD-85 on the HeLa cell line and normal lamb kidney (LK) cells.

Methods: Cells were exposed to various concentrations (8×10^{-4} to 5.6×10^{-6} µg/ml) of ICD-85 at various incubation times (24, 48 and 72 hours). Cell viability was measured by the MTT assay. A morphological study was also carried out using an inverted microscope. Caspase-8 activity was assayed by the Caspase-8 Colorimetric Assay Kit in HeLa cells that were exposed to ICD-85 for 48 hours.

Results: Data analysis showed that ICD-85 has a dose-dependent cytotoxic effect on HeLa cells with an inhibitory concentration 50% (IC_{50}) of 26.62 ± 2.13 µg/ml at 24 hours, 27.33 ± 2.35 µg/ml at 48 hours, and 28.13 ± 2.52 µg/ml at 72 hours. Results also indicated that the cytotoxic effect of ICD-85, at 48 and 72 hours incubation times did not show significant alteration compared to 24 hours of exposure. Interestingly, the minimum concentration of ICD-85 which showed a cytotoxic effect on LK cells was found to be 3500-fold less than the minimum concentration that showed a cytotoxic effect on the HeLa cancer cells. While morphological analysis revealed a significant difference that included the characteristic rounding of dying cells by treatment with ICD-85 compared with untreated HeLa cells, this difference was not observed in normal cells. ICD-85 increased caspase-8 activity in HeLa cells after 48 hours of exposure.

Discussion: ICD-85 has a dose-dependent cytotoxic effect on HeLa cancer cells in contrast with its negligible effect on normal LK cells.

Keywords: Cytotoxic, ICD-85, HeLa cancer cells, MTT assay, normal LK cells.

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Introduction

Oncologists continue to search for new anticancer drugs that are more potent and have less side effects.^{1,2} Most current anticancer agents do not greatly differentiate between cancerous and normal cells, leading to systemic toxicity and adverse effects.³⁻⁵ With the aim of avoiding cancer therapy failure, several approaches such as the design of new anticancer drugs, chemical engineering of conventional drugs, and development of drug delivery systems have been proposed.⁶

A report of a clinical trial of marine-derived anticancer peptide showed the significant role of natural peptides that could be considered as a future hope in cancer treatment.¹ Most contemporary research in the development of anticancer therapeutics from animal venoms have focused on investigating the molecular mechanism by which an agent induces cytotoxicity and apoptosis in cancer cells.⁷⁻¹¹

Our previous studies have proven that ICD-85 (venom-derived peptides) can inhibit the growth of various cancer cell lines, including HL-60 (in press) and MDA-MB231.^{12,13} In another in vivo

study ICD-85 was able to prevent further growth of breast tumors and expand the life expectancy of mice with breast cancer.¹³ However, the action of ICD-85 on HeLa cancer cells is unclear and its cytotoxic effects on normal lamb kidney cells (LK) is unknown. In the present study we aim to determine the cytotoxic effect of ICD-85 on HeLa cells in a comparison with LK, as normal cells.

Materials and Methods

Materials

The cell culture medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco BRL (Life Technologies, Paisley, Scotland). LK cells and the cervical adenocarcinoma cell line (HeLa) were obtained from a cell bank (Razi Vaccine and Serum Research Institute, Karaj, Iran). 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Roche Diagnostics GmbH (Germany).

ICD-85 (venom-derived peptides)

The active fraction of ICD-85 is a combination of three peptides, that range in size from 10000 to 30000 Da and are derived from the venom of the Iranian brown snake (*Agkistrodon halys*) and yellow scorpion (*Hemiscorpius lepturus*). The ICD-85 peptides were selected based on a study of crude venom cytotoxicity and isolation of cytotoxic peptides by two-step purification that included gel filtration and HPLC.^{12,13}

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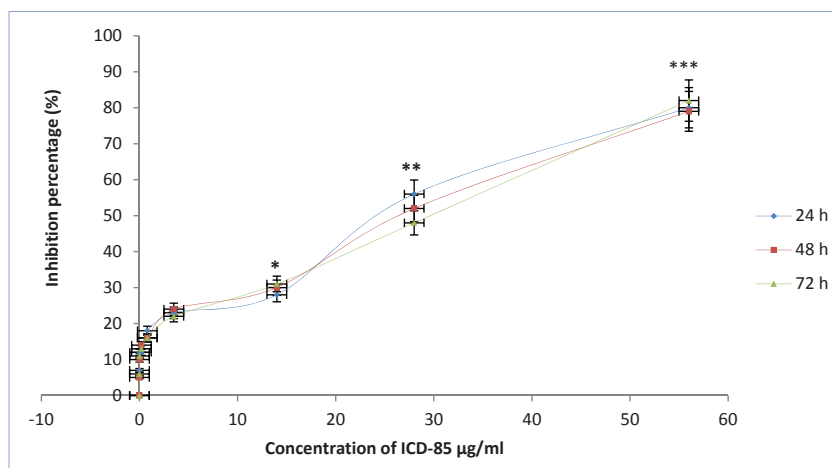


Figure 1. Cytotoxic effects of ICD-85 on HeLa cancer cells at various incubation times (24, 48 and 72 hrs) and concentrations. Growth inhibition of HeLa cells exposed to ICD-85 in concentrations that ranged from 8×10^{-4} to 5.6×10 µg/ml at 24, 48 and 72 hrs, as measured by MTT assay. Data are mean \pm SD from three independent determinations, in triplicate. * $P < 0.05$, ** $P < 0.005$ and *** $P < 0.001$ were considered statistically significant, compared with values from cells incubated in the absence of ICD-85 (controls).

Cell culture

The cells were cultured in DMEM medium with the addition of FBS (10%, v/v), streptomycin (100 µg/ml) and penicillin (100 U/ml). The cells (2×10^4) were seeded, in triplicate, in 96-well plates and incubated at 37°C in 5% CO₂ atmosphere.^{14,15}

Treatment of HeLa cell line and normal LK cells with ICD-85

Cultured cells from previous step were exposed to serial concentrations of ICD-85 (8×10^{-4} to 5.6×10 µg/ml) for 24, 48 and 72 hours. The medium from all wells of the plate were exchanged with fresh medium, after which MTT and DMSO were added and the absorbance of each well was read by an ELISA plate reader.¹⁶

MTT assay for cytotoxicity

After the medium were exchanged with fresh medium, 20 µl of MTT (5 mg/ml) dissolved in phosphate-buffered saline solution (PBS) was added to each well. The plate was covered with aluminium foil and incubated for 4 hours. After removing liquid part from the wells, 100 µl of DMSO was added to each well to solubilize the formazan which formed. Next, Sorensen's glycine buffer (25 µl) was added and the plate was shaken gently for 10 minutes. Immediately, the absorbance of each well was read at 570 nm using an ELISA plate reader. A blank well that contained only culture medium and was used for background correction.¹⁷⁻¹⁹

Determination of inhibitory concentration 50% (IC₅₀) values and cell viability

The concentration that decreased 50% of cell proliferation [inhibitory concentration 50% (IC₅₀)] was determined using serial dilutions of ICD-85 (typically 8×10^{-4} to 5.6×10 µg/ml). Control cells were treated with phosphate buffer solution (PBS) without ICD-85.

The percentage of inhibition was calculated using the following formula:^{20,21}

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

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

Morphological studies

In order to compare the cell morphology in the presence and absence of ICD-85, an inverted microscope (Nikon) was used.

Caspase-8 assay

The extent of caspase-8 activation (also known as FLICE) in HeLa cells treated with ICD-85 was assessed by a commercially available colorimetric assay kit in accordance with the protocol supplied by the manufacturer (BioVision, USA). Briefly, cells were cultured (1×10^6 cells/ml) in 25 cm² flasks (Nunc, Denmark), at a total volume of 5 ml of medium per flask, and incubated with ICD-85 for 48 hours. At the end of treatment, the cell pellet was lysed by the addition of lysis buffer from the kit. The cell lysates were added to 96-well plates (Nunc, Denmark) and incubated with caspase-8 substrate at 37°C for 2 hours. Absorbance in wells was measured at 405 nm. Fold-increase in FLICE activity was determined by comparing the results of treated samples with the level of the uninduced control.

Statistical analysis

Values are expressed as means \pm S.D of six repeats in each group. Data were analyzed using student's t-test with statistical significance of $P < 0.05$.

Results

Cytotoxic effect of ICD-85 on HeLa cancer cells

The cytotoxic effects of cultured HeLa cancer cells treated with various concentrations of ICD-85, for 24, 48, and 72 hours were measured by MTT assay. The results showed that the growth of treated HeLa cells was inhibited compared to HeLa cells that were not exposed to ICD-85. As shown in Figure 1, the IC₅₀ value of ICD-85 for HeLa cells at studied times was: 24 hours (26.62 ± 2.13 µg/ml), 48 hours (27.33 ± 2.35 µg/ml), and 72 hours (28.13 ± 2.52 µg/ml). The results showed the highest inhibition (about 80%) of cancer cells treated with ICD-85 was at a concentration of 5.6×10 µg/ml, whereas the least inhibition (about 0%) was

Table 1. Inhibitory effect of ICD-85 on HeLa cancer cells and normal LK cells.

ICD-85 concentration ($\mu\text{g/ml}$)	8×10^{-4}	1×10^{-3}	1×10^{-2}	8×10^{-1}	35×10^{-1}	1.4×10	2.8×10	5.6×10
Inhibitory effect on HeLa cells (%)	0	6 ± 2.31	13 ± 2.52	15 ± 2.46	24 ± 2.73	31 ± 2.65	47 ± 2.38	83 ± 2.41
Inhibitory effect on LK cells (%)	0	0	0	0	7 ± 2.55	11 ± 2.36	18 ± 2.64	29 ± 2.71
P-value	0	0	0	0	<0.001	<0.001	<0.001	<0.005

Growth inhibition of HeLa cancer cells and LK normal cells exposed to ICD-85 at various concentrations (8×10^{-4} to $5.6 \times 10 \mu\text{g/ml}$) as measured by MTT assay. Data are mean \pm SD from three independent determinations, in triplicate. Comparison was between HeLa and LK cell exposure to ICD-85 at the same concentrations.

noted at a concentration of $8 \times 10^{-4} \mu\text{g/ml}$. However, as seen in Figure 1, no significant differences were noted in the percentage of inhibition observed between 24, 48 and 72 hours of HeLa cell exposure to ICD-85.

Comparative cytotoxic effect of ICD-85 on HeLa cancer cells and normal LK cells

As shown in Table 1 the growth inhibition effect of ICD-85 on the HeLa cell line was about 80% at a concentration of $5.6 \times 10 \mu\text{g/ml}$ while its inhibitory effect on LK cells at the same concentration did not exceed 30%. Additionally, the starting dose for the cytotoxic effect of ICD-85 in HeLa cells was $1 \times 10^{-3} \mu\text{g/ml}$ in contrast with LK cells, which was $35 \times 10^{-1} \mu\text{g/ml}$. When the starting dose of ICD-85 cytotoxicity was compared between LK and HeLa cells, we noted that the cytotoxicity of ICD-85 on LK was

3500-fold less than HeLa cells, which was statistically significant ($P < 0.001$).

Effect of ICD-85 on the morphologies of HeLa and LK cells

Morphological analysis showed a significant difference between HeLa cells unexposed to ICD-85 (control) and those treated with ICD-85. The microscopic cellular images indicated that ICD-85-exposed HeLa cells (Figure 2-B) underwent rounding and granulation compared with unexposed HeLa cells (Figure 2-A) which exhibited polygonal shapes with distinct boundaries and either homogenous or slightly granulated cellular contents. Additionally, LK cells exposed to $2.8 \times 10 \mu\text{g/ml}$ of ICD-85 (Figure 2-D) were similar to unexposed cells (Figure 2-C), with no significant alterations observed.

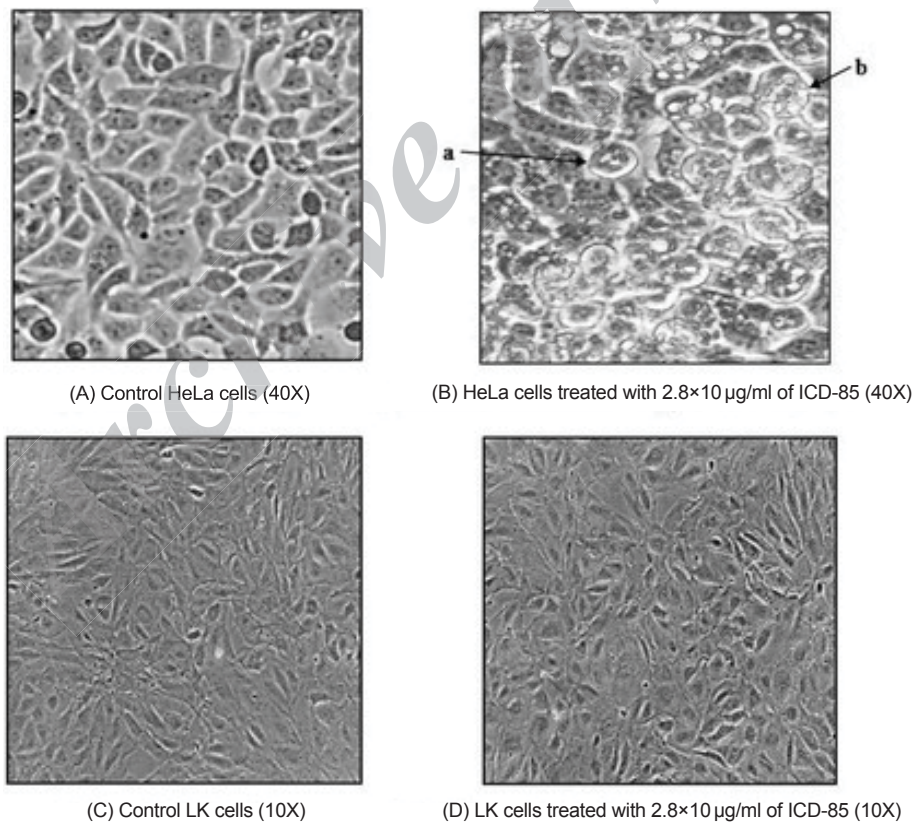


Figure 2. Morphological changes of ICD-85 on HeLa and LK cells. HeLa and LK cells (1×10^6 cells/ml) were cultured in DMEM medium supplemented with 10% FBS and treated in the absence (control cells) or presence of ICD-85 at $2.8 \times 10 \mu\text{g/ml}$ for 24 h at 37°C . Morphological changes of treated cells were observed with an inverted microscope and compared with control cells. The arrows in (B, 40X) showed cell rounding (a) and granulation (b) in HeLa cancer cells treated with ICD-85 compared to untreated HeLa cells (A, 40X). There were no significant morphological changes in normal LK cells treated with ICD-85 (D, 10X) compared to untreated LK cells (C, 10X).

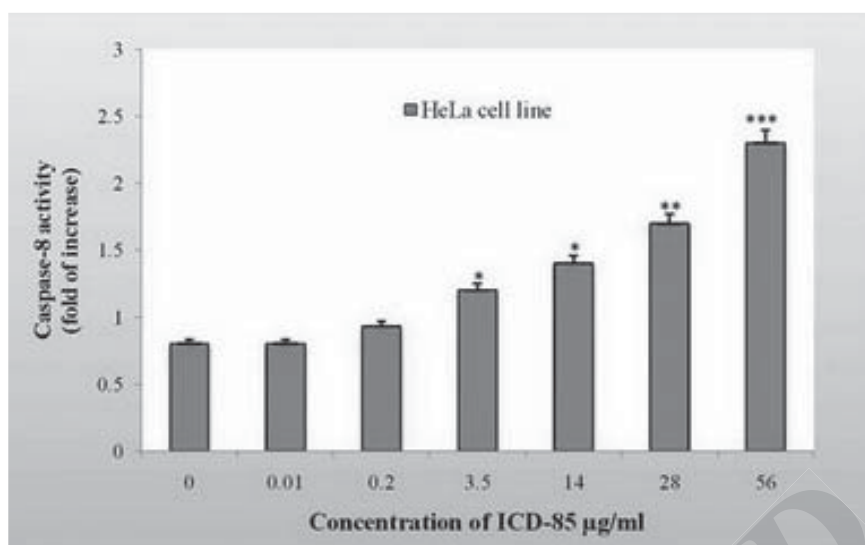


Figure 3. Determination of caspase-8 activity in HeLa cells treated with ICD-85. Caspase-8 activity was evaluated with a colorimetric assay based on the spectrophotometric detection of chromophore p-nitroanilide (pNA). The cells were cultured in DMEM medium supplemented with 10% FBS for 48 hours at 37°C. For determination of caspase-8 activity, HeLa cells were treated in the absence and presence of ICD-85 at concentrations from 1×10^{-2} to 5.6×10^{-1} µg/ml. Data are mean \pm SD from three independent determinations, in triplicate. * $P < 0.05$, ** $P < 0.005$ and *** $P < 0.001$ were considered to be statistically significant when compared with cells incubated in the absence of ICD-85 (controls).

Caspase-8 activity

Treatment of HeLa cells for 48 hours with various concentrations of ICD-85 caused a significant ($P < 0.05$) increase in caspase-8 activity. At an ICD-85 concentration of 2.8×10^{-1} µg/ml, the caspase-8 activity was 1.7-fold and at a concentration of 5.6×10^{-1} µg/ml, it was approximately 2.3-fold (Figure 3). However, the rise in caspase-8 activity was not significant in cells exposed to concentrations less than 2×10^{-1} µg/ml of ICD-85.

Discussion

The search for a cancer cure from natural products (animals and plants) has been ongoing for over a century and the use of purified chemicals to treat cancer still continues.^{22,23} Gomes et al. have suggested that anticancer agent compounds may consist of (i) animal – animal, (ii) animal – plant, or (iii) animal – plant – synthetic.²⁴ The active fraction of ICD-85 used in the present study is a combination of three peptides derived from the venom of two separate species of venomous animals, which appears to have a synergistic effect to suppress the growth of cancer cells.¹²

In this study, we used a HeLa cell line and normal LK cells. The aim of the present study was to compare the cytotoxic effect of ICD-85 between HeLa cancer cells and normal LK cells by MTT assay. The efficacy of this method has been extensively demonstrated.^{17, 25–29} In the current work, the MTT assay confirmed the growth inhibition of HeLa cells by ICD-85 through induction of apoptosis. This result was similar to that reported by Hanif et al.²³ in a study of colon cancer cells. Also, previous studies that utilized the MTT assay have determined that ICD-85 has a cytotoxic effect on the HL-60 cell line through the induction of apoptosis (in press). It has been proven that *Mentha spicata* oil has antiproliferative activity on KB and P388 cell lines as shown by the MTT assay.³⁰ In the current study, according to the MTT assay, ICD-85 had dose-dependent cytotoxicity rather than a time-dependent effect on the HeLa cell line which was in accordance with other re-

ports.^{31–33} Statistical interaction of ICD-85 concentrations showed no significant differences between all concentrations (8×10^{-4} to 5.6×10^{-1} µg/ml) at 24, 48 and 72 hours. Two studies have described that the cytotoxic effect of n-hexane extract of *Curcuma longa*¹⁶ and pure curcumin³⁴ were dose-dependent and not time-dependent on lung cancer cells at different times (24, 48 and 72 hours), which was similar to the current study.

The IC_{50} parameter is defined as the concentration of a chemical that attenuates cell survival to 50%. It is a useful parameter for quantification of the drug effect on cell survival.^{35–37} This study has shown that ICD-85 was selectively cytotoxic against the HeLa cell line, with no significant difference in IC_{50} values obtained at various incubation times (24, 48 and 72 hours).

One of the main obstacles to cancer therapy is the inability to successfully target cancer cells, yet not harm normal cells. Modern medicine desperately needs anticancer molecules that kill cancer cells and leave healthy cells alone.^{38,39} In a previous study by Zare Mirakabadi et al.,¹² it was shown that normal MRC-5 cells treated with ICD-85 at low concentrations (5, 10 and 15 µg/ml) following a 24-hour incubation period had no significant cell damage which confirmed our findings on normal LK cells.

Numerous references point to the effects of cytotoxic agents on the cell morphology and proliferation pattern.^{28,29,40–43} In the present study, ICD-85-induced cytotoxicity in HeLa cancer cells and was involved in the induction of morphological changes. These results were supported by our previous studies on the MDA-MB231 cell line exposed to ICD-85 which showed the shrinkage of cells under light microscopy.¹² Also, in this work, morphological changes were consistent with an apoptotic mechanism of cell death. This phenomenon was supported by the results of caspase-8 activity in the present study. A significant increase in caspase-8 activity by any anticancer agent in cancer cells indicates that the agent works through induction of apoptosis.^{44,45} However when LK cells were exposed to ICD-85 at a concentration similar to HeLa cells, no morphological changes were observed when com-

pared to unexposed cells. This was in accordance with morphological studies by Ardeshiry Iajimi et al. that have demonstrated *Scrophularia striata* extract cause many cancer cells to undergo granulation with no significant effect on normal human fibroblast cell line.¹⁵ Therefore, the results of our morphological studies confirmed data from the MTT assay obtained in a comparison of the cytotoxic effect of ICD-85 on cancer and normal cells. These results indicated that the inhibitory effect of ICD-85 on HeLa cancer cells corresponded to the mechanism of inducing apoptosis.

In conclusion, our present study suggests that ICD-85 has a significant antiproliferative effect, which is dose-dependent on the HeLa cell line in contrast with its non-significant effect on normal LK cells. In view of both our previous and current findings it is evident that ICD-85 functions by a mechanism of selectively inducing apoptosis in cancer cells. Hence, ICD-85 may be considered as a promising chemotherapeutic agent in cancer treatment.

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Conflicts of interest: *There is no conflict.*

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