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Pentoxifylline induces apoptosis *in vitro* in cutaneous T cell lymphoma (HuT-78) and enhances FasL mediated killing by upregulating Fas expression

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ABSTRACT

Constitutive nuclear factor-κB (NF-κB) is known to play an important role in the survival of HuT-78 cells, a cutaneous T cell lymphoma (CTCL) cell line. Here, we have demonstrated that pentoxifylline (PTX), a phosphodiesterase inhibitor, can trigger a series of events leading to apoptosis in HuT-78 cells without affecting NF-κB. Apoptosis was ascertained by sub-G_1 peak analysis and TUNEL assay. Apoptosis induced by PTX in HuT-78 cells involved mitochondrial hyperpolarization, cytochrome *c* release, caspase-3 activation and PARP cleavage. Further, it was found that PTX treatment downregulated Bcl-xl and c-FLIP expression without affecting constitutive NF-κB but upregulated activator protein-1 (AP-1). Low concentration of PTX upregulated Fas and TRAIL expression in HuT-78 cells. In addition, PTX can act as a scavenger of reactive oxygen intermediate and it could enhance FasL mediated killing in HuT-78 cells. Our results taken together indicated that PTX may be a potential agent for killing CTCL cells.

1. Introduction

Cutaneous T cell lymphoma (CTCL) is a group of lymphoproliferative disorders of the skin [1,2]. Approximately 1000–1500 new cases are reported every year in the United States [3]. Mycosis fungoides (MF) and its leukemic counterpart, the Sézary syndrome (SS) are the most frequent forms of this disease. Patients with SS syndrome manifest erythroderma, generalized lymphadenopathy and prominent immunologic defects due to the production of T-helper 2 (Th2) cytokines and by the depressed production of Th1 cytokines [4].

HuT-78, a CTCL cell line constitutively expresses nuclear factor-κB (NF-κB) and tumor necrosis factor alpha, TNF-α [5]. TNF-α, acts as an autocrine growth factor for HuT-78 cells [6], since proliferation of HuT-78 cells can be inhibited by lowering TNF-α level using anti-TNF antibody [5,6].

NF-κB is an important transcription factor involved in immune and inflammatory cellular responses affecting cell growth and survival [7]. NF-κB regulates expression of a large number of genes that are critical for the regulation of programmed cell death or apoptosis [8]. Works from different laboratories have shown that inhibition of constitutive NF-κB induced apoptosis in CTCL cells [9]. Apoptosis induced by TNF-α, cytotoxic drugs and radiation in many cell types can be suppressed by the activation of NF-κB and NF-κB dependent antiapoptotic proteins such as Bcl-2, Bcl-xl, c-FLIP, Bfl-1/A1 and c-IAP [10].

Pentoxifylline (PTX) is a xanthine derived antioxidant known to lower TNF-α in many cells [11]. It is also reported

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Abbreviations: PTX, pentoxifylline; CTCL, cutaneous T cell lymphoma; NF-κB, nuclear factor kappa B; Bcl-xl, B cell lymphoma-x long isoform; c-FLIP, cellular FLICE like inhibitory protein; Fas, fibroblast associated; TRAIL, tumor necrosis factor-related apoptosis inducing ligand.

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that PTX inhibits not only inducible NF-κB but also its constitutive form [12–14]. Moreover, PTX has been shown to enhance antitumor activity of some drugs and can also sensitize tumors cells to radiotherapy [15–18]. Effect of PTX on CTCL cells has not been reported so far.

Present study was undertaken to see the effect of PTX on HuT-78 cells. Here, we provide evidences to show that PTX induces apoptosis in HuT-78 cells and it is not necessary to inhibit constitutive NF-κB to induce apoptosis in these cells. Furthermore, we also showed that PTX scavenges reactive oxygen intermediate, upregulates Fas expression and enhances FasL mediated killing in HuT-78 cells.

2. Materials and methods

2.1. Chemicals and antibodies

Pentoxifylline (PTX), curcumin, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), acridine orange (AO), ethidium bromide (EB), propidium iodide (PI), 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$), carbonyl cyanide 3-chlorophenylhydrazone (CCCP), p-formaldehyde, dihydrorhodamine 123, 2′,7′-dichlorofluorescein diacetate (DCF-DA), recombinant human Fas ligand (FasL), mouse anti-actin antibody and anti-mouse HRP antibody were purchased from Sigma–Aldrich (St. Louis, MO, USA). PI/RNase staining buffer, apoptosis detection kit; APO-BRDU^{TM} kit, mitochondrial membrane sensor kit and antibodies against cytochrome c, PARP, Bcl-xl, cIAP-1/2, caspase-8, PE-labeled CD95 and PE-labeled IgG1 isotype control were purchased from BD PharMingen (San Diego, CA, USA). Antibodies against c-FLIP, p50 and p65 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Caspase-8 inhibitor, Ac-IETD-CHO was purchased from Calbiochem (La Jolla, CA, USA).

2.2. Cell line and cell culture

Human CTCL cell line, HuT-78 was obtained from National Centre for Cell Science (Pune, India). The cells were cultured in RPMI-1640 medium supplemented with 10% heat inactivated Foetal Bovine Serum (GIBCO, Grand Island, NY, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate and 10 mM HEPES. Cells were maintained in an incubator at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. All cell culture reagents were purchased from Sigma–Aldrich. All media and reagents used were endotoxin-free.

2.3. MTT assay

Cytotoxicity of PTX on HuT-78 cells was assessed by MTT as described previously [19]. Briefly, 2×10^4 cells were treated with different concentrations of PTX (0–6 mg/ml) for 24 h. At the end of treatment, 20 μl of MTT (5 mg/ml in phosphate buffered saline, PBS) was added and cells were further incubated for 4 h. Formazan crystals formed were dissolved in 100 μl of lysis solution (20% sodium dodecyl sulfate, 50% dimethylformamide). The absorbance of solubilized formazan was read at 570 nm using ELISA reader (Bio-tek Instruments, Inc.).

2.4. Fluorescence morphological examination

Cell morphology of PTX treated HuT-78 cells was investigated after 48 h of treatment by staining cells with a combination of fluorescent DNA binding dyes AO/EB. The solution containing each dye at 1 μg/ml in PBS was mixed 1:1 with cell suspension. Stained cells were viewed under Zeiss fluorescence microscope using a 20× objective [19].

2.5. Lymphocytes isolation

Human lymphocytes were isolated from peripheral blood of healthy donor by centrifugation in a density gradient of Histopaque as described previously [20]. The viability of the isolated lymphocytes were found to be about 99% as measured by trypan blue exclusion assay.

2.6. Quantification of apoptosis by flow cytometry

Apoptosis induction in PTX treated HuT-78 cells was determined by (i) analysis of hypoploidy, (ii) terminal deoxynucleotidyltransferase (TdT) dUTP nick end labeling (TUNEL). For analysis of hypoploidy, 1×10^5 HuT-78 cells were treated with different concentrations of PTX (0, 1.5, 3 and 6 mg/ml) for 24, 48 and 72 h. After incubation, cells were harvested, fixed in 70% ethanol and stained with PI/RNase staining buffer (5 μg/ml PI, 200 μg/ml RNase) as described [19]. The percentage of apoptotic cells was determined by measuring the fraction of nuclei with a sub-diploid DNA content using Becton Dickinson FACScan with Cell Quest software (Becton Dickinson). Apoptotic cells were also analyzed by TUNEL assay where HuT-78 cells after indicated time points were stained using APO-BRDU^{TM} kit according to manufacturer's protocol. Stained cells were analyzed by Becton Dickinson FACScan using Cell Quest software (Becton Dickinson).

2.7. Analysis of DNA fragmentation

DNA fragmentation was assayed according to the method of Herrmann et al. [21]. Briefly, 1×10^6 cells were treated with PTX (0, 1.5, 3 and 6 mg/ml) for 48 h. Cells were harvested, washed with PBS and pelleted by centrifugation. Cells were lysed in lysis buffer (1% Nonidet P-40, 20 mM EDTA, 50 mM Tris–HCl, pH 7.5) and supernatants were collected after centrifugation at $1600 \times g$ for 5 min. Supernatants were brought to 1% SDS and treated with RNase A for 2 h at 50 °C and subsequently with proteinase K for 2 h at 37 °C. DNA fragments were precipitated with 2.5 volumes of ethanol in the presence of 5 M ammonium acetate. DNA fragmentation was visualized by electrophoresis on a 1% agarose gel.

2.8. Measurement of mitochondrial membrane potential

2.8.1. Mitochondrial membrane potential determination by $DiOC_6$ dye

The change of mitochondrial membrane potential was determined by the retention of the dye $DiOC_6$ [22]. Briefly, 1×10^5 cells were treated with PTX (0, 1.5, 3 and 6 mg/ml) for 12, 24 and 48 h. After treatment cells were harvested, washed twice with PBS and stained with 50 nM $DiOC_6$ at 37 °C for

30 min, the cells were washed again and analyzed by FACScan using Cell Quest software at FL-1 channel.

2.8.2. Mitochondrial membrane potential determination by JC-1 dye

Mitochondrial membrane potential sensor kit was also used to detect changes in the mitochondrial membrane potential as per manufacturer's protocol. Briefly, HuT-78 cells were treated with different concentrations of PTX (0, 1.5, 3 and 6 mg/ml) for 48 h. After treatment cells were harvested, washed with PBS and stained with 5 μg/ml 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolo-carbocyanine (JC-1) for 30 min. Analysis of stained cells was performed at FL-2 channel by Becton Dickinson FACScan using CellQuest software (Becton Dickinson).

2.9. The measurement of cytochrome c release

The release of cytochrome *c* into the cytosol was measured by Western blotting analysis as described previously [23]. Briefly, HuT-78 cells were treated with different concentrations of PTX (0, 1.5, 3 and 6 mg/ml) for 48 h. After treatment cells were harvested, washed twice with ice-cold PBS and gently lysed with ice-cold lysis buffer (250 mM sucrose, 1 mM EDTA, 0.05% digitonin, 25 mM Tris, pH 6.8, 1 mM DTT, 1 μg/ml aprotinin, 1 mM benzamidine and 0.1 mM PMSF). After centrifugation at 12,000 × *g* at 4 °C for 3 min, the supernatants (cytosolic extracts free of mitochondria) were obtained, resolved on a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting using anti-cytochrome *c* antibody.

2.10. Caspase-3 activity assay

Caspase-3 activity was determined using a colorimetric CaspACE assay system according to manufacturer's protocol (Promega, Madison, WI, USA). Cell lysates were prepared after 48 h of treatment with various concentrations of PTX (0, 1.5, 3 and 6 mg/ml) and assayed for caspase-3 activity using colorimetric substrate (Ac-DEVD-pNA). Chromophore p-nitroaniline (pNA) is released from substrate upon cleavage by caspase-3. Absorbance of the samples was read at 405 nm in a microtiter plate reader (Bio-tek Instruments, Inc.).

2.11. Determination of NF-κB activity

NF-κB activity was determined by electrophoretic mobility shift assay (EMSA) as described previously [19]. In brief, 4 μg nuclear extracts prepared from PTX or curcumin treated HuT-78 cells were incubated with ^{32}P-end-labeled 22 mer (5′-AGTTGAGGGGACTTTCCCAGGC-3′; underlining indicates NF-κB binding site) double-stranded NF-κB oligonucleotide (Promega) for 15 min at 37 °C. DNA–protein complex was resolved in a 6.6% native polyacrylamide gel. For supershift assays, nuclear extracts were incubated with antibodies against either p50 or p65 subunits of NF-κB for 30 min at room temperature before the complex was analyzed by EMSA [24]. The specificity of the binding was also examined by competition with unlabelled oligonucleotide. The dried gels were visualized and radioactive bands were quantitated by PhosphorImager (Bio-Rad, Hercules, CA, USA) using Quantity One software.

2.12. Activator protein-1 activation

To determine activator protein-1 (AP-1) activity EMSA was performed as described [24]. Briefly, 6 μg nuclear extracts prepared from PTX treated HuT-78 cells were incubated with ^{32}P-end-labeled 21 mer (5′-CGCTTGATGAGTCAGCCGGAA-3′; underlining indicates AP-1 binding site) double-stranded AP-1 oligonucleotide (Promega) for 30 min at 37 °C. DNA–protein complex was analyzed using 6% native polyacrylamide gel. The specificity of the binding was examined by competition with unlabelled oligonucleotide. Visualization and quantitation of radioactive bands were done as indicated above.

2.13. Western blotting

For Western blot analysis whole cell lysates were prepared from PTX, FasL treated cells as described previously [25]. In brief, at indicated time points, cells were harvested, washed twice with cold PBS and lysed in lysis buffer (20 mM Tris–HCl, pH 7.4, 0.25 M NaCl, 2 mM EDTA, pH 8.0, 0.1% Triton-X-100, 1 mM DTT, 1 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin and 1.25 mg/ml bezamidine). Protein extracts were resolved on 10–12% SDS-PAGE. After electrophoresis, proteins were electrotransferred to nitrocellulose membrane and probed with primary antibodies against either PARP, Bcl-xl, c-FLIP, c-IAP or caspase-8 for overnight at 4 °C and incubated with secondary antibody for 45 min. Protein bands were visualized using enhanced chemiluminescence kit (ECL; Amersham Biosciences, Buckinghamshire, UK) and densitometry of individual bands were determined using Scion Image software (Scion Corporation).

2.14. Ribonuclease protection assay

The ribonuclease protection assay (RPA) is a highly sensitive and specific method for the detection of mRNA species. The expression of various apoptosis associated genes in HuT-78 cells was determined by RPA using human apoptosis multi-probe template set; hStress-1, which included human bcl-x, p53, GADD45, c-fos, p21, bax, bcl-2, mcl-1, L32 and GAPDH or hAPO-3, which included caspase-8, FASL, FAS, FADD, DR3, FAP, FAF, TRAIL, TNFRp55, TRADD, RIP, L32 and GAPDH (BD PharMingen) as per the manufacturer's protocol. Briefly, cells were first treated with PTX for 8 h and then total RNA was isolated using RNA isolation kit (Promega). RNA samples (10 μg) were hybridized with ^{32}P-labeled antisense mRNA probes and digested with RNase and T1 nuclease. The protected hybridized probe fragments were resolved on 4.75% denaturing polyacrylamide gel. The radioactive bands were visualized and quantitated using a Bio-Rad Personal Molecular Imager Fx and the associated Quantity One software. The relative mRNA levels were determined by normalizing band intensities with that of GAPDH probe.

2.15. Analysis of Fas surface expression

The surface expression of Fas/CD95 was studied by flow cytometry as described previously [26]. Briefly, 2×10^5 cells were treated with varying concentrations of PTX (0, 1.5, 3, 4.5 and 6 mg/ml) for 12 or 24 h. After incubation cells were

harvested and washed in FACS buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, 0.02% NaN_3 and 5% foetal bovine serum). Blocking was done by incubation on ice for 30 min using 100 μl of FACS buffer supplemented with 5% normal human serum and 5% normal goat serum. Cells were stained with PE-conjugated mouse IgG1 isotype control or PE-conjugated mouse anti-human CD95 for 1 h on ice followed by three washings with FACS buffer. Cells were analyzed by FACS using Cell Quest software at FL-2 channel.

2.16. *Measurement of reactive oxygen intermediates (ROI)*

2.16.1. *Flow cytometry*

The production of ROI was determined by flow cytometry as described previously [25]. Briefly, 2×10^5 cells were treated with 3 mg/ml PTX for different time periods (0, 1, 2, 4 and 8 h). To detect ROI production cells were exposed to 1 μM dihydrorhodamine 123 for 1 h at 37 °C, washed with PBS three times and resuspended in 0.5 ml of PBS. Rhodamine 123 fluorescence intensity resulting from dihydrorhodamine 123 oxidation was measured using Becton and Dickinson FACScan flow cytometer at FL-1 channel using CellQuest software (Becton and Dickinson).

2.16.2. *Fluorescence microscopy*

In brief, after 8 h of treatment with 3 mg/ml of PTX, cells were incubated with 5 μM of cell-permeable redox-sensitive fluorochrome, DCF-DA for 5 min at 37 °C and subsequently washed twice in cold PBS and viewed using Zeiss fluorescence microscope using a 40× objective [27].

2.17. *PI exclusion assay*

Cell viability was determined by the ability of cells to exclude PI as described earlier [28]. HuT-78 cells (2×10^5 ml^{-1}) were treated with 3 mg/ml PTX or 500 ng/ml FasL either alone or together for 24 h. After treatment, cells were harvested, washed with PBS and resuspended in PBS containing 1 μg/

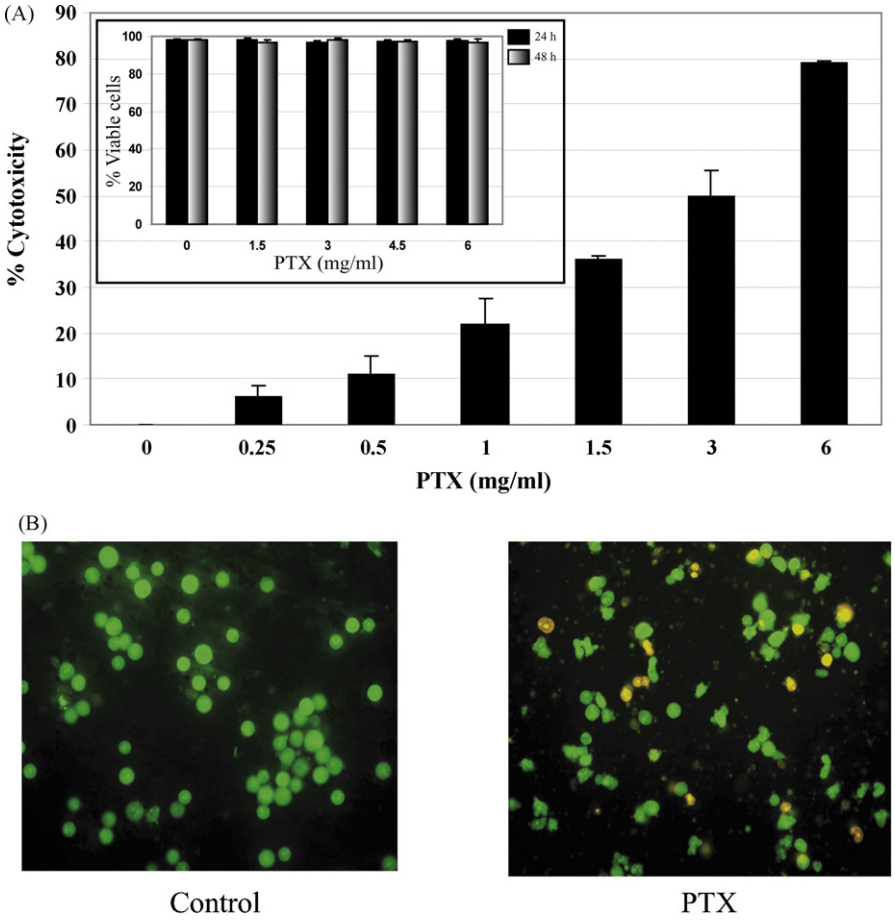

Fig. 1 – Effect of PTX on cell viability. (A) HuT-78 cells were treated with different concentrations of PTX (0–6 mg/ml) for 24 h. The cytotoxicity was evaluated by MTT assay as described in Section 2. Data are expressed as percentage over untreated control. Values are expressed as mean ± S.D. (n = 3). The viability of human lymphocytes after 24 and 48 h of treatment with different concentrations of PTX was determined by trypan blue exclusion assay (inset). Values are expressed as mean ± S.D. (n = 3). Data are expressed as percentage over untreated control. (B) Changes in cell morphology were observed under fluorescence microscope by staining with AO/EB after 48 h of treatment with 3 mg/ml PTX. The viable cells appeared green while apoptotic cells appeared orange. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

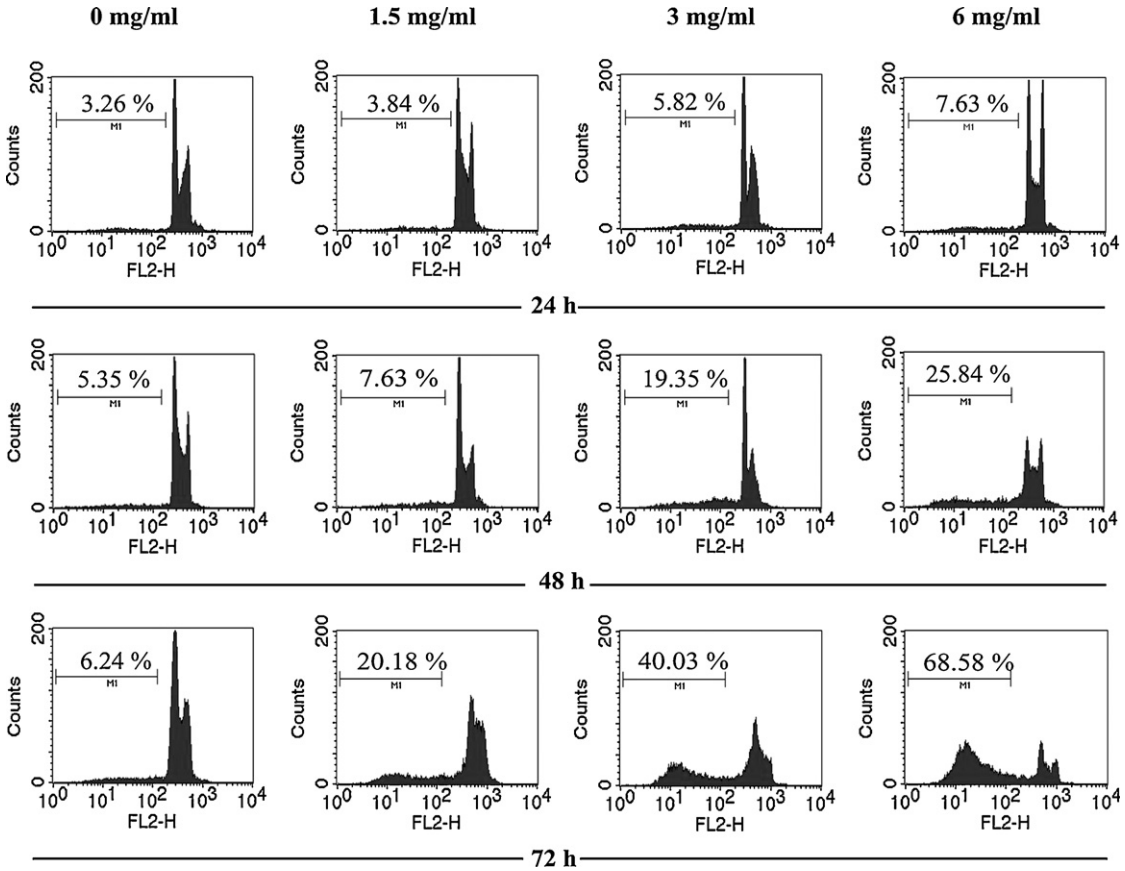

Fig. 2 – Dose and time dependent effect of PTX on sub-G_1 DNA content. HuT-78 cells were treated with PTX (0, 1.5, 3 and 6 mg/ml) for indicated time points (24, 48 and 72 h), stained with PI/RNase staining buffer and assessed for sub-G_1 (hypoploidic) peak by flow cytometry at FL-2 channel, where M1 represents sub-G_1 peak. Data represent the results from one of the three similar experiments.

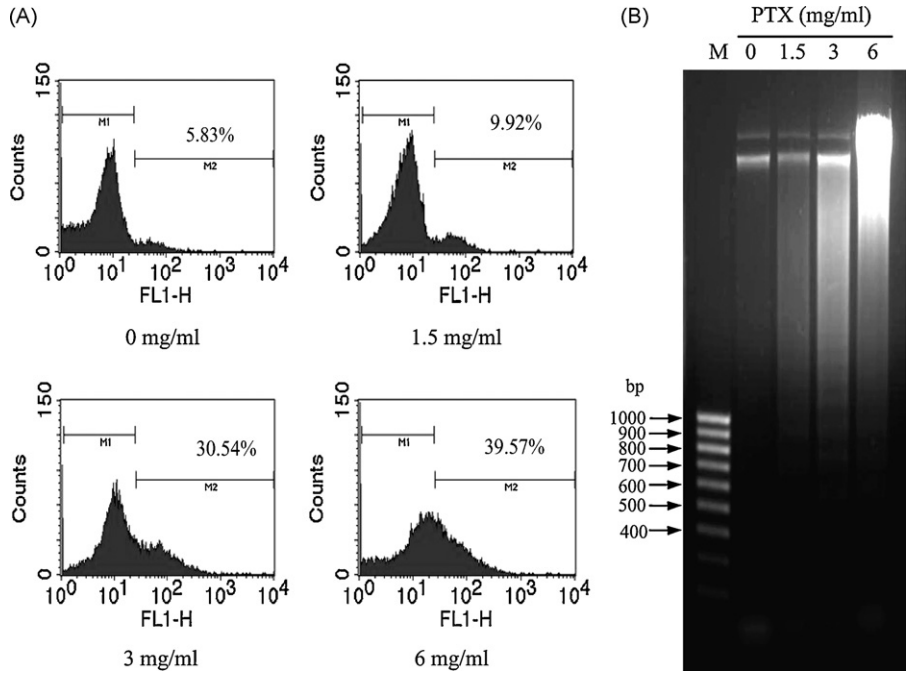

Fig. 3 – PTX induces apoptosis in HuT-78 cells. (A) HuT-78 cells were treated with PTX (0, 1.5, 3 and 6 mg/ml) for 48 h and quantitated for apoptosis by flow cytometry at FL-1 channel using TUNEL assay. The M1 and M2 gates demarcate non-apoptotic and apoptotic populations, respectively. (B) HuT-78 cells were treated with PTX (0, 1.5, 3 and 6 mg/ml) for 48 h and evaluated for DNA fragmentation. M: 100 bp ladder.

ml of PI. The level of PI incorporation was quantitated by flow cytometry on a FACScan flow cytometer.

2.18. Statistical analysis

Statistical significance of the differences was determined by the paired two-tailed Student *t* test using Microsoft Excel software. $P < 0.05$ was considered as significant.

3. Results

3.1. PTX shows cytotoxic activity against HuT-78 cells

Human CTCL cell line, HuT-78 was treated with different concentrations of PTX (0–6 mg/ml) for 24 h. The cytotoxicity of PTX increased in a dose dependent manner with 3 mg/ml inducing 50% cytotoxicity as tested by MTT assay (Fig. 1A). Thereafter, we investigated morphological assay of cell death using AO/EB staining after treatment of cells with 3 mg/ml PTX for 48 h. Uniformly green HuT-78 cells with normal morphology were seen in control cells, whereas orange HuT-78 cells with fragmented chromatin and apoptotic bodies were seen in treated cells, an indicative of apoptosis (Fig. 1B).

We also examined whether PTX is cytotoxic to normal lymphocytes. For this purpose, human lymphocytes isolated from peripheral blood were treated with different concentrations of PTX (0, 1.5, 3, 4.5 and 6 mg/ml) for 24 and 48 h. In all combinations at indicated time points, cell viability was greater than 96% as evaluated by trypan blue exclusion method (Fig. 1A, inset). Thus, we found that even 6 mg/ml PTX is not cytotoxic for normal blood lymphocytes.

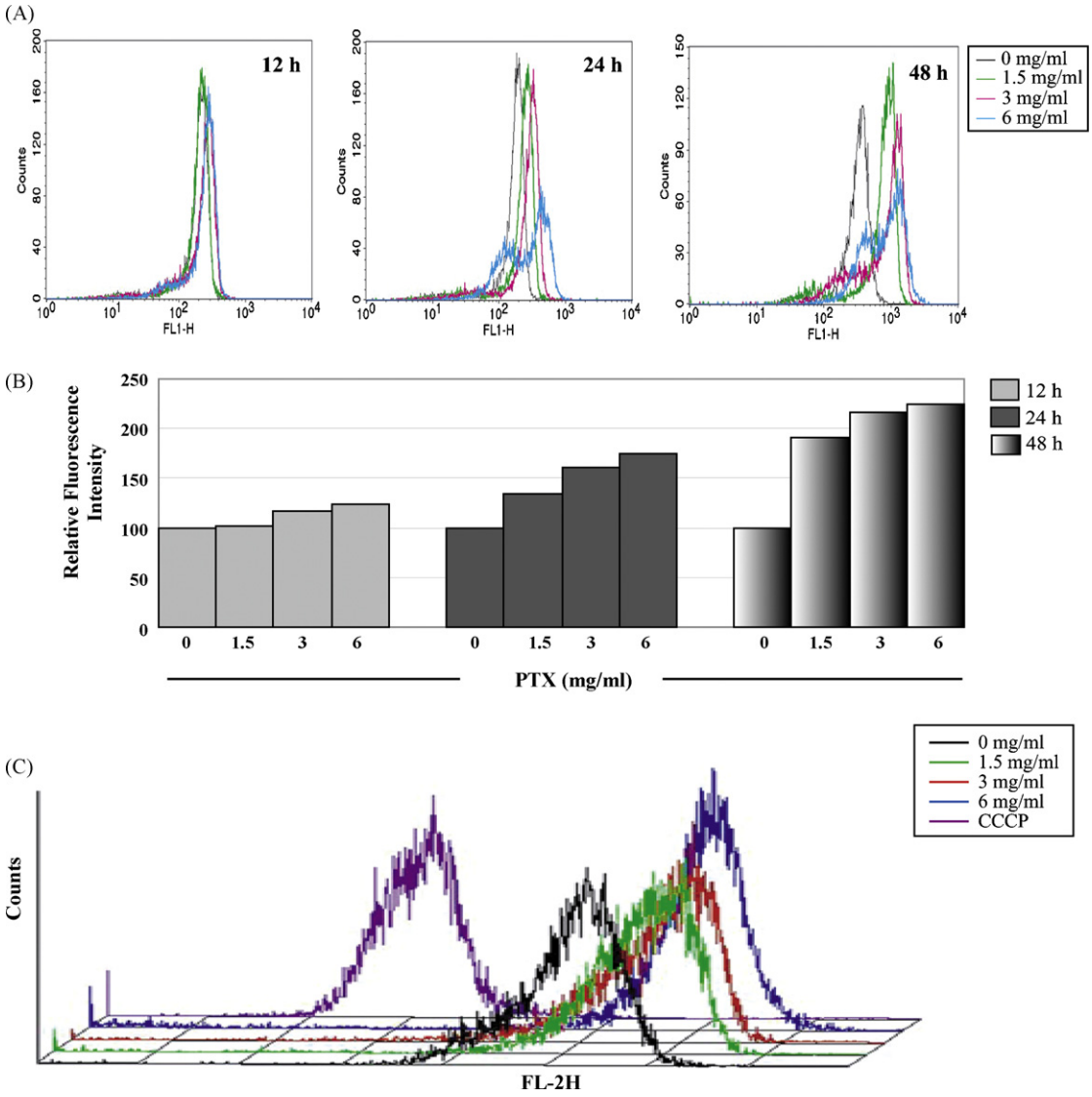

Fig. 4 – PTX induces alterations in MMP. (A) HuT-78 cells were treated with PTX (0, 1.5, 3 and 6 mg/ml) for indicated time points (12, 24 and 48 h). The mitochondrial membrane potential changes were determined by $DiOC_6$ staining using flow cytometry at FL-1 channel. (B) Bar graph represents changes in mitochondrial membrane potential of indicated time points as relative fluorescence intensity of $DiOC_6$ staining. Data represent the results from one of the three similar experiments. (C) Cells treated with PTX (0, 1.5, 3 and 6 mg/ml) for 48 h were incubated with JC-1 dye and changes in MMP were measured by flow cytometry at FL-2 channel. CCCP treated cells served as depolarization control.

3.2. PTX induces apoptosis in HuT-78 cells

HuT-78 cells were treated with different concentrations of PTX (0, 1.5, 3 and 6 mg/ml) and the percentage of sub-G_1 fraction (hypoploidic cells) were quantitated after 24, 48 and 72 h by flow cytometry to detect apoptosis. After 24 h of treatment with PTX, no significant change with respect to control in sub-G_1 fraction was observed. However, a dose dependent increase in the percentage of cells in sub-G_1 fraction was observed after 48 h of treatment with PTX. Further increase in the percentage of cells in sub-G_1 fraction was detected after 72 h of treatment (Fig. 2).

TUNEL assay with PTX treated HuT-78 cells for 48 h were performed to confirm apoptosis. As shown in Fig. 3A, HuT-78 cells showed dose dependent increase in the percentage of apoptotic cells from 5.83% to 9.92%, 30.54% and 39.57% after treatment with 1.5, 3 and 6 mg/ml PTX respectively. Concentration dependent fragmentation of DNA in HuT-78 cells after 48 h of PTX treatment further confirmed apoptosis (Fig. 3B).

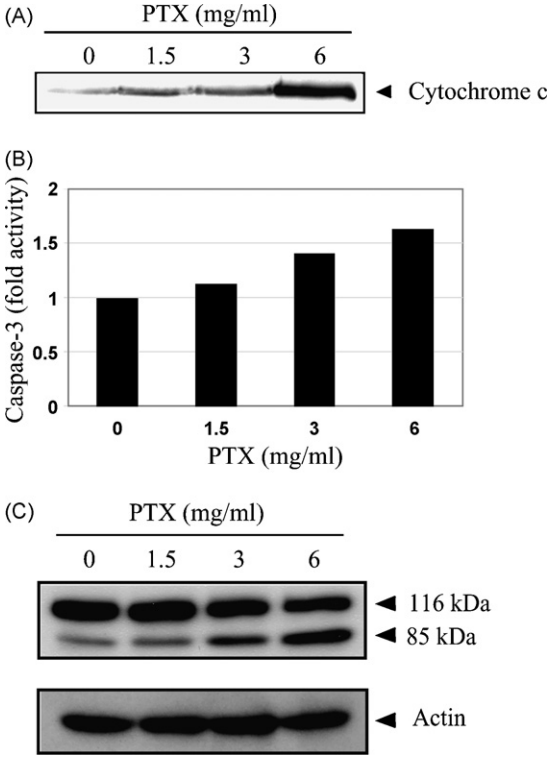

Fig. 5 – PTX causes cytochrome c release, caspase-3 activation and PARP cleavage. (A) HuT-78 cells were treated with different concentrations of PTX (0, 1.5, 3 and 6 mg/ml) for 48 h. The cytosolic fraction was separated and an immunoblot analysis was performed using mouse monoclonal anti-cytochrome c antibody. (B) Cells were treated with PTX (0, 1.5, 3 and 6 mg/ml) for 48 h and analyzed for caspase-3 activation by colorimetric assay as described in Section 2. Similar results were obtained in two separate experiments. (C) PARP cleavage was detected by Western blotting using mouse monoclonal anti-PARP antibody.

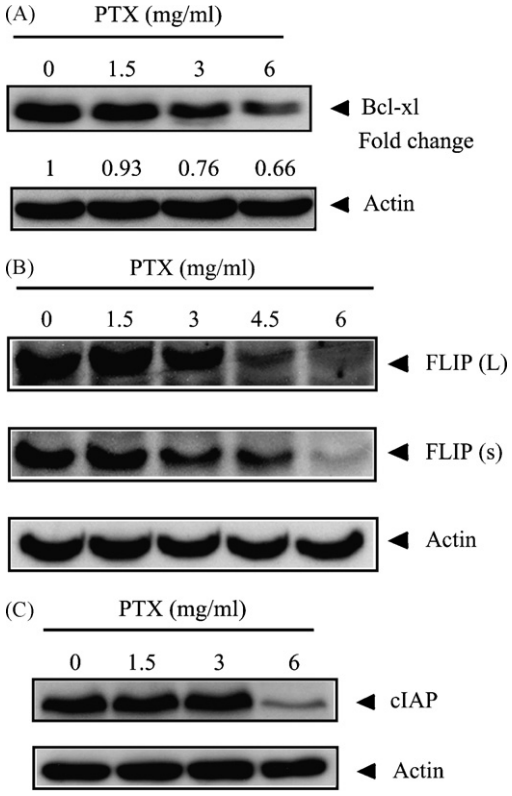

Fig. 6 – PTX inhibits expression of antiapoptotic proteins (Bcl-xl, c-FLIP and c-IAP). (A) HuT-78 cells were treated with varying concentrations of PTX (0, 1.5, 3 and 6 mg/ml) for 48 h. Whole cell lysates were resolved on 12% SDS-PAGE and immunoblotted for Bcl-xl expression using mouse monoclonal anti-Bcl-x antibody. Bcl-xl band intensity was normalized to actin. (B) Whole cell lysates prepared after 18 h of treatment with different concentrations of PTX (0, 1.5, 3, 4.5 and 6 mg/ml) were resolved on 12% SDS-PAGE and immunoblotted for c-FLIP expression using mouse monoclonal anti-c-FLIP$_{S/L}$ antibody. (C) c-IAP expression was detected by Western blotting after 48 h of treatment with PTX (0, 1.5, 3 and 6 mg/ml) on 10% SDS-PAGE using mouse monoclonal anti-c-IAP antibody. Equal loading of protein was confirmed by actin.

3.3. PTX changes mitochondrial membrane potential and causes cytochrome c release

Next, we studied if PTX induced apoptosis involves disruption of mitochondrial membrane potential (MMP). Exposure of HuT-78 cells to different concentrations of PTX (0, 1.5, 3 and 6 mg/ml) for various time points (12, 24 and 48 h) showed a dose dependent increase in MMP at 24 and 48 h indicating mitochondrial hyperpolarization. We did not get much change in MMP at 12 h. At 48 h the depolarization peak also increased with increasing concentrations of PTX. The depolarization corresponds to lower fluorescence which indicates the dysfunction of mitochondria (Fig. 4A and B).

The increase in MMP by PTX was further verified by staining with JC-1 dye, a potentially sensitive fluorescent dye that

detects polarized mitochondria giving red fluorescence at FL-2 channel. HuT-78 cells were treated with different concentrations of PTX (0, 1.5, 3 and 6 mg/ml) for 48 h and then stained with JC-1 dye. Fig. 4C showed that red fluorescence in PTX treated HuT-78 cells shifted towards right compared to untreated cells in a dose dependent manner. On the other hand, 100 μM CCCP treated cells, a control for depolarization, shifted the intensity of fluorescence to left indicative of depolarized MMP.

Next, we examined the release of cytochrome c in PTX treated cells after 48 h of treatment. PTX treatment induced cytochrome c release into the cytosol (Fig. 5A) as detected by Western blot analysis, indicating that mitochondrial pathway is associated with PTX mediated apoptosis.

3.4. *PTX induces caspase-3 activation and PARP cleavage in HuT-78 cells*

Colorimetric assay was performed to see the activation of caspase-3 in PTX treated HuT-78 cells. Our results revealed the dose dependent activation of caspase-3 in HuT-78 cells after treatment with different concentrations of PTX (0, 1.5, 3 and 6 mg/ml) for 48 h as evident by colorimetric assay (Fig. 5B). The specificity of caspase-3 activity was checked by using caspase-3 specific inhibitor (data not shown).

PARP is a substrate for caspase-3 and its cleavage is also an indicator of apoptosis. For studying PARP cleavage, whole cell lysates were prepared after 48 h of treatment with different concentrations of PTX (0, 1.5, 3 and 6 mg/ml) and PARP cleavage was detected by immunoblotting using anti-PARP antibody which recognizes both 116 kDa intact and 85 kDa cleaved forms of PARP. A gradual increase in 85 kDa cleavage product with increasing concentrations of PTX was observed (Fig. 5C).

3.5. *PTX suppresses Bcl-xl and c-FLIP protein expression in HuT-78 cells*

Expression of NF-κB dependent proteins, Bcl-xl, c-FLIP and c-IAP in PTX treated HuT-78 cells were studied. c-FLIP is an antiapoptotic protein responsible for cell resistance to apoptosis by inhibiting activation of procaspase-8 at the death-inducing signaling complex (DISC). c-FLIP$_L$ (long form) and c-FLIP$_S$ (short form) are two main known isoforms of c-FLIP protein [29]. Western blot analysis of cell lysates

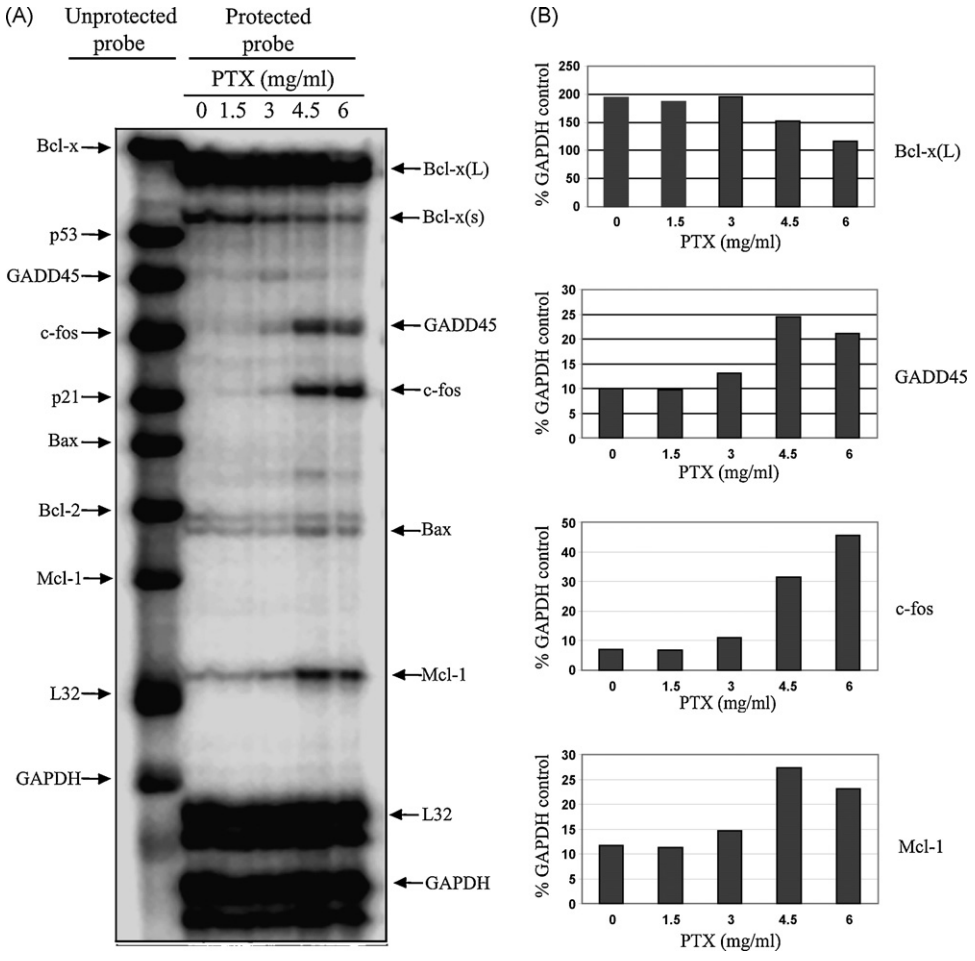

Fig. 7 – PTX treatment affects Bcl-xl, GADD45, c-Fos and Mcl-1 mRNA expression. (A) Cells were treated with varying concentrations of PTX (0, 1.5, 3, 4.5 and 6 mg/ml) for 8 h. Total RNA was isolated and analyzed by RPA as described in Section 2. (B) Results of RPA with a graphical representation of Bcl-xl, GADD45, c-Fos and Mcl-1 mRNA expression after normalization to GAPDH. Similar results were obtained in two separate experiments.

prepared after 48 h of treatment with different concentrations of PTX (0, 1.5, 3 and 6 mg/ml) showed dose dependent inhibition of Bcl-xl expression (Fig. 6A). Treatment of HuT-78 cells with different concentrations of PTX for 18 h inhibited the expression of c-FLIP$_L$ and c-FLIP$_S$ dose dependently (Fig. 6B). PTX treatment failed to show any effect on c-IAP expression up to 3 mg/ml concentration. However, when cells were exposed to 6 mg/ml PTX, c-IAP expression was lowered (Fig. 6C).

3.6. *PTX affects Bcl-xl, GADD45, c-Fos and Mcl-1 mRNA expression*

Here, we examined mRNA expression of several Bcl-2 family members as well as human cell cycle regulator molecules by RPA using hStress-1 multiprobe template set (BD PharMingen). For this purpose, cells were treated with various concentrations of PTX (0, 1.5, 3, 4.5 and 6 mg/ml) and total RNA was isolated after 8 h of treatment. HuT-78 cells express high level

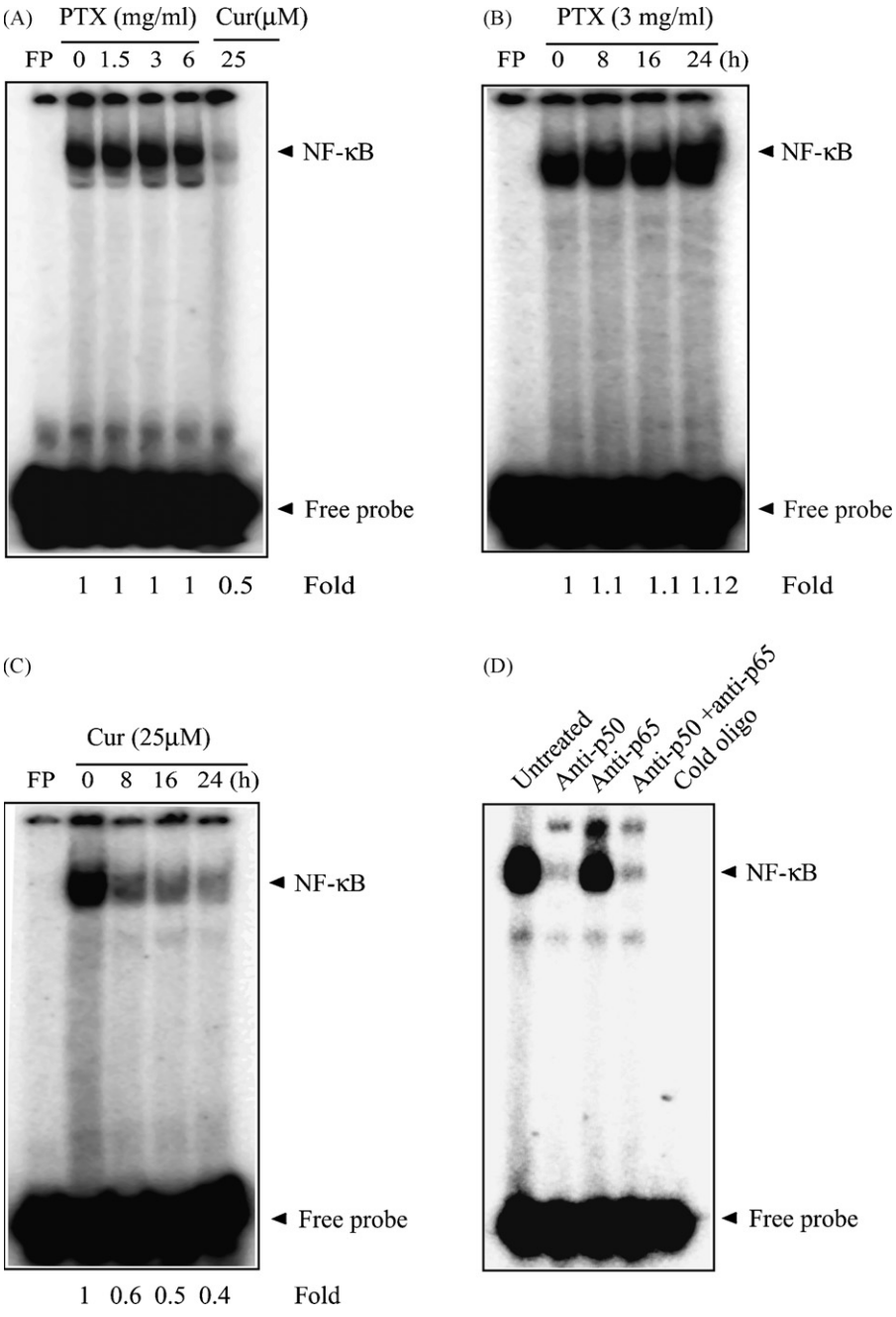

Fig. 8 – Effect of PTX on NF-κB activation. (A) HuT-78 cells were treated with varying concentrations of PTX (0, 1.5, 3 and 6 mg/ml) or curcumin, Cur (25 μM) for 16 h. Nuclear extracts were prepared and assayed for NF-κB by EMSA as described in Section 2. (B and C) HuT-78 cells were treated with 3 mg/ml PTX or 25 μM Cur for indicated time points and determined for NF-κB activity in nuclear extracts by EMSA. FP represents free probe alone (no nuclear extracts). (D) Binding of NF-κB to the DNA is specific and consists of p50 and p65 subunits. Nuclear extracts were prepared from HuT-78 cells, incubated for 30 min with different antibodies or unlabeled NF-κB oligo and then assayed for NF-κB by EMSA.

of Bcl-xl mRNA [19]. We did not find any inhibition of Bcl-xl mRNA expression up to 3 mg/ml PTX concentration, but PTX downregulated Bcl-xl mRNA expression by 0.78 and 0.6 fold in 4.5 and 6 mg/ml PTX treated cells respectively. PTX upregulated DNA damage inducible gene, GADD45 (growth arrest and DNA damage) mRNA expression and maximum increase was observed with PTX 4.5 mg/ml. c-Fos mRNA expression was increased with increasing concentrations of PTX although exposure to 1.5 mg/ml PTX failed to induce c-Fos mRNA expression. Enhancement of Mcl-1 mRNA expression in 4.5 mg/ml PTX treated cells was detected. We did not find any significant change of p53, p21, Bax and Bcl-2 mRNA expression (Fig. 7A and B).

3.7. *PTX does not have any effect on constitutive NF-κB activation*

It has been reported that HuT-78 cells constitutively express NF-κB which plays an important role in cell survival and make these cells resistance to apoptosis [9,19]. We examined whether PTX has any role on constitutive NF-κB expression. For this purpose, HuT-78 cells were treated either with different concentrations of PTX (0, 1.5, 3 and 6 mg/ml) or 25 μM curcumin (used as a control NF-κB inhibitor) for 16 h. EMSA was performed to examine NF-κB in the nucleus. Our results showed that PTX did not affect NF-κB level, whereas curcumin treatment suppressed NF-κB expression (Fig. 8A). Next, we studied the effect of PTX and curcumin on NF-κB level at different time points (0, 8, 16 and 24 h). No significant change in NF-κB compare to untreated control at any time point was observed (Fig. 8B) but curcumin showed inhibition at all time points (Fig. 8C). These results clearly indicated that NF-κB is not involved in PTX induced apoptosis in HuT-78 cells.

Supershift assays using antibodies to either p50 or p65 subunits of NF-κB were performed. Both antibodies shifted the band to a higher molecular mass, thus suggesting that the major NF-κB band in HuT-78 cells consisted of p50 and p65 subunits. In addition, excess unlabelled NF-κB (cold oligonucleotide; 100 fold) caused complete disappearance of the band (Fig. 8D).

3.8. *PTX enhances AP-1 DNA binding activity*

Besides NF-κB, we also studied the DNA binding activity of AP-1. In order to study the effect of PTX on AP-1, HuT-78 cells were treated with various concentrations of PTX (0, 1.5, 3, 4.5 and 6 mg/ml) for 12 h and the AP-1 activity in nuclear extracts were assessed by EMSA. Our results revealed that PTX enhances AP-1 DNA binding activity in a dose dependent manner up to 4.5 mg/ml PTX and thereafter slight decrease was seen with 6 mg/ml PTX (Fig. 9).

3.9. *PTX upregulates Fas and TRAIL mRNA expression*

We also analyzed the mRNA expression of several molecules those play important role in apoptosis by RPA using hAPO-3 multiprobe template set (BD PharMingen) after 8 h of treatment with various concentrations of PTX (0, 1.5, 3, 4.5 and 6 mg/ml). It was observed that PTX at 1.5 and 3 mg/ml concentration upregulated Fas expression by 2 fold as compared to untreated cells, thereafter with increase in PTX concentrations Fas mRNA expression decreased. It was also found that TRAIL mRNA expression increased in 1.5 mg/ml PTX (1.3 fold) treated cells thereafter the expression decreased with increase in concentrations of PTX (Fig. 10A and B).

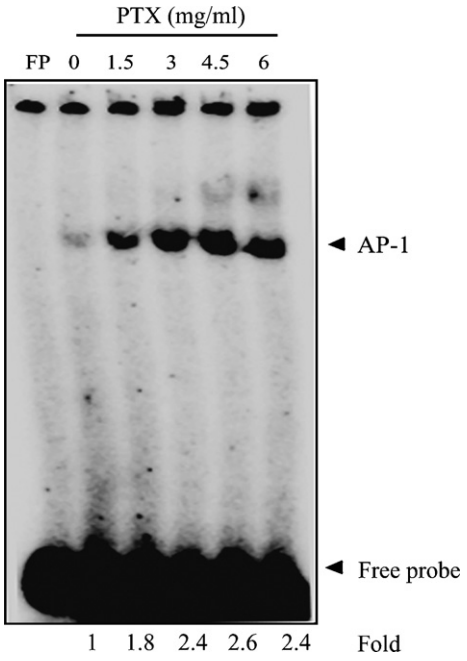

Fig. 9 – Effect of PTX on AP-1 level. Cells were treated with different concentrations of PTX for 12 h and were examined for AP-1 activity in nuclear extracts by EMSA. FP represents free probe alone (no nuclear extracts).

3.10. *PTX upregulates Fas expression at cell surface*

FACS analysis was performed with PTX treated cells at various time points. Mean fluorescence intensity (MFI) value indicated the increase in surface Fas expression in cells treated with 1.5 as well as 3 mg/ml PTX in comparison to control untreated cells. However, further increase in PTX concentrations showed decrease in MFI value (Fig. 11) which correlates with Fas mRNA expression in PTX treated HuT-78 cells (Fig. 10A).

3.11. *PTX scavenges reactive oxygen intermediate*

It is known that HuT-78 cells produce ROI, which plays antiapoptotic role in these cells [6]. Here, we examined the effect of PTX on ROI level. For this purpose, HuT-78 cells were treated with 3 mg/ml PTX for various time points (0, 1, 2, 4 and 8 h) and analyzed by flow cytometry at FL-1 channel. Our results revealed basal high level of ROI in HuT-78 cells which gets lowered on treatment with PTX. HuT-78 cells showed a MFI of 328 which decreased by 1.9 fold (MFI of 174) within 1 h by PTX. Further treatment with PTX for 2, 4 and 8 h decreased ROI by nearly 2.3 fold (Fig. 12A). This result was further substantiated by fluorescence microscopy where PTX treated cells showed less green fluorescence as compared to control

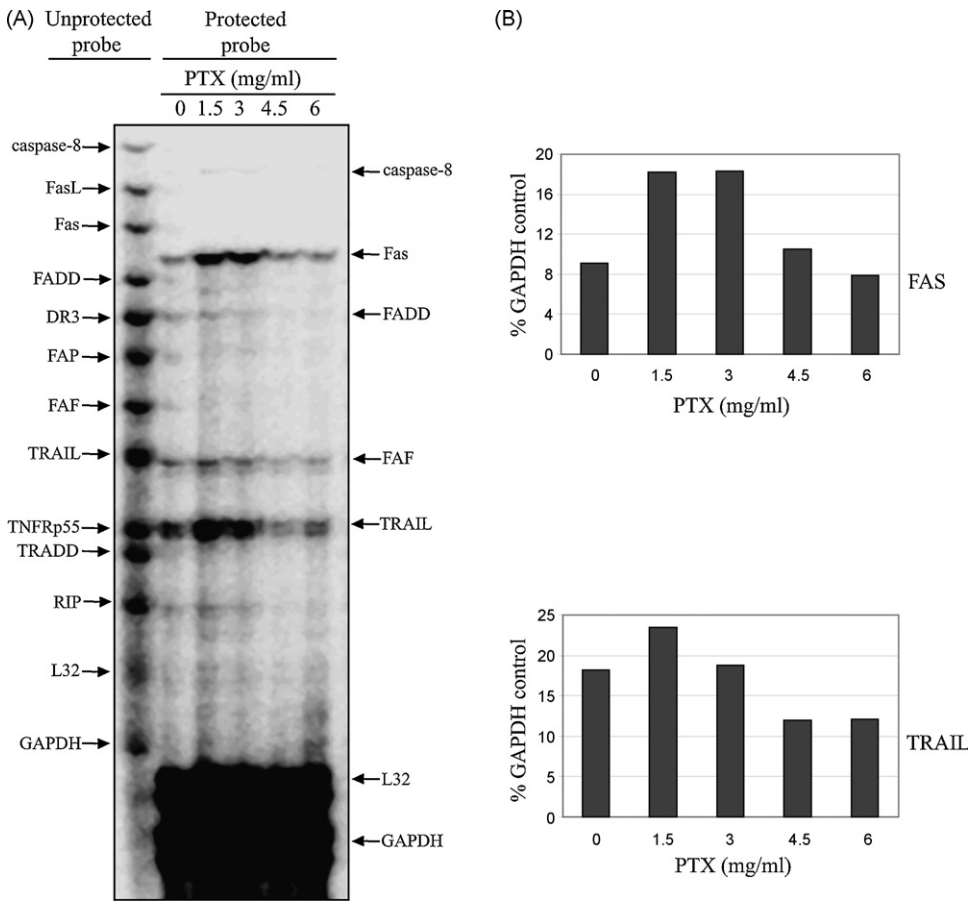

Fig. 10 – PTX treatment upregulates Fas and TRAIL mRNA expression. (A) Cells were treated with varying concentrations of PTX (0, 1.5, 3, 4.5 and 6 mg/ml) for 8 h. Total RNA was isolated and analyzed by RPA as described in Section 2. (B) Results of RPA with a graphical representation of Fas and TRAIL mRNA expression after normalization to GAPDH. Similar results were obtained in two separate experiments.

cells after 8 h of treatment. Thus, PTX acts as a scavenger of ROI in HuT-78 cells (Fig. 12B).

3.12. *PTX enhances FasL mediated killing and apoptosis*

The sensitivity of HuT-78 cells to agonistic CD95 antibody, CH-11 and soluble FasL has been demonstrated [4,30]. As mentioned above, our results showed increase in Fas surface expression by PTX. Therefore, we attempted to see whether PTX could enhance FasL mediated killing. We assessed the viability of HuT-78 cells after 24 h of treatment, either alone or together with 3 mg/ml PTX and 500 ng/ml FasL by flow cytomtery using PI exclusion method (Fig. 13A). As compared to control cells, PTX and FasL alone increased percentage of dead cells from 5.5% to 11% and 17% respectively. Interestingly, combined treatment with PTX and FasL increased the percentage of dead cells to 46.2%, which is 1.65 fold higher than sum of percentage of dead cells by either agent alone. This observation indicated that PTX treatment could enhance FasL mediated killing in HuT-78 cells.

Next, we investigated whether enhancement in FasL mediated killing involved apoptosis. For this purpose, HuT-78 cells were treated either alone or together with 3 mg/ml PTX and 500 ng/ml FasL for 24 h and analyzed for apoptosis by TUNEL assay. When HuT-78 cells were exposed to both PTX and FasL, 20.7% apoptotic cells were detected compared to 2.87% in only PTX treated cells and 6.47% in only FasL treated cells (Fig. 13B). Therefore, PTX enhances FasL mediated apoptosis in HuT-78 cells.

Further, we studied caspase-8 activity in HuT-78 cells treated either alone or together with 3 mg/ml PTX and 500 ng/ml FasL. Although, PTX alone failed to activate caspase-8 but FasL alone or in combination with PTX activated caspase-8. Addition of caspase-8 inhibitor, Ac-IETD-CHO (100 μM) blocked caspase-8 activation in PTX and FasL treated HuT-78 cells (Fig. 13C).

4. Discussion

PTX, a phosphodiesterase inhibitor is a methylxanthine derivative which is generally used as therapeutic drug for the treatment of hematological disorders. Moreover, it has been documented that PTX enhances antitumor activity of many chemotherapeutic agents and sensitizes tumor cells to radiotherapy [15–18]. Based on the experimental observations

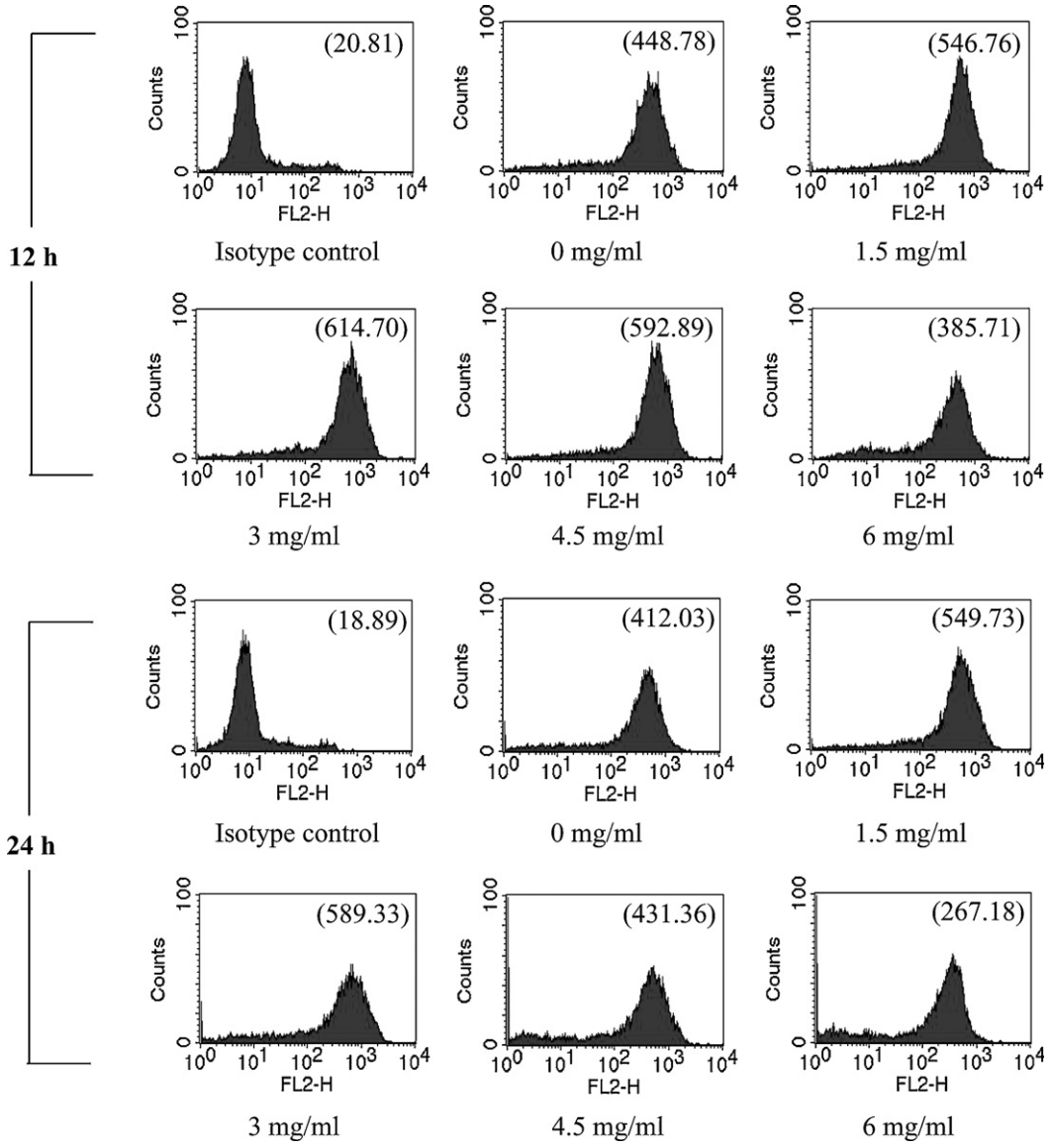

Fig. 11 – PTX upregulates Fas expression on the surface of HuT-78 cells. Cells were treated with varying concentrations of PTX (0, 1.5, 3, 4.5 and 6 mg/ml) for indicated time points, surface stained for Fas expression and analyzed by flow cytometry as described in Section 2. Values shown in the parentheses are the MFI scores. Data represent the results from one of the two similar experiments.

we report here that PTX is cytotoxic to HuT-78 cells and initiates a series of events leading to apoptosis. First, PTX treated cells showed dose dependent as well as time dependent increase in sub-G_1 peak. Second, PTX treatment for 48 h showed concentration dependent increase in percentage of apoptotic cells as well as DNA fragmentation. Third, PTX affects mitochondria by inducing hyperpolarization of the MMP, causes cytochrome *c* release, activates caspase-3 and leads to PARP cleavage.

Exposure to PTX causes downregulation of Bcl-xl and c-FLIP protein expression in HuT-78 cells which otherwise constitutively express high level of Bcl-xl and c-FLIP [19,30]. Bcl-xl is an antiapoptotic member of Bcl-2 family which promotes cell survival and regulates MMP [31]. Downregulation of Bcl-xl protein by PTX probably explained mitochondrial dysfunction caused by PTX in HuT-78 cells.

Next, we studied the effect of PTX on mRNA expression of apoptosis specific genes belonging to Bcl-2 family and on p53 regulated genes. Although PTX showed dose dependent inhibition of Bcl-xl at protein level after 48 h of exposure but at mRNA level (after 8 h of treatment) inhibition was apparent only with high concentrations of PTX (4.5 and 6 mg/ml). Upregulation of GADD45 mRNA expression was observed in HuT-78 cells treated with PTX. GADD45 is a nuclear protein widely expressed in normal tissues. Expression of GADD45 is significantly reduced in cancer cells and its reexpression has been shown to cause apoptosis [32]. It is possible that reexpression of GADD45 after treatment with PTX may induce

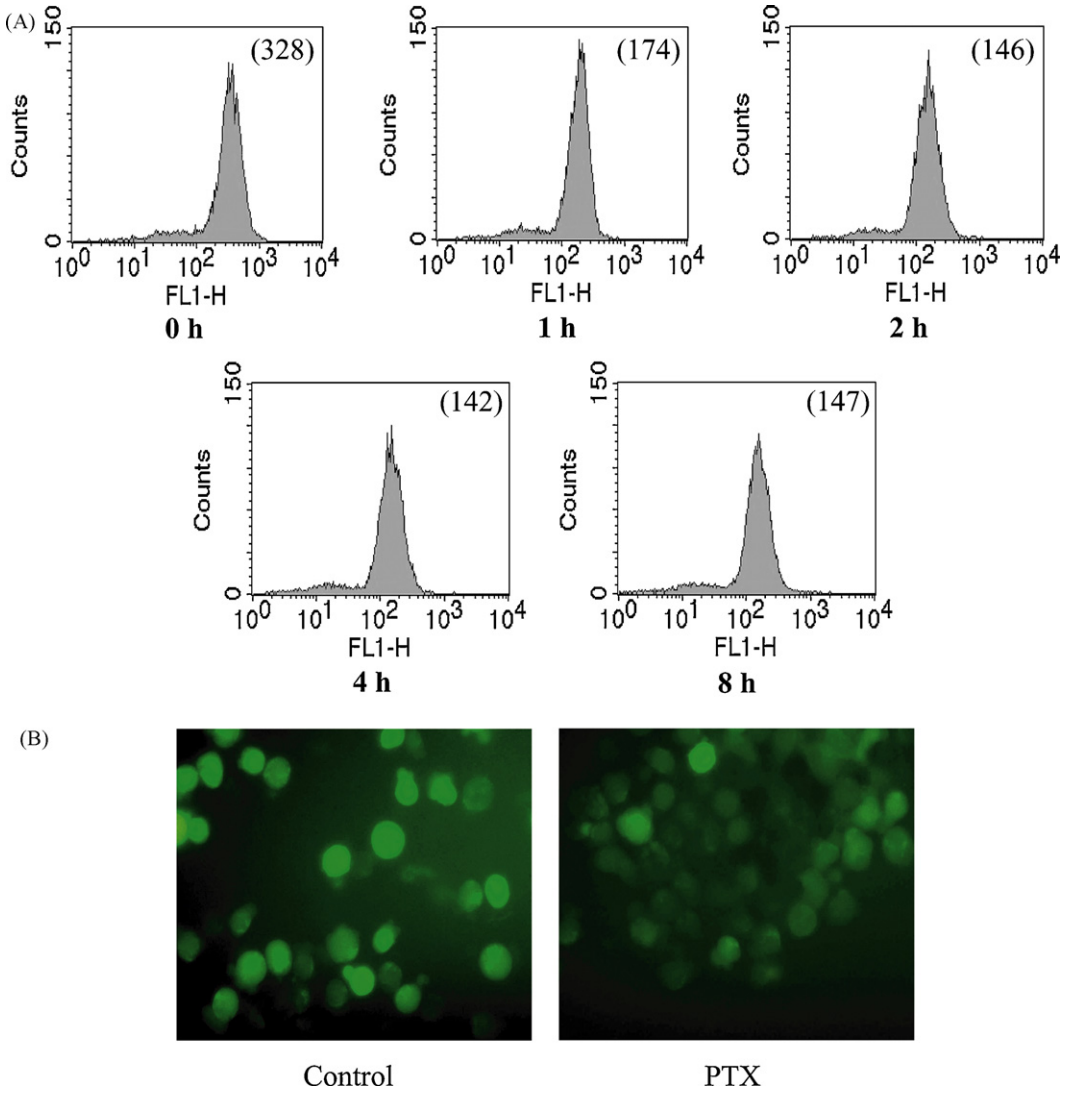

Fig. 12 – PTX scavenges ROI. (A) Cells were treated for indicated time points with 3 mg/ml PTX and exposed to dihydrorhodamine 123. Fluorescence intensity resulting from dihydrorhodamine 123 oxidation by ROI was measured by flow cytometry. Values shown in the parentheses are the MFI scores. (B) Changes in ROI level were visualized under fluorescent microscope by staining with DCF-DA after 8 h of treatment with 3 mg/ml PTX.

apoptosis in HuT-78 cells. In addition, upregulation of c-Fos and Mcl-1 mRNA were detected in RPA. p53 expression could not be detected in HuT-78 cells at any condition. Earlier Tolomeo et al. also reported that HuT-78 cells do not express p53 protein [33].

It is known that NF-κB is constitutively expressed in CTCL cells and it regulates many antiapoptotic molecules [9,19]. It has been reported that treatment of CTCL cell lines with NF-κB inhibitors viz. MG132, gliotoxin, BAY 11-7082, BAY 11-7085, bortezomib can induce apoptosis in these cells [9,34]. We investigated the effect of PTX on constitutive NF-κB in HuT-78 cells. PTX treatment showed hardly any change in NF-κB expression. Therefore, NF-κB is not involved in apoptosis induced by PTX. Recently we have reported [19] that sodium nitroprusside (SNP), a nitric oxide generating compound induces apoptosis in HuT-78 cells by downregulating NF-κB level. Present study clearly demonstrated that mode of action of SNP and PTX are not the same. cAMP analogues; dibutyryl cAMP and 3-isobutyl-1-methylxanthine failed to show any cytotoxic effect on HuT-78 cells as assessed by flow cytometry (data not shown). Therefore, cAMP is not involved in PTX mediated apoptosis of HuT-78 cells.

Our results revealed antioxidant nature of PTX and showed that ROI has very little effect on NF-κB activation as inhibition of ROI by PTX failed to show any effect on NF-κB level. Thus, our results emphasize on ROI independent activation of NF-κB in HuT-78 cells, although Giri and Aggarwal had demonstrated that ROI plays a major role in maintaining constitutive NF-κB level in HuT-78 cells which ultimately prevents apoptosis [6].

Downregulation of NF-κB dependent antiapoptotic proteins, Bcl-xl and c-FLIP by PTX without affecting NF-κB pointed towards involvement of another NF-κB independent pathway operative in HuT-78 cells. It is known that other than NF-κB, transcription factors like signal transducers and activators of

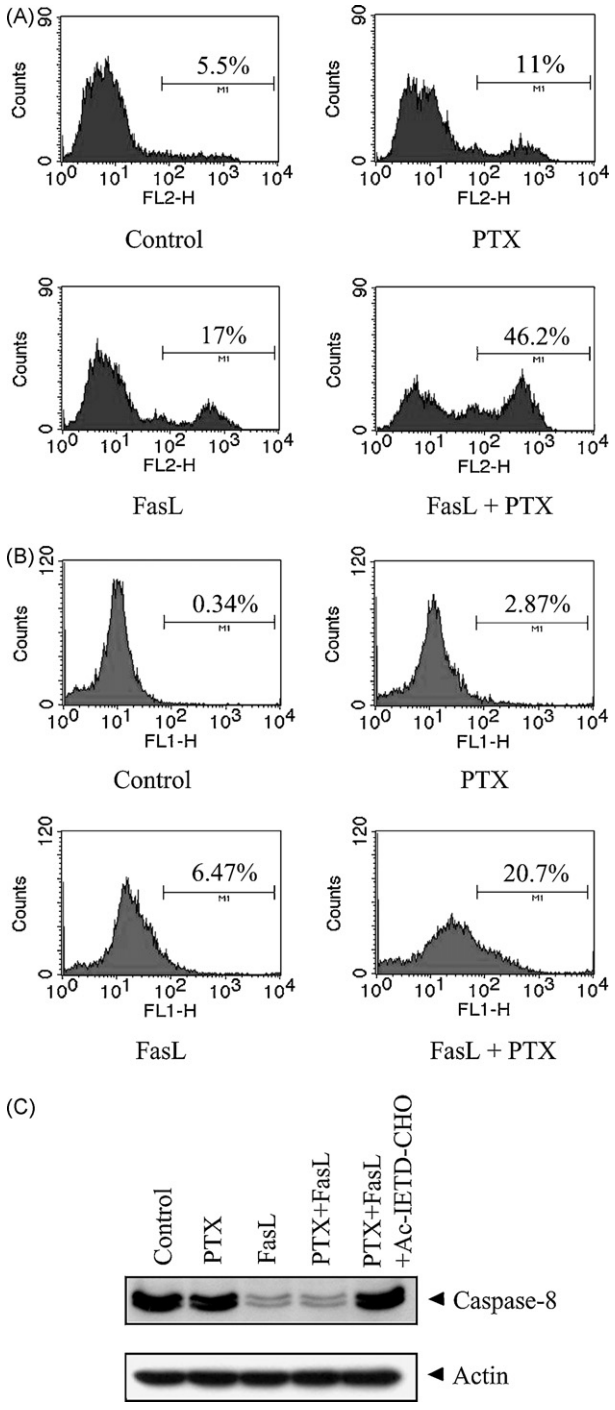

Fig. 13 – PTX enhances FasL mediated killing and apoptosis. Cells were treated or not with PTX (3 mg/ml) and/or FasL (500 ng/ml) for 24 h and then examined for (A) cell viability by PI exclusion method and (B) apoptosis by TUNEL assay. (C) Cell lysates were analyzed for caspase-8 activation by immunoblotting using mouse monoclonal anti-caspase-8 antibody which recognizes procaspase-8 (55 kDa) band. Data represent the results from one of the two similar experiments.

transcription (STAT), Ets and AP-1 can regulate Bcl-xl expression [35]. Significant increase in AP-1 expression was detected in PTX treated HuT-78 cells. Transcription factor c-Fos heterodimerizes with members of Jun family and forms AP-1 complex. It is known that many compounds activate AP-1 activity in association with apoptosis [36,37]. It is therefore possible that AP-1 instead of NF-κB is involved in PTX mediated apoptosis in HuT-78 cells.

Next, we studied the effect of PTX on mRNA level of various molecules involved in death receptor mediated apoptosis. We found that low concentration of PTX that did not induce apoptosis but significantly upregulated Fas and TRAIL expression. Recently it has also been shown that c-Fos acts as a proapoptotic agent by repressing c-FLIP$_L$ expression upon binding to promoter region of c-FLIP$_L$ and potentiates TRAIL induced apoptosis in prostate cancer cells [38]. CTCL cells are generally resistant to TRAIL mediated killing [30]. It will be interesting to know whether HuT-78 cells could be sensitized to TRAIL mediated killing by downregulating c-FLIP expression without affecting constitutive NF-κB.

It is reported that ROI provides beneficial effects to tumor cells and its reduction by antioxidants increase sensitivity of myeloma cells to Fas mediated apoptosis [39]. Many cytotoxic agents known to increase cellular oxidative stress downregulates c-FLIP expression and sensitize cells to Fas induced apoptosis in a ROI dependent manner [40]. In contrast cytotoxic agent PTX scavenged ROI, increased surface Fas expression and augmented FasL mediated killing and apoptosis.

Presently, only limited compounds are approved by Food and Drug Administration (FDA) for treatment of CTCL cells, which includes; Bexarotene, a synthetic rexinoid, Vorinostat or suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor and Denileukin diftitox, a recombinant fusion protein [3,41]. Besides these, cytokine therapy and extracorporeal photochemotherapy has also been reported for the treatment of CTCL cells [42–44].

It has been shown that decreased Fas expression on peripheral blood CD4+ T lymphocytes in MF and SS patients and progression into aggressive CTCL was associated with absence of Fas expression [45]. In addition, in comparison to healthy donors, CD4+ cells from SS patients showed varied sensitivity towards FasL, with some of the SS patients CD4+ cells exhibiting complete resistance towards FasL mediated killing [4]. Furthermore, as mentioned, our studies highlighted the increase in cell surface Fas expression and enhancement in FasL mediated killing by PTX. Thus, outcome of this study may hold PTX as a potential chemotherapeutic agent for treatment of CTCL patients.

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