

Necrotic Effect versus Apoptotic Nature of Camptothecin in Human Cervical Cancer Cells

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Abstract

Background: Functional defects in mitochondria are involved in the induction of cell death in cancer cells. The process of programmed cell death may occur through the mechanisms of apoptosis. Several potential lead molecules such as Camptothecin (CPT) and its analogues have been isolated from plants with anticancer effect. The aim of the present study was to understand the necrotic effect versus apoptotic nature of CPT in HeLa cancer cells.

Methods: The anti-proliferative activity of CPT was estimated through 3-(4, 5-Dimethyl Thiazol-2-yl)-2, 5-diphenyl Tetrazolium bromide (MTT) assay and DNA fragmentation analysis using gel electrophoresis. Lactate Dehydrogenase (LDH) activity and cell morphology were assessed under control and CPT exposed conditions to evaluate the necrotic effect of CPT.

Results: The results showed that CPT inhibited the proliferation of HeLa cells in a dose-dependent manner with an Inhibitory Concentration 50% (IC₅₀) of 0.08±0.012 µg/ml. However the significant (p<0.05) increase happens in LDH activity at concentrations 1×10⁻¹ µg/ml and above. Morphological changes showed that CPT in low concentrations induced an apoptotic mechanism of cell death, such as cell shrinkage and characteristic rounding of dying cells, while at high concentrations showed necrosis changes. The characteristic DNA ladder formation of CPT-treated cells in agarose gel electrophoresis confirmed the results obtained by light microscopy and LDH assay.

Conclusion: Camptothecin as an anticancer drug may have anti-proliferative effect on HeLa cancer cells in low concentrations, through its nature of induction of apoptosis. The border line between necrotic effect and apoptotic nature of CPT in HeLa cancer cells has been found to be at concentration of 1×10⁻¹ µg/ml.

Keywords: Cell death; Camptothecin; HeLa cells; Necrosis; Apoptosis

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Introduction

Apoptosis and necrosis represent two fundamental types of cell death [1-4]. Apoptosis is an important and active regulatory pathway of cell growth and proliferation in which cells respond to specific induction signals by initiating intracellular processes that result in characteristic physiological changes occurring over hours or days [5-10]. In contrast, necrosis occurs suddenly with mitochondrial and cellular swelling and ensuing plasma membrane disruption [11]. However, cell death, through necrosis or apoptosis mechanisms, is currently the subject of a considerable number of investigations [12-14].

Some natural and chemical compounds are said to be cytotoxic to cell that cause its death [3, 4]. In the

early sixties, the discovery of Camptothecin (CPT) by Wall and Wani, as an anticancer drug with a unique mode of action, which is inhibition of DNA topoisomerase I, was an entirely new dimension in chemotherapy [15-17]. This natural alkaloid was first extracted from the stem wood of the Chinese ornamental tree *Camptotheca acuminata* [17-20]. There are many studies that confirmed anti-proliferative and cytotoxic effect of CPT in different cancer cells [17, 21-25].

Although CPT showed strong antitumor activity among cancer patients, it also caused unpredictable and severe adverse effects including myelosuppression, vomiting, diarrhoea, and severe haemorrhagic cystitis [17]. However, at present, CPT is not used as a drug of choice due to its severe

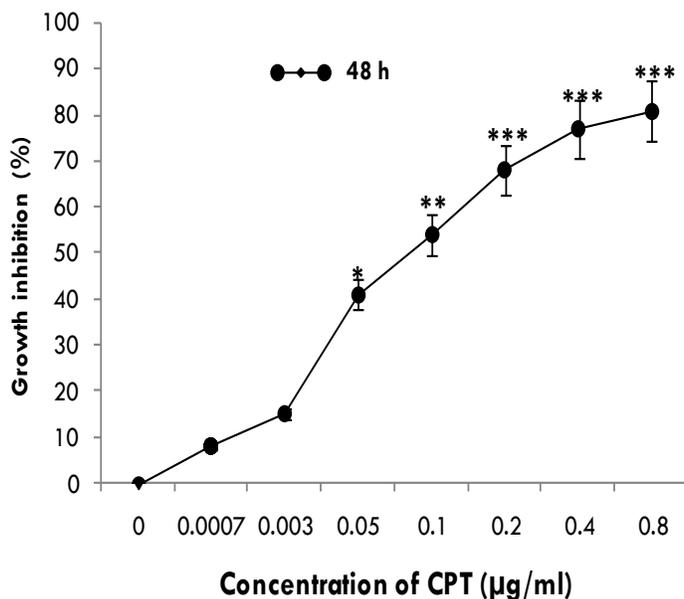


Figure 1. Anti-proliferative effect of CPT on HeLa cells. Growth inhibition of HeLa cells exposed to CPT in concentrations ranging from 7×10^{-4} to 8×10^{-1} µg/ml at 48 hrs and measured by MTT assay. Data are mean \pm SD from three independent determinations in triplicate. * $p < 0.05$, ** $p < 0.005$ and *** $p < 0.001$ were considered to be statistically significant, compared with values from cells incubated in the absence of CPT (control).

toxicity effect in deferent cells. Thus, several groups have been tried to synthesize derivatives with a lower toxicity [26-29]. The determination of border line concentration between necrotic effect and apoptotic nature of CPT in cancer cells is not clear so we attempt in the present study to determine it.

Materials and Methods

Materials

Streptomycin, Penicillin, Ambistyrin, Tris- Acetate-Ethylenediaminetetraacetic acid (TAE) buffer, Trypsin, Phosphate Buffered Saline (PBS) and Sodium Dodecyl Sulphate were purchased from Himedia Laboratories Pvt. Ltd. (India). Dulbecco's Modified Eagle Medium (DMEM) and 3-(4, 5- Dimethyl Thiazol-2-yl)-2, 5-diphenyl Tetrazolium bromide (MTT) were purchased from Sigma chemical company (St. Louis, MO. USA). The Fetal Bovine Serum (FBS) was also purchased from Gibco BRL (Life Technologies, Paisley, Scotland). Human cervical carcinoma HeLa cells were obtained from cell bank (Razi Vaccine and Serum Research Institute, Karaj, Iran). Camptothecin and Lactate Dehydrogenase (LDH) assay Kit were purchased from Sigma-Aldrich (USA).

Cell Culture and Drug Treatment

The HeLa cells (density= 1×10^6 cells/ml) were cultured in DMEM, supplemented with 10% FBS, 50 U/ml penicillin and 5 mg/ml streptomycin, at an incubator setting of 5% CO₂ and 37°C. The cultured cells were exposed to various concentrations of CPT (7×10^{-4} to 8×10^{-1} µg/ml) for 48 hrs. The medium of all wells of plate were exchanged with fresh medium and then MTT and Dimethyl Sulfoxide (DMSO) were added and absorbance of each well was read using ELISA plate reader [30, 31].

Assessment of Cytotoxicity

The effect of CPT on HeLa cells was determined with MTT (3-(4, 5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) assay. Anticancer drugs/cytotoxic compounds damage the cancerous cells and change the mitochondrial activity. When tetrazolium salts come in contact with the cancerous cells, salts are taken up into the mitochondria due to its net positive charge and plasma membrane potential.

In mitochondria, these coloured tetrazolium salts get reduced to formazan dye by NADH⁺ dependent reaction catalysed by the Mitochondrial Succinate Dehydrogenase Enzymes (MSDE). As this conversion takes place only in the living cell mitochondria, the amount of formazan formed is proportional to the

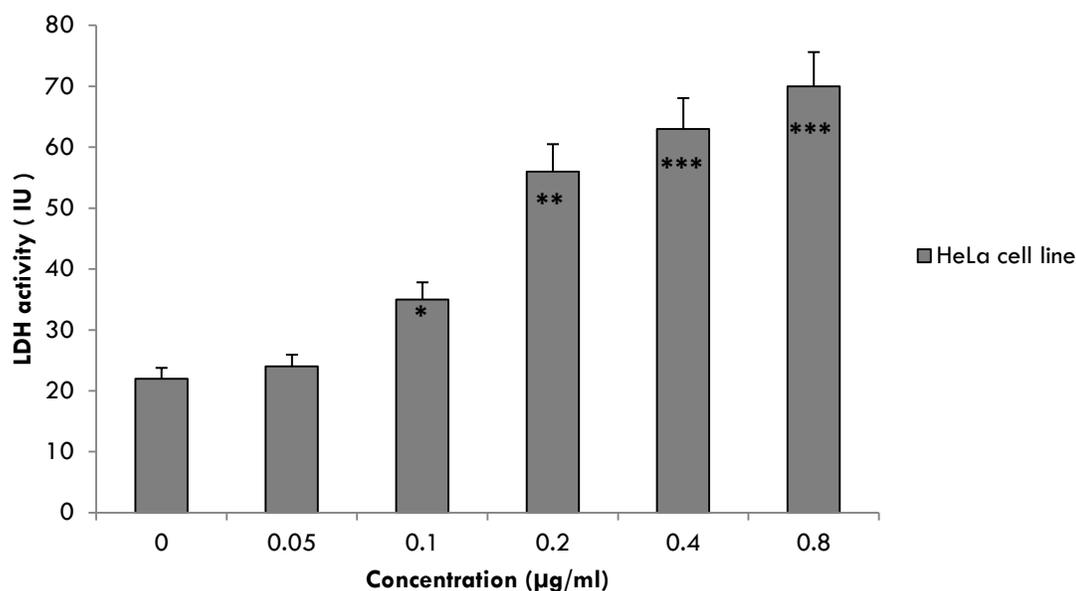


Figure 2. Lactate Dehydrogenase (LDH) activity. Activity of cytosolic enzyme Lactate Dehydrogenase (LDH) after treatment of HeLa cells in the presence and in the absence (control cells) of CPT at various concentrations of 5×10^{-2} to 8×10^{-1} µg/ml. The cells were cultured in DMEM medium supplemented with 10% FBS for 48 hrs at 37°C. 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, compared with values from cells incubated in the absence of CPT (control).

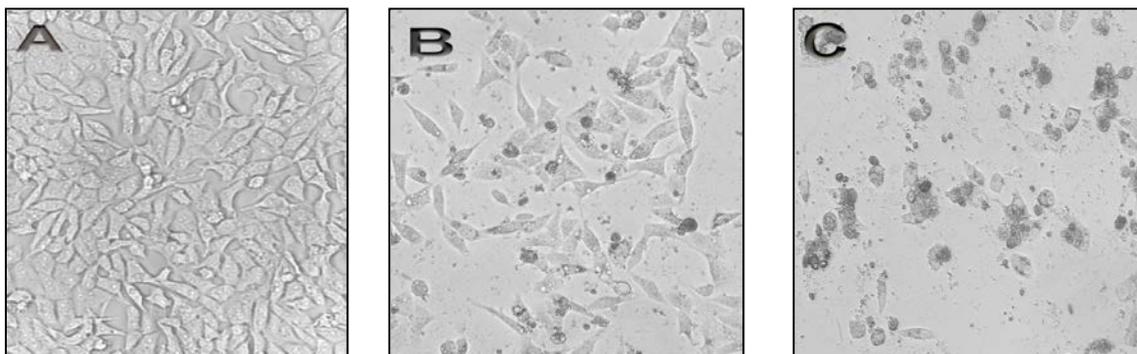


Figure 3. Effect of CPT on morphology of HeLa cells. HeLa cells (1×10^6 cells/ml) were cultured in DMEM medium supplemented with 10% FBS and treated in the absence (control cells), or in the presence of CPT at 5×10^{-2} and 4×10^{-1} µg/ml for 48 hrs at 37°C. Morphological changes of treated cells were observed by invert microscopy and compared with control cells. (A) Control of HeLa cells. (B) HeLa cells treated with 5×10^{-2} µg/ml of CPT. (C) HeLa cells treated with 4×10^{-1} µg/ml of CPT. CPT= Camptothecin.

number of living cells. Quantification of viable cells is made possible by quantification of formazan at 570 nm by a spectrophotometer [32-35]. Cells were seeded in a 96-well flat-bottom plate (10,000 cells/well) and left to adhere for 24 hrs at 37°C with 5% CO₂. Various concentrations of CPT were added

and incubated for further 48 hrs and then MTT (5mg/ml) was added. The absorbance of each well was determined at 570 nm using an ELISA plate reader. The percentage of growth inhibition was calculated using the following formula [36, 37].

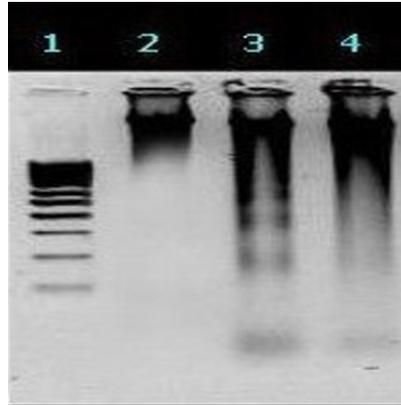


Figure 4. Shows the DNA fragmentation analysis. Lane 1: Molecular weight marker, Lane 2: Untreated HeLa cells, Lane 3: CPT ($5 \times 10^{-2} \mu\text{g/ml}$) treated HeLa cells, Lane 4: CPT ($4 \times 10^{-1} \mu\text{g/ml}$) treated HeLa cells. CPT= Camptothecin.

$\text{Growth inhibition (\%)} = 100 - [\text{OD of individual test group} / \text{OD of control group}] \times 100$

LDH Determination

LDH is a cytoplasmic enzyme retained by viable cells with intact plasma membranes, but it released from necrotic cells with damaged membranes. Cytotoxicity induced by CPT was also assessed by LDH leakage into the culture medium. Following exposure to CPT, the cells were harvested and LDH activity was assayed spectrophotometrically at 340 nm by LDH assay kit [38, 39].

Light Microscopy

HeLa cells (1×10^6 cells/ml) were cultured in the absence (control cells), and presence of CPT at concentrations 5×10^{-2} and $4 \times 10^{-1} \mu\text{g/ml}$ for 48 hrs in DMEM medium supplemented with 10% FBS at 37°C . Morphological changes of treated cells were observed by invert microscopy and compared with control cells [40].

DNA Fragmentation Assay

For DNA fragmentation assay, 2×10^6 HeLa cells, $2 \mu\text{l}$ trypsin and CPT at concentrations of 5×10^{-2} and $4 \times 10^{-1} \mu\text{g/ml}$ were added to the medium and final volume was adjusted to 2ml. The cells incubated at 37°C for 48 hrs, and they were then centrifuged at 10,000 rpm for 10mins. After discarding the medium, the cells were washed twice in NaCl-Tris-Ethylenediaminetetraacetic acid (NTE) buffer. The cells suspended in 2ml NTE buffer and 2% trypsin ($100 \mu\text{g/ml}$), 20% Sodium Dodecyl Sulfate (SDS) ($25 \mu\text{l/ml}$) and proteinase K ($100 \mu\text{g/ml}$) were added. After overnight incubation at 37°C , saturated phenol and chloroform were added and

after shaking the vials, they were centrifuged at 10,000 rpm for 10 min. Having transferred the upper portion to another vial and adding 1ml chloroform, it was repeated for 4 times. RNase was added and incubated at 35°C for 2 hrs. Finally it was centrifuged at 10,000 rpm for 10 min and decanted the solvent. The pellet (DNA) was dissolved in Tris-Acetate- Ethylenediaminetetraacetic acid (TAE) buffer and subjected to horizontal electrophoresis [41, 42].

Statistical Analysis

Values are expressed as means \pm SD of three repeats in each group. Data were analysed using students' t-test with a statistical significance of $p < 0.05$.

Results

CPT Toxicity Against HeLa Cells

The inhibitory effects of CPT on the proliferation of HeLa cells were tested at different concentrations (7×10^{-4} to $8 \times 10^{-1} \mu\text{g/ml}$) for 48 hrs using MTT assay and the IR% was determined (Figure1). Data analysis showed that the growth of HeLa cells was significantly ($p < 0.05$) inhibited as compared to control cells (CPT-unexposed cells) in dose-dependent manner. The starting dose which inhibited the HeLa cells proliferation was $7 \times 10^{-4} \mu\text{g/ml}$, and nearly 80% of HeLa cells were inhibited by $8 \times 10^{-1} \mu\text{g/ml}$ CPT. The Inhibitory Concentration 50% (IC_{50}) was found to be $0.08 \pm 0.012 \mu\text{g/ml}$.

Lactate Dehydrogenase (LDH) Activity

CPT-induced cell membrane damage was assessed by the LDH release assay. Cells were treated with CPT for 48 hrs resulted in a significant ($p < 0.05$) increase in LDH release relative to the

untreated cells (Figure 2). When CPT concentration increased to 1×10^{-1} $\mu\text{g/ml}$ and above, the LDH activity in the cultured media increased significantly ($p < 0.05$). However, CPT caused highly significant ($p < 0.005$) increase in the activity of LDH enzyme at concentration of 2×10^{-1} $\mu\text{g/ml}$ by 2.5 folds and at maximum concentration (8×10^{-1} $\mu\text{g/ml}$) by 3.2 folds as compared with unexposed cells. By contrast, treatment of HeLa cells with CPT at concentrations below 1×10^{-1} $\mu\text{g/ml}$ did not significantly increase LDH release (Figure 2).

Cell Morphology

To assess alterations of cell morphology, HeLa cells treated with CPT for 48 hrs and morphology of cells observed by invert microscopy and compared with untreated cells (control cells, Figure 3-A). Figure 3-B shows that the HeLa cells treated with CPT at 5×10^{-2} $\mu\text{g/ml}$ were changed into round shapes as compared to untreated HeLa cells (Figure 3-A). The untreated cells also showed a high confluency of monolayer cells (Figure 3-A) compared to CPT-treated cells (Figure 3-B and C), which Figure 3-B showed a reduction in cell volume and cell shrinkage that finally resulted in generation of apoptotic bodies. As shown in Figure 3-C, significant morphological changes were observed at concentration of 4×10^{-1} $\mu\text{g/ml}$ after CPT exposure, making features of necrosis such as loss of membrane integrity, no vesicle formation and complete lysis as compared to control cells (Figure 3-A).

Effect of CPT on DNA Fragmentation

DNA fragmentation was observed from the HeLa cells cultured in the presence of CPT at various concentrations for 48 hrs as to compare with untreated cells (Figure 4, lane 2, 3, 4). Data analysis showed that CPT at 5×10^{-2} $\mu\text{g/ml}$ concentration clearly produced characteristic DNA laddering (Figure 4, lane 3). However, as shown in lane 4 no DNA laddering is observed when HeLa cells exposed to CPT at concentration of 4×10^{-1} $\mu\text{g/ml}$.

Discussion

Many studies showed that CPT and its analogues are used clinically against many forms of cancers including lung, breast, ovarian, and colorectal cancer, because they cause cell death and inhibition mechanism of DNA topoisomerase I [43-45]. Although, CPT was considered as a well anti-proliferative agent in cancer cells but at present, it is not used as a drug of choice due to its severe toxicity effect on cells [46-49]. Recently, many CPT analogues are under investigation as cell cancer

inhibitors [50, 51]. Finding the border line between necrotic effect and apoptotic nature of CPT may be useful to find the cause of high toxicity of this agent in cancer patients. This study was designed to investigate the border line between necrotic effects versus apoptotic nature of CPT in human cervical cancer cells (HeLa).

In the present study, HeLa monolayer cell line was used. The use of monolayer cells, instead of floating cells, has some advantages as they are much easier to maintain and handle [52]. Various concentrations of CPT are proved to be cytotoxic in MTT assay (Figure 1). The MTT assay has been used in many experiments for assessment of cytotoxic effects to test agents. In this method the MTT dye, is reduced by living cells and this reaction is used as the end point in a rapid drug-screening assay [36, 53]. The efficacy of this method has been extensively demonstrated [54-57]. In our study, the MTT assay showed that CPT has anti-proliferative effect on HeLa cells in a dose-dependent manner with an IC_{50} of 0.08 ± 0.012 $\mu\text{g/ml}$. IC_{50} is a useful parameter for quantification of drug effect on cell survival [58-60]. Also, the MTT clearly demonstrated that the growth inhibition of HeLa cells by CPT is through induction of apoptosis, at concentrations below 1×10^{-1} $\mu\text{g/ml}$. Recent studies have shown that CPT and its analogues can strongly induce apoptosis in human leukemic cells [27, 50], prostate [26, 50], colon [29] and breast [28, 51] cancer cells as well as glioma cells [61].

LDH is a cytoplasmic enzyme retained by viable cells with intact plasma membranes, but it is released from necrotic cells with damaged membranes [38, 39]. We also studied released Lactate Dehydrogenase (LDH) activity in HeLa cells. Our results showed an increase in LDH activity in culture media of HeLa cells exposed to CPT at concentration above 1×10^{-1} $\mu\text{g/ml}$, while no significant rise was observed in LDH release below concentration of 5×10^{-2} $\mu\text{g/ml}$. It has been well documented that lactate dehydrogenase levels, as a marker of necrosis in the cell medium, elevate when cells are exposed to high concentration of anticancer agents [62, 63]. Also, it is a well known phenomenon that cytotoxic drugs, which can induce apoptosis, promote necrosis when they are administered at higher concentrations which can induce toxicity to normal cells [64-67].

In this study, treatment of the HeLa cells with CPT induced morphological changes and apoptosis, including rounding of cells, reduction in cell volume and cell shrinkage at concentration of 5×10^{-2} $\mu\text{g/ml}$. In contrast, morphological studies on cells treated

with CPT at concentration of $4 \times 10^{-1} \mu\text{g/ml}$, revealed loss of membrane integrity, no vesicle formation and complete lysis of cells. Cell death has been characterized as either apoptotic or necrotic, based on morphological features [4, 68, 69]. Two prominent morphological changes which distinguish apoptosis from necrosis are the formation of apoptotic bodies, and the maintenance of cell membrane integrity [70-74]. However, these morphological features described and confirmed necrotic effect of CPT at high concentrations.

Also DNA fragmentation was analysed in HeLa cells treated with CPT. The results suggest that CPT induces apoptosis in HeLa cells at down stairs concentration ($5 \times 10^{-2} \mu\text{g/ml}$). However, no DNA ladder was observed when the cells were exposed to high concentrations of $4 \times 10^{-1} \mu\text{g/ml}$ CPT. Recent studies have suggested that during apoptosis, a specific nuclease cuts the genomic DNA in nucleosomes to generate DNA fragments. The presence of this ladder has been extensively used as a marker for apoptotic cell death [75, 76].

Conclusion

In conclusion, CPT has an anti-proliferative effect on HeLa cells through apoptosis at a low concentration which is very close to the concentration that cause necrosis to cells. The narrow border line between necrotic and apoptotic effects of this drug was the main reason for its high toxicity in patients. Therefore, a close monitoring of both modes of cell death is vital to evaluate accurately the cytotoxic effects of antitumor agents.

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Conflict of Interest

There is no conflict of interest in this article.

Authors' Contribution

Abbas Zare-Mirakabadi reviewed the manuscript. Ali Sarzaeem designed the study and wrote the manuscript. Saeed Moredhaseli reported the results. Aida Sayad and Masoud Negahdary analyzed the data.

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