

PKC-Inhibitors Induce Apoptosis by Inhibiting Hsp70-Mediated Protection of Anti-Apoptotic BCL₂ Proteins in Dalton's Lymphoma Cells

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ABSTRACT

BACKGROUND: Proteins of the BCL₂ family regulate the mitochondria-dependent apoptotic pathway, whose down regulation induced apoptosis for which the basis is poorly understood. Therefore, aims of the present investigation were to study the effect of PKC inhibitors (chelerythrine and staurosporine) on the expression of BCL₂ proteins in Dalton's Lymphoma (DL) cells and to identify whether exogenous application of tumor derived hsp70 protects DL cells from PKC-inhibitor-mediated apoptosis.

METHODS: To investigate this, cells were harvested from tumor bearing mice after 18 days of post transplantation and cultured for 48hrs in RPMI-1640 supplemented with 10% FBS and 5µg/mL pen/strep in 5% CO₂ at 37°C. Cells were then treated with PKC inhibitors (CHE-10µM/STS-1µM) in the presence of HS⁺, cHS⁺ and tumor derived hsp70, immunocytochemistry, western blots, RT-PCR, caspase assay, and flowcytometry were carried out.

RESULTS: Our results showed that PKC inhibitors (CHE/STS) treatment resulted in depressed expression of BCL₂ and Bcl-xL proteins in hsp70-mediated protection, while Bax and Bcl-xL were found to be upregulated significantly. Altered expression of BCL₂ proteins induced activation of caspase-3 and apoptosis.

CONCLUSION: PKC inhibitors (CHE/STS) treatment may contribute to develop effective therapeutic regimen against Dalton's lymphoma.

Key words: Apoptosis; Anti- & pro-apoptotic BCL₂ proteins; Chelerythrine; DL cells; Staurosporine

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INTRODUCTION

Apoptosis is the genetically programmed cell death required for the growth, differentiation, development and cellular homeostasis in all organisms. Malfunctions in apoptosis result, in severe side effects including cancer, myocardial infarction, neurodegenerative disease and ischemic stroke^[1]. Activation of the intrinsic apoptotic pathway(s) induces release of cyt-c, activation of effectors caspases-3/6/9, and loss of mitochondrial potential^[2]. However, studies show that hsp70 can interact in apoptotic signaling at several points, such as the release of cyt-c, formation of apoptosome, activation of caspases, and inhibition of apoptosis^[2,3].

Hsp70 is an important stress response protein that has been shown to protect cells from a variety of apoptotic stimuli, such as heat shock, tumor burden, growth factor withdrawal, and oxidative stress^[2-4]. Additionally, research has shown that hsp70 is a promising molecule in controlling and regulating apoptosis^[1,2]. Inhibition/depletion of hsp70 disrupts heterodimerization with anti-apoptotic BCL₂ proteins (BCL₂/Bcl-xL), leading to the release of cytochrome-c, the activation of caspase-3/6/7 and ultimately apoptosis^[1-4]. However, it was reported that enhanced expression of hsp70 protects anti-apoptotic BCL₂ proteins by organizing complex structures with BCL₂ and therefore inhibiting over expression of pro-apoptotic BCL₂ proteins (Bax, Bcl-Xs), resulting in increased cell survival^[2]. However, the mechanism by which hsp70 protects anti-apoptotic BCL₂ proteins and prevents release of cyt-c is not well defined.

Proteins of the BCL₂ family regulate mitochondrial integrity and activation of the mitochondria-dependent apoptotic pathway^[2]. Therefore, on the basis of conserved sequence motifs, proteins of

BCL₂ family can be divided into anti-apoptotic (BCL₂/Bcl-xL), pro-apoptotic (Bax/Bcl-Xs) and the members of BH₃-only proteins (Bid, Bik, Bim, Bad)^[2-4]. The BH-3 only proteins can alter the function of two previously described multidomain BCL₂ family proteins (anti-apoptotic and pro-apoptotic)^[2-5]. Anti-apoptotic proteins interact with pro-apoptotic proteins and provide a hydrophobic groove by BH₁-BH₃ domains, which are docking sites for BH₃ domains of pro-apoptotic proteins^[6,7]. BH₃ domains of Bax, Bak, and Bcl-Xs are crucial for interaction with anti-apoptotic BCL₂ proteins (BCL₂/Bcl-xL)^[8]. Heterodimerization of BCL₂ and Bcl-xL proteins with hsp70 on the mitochondrial surfaces prevents the release of cytochrome-c and the downstream activation of caspases and therefore maintains mitochondrial integrity^[9-11]. In contrast to BH₃, BH₄ domain of BCL₂ resides in its N-terminal α -helix but its role is poorly understood in comparison to the other three BH domains. Whether or not BH₄ domain is essential for binding to pro-apoptotic proteins is still not clear, but deletion of BH₄ domain causes conversion of BCL₂ into a pro-apoptotic protein without altering the homo- and heterodimerization of BCL₂ proteins, suggesting its anti-apoptotic role may be different from its pro-apoptotic one^[12].

A multistep broad spectrum, yet simple signaling cascade is the release of one or more different apoptosis inducing proteins. These proteins are in the family of BH₃-only proteins that migrate to the outer mitochondrial membrane. BH₃-only proteins prevent mitochondrial integrity because they organize a complex structure with anti-apoptotic proteins (BCL₂/Bcl-xL)^[6-8,13-15]. BH₃-only proteins (Bim and Bid) also induce activation of multidomain pro-apoptotic proteins^[14-16]. Furthermore, the relative proportion of Bax: BCL₂ plays an important role in maintaining cellular equilibrium^[14,15-16].

Bax/Bcl-Xs and Bak are important regulators of the mitochondrial-dependent apoptotic pathway^[8,15,17]. Cells lacking in one or more such proteins showed signs of apoptosis. However, loss of both proteins exhibited abnormalities in murine fibroblast cells (MEFs)^[13]. In addition, Bax/Bcl-Xs double knockout mice have never been shown to be resistant to apoptosis. The ability of survival of Bax/Bcl-Xs double knockout mice suggested the existence of alternate modes of cell death^[18]. Anti-cancer agents induced dual mode of apoptosis in Bax/Bcl-Xs lacking cells^[13]. Thus, treatment with etoposide and staurosporine showed caspase-independent apoptosis in MEFs cells but not Bax-dependent variants.

Furthermore, it was found that PKC inhibitors (CHE and STS) induce the release of cyt-c; however, only chelerythrine induced the release of cyt-c from isolated mitochondria^[19]. Chelerythrine induces activation of JNK/p38 pathways and blocks Bcl-xL function in cancer cells^[20-24]. In this context, the effect of PKC inhibitors (CHE & STS) on the expression of BCL₂ proteins in hsp70-mediated cytoprotection was elucidated. It was observed that PKC inhibitors (CHE & STS) treatment in the presence of HS⁺, cHS⁺ and tumor derived hsp70 resulted in decreased expression of anti-apoptotic BCL₂ proteins (BCL₂/Bcl-xL) and increase expression of pro-apoptotic Bcl₂ proteins (Bax/BCL₂), activation of caspase-, thereby apoptosis in target cell. Therefore, PKC inhibitors (CHE & STS) may be used as cancer therapeutic regimens.

MATERIALS AND METHODS

Reagents

RPMI-1640 culture medium and ALP-conjugated goat anti-mouse antibody (IgG) were purchased from Bangalore Genie, Mumbai, India. Fetal bovine serum (FBS) was obtained from Invitrogen, Grand Island, NY, USA. Mouse anti-BCL₂, anti-Bcl-xL, anti-Bax,

and anti-Bcl-Xs were purchased from Cell Signaling Technology, Danvers, MA, USA. PVDF (polyvinylidene fluoride) membrane was purchased from Millipore, Bangalore, India. Total RNA isolation kit, first strand cDNA synthesis kit and primers (BCL₂, Bcl-xL, Bax and Bcl-Xs) were obtained from Medox Biotech Pvt. Ltd., Chennai, India. DNA ladder was purchased from Promega, New Delhi, India. Associated chemicals stated otherwise were obtained from Qualigens, SDFine and HiMedia, Mumbai, India or Super Religare Laboratory (SRL), Mumbai, India.

Cell culture and inhibitor treatment

DL cells were cultured in RPMI-1640 supplemented with 10% FBS, 5mg/Liter pen/strep at 37°C in 5% CO₂ in a humidified chamber. Single cell suspension of non-adherent DL cells was prepared. Cells (100% viable) at the density of 1.0×10^6 in 1.0 mL complete culture medium were treated with chelerythrine-10 μ M and staurosporine-1 μ M in the presence of heat shock at 42°C for 1 hr (HS⁺), concurrent heat shock (cHS⁺); heat shock at 42°C for 1 hr followed by 1hr recovery period then again heat shock at 42°C for 1 hr and tumor derived-hsp70 (10 μ g) for 6 hr. Control cells were cultured without any treatment; however, cells treated with heat shock were used as the positive control. Animal cell lines were used under the ethical guidelines by Indian Council of Medical Research (ICMR), New Delhi.

Immunocytochemistry

Cellular localization of anti-apoptotic BCL₂ proteins (BCL₂/Bcl-xL) and pro-apoptotic BCL₂ proteins (Bax/Bcl-Xs) in DL cells was examined. For this, cells were treated with PKC inhibitors, CHE-10 μ M and STS-1 μ M in the presence of HS⁺, cHS⁺ and tumor derived-hsp70 (10 μ g), washed and uniformly smeared on clean slides. Cells were fixed and permeabilized in 4% paraformaldehyde solution for 10-15 min. Cells were incubated with mouse anti-BCL₂, anti-Bcl-xL, anti-Bax and anti-Bcl-Xs (1:1000) antibodies overnight at 4°C followed by incubation with a secondary antibody (1:5000) for 1-2 hrs at room temperature. Localization of BCL₂, Bcl-xL, Bax and Bcl-Xs was detected using liquid substrate system of BCIP/NBT (5-bromo, 4-chloro, 3-indolyl phosphate/nitroblue tetrazolium) for 5-10 min. Cells were observed under light microscope (Lica, MD 2000, Switzerland).

Western blots and Densitometric analysis

Treated cells were lysed and the estimated concentration of total protein (40-50 μ g/lane) was resolved on 14% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands were transferred to polyvinylidene fluoride (PVDF) membrane. BSA (3%) in Tris-buffered saline (pH 8.5 and 0.1% Triton-X100) for 1-2 h at 4°C was used to block nonspecific binding. Membranes were incubated with monoclonal antibodies of mouse anti-BCL₂, anti-Bcl-xL, anti-Bax and anti-Bcl-Xs (1:1000) overnight at 4°C. Cells were washed and incubated with rabbit anti-mouse ALP-conjugated antibody (1:5000) for 1-2 hr at room temperature. Protein bands of BCL₂, Bcl-xL, Bax and Bcl-Xs on membranes were detected by liquid substrate system of BCIP/NBT (5-bromo, 4-chloro, 3-indolyl phosphate/nitroblue tetrazolium) for 5-10 min at room temperature and percent units of expression were determined using AlphaImager 2200 software.

Semi-quantitative RT-PCR

Cells were treated in aforementioned conditions and total RNA was isolated. For cDNA synthesis, DNase was inactivated and total RNA (1-3 μ g) with random primers (1 μ L of 100 pmol) (Table 1) was

mixed and incubated at 70°C for 5 min and thereafter 4 µL of 5× reaction buffer, 0.5 µL of (40 U/µL) nuclease inhibitor and 2 µL of 10 mM dNTPs mixture were added, spun, and incubated at 42°C for 5 min. Further, AMV reverse transcriptase (2 µL of 10 U/µL) was mixed and incubated for 10 min at 25°C followed by 42°C for 60 min and then 70°C for 10 min to stop the reaction. First strand cDNA was synthesized successfully which can be used for amplification.

For Polymerase Chain Reaction (PCR), 5 µL of 10× PCR buffer, 5 µL of 2 mM dNTP solution, 3 µL of 25 mM MgCl₂, 2 µL of 10 pmole primers of each genes (Table 1), 2 µL of first strand cDNA, and 1 µL of (5 U/µL) Taq DNA polymerase were added to each PCR tube and run for 2 min at 95°C, 45 sec at 94°C, 1.5 min at 53°C, and 7 min at 72°C for 30 cycles. The amplified product was resolved on 2% agarose gel and percent units of expression were determined using AlphaImager 2200 software.

Assessment of caspase-3

Cells (1.0×10^6) were treated with PKC inhibitors, CHE-10 µM and STS-1 µM in the presence of HS⁺, cHS⁺ and tumor derived-hsp70 (10 µg), washed and lysed. For assessment of caspase-3, 50 µL of lysate, 50 µL of 2× reaction buffers and 5 µL of 4mM DEVD-pNA substrate were mixed gently in 96-well plate. Intensity of color product was read using an ELISA reader (Bio-Rad, CA, USA) at 400-405 nm. Difference in absorbance of DEVD-pNA from an apoptotic sample with respect to control allows us to know about fold increase in the activation of caspase-3.

Annexin V-FITC and PI staining

Cells treated with PKC inhibitors, CHE-10 µM and STS-1 µM in the presence of HS⁺, cHS⁺ and tumor derived-hsp70 (10 µg) were washed and resuspended in annexin V-FITC binding buffer with Annexin V-FITC (5 µL) and a counter stain, PI (5 µL) for 15 min at room temperature. Thereafter, cells were washed properly and resuspended in 200 µL of FACS buffer. Cells were observed under flow cytometry. Emission of Annexin V-FITC was detected in FL1 using a 488/515 nm band pass filter and PI in FL2 using a 515/575 nm band pass filter. Data were analyzed using Win MDI software.

Statistical Analysis

Data was analyzed using One Way ANOVA followed by Bonferroni *t*-test and Dunnett's Method as post hoc test. Values of $p < 0.05$ were taken as statistically significant. All statistical analyses were performed on Sigma plot Version 12.0 (Systat Software Inc., San Jose, CA, USA).

RESULTS

Effect of PKC inhibitors on the expression of anti-apoptotic BCL₂ proteins in hsp70-mediated protection in DL cells

In order to investigate the effectiveness of PKC inhibitors (CHE &

STS) on the expression of anti-apoptotic BCL₂ proteins (BCL₂/Bcl-xL) in hsp70-mediated protection, immunocytochemistry, western blots and RT-PCR were carried out. On the basis of three independent observations, it was found that treatment with PKC inhibitors (CHE & STS) in the presence of HS⁺, cHS⁺ and tumor derived-hsp70 resulted in the decreased number of BCL₂/Bcl-xL positive cells as compared to the control cells (Figure 1). However, the number of BCL₂/Bcl-xL positive cells was increased after treatment with PKC inhibitors in the presence of cHS⁺ and tumor derived-hsp70 (Figure 1). Furthermore, a densitometric analysis of anti-apoptotic BCL₂ proteins (BCL₂/Bcl-xL) revealed that PKC inhibitors (CHE & STS) treatment down regulated the expression of anti-apoptotic BCL₂ proteins (BCL₂/Bcl-xL) in hsp70-mediated protection (Figure 2A and 2B). Although heat shock and concurrent heat shock treatments increased the expression of hsp70, which interacts with anti-apoptotic BCL₂ proteins and protects them from PKC inhibitor (CHE & STS)-mediated apoptosis, the effect of hsp70 mediated protection was found attenuated at this time. Additionally, tumor derived-hsp70 failed to protect cells from PKC inhibitor (CHE & STS)-mediated apoptosis. In fact, the expression of anti-apoptotic BCL₂ proteins (BCL₂/Bcl-xL) at the RNA level was observed to correspond with the expression of anti-apoptotic BCL₂ proteins (BCL₂/Bcl-xL) at the protein level (Figure 2A and 2B). However, treatment with STS showed time-dependent inhibition of anti-apoptotic BCL₂ proteins (BCL₂/Bcl-xL) expression at protein and RNA levels in hsp70-mediated protection in DL cells (Figure 2A and 2B). However, exogenous application of tumor derived hsp70 showed weaker protection of the anti-apoptotic BCL₂ proteins (BCL₂/Bcl-xL) as compared with intracellular hsp70-mediated cytoprotection (Figure 2A and 2B).

Effect of PKC inhibitors on the expression of pro-apoptotic BCL₂ proteins in hsp70-mediated inhibition in DL cells

To examine the effect of PKC inhibitors (CHE & STS) on the expression of pro-apoptotic BCL₂ proteins (Bax/Bcl-Xs) in hsp70-mediated inhibition, immunocytochemistry, western blots and RT-PCR were performed. Results show that PKC inhibitors (CHE & STS) treatment in the presence of HS⁺, cHS⁺ and tumor derived-hsp70, caused an increase in number of Bax/Bcl-Xs positive cells as compared to the control (Figure 3). A densitometric analysis of pro-apoptotic BCL₂ proteins (Bax/Bcl-Xs) revealed that treatment with PKC inhibitors (CHE & STS) resulted in the significant increased expression of pro-apoptotic BCL₂ proteins (Bax/Bcl-Xs) in terms of percent expression units in hsp70-mediated inhibition as compared to the control (Figure 4A and 4B). Heat shock and concurrent heat shock treatment induced the expression of intracellular hsp70, which inhibited the expression of pro-apoptotic proteins up to a certain extent. Further, exogenous application of tumor derived hsp70 showed weaker inhibition as compared to intracellular hsp70 (Figure 4A and 4B). In addition, treatment with PKC inhibitors (CHE & STS) showed almost similar expression of pro-apoptotic BCL₂ proteins (Bax/Bcl-

Table 1 Primer sequence, length, T_m values and number of cycles.

S.R.	Gene	Primers	Length	T _m	No. of cycle	
1.	Bcl2	sense	5'-ATC TTC TCC TTC CAG CCT GA-3'	20-mer	57.3°C	30
		antisense	5'-TCA GTC ATC CAC AGG GCG AT-3'	20-mer	59.4°C	30
2.	Bcl-xL	sense	5'-GGA GAG CGT TCA GTC ATC-3'	18-mer	56.0°C	30
		antisense	5'-CAA TGG TGG CTG AAG AGA-3'	18-mer	53.7°C	30
3.	Bax	sense	5'-CCA GGA TGC GTC CAC CAA GA-3'	20-mer	61.4°C	30
		antisense	5'-GGT GAG GAC TCC AGC CAC AA-3'	20-mer	61.4°C	30
4.	Bcl-Xs	sense	5'-CAT GGC AGC AGT AAA GCA AG-3'	20-mer	57.3°C	30
		antisense	5'-GCA TTG TTC CCA TAG AGT TCC-3'	21-mer	57.9°C	30
5.	GAPDH	sense	5'-GGA GAA AGC TGC CAA ATA T-3'	19-mer	52.4°C	30
		antisense	5'-CAG GAA ATG AGC TTG ACA A-3'	19-mer	52.4°C	30

Xs) at the RNA level as it was observed at the proteins level (Figure 4A and 4B). However, treatment with STS resulted in increased expression of pro-apoptotic BCL₂ proteins (Bax/Bcl-Xs) in a time-dependent fashion at protein and RNA levels (Figures 3 and 4A/B).

Effect of PKC inhibitors on the activation of caspase-3 and apoptosis in hsp70-mediated inhibition in DL cells

In order to examine the effect of PKC inhibitors (CHE & STS) on the activation of caspase-3 in hsp70-mediated inhibition and apoptotic like changes in DL cells, colorimetric assay and flow cytometry were carried out. It was observed that treatment with PKC inhibitors (CHE & STS) in the presence of HS⁺, cHS⁺ and tumor derived-hsp70 showed significant increased activity of caspase-3 as compared to the control (Figure 5). However, exogenous application of tumor derived-hsp70 results in attenuated inhibition of caspase-3 activation (Figure 5). In addition, treatment with staurosporine resulted in a significantly enhanced expression of caspase-3 in a time dependent manner. No significant impact was made by tumor derived hsp70 on caspase-3 activation when it was administered exogenously (Figure 5).

To confirm PKC inhibitor (CHE & STS)-mediated apoptosis,

apoptotic like changes such as PS externalization and DNA fragmentation were studied. It was observed that treatment with PKC inhibitors (CHE & STS) induced PS externalization and subsequent DNA fragmentation in DL cells (Figure 6). Cells exhibiting these characteristics were identified by Annexin-V FITC/PI staining. Annexin-V FITC/PI discriminated cells into three different populations: first, Annexin-V FITC positive (showed Phosphatidylserine externalization, but normal DNA content) on FL1 band pass filter (488-515 nm); second, PI-positive (dead cells showed damaged DNA) on FL2 band pass filter (515-575 nm); and third, double positive/hypodiploid cells (PS-externalization and DNA fragmentation) (Figure 6).

DISCUSSION

Heat shock response enhances the synthesis of intracellular heat shock protein (hsp70) that confers a protective effect against a wide range of cellular stresses such as chemotherapy, tumor burden and malignancies^[1-4,19,25]. Therefore, heat shock and concurrent heat shock treatments were applied to induce the expression of intracellular

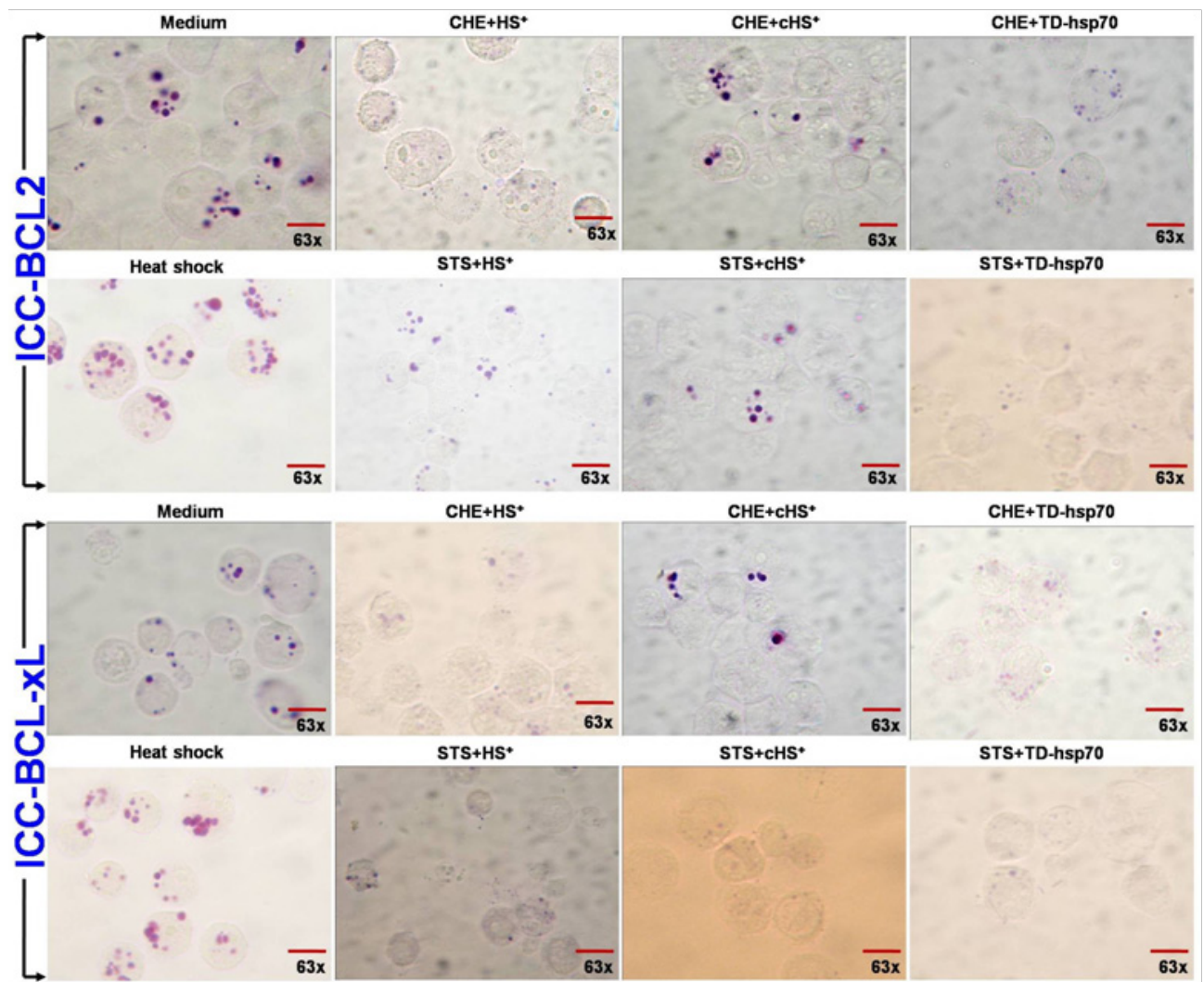


Figure 1 Effect of PKC inhibitors (CHE & STS) on cellular localization of anti-apoptotic BCL₂ proteins (BCL₂/Bcl-xL) in hsp70-mediated protection in DL cells. Cells were treated with PKC inhibitors (CHE-10µM & STS-1 µM) in the presence of HS⁺, cHS⁺ and tumor derived hsp70 for 6 hr, as mentioned in materials and methods, and the localization of anti-apoptotic BCL₂ proteins (BCL₂/Bcl-xL) in DL cells was examined. Cells treated with heat shock were used as positive control and untreated cells served as the negative control.

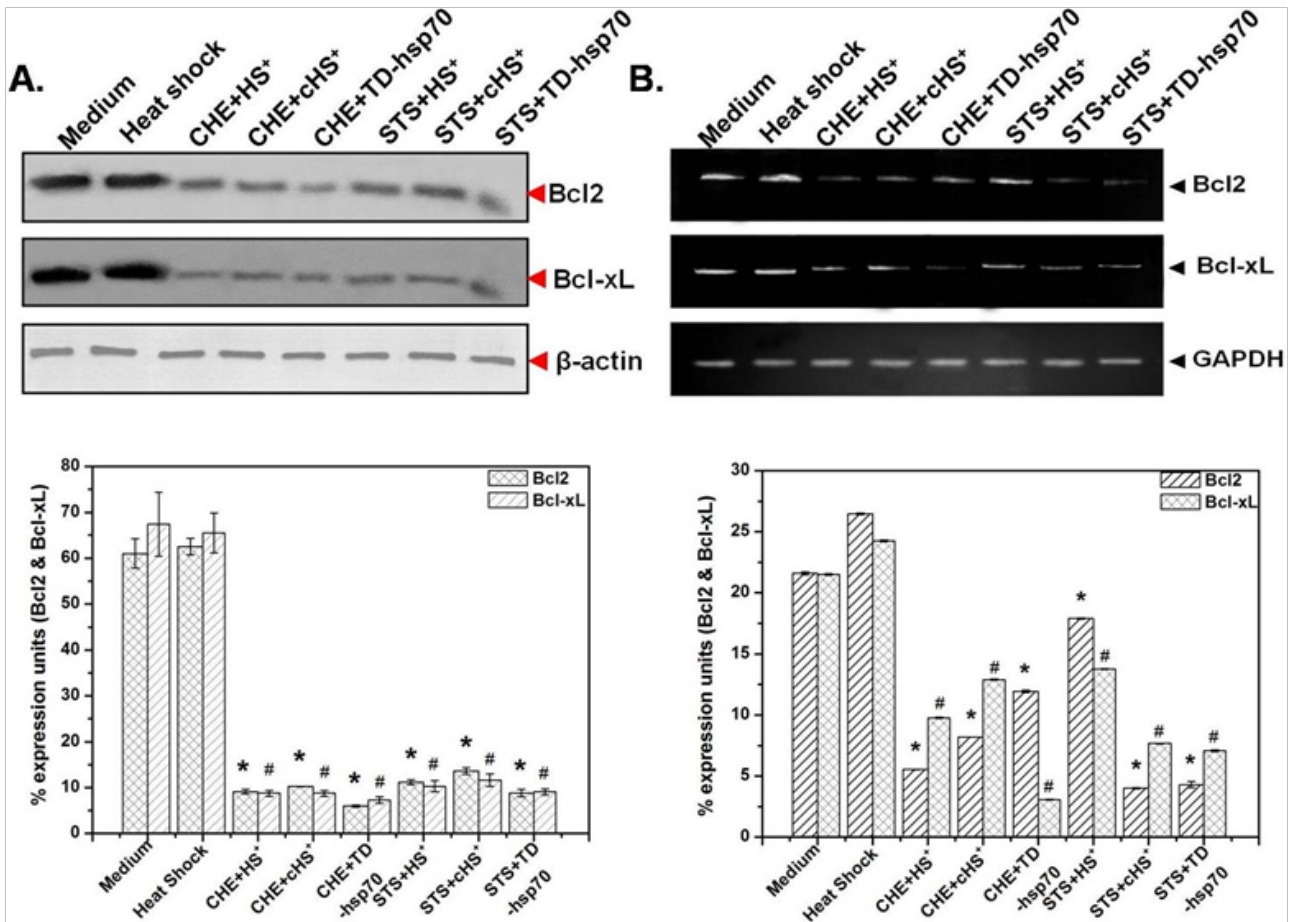


Figure 2 Effect of PKC inhibitors (CHE & STS) on the expression of anti-apoptotic BCL₂ proteins (BCL₂/Bcl-xL) in hsp70 mediated protection in DL cells. Cells were treated with PKC inhibitors (CHE-10 μ M & STS-1 μ M) in the presence of HS+, cHS+ and tumor derived hsp70 for 6 hr, as mentioned in materials and methods, and expression of anti-apoptotic BCL₂ proteins (BCL₂/Bcl-xL) in DL cells at protein and RNA levels were examined. Untreated cells were used as negative control while cells treated heat shock served as the positive control. Figure 2 A shows percent expression units of BCL₂/Bcl-xL at protein level. Figure 2 B shows percent expression units of BCL₂/Bcl-xL at RNA level. Mean BCL₂/Bcl-xL values \pm SEM were taken as significant at $p < 0.05$ (* & # indicates $p \leq 0.001$; control versus experimental).

hsp70 in DL cells. In addition, isolated and purified tumor derived-hsp70 was used exogenously, as previously described (methods, to study BCL₂ proteins expression against PKC inhibitors (CHE & STS) mediated apoptosis)^[2]. However, it is clear that hsp70 has a general anti-apoptotic function for which the mechanism is poorly understood. Previous studies show that hsp70 interferes in apoptotic signalling at several points and inhibits the release of cytochrome-c, activation of caspases and release of AIF from mitochondria^[1-4,26,27]. In this view, it was demonstrated that hsp70 prevents PKC inhibitor (CHE & STS) mediated down regulation of anti-apoptotic BCL₂ proteins (BCL₂/Bcl-xL) and thereafter apoptosis, up to a certain extent but beyond its threshold limit, was induced. Furthermore, exogenous application of tumor derived hsp70 showed insignificant protection against PKC inhibitor (CHE & STS)-mediated apoptosis. It was reported that enhanced expression of hsp70 during treatment with chemotherapeutic drugs promotes tumorigenesis^[28]. These data points suggest that hsp70 inhibited apoptosis at multiple points but treatment with PKC inhibitors (CHE & STS) significantly attenuated hsp70-mediated protection to anti-apoptotic BCL₂ proteins (BCL₂/Bcl-xL) and induced apoptosis in DL cells^[26,29,28].

Cytochrome-c, a mitochondrial protein, is released into cytosol during apoptosis upon treatment with cytotoxic drugs that induce DNA fragmentation^[19,30]. Further, cyt-c with Apaf-1, and pro-caspase-9 forms a complex structure, Apoptosome, which further

induced cleavage of pro-caspase-3 into active caspase-3^[26,29]. However, studies suggest that stress induced expression of hsp70 prevented release of cytochrome-c and activation of caspases^[1-4,19,30]. Anti-apoptotic BCL₂ proteins (BCL₂/Bcl-xL) are localized at the cellular membranes, particularly in mitochondria, where they stabilize mitochondrial integrity and inhibit the release of apoptogenic proteins such as cyt-c and AIF^[1-4,31,32]. Opposite to this, it was reported that treatment with PKC inhibitor (CHE) blocked Bcl-xL function and induced apoptosis in SH-SY5Y and MCF-7 cells^[26,30]. These findings are in corroboration with previous data points which suggests that cells were found highly sensitive to chelerythrine in hsp70-mediated cytoprotection. Furthermore, staurosporine showed parallel sensitivity to cells in hsp70-mediated protection at one tenth concentrations as compared to chelerythrine but it takes a longer time. These results supported our previous studies^[1-4,33]. In addition, treatment with PKC inhibitors (CHE & STS) led to the significantly reduced expression of anti-apoptotic BCL₂ proteins (BCL₂/Bcl-xL) during hsp70 mediated protection; however, staurosporine showed decreased expression of anti-apoptotic BCL₂ proteins (BCL₂/Bcl-xL) in a time-dependent manner^[32]. It was reported that reduced expression of anti-apoptotic BCL₂ proteins (BCL₂/Bcl-xL) resulted in enhanced expression of pro-apoptotic BCL₂ proteins (Bax/Bcl-xS) and vice versa^[6,34,35]. Correspondingly, PKC inhibitors (CHE & STS) induced the expression of pro-apoptotic BCL₂ proteins (Bax/

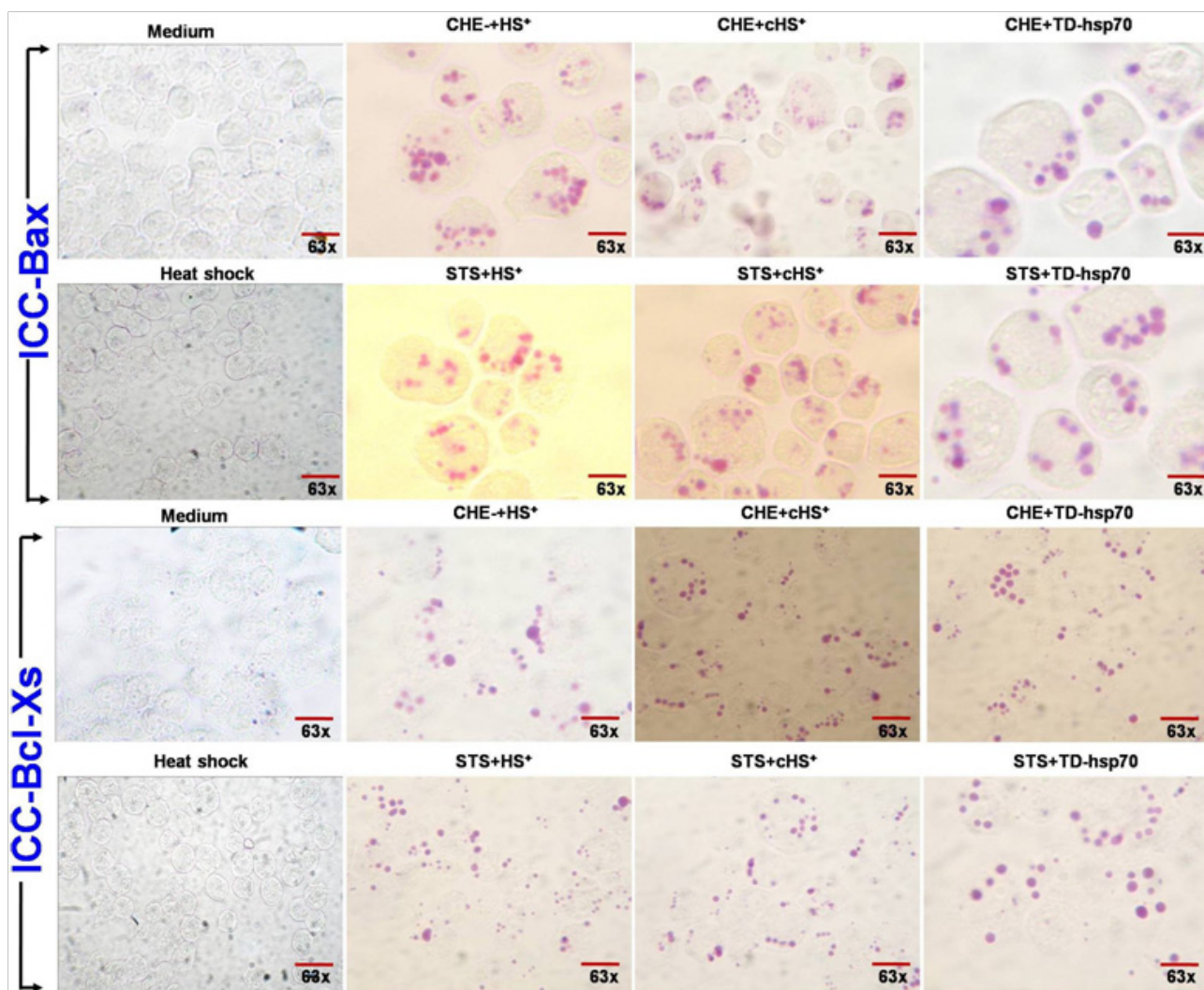


Figure 3 Effect of PKC inhibitors (CHE & STS) on cellular localization of pro-apoptotic BCL₂ proteins (Bax/Bcl-Xs) in hsp70-mediated inhibition in DL cells. Cells were treated with PKC inhibitors (CHE-10 μ M & STS-1 μ M) in the presence of HS⁺, cHS⁺ and tumor derived hsp70 for 6hr, as mentioned in materials and methods, and localization of pro-apoptotic BCL₂ proteins (Bax/Bcl-Xs) in DL cells was observed. Heat shock treated cells were considered as positive control cells and untreated cells served as the negative control.

Bcl-xS) in hsp70 mediated inhibition in DL cells. Staurosporine enhanced expression of pro-apoptotic BCL₂ proteins (Bax/Bcl-Xs) in time-dependent fashion than that of chelerythrine in hsp70-mediated inhibition. These results are in agreement with the previous findings of other co-workers, who compared the effect of chelerythrine and sanguinine on human prostate cancer cell lines, LNCaP, and DU-145 and human normal gingival fibroblast cells (Figure 7)^[23,36].

Differences in the expression of pro-apoptotic proteins (Bax/Bcl-Xs) in hsp70-mediated inhibition with studied compounds may be due to the change in the docking site for BH3 binding^[23,26]. These findings corresponded well with other colleagues^[32,33,37]. In addition, treatment with PKC inhibitors (CHE & STS) not only increased the expression of pro-apoptotic proteins (Bax/Bcl-Xs) but also induced the release of cytochrome-c and activated caspase-3 in DL cells which further led to the activation of nucleases like DNase^[1-4,38]. Activated nucleases induced DNA fragmentation and externalization of phosphatidylserine (PS) on the cell surface; these cells can be counted as part of the apoptotic cell population^[33,39]. The number of Annexin-V FITC positive and double positive/hypodiploid cells observed was higher than those of control cells, suggesting that both

studied compounds caused morphological changes in hsp70 mediated protection, corresponding to apoptosis. Therefore, treatment with PKC inhibitors (CHE & STS) showed a remarkable percentage of apoptotic cell population.

Although previous findings have demonstrated that increased expression of pro-apoptotic BCL₂ proteins (Bax/Bcl-Xs) directly induced activation of effector caspases and apoptosis, it was reported that chelerythrine induced Bax/Bak independent apoptosis in WT (wild type) and DKO (double knock-out) MEFs (murine embryonic fibroblasts) cells. In this context, chelerythrine in comparison with staurosporine showed strong pro-apoptotic potential in hsp70-mediated protection against Dalton's lymphoma^[24]. These results confirmed that PKC inhibitors (CHE & STS) significantly repressed the expression of anti-apoptotic BCL₂ proteins (BCL₂/Bcl-xL) in hsp70-mediated protection while the expression of pro-apoptotic BCL₂ proteins (Bax/Bcl-Xs) was upregulated in DL cells.

In conclusion, treatment with PKC inhibitors (CHE & STS) down regulated the expression of anti-apoptotic BCL₂ proteins (BCL₂/Bcl-xL) in hsp70-mediated protection in DL cells. Further, treatment

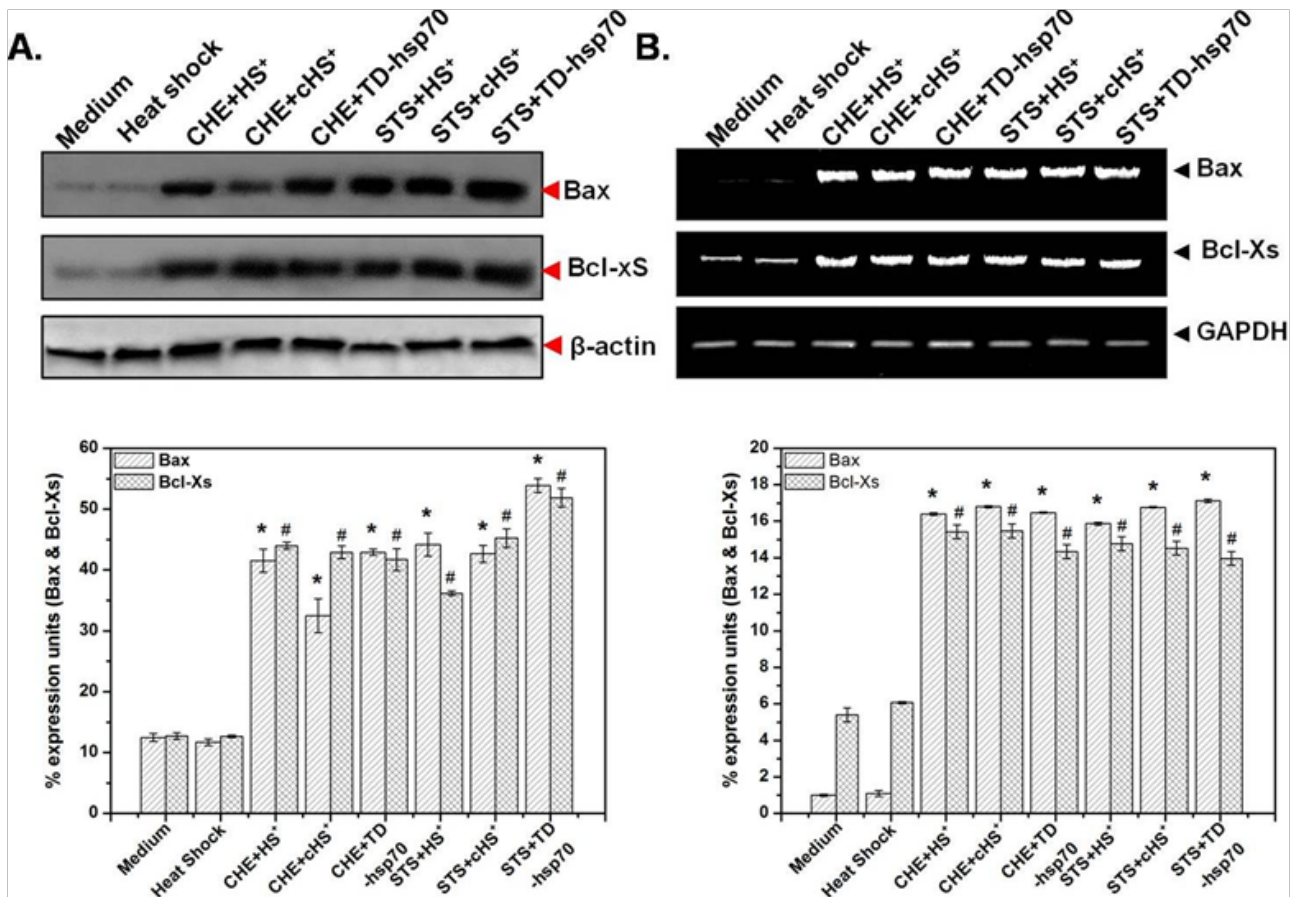


Figure 4 Effect of PKC inhibitors (CHE & STS) on the expression of pro-apoptotic BCL₂ proteins (Bax/Bcl-Xs) in hsp70 mediated inhibition in DL cells. Cells were treated with PKC inhibitors (CHE-10 μM & STS-1 μM) in the presence of HS⁺, cHS⁺ and tumor derived hsp70 for 6hr, as mentioned in materials and methods, and expression of Bax/Bcl-Xs at protein and RNA levels were examined in DL cells. Untreated cells were served as the negative control and heat shock treated cells served as the positive control. Figure 4 A shows percent expression units of Bax/Bcl-Xs at protein level. Figure 4 B shows percent expression units of Bax/Bcl-Xs at RNA level. Data was considered significant at $p < 0.05$ as applicable (* & # indicates $p \leq 0.001$; control versus experimental).

resulted in enhanced expression of pro-apoptotic BCL₂ proteins (Bax/Bcl-Xs) and caspase-3, and thereafter induction of apoptosis. This data suggests that PKC-inhibitors (CHE & STS) induced apoptosis by blocking hsp70-mediated protection of anti-apoptotic BCL₂ proteins in DL cells; however, hsp70 plays an important anti-apoptotic role in maintaining cellular equilibrium. Therefore, PKC inhibitors (CHE & STS) may pave the foundation for the development of new therapeutic approaches. However, additional studies are needed to explore the underlying mechanism.

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CONFLICT OF INTERESTS

There is no conflict of interest among the authors. The authors alone are responsible for the content and writing of the paper.

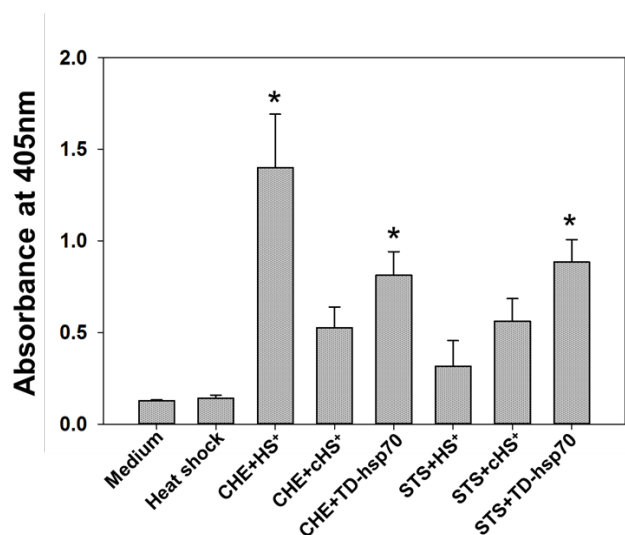


Figure 5 Effect of PKC inhibitors (CHE & STS) on the activation of caspase-3 in hsp70 mediated inhibition in DL cells. Cells were treated with PKC inhibitors (CHE-10 μM & STS-1 μM) in the presence of HS⁺, cHS⁺ and tumor derived hsp70 for 6hr and activation of caspase-3 in DL cells was examined by colorimetric assay. Heat shock treated cells were used as positive control and untreated (medium) cells served as the negative control. Data was taken as significant at $p < 0.05$ as applicable (* indicates $p < 0.001$; control versus experimental).

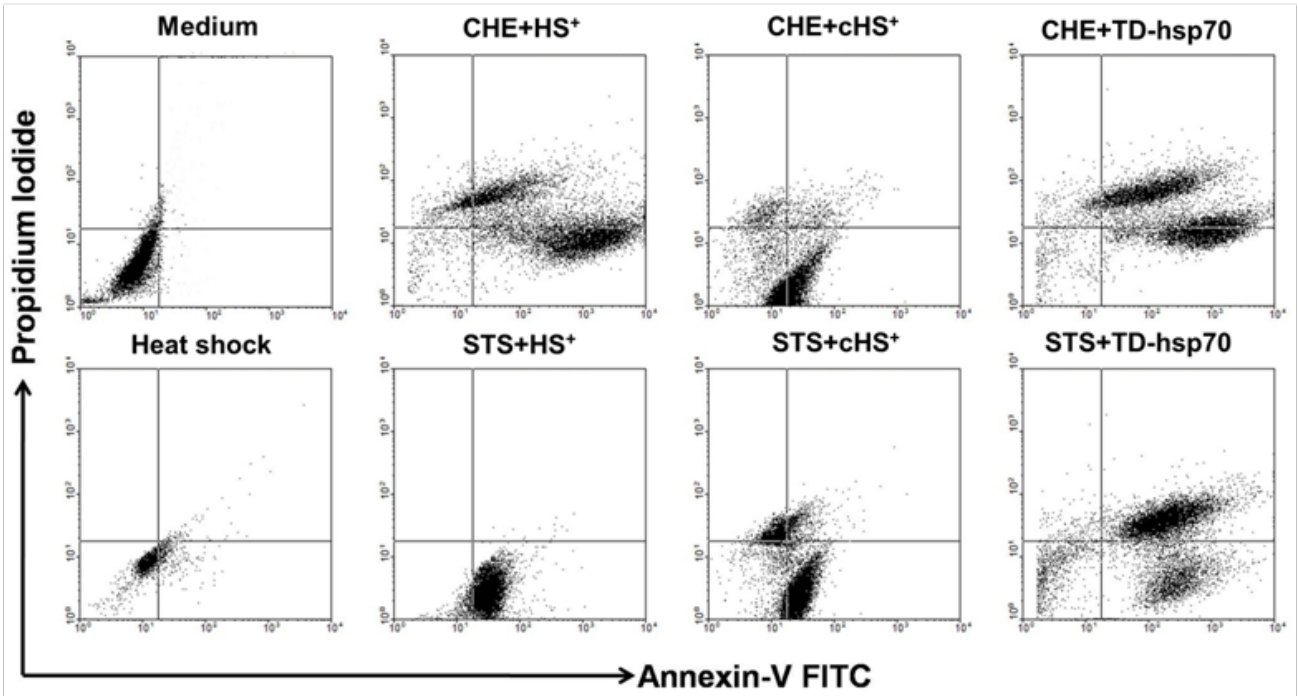


Figure 6 Effect of PKC inhibitors (CHE & STS) on apoptosis in hsp70-mediated protection in DL cells. Cells were treated with PKC inhibitors (CHE-10 μ M & STS-1 μ M) in the presence of HS⁺, cHS⁺ and tumor derived hsp70 and Annexin V FITC staining was carried out. Cells were stained with Annexin-V FITC and a counter stain propidium iodide (PI) and observed under flow cytometry. Heat shock was used as positive control and untreated cells (medium) was taken as the negative control.

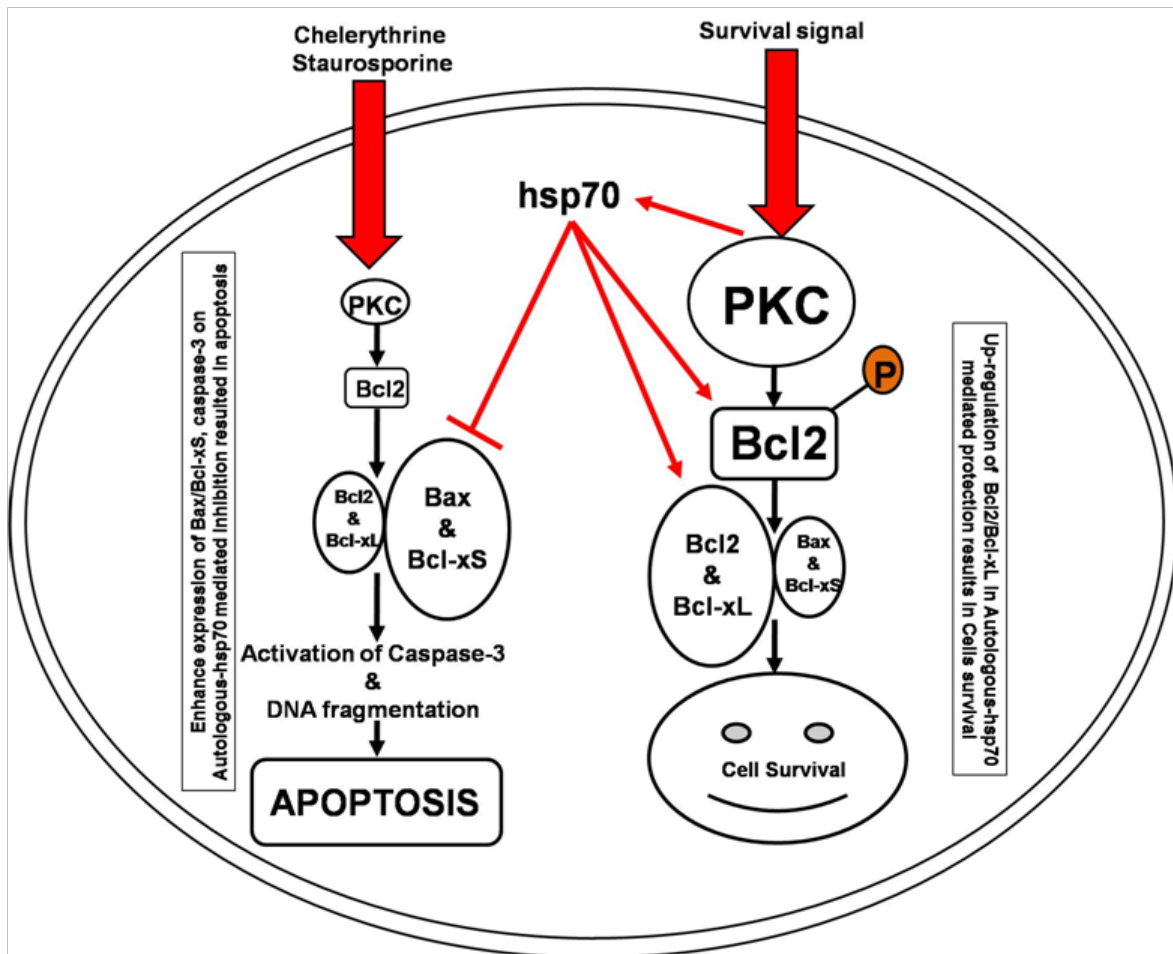


Figure 7 Schematic representation of PKC inhibitors (CHE & STS) modulated survival versus death pathway(s) in DL cells. Treatment with PKC inhibitors (CHE-10 μ M & STS-1 μ M) in the presence of HS⁺, cHS⁺ and tumor derived hsp70 showed activation of pro-apoptotic BCL2 proteins (Bax/Bcl-xS) and activation of caspase-3 in DL cells that resulted in degradation of DNA into fragments and apoptosis. Opposite to this, increase expression of anti-apoptotic BCL2 proteins (BCL2/Bcl-xL) in hsp70-mediated protection leads to cells survival^[1,27,32,35-37,41].

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