A benzophenanthridine alkaloid, chelerythrine induces apoptosis in vitro in a Dalton's lymphoma

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Abstract

Purpose: The aim of this study was to investigate the effect of chelerythrine on DL cell apoptosis in an in vitro experimental setup.

Materials and Methods: For tumor model, spontaneous occurring T-cell lymphoma designated as Dalton's lymphoma (DL) was selected. Double staining, transmission electron microscope (TEM), fluorescence microscopy, Western blotting, Reverse Transcriptase-Polymerase Chain Reaction, and DNA fragmentation assay were used to detect heat shock factor 1 (HSF1) and hsp70 expression and PKC phosphorylation, and apoptotic characteristic of DL cells.

Results: Chelerythrine exposure resulted in significant morphological alteration comparable to that of apoptosis. Furthermore, it was confirmed by fluorescence microscopy, TEM analysis, and DNA fragmentation assay that 10°g/mL of chelerythrine is capable of inducing apoptosis in DL cells. The suppression in HSF1
expression and subsequent inhibition of hsp70 expression in chelerythrine-treated DL cells suggest that chelerythrine induces apoptosis in DL cells by inhibiting the expression of these cytoprotective proteins.

**Conclusion:** Chelerythrine is capable of inducing apoptosis DL cells in vitro and therefore, it could be useful in combating tumor growth and progression.

**Keywords:** Apoptosis, chelerythrine, Dalton’s lymphoma, DNA fragmentation, protein kinase C inhibitor

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> Introduction

Chelerythrine is a benzophenanthridine alkaloid derived from *Chelidonium majus* (*L*). (Papaveraceae) plants. Structurally, it is 1, 2-dimethoxy-3-methyl (1,3)benzodioxolo (5, 6-c) phenanthridinium chloride (C₂₁H₁₈ClNO₄ ). It is a potent selective and specific inhibitor of protein kinase C (PKC), which interacts with PKC catalytic domain and inhibits the translocation of PKC from the cytosol to the membrane. PKC is activated under stress and leads to phosphorylation of heat shock factor 1 (HSF1), resulting in the homotrimerization, activation, and nuclear translocation of HSF1 from the cytosol to the nucleus. The activated HSF1 in the nucleus binds to heat shock elements of the heat shock protein (HSP) gene and induces transcriptional activation of this gene expression. Overexpression of hsp70 plays a vital role in cellular defense mechanism against stresses. Stress induces accumulation of damaged/misfolded proteins in intracellular milieu that might cause an imbalance in the ratio of protective to damaged proteins, leading to programmed cell death or apoptosis. HSF1 promotes the cancer phenotype due to dysregulation in signaling and alteration in DNA, protein and energy metabolism associated with oncogenesis via up-regulating the expression of heat responsive proteins. HSF1-induced overexpression of heat shock response proteins such as hsp70 functions like a pro-survival protein in several tumor models and their cell lines.

As discussed, the tumor cells utilize HSPs to inhibit apoptosis due to stress in a host. The expression of HSPs is regulated by the activation of HSF1 which contains serine/threonine residues in its active site and is activated by a serine/threonine kinase-
HSF1 activation involves the conversion of HSF1 from a latent cytoplasmic monomer to a trimeric nuclear protein complex that controls transcription of heat shock genes. Therefore, blocking the activation of HSF1 by a serine/threonine kinase inhibitor such as chelerythrine might be a novel strategy in cancer therapeutics. Inhibition of HSF1 is also a target of antisense oligonucleotide therapy or other manipulations with possibilities for anticancer therapy with least side effects. Further studies on HSF1 inhibition suggest that triptolide, a plant product, is capable of blocking HSF1 activation and would be of great value in the treatment of cancer. However, more potent PKC inhibitors have not been well studied in this respect. Therefore, chelerythrine has been selected for its specificity, ease, and lowest side effect with minimal toxicity. Chelerythrine inhibits PKC phosphorylation and subsequent activation of HSF1 in a very specific manner, and thus known as a selective inhibitor for group A and B isoforms of PKC. Recent reports have suggested the growth inhibitory effect of chelerythrine against various tumor cell lines as it exerts an antiproliferative and antitumor effect against several human tumor cell lines tested in vitro. Therefore, in this study, we sought to investigate the effect of chelerythrine on the apoptosis of Dalton's lymphoma (DL), a type of spontaneous murine T-cell lymphoma that has its own unique etiology and is characterized by highly deleterious, invasive, and rapid growth in murine models.

Materials and Methods

MTT (3(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) assay was carried out to evaluate the effect of chelerythrine (Sigma Chemical Company, Bangalore, India) on DL cells proliferation, if any. DL cells were harvested from the DL-bearing mice after 18 days of post-DL cell transplantation, where the yield of DL cells is maximum. Single cell suspension of DL cells containing $1 \times 10^5$ DL cells/mL was incubated in medium alone or medium containing 10 μg/mL PMA (phorbol 12-myristate 13-acetate) as a positive control or 10 μg/mL chelerythrine with Con-A stimulation. Briefly, MTT was dissolved in phosphate-buffered saline (PBS) at a concentration of 5.0 mg/mL. To each well of the culture plate, 10 μL of MTT solution was added and the plate incubated for 5-6 hrs at 37°C in various experimental conditions such as before heat shock, after heat shock, and 6 h and 12 h without heat shock. The plate was centrifuged for 5 min at 100 × g at 4°C. The supernatant was then carefully removed without disturbing the darkblue formazan crystals. Then, 100 μL of DMSO was added to each well to dissolve the formazan crystals and the absorbance was read at 540 nm of wavelength.

To assess the PKC activity in DL cells, lysate was prepared from both cytosol and membrane bound cell fractions. Briefly, DL cells were harvested, treated with chelerythrine in different conditions, lysed, and centrifuged at 1, 00,000 × g at 4°C. The supernatant was collected, membrane fraction was further lysed, and protein was estimated using the Bradford method. PKC activity was measured by phosphorylation of specific TMB substrate, Leu....X....Ser....Gly. To each well of the microtiter plate, 50 μL lysate and 50 μL kinase assay buffer were added and incubated for 10 min at room temperature, and the negative control was left blank. Furthermore, 10 μL of ATP working solution was added to each well in the plate provided with the assay kit (Enzo life sciences, Exeter, UK) and kept at 30°C for 90 min at 60 rpm. The plate was inverted on a clean paper for 10 min and 40μL of phospho-specific substrate
antibody was added to each well and incubated for 60 min at room temperature and washed. Thereafter, 40 μL of phospho-specific HRP-conjugated antibody was added to each well and the plate incubated for 30 min at room temperature. Again, the wells were washed four times and finally 60 μL of substrate was mixed for 30-60 min allowing color to develop, and absorbance was measured by adding acid stop solution at 450 nm. The relative activity of PKC was calculated using the following formula and expressed in terms of ng/assay.

Relative kinase activity in DL cell lysate =

\[
\frac{\text{Average absorbance (sample)} - \text{Average absorbance (Blank)}}{\text{Quantity of crude protein used per assay}}
\]

To show the expression of HSF1 and hsp70, DL cells were fixed in 4% paraformaldehyde solution for 10 min. Slides were dipped in 3% bovine serum albumin (BSA) for 2 h at 4°C and washed in PBS-T20 for 10 min at room temperature. Cells were incubated with monoclonal primary mouse antibody anti-HSF-1 and anti-hsp70 antibodies (Cell Signaling, Danvers, MA, USA) overnight at 4°C at a dilution of 1:1,000. The slides were then washed in sterile PBS-T20 and incubated with goat anti-rabbit ALP (alkaline phosphatase-conjugated) IgG (immunoglobulin G) secondary antibody (HiMedia, Mumbai, India) for 1-2 h at room temperature at a dilution of 1:5000. The slides were incubated with 5-bromo, 4-chloro, 3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate for 5-10 min and observed under simple light microscope (Lica, MD 2000, Switzerland).

Western blot analysis was performed and DL cell lysate containing 30 μg/well of total protein onto 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was resolved. The bands were transferred onto polyvinylidene fluoride membrane (Immobilon-P, Millipore, Bangalore, India). The membrane was blocked by incubating it in Tris-buffered saline, pH 8.5, containing 3% BSA and 0.1% Tween-20 for 1-2 h to avoid nonspecific binding and thereafter incubated overnight with antimouse anti-HSF-1 and anti-hsp70 at 4°C at a dilution of 1:1000 in separate experiments. The membranes were washed thrice with PBS-T20 and incubated with anti-rabbit ALP-conjugated IgG secondary antibody for 1 h at room temperature at a dilution of 1:5000. Furthermore, the membranes were incubated with BCIP/NBT substrate for 5-10 min.

For RT-PCR analysis, adherence-purified DL cells were treated in different conditions, lysed, and total mRNA was isolated. DNase was inactivated by heating the cells at 75°C for 15 min in the water bath. Then, 5 μL (1×) of Polymerase chain reaction-buffer (PCR-buffer) buffer, 5 μL (0.2mM) of dNTPs (deoxy nucleotide triphosphates) mixture, 2 μL (0.4mM) of PCR primer and 2 μL (~30 ng) of mRNA, 1 μL (0.1 units/ μL) of reverse transcriptase, 1 μL of RNase inhibitor, and 34 μL nuclease-free water were added to PCR tubes and the tubes placed in the thermal cycler when the cycler reaches 95°C. PCR was run for 45 s at 94°C and 53°C, 1.5 min at 72°C, and 7 min at 72°C for 30 cycles. The amplified product was evaluated by 2% agarose gel electrophoresis.

In order to study the effect of chelerythrine on DL cell morphology, the cells were
harvested, washed, and incubated with Roswell Park Memorial Institute medium (RPMI)-1640 supplemented with 10% fetal calf serum (FCS) (fetal calf serum) (Invitrogen, Grand Island, NY, USA) for 2-3 h, and thereafter non-adherent cell population was treated with 10 μg/mL chelerythrine in different conditions. The DL cells incubated in a medium only act as a negative control, whereas the DL cells incubated with 10 μM H₂O₂ act as a positive control. The DL cells were double stained with Wright-Grönwald-Giemsa and eosin stains and the slides were examined under inverted light microscope (Olympus India, Bangalore, India) at different magnifications. For transmission electron microscope (TEM) analysis, treated DL cells were washed, fixed in 2.5% glutaraldehyde solution, and washed with 0.1M PBS (NaH₂PO₄, Na₂HPO₄, NaCl, and KCl) repeatedly after 45 min. The DL cells were sectioned and observed under TEM (Leica, Alwarpet, Chennai, India) at different magnifications.

Apoptotic DL cells were evaluated by Hoechst 33258 staining. Briefly, adherent-purified DL cells were treated with IC₅₀ 10 μg/mL chelerythrine in different experimental conditions. The cells were washed thrice in sterile ice-cold PBS and fixed in 4% paraformaldehyde freshly prepared in PBS for 1 h at 4°C. The fixed DL cells were stained with 10 mM/L of Hoechst-33258, a kind of blue fluorescent dye (excitation/emission maxima~350/461 nm) used commonly for labeling DNA, nuclei, and mitochondria, respectively. Washing was repeated with ice-cold sterile PBS, and the DL cells were observed under fluorescence microscope (Zeiss India Pvt. Ltd., Bangalore, India).

To show the apoptosis in DL cells, adherence-purified DL cells (1.5 × 10⁵ cells/mL) were treated with IC₅₀ 10 μg/mL chelerythrine under different experimental conditions, and DNA fragmentation assay was performed. Treated DL cells were washed thrice with PBS and centrifuged at 1,200xg for 15 min at 4°C. Cell pellet was resuspended in 1 mL of DNA lysis buffer containing 20 mM of EDTA, 10μg/mL proteinase-K, 5μL DNA inhibitor, and RNase. The cells were kept at 55°C overnight, mixed gently, and centrifuged for 30 min at 10,000xg. Supernatant was collected and the pellet was discarded. One half of volume of 10 mL of ammonium acetate and 2.5 volume of cold ethanol were added to the supernatant. Finally, DNA was precipitated in isopropanol at high salt buffer and centrifuged at 10,000xg. Supernatants were discarded and the pellet was dried in oven. Dried DNA pellet was then suspended in Tris-borate-EDTA buffer (TBE) (tris/borate/EDTA) buffer, and 10 μL of this sample was run on 2% agarose gel to analyze the extent of DNA fragmentation.

Student's t-test was used to test the significance of data obtained in experimental settings. The data were taken as significant at *P <0.05. All statistical analyses of data were performed on Sigma plot Version 8.0 (Systat Software Inc., San Jose, CA, USA).

> Results

DL cells were treated with increasing concentrations of chelerythrine with IC₅₀ 2, 4, 6, 8, and 10 μg/mL and viability was measured according to previous studies. It was found that the viability of DL cells significantly reduced with IC₅₀ 10 μg/mL concentration of chelerythrine [Figure 1a], but further increased in its concentration-
accelerated necrotic cell death mechanism. Therefore, we used IC$_{50}$ 10 µg/mL of chelerythrine in all experiments. Time-dependent effect of chelerythrine on DL cells viability was also evaluated. Results from time kinetics of chelerythrine in different experimental conditions such as before heat shock, after heat shock, and 6 and 12 h without heat shock suggest that chelerythrine significantly reduced DL cell viability during 6 and 12 h without heat shock [Figure 1]b. We further increased the incubation period of chelerythrine for 18 h which resulted in increased number of DL cells undergoing necrosis rather than increased number of DL cells undergoing apoptosis (data not shown). Therefore, our observations suggest that chelerythrine treatment significantly reduces DL cell viability, whereas treatment after heat shock showed a comparatively low reduction in viability than that without heat shock [Figure 1]b. Thus heat shock might prevent DL cells from apoptosis, but up to a certain limit beyond its threshold capacity DL cells adopt either apoptotic pathway most probably or necrotic pathway, question remains to be answered. The effect of chelerythrine on DL cell proliferation was also determined by MTT assay, as described in the "Materials and Methods" section. Results indicate that DL cell proliferation was significantly pronounced in Con-A stimulated condition, whereas chelerythrine treatment showed significant inhibition of DL cell proliferation in DL cells treated with chelerythrine before heat shock, and 6 and 12 h without heat shock [Figure 1]c.

Earlier repsrts revealed that chelerythrine is a strong inhibitor of PKC in vitro and SQ-20B, WEHI-231 cells in vivo. [24][25]. To evaluate the effect of chelerythrine on PKC modulation, we used commercial kinase assay to determine the activity of PKC in DL cell lysate. Results show that PKC activity in cytosol was significantly decreased with12chelerythrine treatment, whereas membrane bound fraction of DL cells show more significant inhibition of PKC translocation, which suggests that chelerythrine exerts a dominant effect on PKC activity and its migration from cytosol to membrane. Furthermore, inhibition of PKC activity in cytosol and membrane bound fraction [Figure 2]a and b indicates that synaptosome formation was not significant, suggesting that phosphorylation might be prevented by chelerythrine. Chelerythrine treatment with IC$_{50}$ 10µg/mL modulates PKC activity in DL cells significantly compared to that in
untreated DL cells. Therefore, it can be suggested that PKC inhibition is associated with transcriptional activation of HSF1 that might be hampered, leading to interruption in hsp70 expression.

Figure 2: Effect of chelerythrine on protein kinase C (PKC) activity. In all, 1.0 x 10^5 DL cells were treated with chelerythrine IC_{50} 10μg/mL under different experimental conditions as described. Dalton’s lymphoma cells were lysed, centrifuged at 100,000×g, and relative kinase was determined in terms of ng/assay as described in the “Material and Methods” section. (a) Indicates PKC activity in cytosolic fraction. (b) Indicates PKC activity in membrane bound fraction. Data are representative of three independent experiments.

Expression of hsp70 is regulated by activation/inactivation of HSF1, a transcription factor. HSF1 is itself switched on/off by the activation of PKC. Therefore, to evaluate the effect of chelerythrine on transcriptional activation of HSF1, immunocytochemistry and Western blot of cytosolic fraction (cHSF1) and nuclear fraction (nHSF1) were performed by using mouse anti-HSF1 monoclonal antibody. It was observed that chelerythrine treatment significantly reduced the nuclear localization of nHSF1 compared to untreated or positive control group of DL cells, whereas cHSF1 has a tendency to increase in cytosolic fraction compared to that of untreated or positive control group of DL cells. On increasing the incubation period, nHSF1 progressively decreases. On the contrary, cHSF1 progressively increases in the cytosolic fraction. Correspondingly, the level of hsp70 proteins tends to decrease progressively upon increase in incubation period of DL cells treated with chelerythrine [Figure 3]a, as analyzed by optical density of protein bands [Figure 3]b. Corresponding result was observed when RT-PCR analysis of HSF1 and hsp70 genes was performed [Figure 3]c.

Figure 3: Expression of HSF1 and hsp70 in Dalton’s lymphoma cells. In all, 1.0 x 10^5 DL cells were treated with or without chelerythrine IC_{50} 10 μg/mL in different experimental conditions and the expression of nHSF1 and cHSF1 and hsp70 was determined by Western blotting and RT-PCR. L1 shows negative control, L2 positive control with PMA, L3 chelerythrine before heat shock, L4 chelerythrine after heat shock, L5 chelerythrine 6 h without heat shock, L6 and chelerythrine 12 h without heat shock. Each data point is representative of at least three independent experiments. (a) Shows the Western blot of nHSF1, cHSF1, and hsp70 protein. (b) Shows the relative band intensity of blots; and (c) shows the RT-PCR analysis for the expression of HSF1 and hsp70.
Inhibition of transcriptional activation of HSF1 was further revealed by immunolocalization by immunocytochemical observations. The number of purple granules observed was significantly low in treated DL cells [Figure 4] c, d, e and f compared to that in untreated DL cells, functioning as a negative control [Figure 4]a and as a positive control [Figure 4]b. These biochemical observations suggest the role of serine/threonine kinase inhibitor, chelerythrine, in the regulation of transcriptional activation of HSF1 in DL cells. Corresponding observation was made when DL cells were immunostained for hsp70 expression in different experimental conditions [Figure 5].

Figure 4: HSF1 localization in Dalton's lymphoma (DL) cells. In all, 1.0 × 10^5 DL cells were treated with chelerythrine IC_{50} 10μg/mL in a similar experimental condition as in previous experiments. DL cells were fixed on slides and incubated with mouse anti-HSF1 and then ALP conjugated secondary antibody. Then, the slides were incubated with substrate NBT/BCIP and color intensity was detected in terms of the number of purple granules under light microscope. Data are representative of three independent experiments. (a) Negative control (b) Positive control with H_2O_2, (c) chelerythrine treatment before heat shock, (d) chelerythrine treatment after heat shock (e) 6-h chelerythrine treatment without heat shock, (f) 12-h chelerythrine treatment without heat shock.

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Figure 5: Hsp70 localization in Dalton's lymphoma (DL) cells. In all, 1.0 × 10^5 DL cells were treated with chelerythrine IC_{50} 10μg/mL in an experimental condition similar to previous experiments. DL cells were fixed on slides and incubated with mouse anti-hsp70 and then ALP-conjugated secondary antibody. Then, the slides were incubated with substrate (NBT/BCIP) and color intensity was detected in terms of the number of purple granules under light microscope. Data are representative of three independent experiments. (a) Denotes medium only. (b) Positive control with H_2O_2. (c) Chelerythrine treatment before heat shock. (d) Chelerythrine treatment after heat shock. (e) 6-h chelerythrine treatment without heat shock. (f) 12-h chelerythrine treatment without heat shock.

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DL cells treated in vitro with 10 μg/mL chelerythrine showed characteristics of
morphological alteration that correspond to cells undergoing apoptosis such as eccentric nuclei, chromatin condensation, and formation of apoptotic bodies. Chelerythrine-treated DL cells with heat shock showed comparatively lower number of cells that has apoptotic features [Figure 6]a and b compared to DL cells with 6-and 12-h treatment of chelerythrine without heat shock [Figure 6]c and d. Chelerythrine treatment resulted in significant number of cells undergoing apoptosis compared to negative control [Figure 6]e. Correspondingly, on increasing the incubation period, number of apoptotic DL cells increased, and maximum number of DL cells undergoing apoptosis was observed at 12 h of incubation without heat shock. Further increase in concentration and incubation period of chelerythrine increased the number of necrotic cells [Figure 6]h; however, the fraction of such cells was comparatively very low [Figure 6]h in comparison to the number of cells undergoing apoptosis [Figure 6]g, but insignificant number of cells were detected as necrotic in culture as observed by morphologic changes. Furthermore, to test nuclear changes that occur during apoptosis, treated cells were observed under TEM. TEM is a highly specific and valuable technique for imaging at a significantly higher resolution than light microscopy. DL cells incubated in medium alone showed normal nuclear architecture [Figure 7]a. The ultra/ internal and nuclear changes of apoptotic DL cells were emarginated, eccentric condensed chromatin within the nucleus, and membrane blebbing upon chelerythrine treatment. Corresponding to the observation of double-stained DL cells, an intense apoptotic feature was found in the DL cells treated with chelerythrine 6 and 12 h without heat shock [Figure 7]e and f b compared to the DL cells treated with chelerythrine before and after heat shock [Figure 7]c and d. Upon increasing the incubation period, significantly more number of DL cells showed apoptotic features by nuclear membrane disintegration. The DL cells treated with chelerythrine for 12 h without heat shock showed completely disintegrated nuclear membrane [Figure 7]f compared to that of the positive control [Figure 7]b.

DL cells incubated with chelerythrine before heat shock, after heat shock, 6h and 12h without heat shock [Figure 8]c, d,e, and f exhibited a brighter and eccentric nucleus compared to [Figure 8]a but significantly higher in [Figure 9]e and f suggesting maximum number of DL cells undergoing apoptosis. Darker nucleus shows normal morphology of DL cells as well as nucleus. However, DL cells treated with chelerythrine for 6 h without heat shock showed enhanced apoptotic features in comparatively larger number of DL cells [Figure 8]b. On increasing the incubation period of treated DL cells, the number of cells undergoing apoptosis increased significantly as observed by intense fluorescence [Figure 8] d, e and f. Further increase in the period of incubation and concentration of chelerythrine induces necrotic cell death, which is distinct from normal physiologic cell death.

**Figure 6:** Effect of chelerythrine on the Dalton's lymphoma (DL) cell morphology and apoptosis. In all, 1.0 × 10^5 DL cells were treated with chelerythrine IC_{50} 10 μg/mL under different conditions. (a) Shows medium only. (b) Positive control with H_{2}O_{2}. . (c) Chelerythrine treatment before heat shock. (d) Chelerythrine treatment after heat shock. (e) 6-h chelerythrine treatment without heat shock (f) 12-h chelerythrine treatment without heat shock double stained with Giemsa and eosin, (g) Denotes the percent apoptotic DL cells, and (h) Denotes the percent of DL cells undergoing necrosis.. The DL cells were then examined under light microscope. Data
are significant at $P>0.05$ and are presented as mean number of cells ± SEM of at least three independent experiments in triplicate.

**Figure 7:** Transmission electron microscope analysis of Dalton's lymphoma (DL) cells. In all, $1.0 \times 10^5 < DL$ cells were treated with chelerythrine IC$_{50}$ $10\mu g/mL$ in experimental conditions similar to that in Figure 1, and sectioning was performed and examined under TEM for morphologic analysis. (a) Shows medium only ($\times 21,000$) (b) Positive control with H$_2$O$_2$ ($\times 15,000$) (c) Chelerythrine treatment before heat shock ($\times 26,000$). (d) Chelerythrine treatment after heat shock ($\times 26,000$). (e) 6-h chelerythrine treatment without heat shock ($\times 15,000$), (f) 12-h chelerythrine treatment without heat shock ($\times 21,000$). Results are representative of three independent experiments.

**Figure 8:** Hoechst staining of Dalton's lymphoma (DL) cells. In all, $1.0 \times 10^5$ DL cells were treated with chelerythrine IC$_{50}$ $10\mu g/mL$ under different experimental conditions as described in Figure 1 and stained with nuclear fluorescent stain Hoechst 33258, as described in the "Materials and Methods". Stained DL cells were examined under fluorescent microscope. (a) Denotes medium only, (b) Positive control with H$_2$O$_2$, (c) Chelerythrine treatment before heat shock, (d) Chelerythrine treatment after heat shock, (e) 6-h chelerythrine treatment without heat shock.. (f) 12-h chelerythrine treatment without heat shock. Results are representative of three independent experiments.

**Figure 9:** Effect of chelerythrine on DNA fragmentation in Dalton's lymphoma (DL) cells. In all, $1.0 \times 10^5$ DL cells were treated with chelerythrine IC$_{50}$ $10\mu g/mL$ under different experimental conditions as described previously, and DNA was isolated as described in the "Materials and Methods" and solubilized in TBE buffer and resolved onto 2% agarose gel electrophoresis. (a) Represents the neutral gel electrophoresis of DNA. (b) Represents formaldehyde neutral gel electrophoresis of DNA. (c) Represents alkaline gel electrophoresis of DNA isolated from chelerythrine-treated DL cells. Results are representative of three independent experiments.
DNA fragmentation showed that chelerythrine induces the appearance of short DNA fragments (mean strand break of about 180-200bp), as shown in [Figure 9]a-c. DL cells under experimental conditions such as before heat shock, after heat shock, and 6 and 12 h without heat shock show DNA fragmentation compared to untreated DL cells. But the appearance of DNA fragments has been found to be greater after 6 and 12 h exposure of DL cells to chelerythrine. On increasing the incubation period, DNA fragmentation was observed to be higher, but up to a certain limit beyond threshold limit, the bands disappeared and only smear was detected (data not shown) in both neutral and alkaline agarose gels. Significant DNA fragmentation was observed after 12h incubation of DL cells with chelerythrine without heat shock. DNA fragmentation evaluated by alkaline sucrose linear gradient agarose gel electrophoresis showed DNA fragments shorter than $1 \times 10^3$ kDa [Figure 9]a, whereas in neutral agarose gel electrophoresis similar pattern of DNA bands was observed [Figure 9]b, indicating that internucleosomal DNA cleavage is similar to that appeared in alkaline sucrose gradient gel electrophoresis. When same DNA samples were resolved by denatured gel electrophoresis, ladder appeared in the gel have DNA fragments of different sizes that correspond to mean strand break of about 180-200 bp. Furthermore, to test DNA fragments, formaldehyde gel electrophoresis and neutral pH formaldehyde plus agarose gel electrophoresis of heat denatured DNA isolated from apoptotic DL cells, were performed [Figure 9]c. DNA was digested with Eco-R1+Hind-III using as a positive control in all three types of gel electrophoresis [Figure 9]d. Observations from experimental data suggested that chelerythrine induces significant change in treated DL cells in contrast to that in control. These results support our observation that treatment of chelerythrine induces apoptosis of DL cells.

> Discussion

Results from many studies employing pharmacologic agents that affect PKC activity have indicated PKC-responsive signaling mechanism might be involved in regulation of heat shock response. However, the precise relationship between PKC activation and induction of heat shock response in DL cells has not been investigated yet. We have shown in this study that chelerythrine treatment blocked PKC activation that resulted in transcriptional inhibition of HSF1, which led to the inhibition of heat shock response by inhibiting hsp70 expression in DL cells. PKC-responsive signaling and the effector machinery involved in the activation of HSF1 lead to an enhanced heat shock response by overexpression of hsp70. However, chelerythrine treatment with heat shock results in comparatively enhanced viability of DL cells. The effect of chelerythrine on DL cell proliferation was evaluated, which suggests that proliferation of DL cells was reduced in DL cells treated with chelerythrine before heat shock and 6 and 12 h without heat shock, but more significantly in 12 h without heat shock. These observations indicate that chelerythrine reduces not only DL cell viability but also proliferation of DL cells. However, no other report is available to show the effect of chelerythrine on DL cell proliferation. Furthermore, chelerythrine treatment induces characteristic morphological alterations in DL cells such as plasma membrane blebbing, chromatin condensation, and
eccentric nuclei as seen in double staining, TEM analysis, and Hoechst staining. Results indicate intense fluorescence of DL cells in 6 and 12 h without heat shock, enhanced apoptosis of DL cells comparable to that in those treated with chelerythrine after heat shock. Therefore, heat shock may delay DL cell apoptosis, but chelerythrine treatment does not increase DL cell viability and survival. Maximum number of DL cells undergoing apoptosis under 12h incubation with IC_{50} 10μg/mL indicates that chelerythrine induces apoptosis in DL cells not only in a dose-dependent manner but also depending on time of incubation period. The results corroborate the earlier observation demonstrating that PKC activator 12-O-tetradecanoylphorbol-13-acetate inhibits the apoptosis of tumor cells, whereas the treatment of PKC inhibitor chelerythrine or Ro-31-8220 induces apoptosis and restricts growth and progression of colon cancer cells.\textsuperscript{[22]} It has been reported that chelerythrine treatment has a cytostatic and cytotoxic effect on DL cells in both in vitro and in vivo experimental settings (data not shown).\textsuperscript{[23]} Furthermore, chelerythrine induces DNA laddering in DL cells. The comparatively low intensity of DNA bands indicates that heat shock results in the expression of such factors, which are capable of neutralizing the effect of PKC inhibition in the chelerythrine-treated DL cells, as discussed previously. The DL cells incubated with chelerythrine for 12 h without heat shock resulted in maximal fragmentation of DNA in comparison to DL cells incubated with chelerythrine for 6 h without heat shock and before heat shock. DNA bands appeared in multiple of 180-200bp DNA fragments, which supports the evidence that chelerythrine treatment leads to the generation of short DNA fragments characteristics of apoptotic nuclei. However, appearance of short DNA fragments in alkaline sucrose linear gradient analysis that corresponds to shorter than 1 × 10^{-3} kD suggests that chelerythrine induces internucleosomal DNA cleavage in DL cells. Furthermore, increased band intensity indicates that chelerythrine effectively induces DNA fragmentation in DL cells at 10 μg/mL (IC_{50}) , which corroborates with the findings of other similar studies in different tumor models.\textsuperscript{[25],[26]} However, progressive increase in incubation time and dose of chelerythrine induces cell killing by necrosis. In conclusion, chelerythrine is a strong inhibitor of PKC activity that can modulate the activation of HSF1 and subsequent expression of hsp70 in DL cells. Inhibition of hsp70 expression might induce apoptosis of DL cells, but the mechanism of action needs to be investigated in detail. Recently, it has been shown that besides the primary role of chelerythrine in PKC inhibition, it may also lead to posttranslational modification and/or degradation of vital proteins in several tumor cell lines that may further result in apoptosis.\textsuperscript{[1],[21],[31],[33],[34],[35]}

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Figures

[Figure 1], [Figure 2], [Figure 3], [Figure 4], [Figure 5], [Figure 6], [Figure 7], [Figure 8], [Figure 9]