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Pentoxifylline augments TRAIL/Apo2L mediated apoptosis in cutaneous T cell lymphoma (HuT-78 and MyLa) by modulating the expression of antiapoptotic proteins and death receptors

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ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo2L) is a promising anticancer agent but cutaneous T lymphoma cells (CTCL) are less sensitive to TRAIL-induced apoptosis. Here, we report that pentoxifylline (PTX), a phosphodiesterase inhibitor, augments TRAIL-mediated apoptosis in HuT-78 and MyLa cells through modulating extrinsic death receptors and intrinsic mitochondria dependent pathways. Our results clearly show that PTX augments TRAIL-mediated activation of caspase-8 and induces cleavage of Bid, although PTX alone cannot activate caspase-8. This is followed by cytochrome *c* release and subsequent, activation of caspase-9 and caspase-3 and cleavage of poly (ADP ribose) polymerase (PARP). Combined treatment downregulates the expression of various antiapoptotic proteins including c-FLIP, Bcl-xl, cIAP-1, cIAP-2 and XIAP. PTX induces the expression of death receptors DR4 and DR5 on cell surface of both the cell types where c-Jun NH2-terminal kinase (JNK) pathway plays an important role. Moreover, combined silencing of DR4 and DR5 by small interfering RNA abrogates the ability of PTX to induce TRAIL-mediated apoptosis. Thus, this is the first demonstration that PTX can potentiate TRAIL-mediated apoptosis through downregulation of cell survival gene products and upregulation of death receptors.

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1. Introduction

Cutaneous T cell lymphomas (CTCL) are lymphoproliferative disorders of the skin. The two most common forms of CTCL are Mycosis fungoides (MF) and Sézary syndrome (SS) as its leukemic form, together they account for majority of cutaneous lymphoma [1]. CTCL cells show defects in their death inducing signaling complex due to altered expression of death receptors which make them less sensitive toward TNF superfamily molecules [2,3]. It has been reported that circulating CD4+ cells from SS patients are resistant to soluble TRAIL [3].

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo2L) has recently emerged as a novel anticancer agent based on its ability to induce apoptosis in tumor cells, without showing toxicity to normal cells [4]. TRAIL binds to four membrane bound receptors DR4/TRAIL-R1, DR5/TRAIL-R2, DcR1/TRAIL-R3, DcR2/TRAIL-R4 and a soluble receptor osteoprotegerin (OPG). TRAIL transduces its signal only through DR4 and DR5 which contain cytoplasmic region called the death domain. DcR1 and DcR2 do not have functional cytoplasmic death domain and functions as blocker of TRAIL-induced apoptosis [5]. Binding of TRAIL to DR4 and DR5 leads to the formation of death inducing signaling complex (DISC) with the binding of caspase-8 which is then activated. Activation of caspase-8 ultimately activates caspase-3 leading to cell death. Alternatively, TRAIL can also activate caspase-3 through caspase-9 pathway where mitochondria are involved [6]. The presence of intracellular apoptosis inhibitory substances (Bcl-xl, c-FLIP, cIAP etc.) have been shown to play an important role in TRAIL resistance in different cancer cells [7]. Numerous studies demonstrated that combination of TRAIL with chemotherapeutic drugs or ionizing radiation can induce synergistic tumor cell apoptosis by upregulating DR4 and/or DR5 or by inhibiting intracellular cell survival proteins [5,8]. Earlier, we reported that PTX, a methylxanthine derivative induces FasL mediated killing in human sézary CTCL cell line, HuT-78 by upregulating the expression of Fas receptors on their cell surface [9].

Cyclic nucleotide phosphodiesterase inhibitor, Pentoxifylline (PTX), has been shown to beneficially influence a large number of inflammatory skin diseases and now being clinically used as peripheric vasodilator [10,11]. PTX has been shown to enhance

Abbreviations: CTCL, cutaneous T cell lymphoma; MF, mycosis fungoides; SS, sézary syndrome; PTX, pentoxifylline; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; c-FLIP, cellular FLICE like inhibitory protein; cIAP, cellular inhibitor of apoptosis protein; XIAP, X-linked inhibitor of apoptosis; JNK/MAPK, c-Jun NH2-terminal kinase/mitogen-activated protein kinase; DR4, death receptor 4; DR5, death receptor 5; DcR, decoy receptor.

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tumor sensitivity of many chemotherapeutic agents as well as sensitize tumor cells to ionizing irradiation [12–15]. Recently, PTX in combination with Vitamin E have been found beneficial for radiation induced fibrosis and survival in patients with non-small cell lung cancer [10]. Presently, only limited compounds are approved by Food and Drug Administration (FDA) for the treatment of CTCL patients and therapies available are usually palliative [16–18]. Therefore, alternative or complementary therapies, especially for advanced MF/SS, are urgently required [19]. Earlier, gene expression profiling of aggressive CTCL patients showed an overexpression of TRAIL, but the expression of TRAIL receptors has not been correlated with TRAIL sensitivity in SS tumor cells [3].

Present work was undertaken to see the effect of PTX on TRAIL-induced apoptosis in CTCL cell lines (HuT-78 and MyLa). Here, we report for the first time, that PTX augments TRAIL-mediated caspase-8 activation and potently induces TRAIL-mediated apoptosis in both the cell lines by downregulating different antiapoptotic proteins and upregulating DR4 and DR5.

2. Materials and methods

2.1. Chemicals and antibodies

Human CTCL cell lines, HuT-78 was obtained from National Centre for Cell Science (Pune, India) and MyLa was generously provided by Dr. Emmanuel Contassot (Department of Dermatology, Zürich University Hospital, Zürich, Switzerland). Both the cell lines were maintained in RPMI-1640 medium, containing 10% heat inactivated Fetal Bovine Serum (GIBCO, Grand Island, NY) at 37 °C and in the presence of 5% CO_2. Human recombinant TRAIL was purchased from Millipore/Chemicon (Billerica, MA). JNK inhibitor, SP600125, caspase inhibitors, Z-VAD-FMK and Z-IETD-FMK were purchased from Calbiochem (La Jolla, CA). Antibodies against c-FLIP, t-Bid, ERK, p-ERK, p38, p-p38, DR4 and DR5 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PI/RNase staining buffer, annexin V-FITC apoptosis detection kit, APO-BRDU™ kit and antibodies against cytochrome *c*, PARP, Bcl-xl, Apaf-1, cIAP-1, cIAP-2, XIAP, JNK1/JNK2, JNK/SAPK (pT183/pY185), PE-labeled IgG1 isotype control were purchased from BD PharMingen (San Diego, CA). PE-labeled TRAIL-R1/DR4, TRAIL-R2/DR5, TRAIL-R3/DcR1 and TRAIL-R4/DcR2 antibodies for flowcytometry and neutralizing human anti-TRAIL antibody (RIK-2) were purchased from e-Biosciences (San Diego, CA). Quick-Load™ 2-Log DNA Ladder was purchased from New England Biolabs (Ipswich, MA). Pentoxifylline (PTX), acridine orange (AO), ethidium bromide (EB), propidium iodide (PI), p-formaldehyde, 4,6-diamidino-2-phenyl-indole (DAPI), anti-actin antibody, anti-mouse HRP, anti-rabbit HRP and anti-goat HRP antibodies were purchased from Sigma–Aldrich (St. Louis, MO). All media and reagents used were endotoxin free.

2.2. PI exclusion assay for cell viability

Cell viability was determined by PI exclusion assay [9]. Briefly, 2×10^5 HuT-78 and MyLa cells each were treated with different concentrations of PTX (0, 1.5 and 3 mg/mL) or TRAIL (0, 25, 50 and 100 ng/mL) either alone or together for 24 h. After treatment, cells were harvested, washed with phosphate buffered saline (PBS), and resuspended in PBS containing 1 μg/mL of PI. The level of PI incorporation was quantified by flow cytometry on a FACSCalibur (Becton Dickinson).

2.3. Fluorescence morphological examination

Cell morphology was investigated by staining cells with a combination of fluorescent DNA binding dyes AO/EB. Briefly, 2.5×10^5 HuT-78 cells were treated with 3 mg/mL PTX or 100 ng/mL TRAIL either alone or together for 24 h. The solution containing each dye at 1 μg/mL in PBS was mixed 1:1 with cell suspension. Cell viability was determined under Zeiss fluorescence microscope using a 40× objective by counting live (green) and dead (red) cells [9].

2.4. Quantification of apoptosis by flow cytometry

2.4.1. Analysis of hypoploidy

1×10^5 HuT-78 cells were treated with 3 mg/mL PTX or 100 ng/mL TRAIL either alone or together for 24 and 48 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 [20]. The percentage of apoptotic cells was determined by measuring the fraction of nuclei with a sub-diploid DNA content using Becton Dickinson FACSCalibur with Cell Quest software (Becton Dickinson).

2.4.2. Annexin V staining

HuT-78 cells were treated with 3 mg/mL PTX or 100 ng/mL TRAIL either alone or together for 24 and 48 h. Cells were harvested, washed and stained with 5 μL of fluorescein isothiocyanate (FITC)-conjugated annexin V using Annexin V staining kit (BD Pharmingen) according to manufacturer's protocol. Stained cells were immediately analyzed with a Becton Dickinson FACSCalibur and further analyzed using CellQuest software (Becton Dickinson).

2.4.3. Terminal deoxynucleotidyltransferase dUTP nick end labelling (TUNEL)

To measure the DNA strand breaks during apoptosis, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay was performed using APO-BRDU™ kit (BD Pharmingen) according to manufacturer's protocol. In brief, 5×10^5 HuT-78 and MyLa cells each were treated with 3 mg/mL PTX or 100 ng/mL TRAIL either alone or together for 24 and 48 h. Cells were harvested, washed with PBS and stained by incubating with reaction mixture. Stained cells were analyzed with flow cytometer (FACSCalibur, Becton Dickinson).

2.5. DAPI staining

For analysis of nuclear morphology, cells were harvested after treatment, washed twice with PBS and fixed in 1% p-formaldehyde. After fixation, staining was carried out by incubating cells with 10 μL of 4 μg/mL DAPI for 10 min in dark. Later samples were visualized under Zeiss fluorescence microscope [21].

2.6. DNA fragmentation analysis

Cells were harvested after treatment and DNA fragmentation was assayed according to the method described by Herrmann et al. [22].

2.7. Measurement of caspase activity

2.7.1. Caspase-8 and caspase-3 activity assay

Caspase-8 (Sigma–Aldrich, St Louis, MO) and caspase-3 (Promega, Madison, WI) activities were determined according to manufacturer's protocol. Cell lysates were prepared after treatment with 3 mg/mL PTX or 100 ng/mL TRAIL either alone or together for 24 h. After treatment cells were harvested and assayed for caspase-8 and caspase-3 activity using colorimetric substrates. The specificity of caspase-8 and 3 activities were checked by using their specific inhibitors Z-IETD-FMK and Z-VAD-FMK respectively.

2.7.2. Caspase-9 activity assay

Caspase-9 activity was measured using a Caspase-Glo® 9 assay system according to manufacturer's instructions (Promega, Madison, WI). Briefly, 2×10^4 HuT-78 and MyLa cells each were seeded in 96 well plate and treated with 3 mg/mL PTX or 100 ng/mL TRAIL either alone or together for 24 h. After incubation plate containing cells was allowed to equilibrate to room temperature. 100 μL of luminogenic caspase-9 substrate (Caspase-Glo® 9 reagent) was added to each well and incubated for 1 h at room temperature. The luminescent signal generated by caspase mediated cleavage of substrate was measured by MicroBeta® TriLux (PerkinElmer, Finland).

2.8. Measurement of cytochrome c release

HuT-78 cells were treated with 3 mg/mL PTX or 100 ng/mL TRAIL either alone or together for 24 h and cytosolic extracts were prepared according to the method as described previously by Miyoshi et al. [23]. The release of cytochrome *c* into cytosol was analyzed by Western blotting using anti-cytochrome *c* antibody. Same cytosolic extracts were used for analyzing the level of t-Bid and Apaf-1 proteins.

2.9. Western blot analysis

For Western blot analysis cell lysates were prepared after treatment as described previously [24]. In brief, cells were harvested at indicated time points, washed twice with ice cold PBS and lysed in lysis buffer (20 mM Tris–HCl pH 7.4, 0.25 mM 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 10–15% SDS-PAGE and analyzed by Western blotting using specific antibodies against either PARP, Bcl-xl, c-FLIP, cIAP-1, cIAP-2, XIAP, DR4, DR5, p-ERK, ERK, p-p38, p38, JNK1/JNK2 and JNK/SAPK. Densitometry of individual bands was determined by PhosphorImager (Bio-Rad, Hercules, CA).

2.10. Analysis of cell surface expression of TRAIL receptors

Treated and untreated cells were stained with PE-conjugated mouse IgG1 isotype control or PE-conjugated mouse anti-human TRAIL-R1/DR4, TRAIL-R2/DR5, TRAIL-R3/DcR1 and TRAIL-R4/DcR2 (e-Biosciences, San Diego, CA) for 1 h on ice according to the method as described previously by Rishi et al. [9]. Stained cells were analyzed with flow cytometer (FACSCalibur, Becton Dickinson).

2.11. RT-PCR for TRAIL receptors

Total RNA was isolated from PTX or TRAIL treated cells at 12 h time point using RNA isolation kit (Promega, Madison, WI). RT-PCR was carried out in a volume of 50 μL using Access RT-PCR system (Promega, Madison, WI) according to manufacturer's instructions. The primer sequences for human TRAIL-R1 were sense: 5′-CTG AGC AAC GCA GAC TCG CTG TCC AC-3′; antisense: 5′-TCC AAG GAC ACG GCA GAG CCT GTG CCA T-3′ and for human TRAIL-R2 sense: 5′-GCC TCA TGG ACA ATG AGA TAA AGG TGG CT-3′; antisense: 5′-CCA AAT CTC AAA GTA CGC ACA AAC GG-3′ [25]. β-Actin, sense: 5′-AAG AGA GGC ATC CTC ACC C-3′; antisense 5′-TAC ATG GCT GGG GTG TTG AA-3′ [26]. Amplification conditions for TRAIL-R1 and TRAIL-R2 were initial denaturation of 1 min at 94 °C, 55 °C for 1 min, and 72 °C for 1 min for 30 cycles. β-Actin was amplified at initial denaturation of 5 min at 94 °C and 35 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1.5 min, followed by a final amplification at 72 °C for 5 min. Samples were resolved on a 2% agarose gel and visualized with ethidium bromide.

2.12. Electrophoretic mobility shift assay

NF-κB and AP-1 DNA binding activities were determined by electrophoretic mobility shift assay (EMSA) as described previously [9].

2.13. Transfection with siRNA

2×10^5 HuT-78 cells were transfected with either DR4, DR5 specific siRNA or with control (scrambled) siRNA according to manufacturer's protocol (Santa Cruz, CA). After 48 h of transfection, cells were washed with PBS, culture medium was replaced and cells were treated with 3 mg/mL PTX or 100 ng/mL TRAIL either alone or together. Cells were harvested either 12 h after treatment for determination of cell surface receptor expression by flowcytometry or 24 h after treatment for PI staining followed by flowcytometry analysis.

2.14. 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. Pentoxifylline sensitizes TRAIL-induced cytotoxicity in HuT-78 and MyLa cells

In order to see whether PTX is able to sensitize TRAIL-induced cytotoxicity, HuT-78 cells were treated with PTX (either 1.5 mg/mL or 3 mg/mL) along with different concentrations of TRAIL (25–100 ng/mL) for 24 h. Cell viability was detected by live PI dye exclusion method. As shown in Fig. 1A, when different concentrations of TRAIL (25–100 ng/mL) were added to HuT-78 cells, <10% cell viability was lost after 24 h, on the other hand 3 mg/mL concentration of PTX showed 14.1% cytotoxicity alone. Dose dependent cytotoxic effect was observed (17.3%, 32.4% and 52.5%) when HuT-78 cells were treated with 3 mg/mL PTX along with various concentrations of TRAIL. We did not find any synergistic effect when 1.5 mg/mL PTX was used with varying concentration of TRAIL (Fig. 1A, left panel). To determine whether the potentiation of TRAIL-induced cytotoxicity by PTX was only with sézary CTCL cell line HuT-78, an additional CTCL cell line of Mycosis fungoid, MyLa, was also tested similarly. As shown in Fig. 1A, MyLa cells were seemed to be weakly sensitive to TRAIL as HuT-78 cells. Interestingly, enhanced synergistic cytotoxic effect was observed when MyLa cells were treated with 3 mg/mL PTX along with different concentrations of TRAIL, while 1.5 mg/mL PTX did not have any effect on TRAIL-mediated killing (Fig. 1A, right panel). Pre-treatment of HuT-78 cells with neutralizing human anti-TRAIL/Apo2L antibody (RIK-2) restored the cell viability to 90% (Fig. 1B). This suggests that PTX-induced TRAIL-mediated cytotoxicity was TRAIL/Apo2L specific.

3.2. The sensitization of TRAIL-induced cytotoxicity by PTX is associated with apoptosis in HuT-78 and MyLa cells

Next, experiments were performed to see whether the synergistic cytotoxic effect seen by the combined treatment of PTX with TRAIL was associated with apoptosis. We treated HuT-78 cells with PTX and TRAIL alone or in combination for 24 and 48 h time points and the percentage of sub-G_1 population (hypoploidic cells) were quantified by flow cytometry to detect apoptosis. In Fig. 2A, PTX and TRAIL individually caused 6.9% and 5.3% apoptosis respectively, while, combined treatment significantly induced

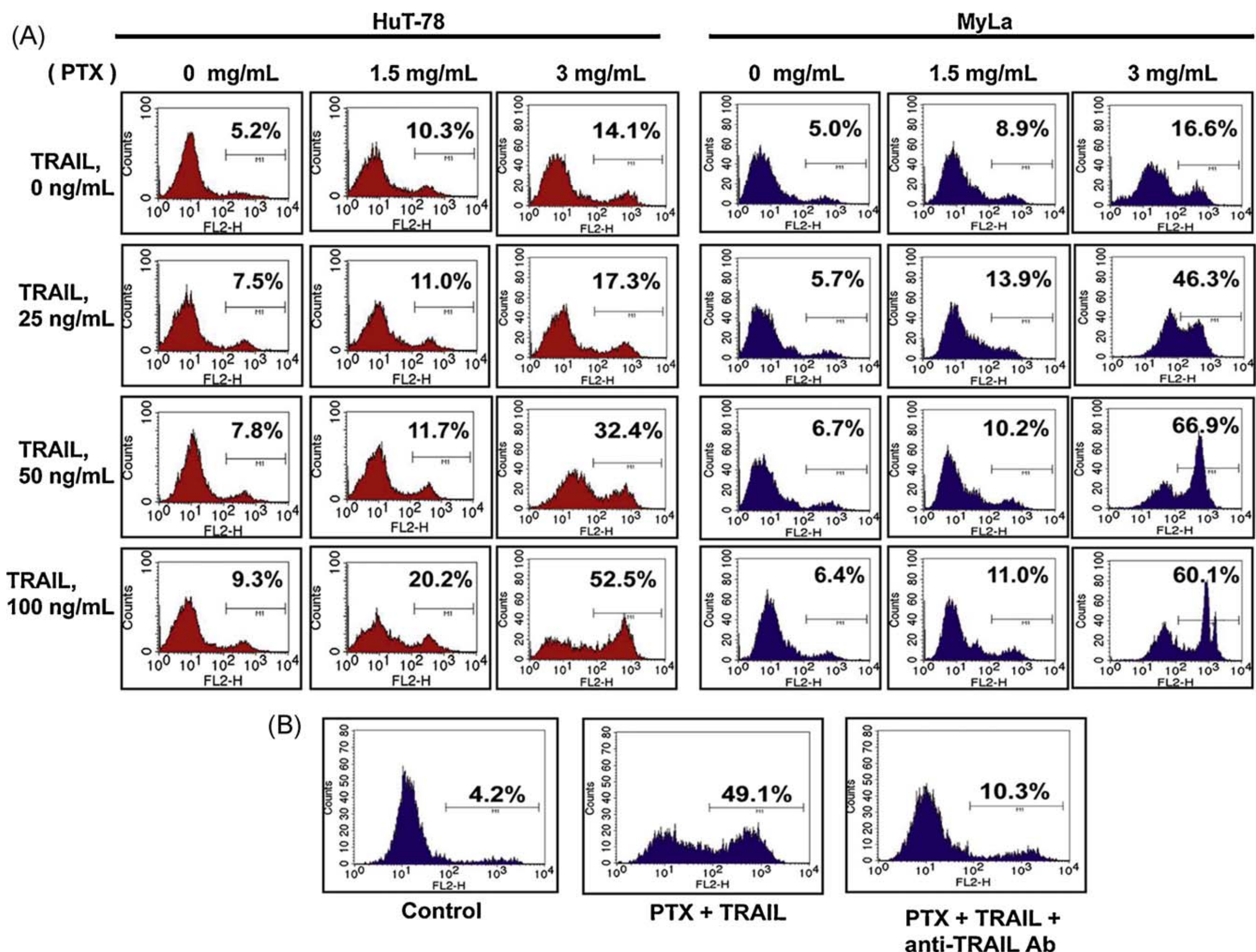

Fig. 1. PTX sensitizes TRAIL-mediated killing in HuT-78 and MyLa cells. (A) HuT-78 and MyLa cells were treated or not with PTX (0, 1.5 and 3 mg/mL) and/or TRAIL (25–100 ng/mL) for 24 h and then examined for cell viability by PI exclusion method, where M1 represent % dead cells. (B) HuT-78 cells were treated with PTX (3 mg/mL) and TRAIL (100 ng/mL) in combination for 24 h in the presence or absence of neutralizing anti-TRAIL antibody (10 μg/ml). Cell viability was measured by PI exclusion method, where M1 represent % dead cells. Data represent the results from one of the three similar experiments.

apoptosis with 26.8% after 24 h. Moreover, the percentage of sub-G_1 fraction was increased up to 45.4% with combined treatment as compared to individual apoptosis of 14.4% with PTX and 17.7% with TRAIL at 48 h (Fig. 2A).

Annexin V-FITC staining was done to further confirm the apoptosis induced by combination of PTX and TRAIL in HuT-78 cells. In Fig. 2B, annexin V staining clearly showed a greater proportion of apoptotic cells (24.2% and 52.6%) with combined treatment at 24 and 48 h respectively as compared to PTX or TRAIL alone treatment. Further, we performed TUNEL assay to detect the apoptosis (Fig. 2C). PTX potentiated TRAIL-induced apoptosis in HuT-78 cells, from 6.2% and 10.9% with PTX and TRAIL alone respectively, to 28.5% when used in combination at 24 h which further enhanced upto 49.6% with individual apoptosis of 12.9% with PTX and 14.1% with TRAIL at 48 h. Similar experiments were also done in MyLa cells. Fig. 2C, indicated that combined treatment of PTX and TRAIL resulted into 29.6% and 45.0% apoptosis at 24 and 48 h respectively in MyLa cells.

Next, we also performed AO/EB staining of HuT-78 cells after 24 h treatment. In Fig. 2D, morphological observation also supported that control untreated cells were seen uniformly green with normal morphology, while the orange dead cells with fragmented chromatin and apoptotic bodies were seen in treated cells, an indicative of apoptosis (Fig. 2D, upper panel). DAPI staining and DNA fragmentation analysis further supported our data of apoptosis. DAPI staining with PTX and TRAIL together showed enhanced chromatin condensation with nuclear fragmentation as compared to alone treatment (Fig. 2D, lower panel). An enhanced DNA laddering pattern was also observed in Fig. 2E, when HuT-78 cells were treated with PTX and TRAIL together for 24 h.

3.3. PTX augments TRAIL-induced apoptosis through activation of caspase-8, bid truncation and cytochrome c release followed by caspase-9 activation

As apoptosis is usually associated with the activation of caspase cascade, we were further interested to see the caspase-8 activation which plays an extensive role for extrinsic apoptotic pathway. As shown in Fig. 3A, treatment of 100 ng/mL TRAIL alone caused slight activation of caspase-8 while upon addition to 3 mg/mL PTX a drastic enhancement of caspase-8 activity was found in HuT-78 and MyLa cells after 24 h treatment as evident by colorimetric assay. However, PTX alone did not activate caspase-8. The specificity of caspase-8 activity was checked by using caspase-8 specific inhibitor Z-IETD-FMK (data not shown).

The cleavage of Bcl-2 family member Bid to t-Bid, a substrate for caspase-8, is essential for linking death receptor mediated extrinsic pathway to mitochondrial pathway [27]. Therefore, we measured, whether PTX and TRAIL treatment caused cleavage of Bid in HuT-78 cells. As shown in Fig. 3B, an enhanced level of truncated Bid was observed in the cytosolic extract of PTX and

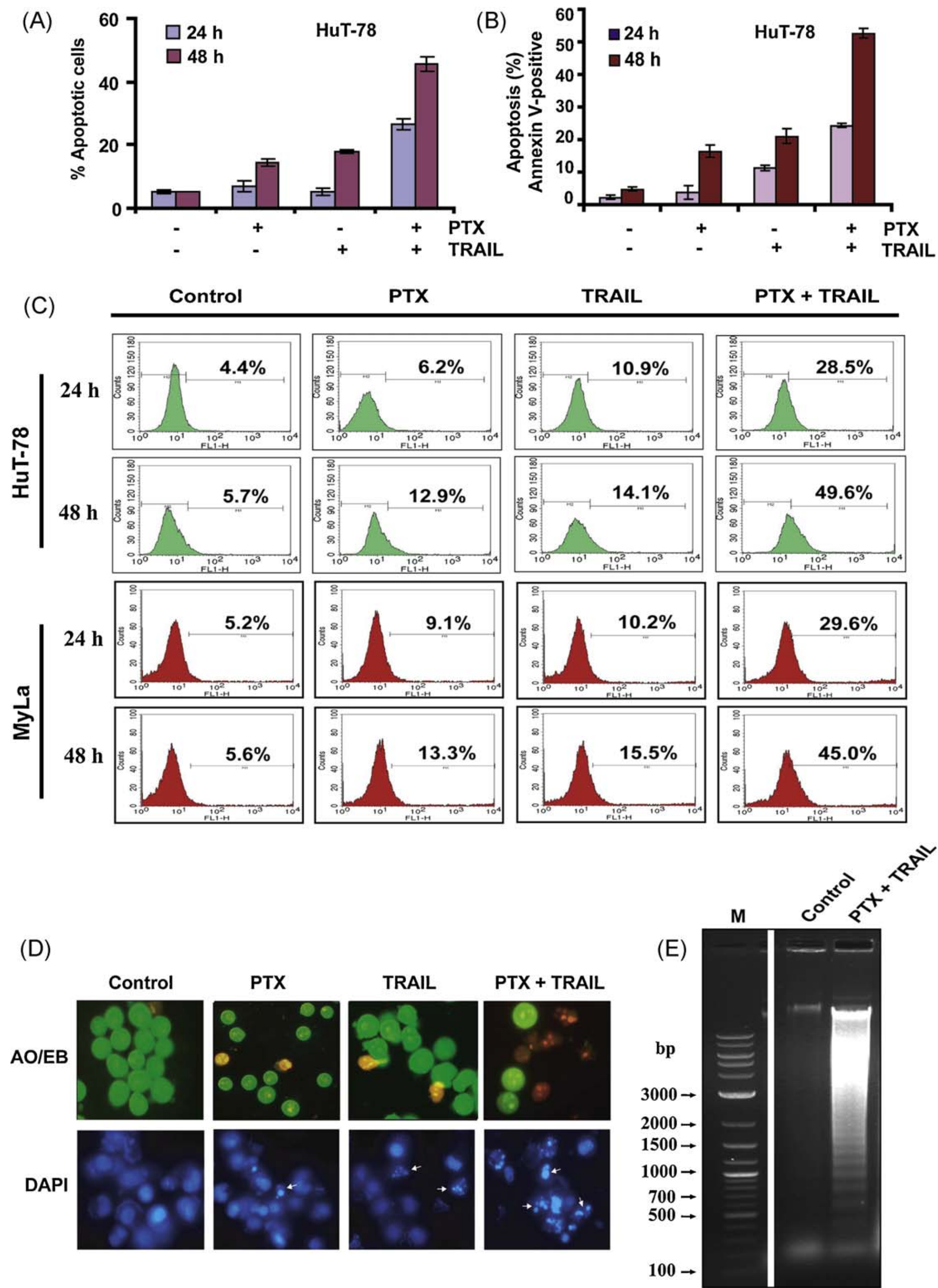

Fig. 2. PTX and TRAIL combined treatment induces apoptosis in HuT-78 and MyLa cells. (A and B) HuT-78 cells were treated with PTX (3 mg/mL) or TRAIL (100 ng/mL) either alone or together for indicated time points (24 and 48 h) and apoptosis was studied by (A) sub-G_1 (hypoploidic) peak analysis, data show mean values $\pm$ S.D. of three similar experiments. (B) Annexin V staining, data show mean values $\pm$ S.D. of three similar experiments. (C) HuT-78 and MyLa cells were treated similarly for 24 and 48 h and apoptosis was detected by TUNEL assay through flow cytometry at FL-1 channel, where, M1 gate demarcates apoptotic population. Data represent the results from one of the two similar experiments. (D) Changes in morphology of HuT-78 cells were observed under fluorescence microscope by staining with AO/EB after 24 h treatment with PTX (3 mg/mL) or TRAIL (100 ng/mL) either alone or together. Green stained cells indicated viable healthy cells and orange cells indicated apoptotic cells (D, upper panel). Under similar condition nuclear morphology was examined by DAPI staining after 24 h. The arrows indicate apoptotic cells (D, lower panel). (E) HuT-78 cells were treated with PTX (3 mg/mL) and TRAIL (100 ng/mL) in combination for 24 h and evaluated for DNA fragmentation. M: 2-Log DNA Ladder. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

TRAIL combined treated cells after 24 h as compared to PTX or TRAIL alone treatment.

Next, we examined the induction of cytochrome *c* release from the mitochondria with PTX and TRAIL treatment in cytosolic extracts. Our result clearly revealed that, combined treatment significantly induced cytochrome *c* release into the cytosol as compared to PTX or TRAIL alone treatment at 24 h time point as detected by Western blot analysis. An enhanced expression of

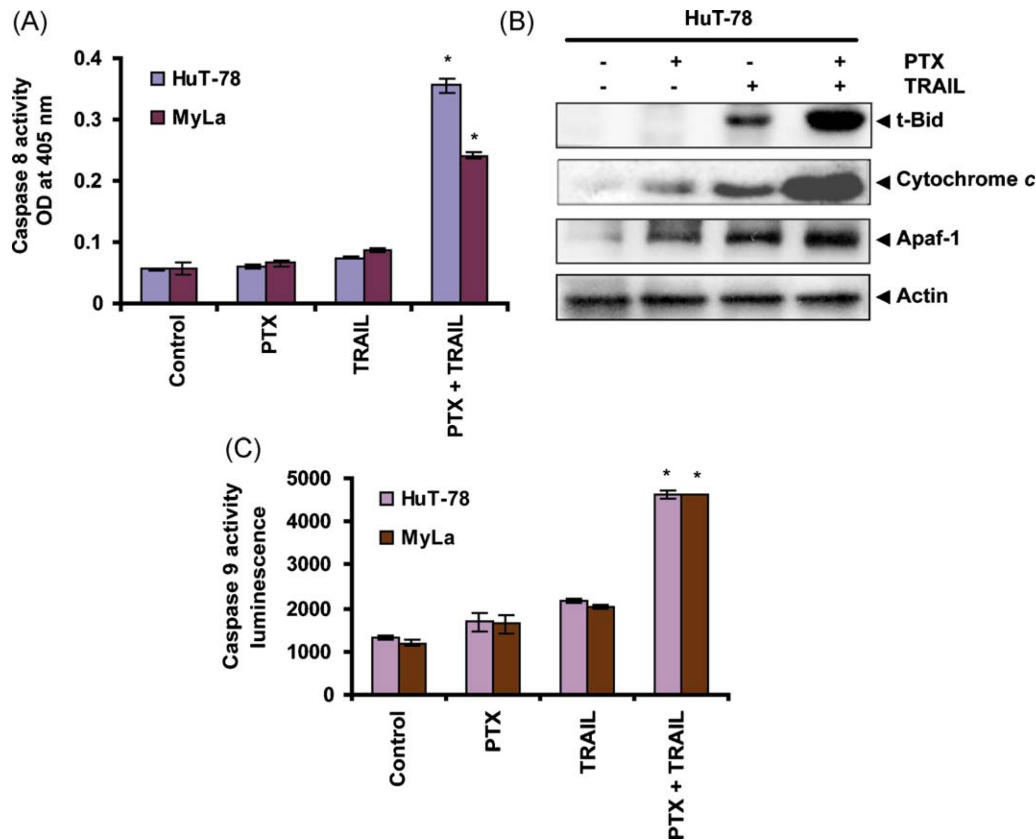

Fig. 3. PTX induces TRAIL-mediated caspase-8 activation, Bid truncation, cytochrome *c* release and activates caspase-9. (A) HuT-78 and MyLa cells were treated with PTX (3 mg/mL) or TRAIL (100 ng/mL) either alone or together for 24 h and caspase-8 activation was determined by colorimetric assay as described in Section 2. Data represent mean values ± S.D. of three similar experiments. (*$P < 0.05$ vs. control.) (B) Cytosolic fraction was separated from treated cells, resolved on 15% SDS-PAGE and analyzed for t-Bid, cytochrome *c* and Apaf-1 using specific antibodies by immunoblotting. (C) HuT-78 and MyLa cells were treated with PTX (3 mg/mL) and TRAIL (100 ng/mL) either alone or together for 24 h and caspase-9 activity was determined by cell based luminescence assay as described in Section 2. Values are expressed as mean ± S.D. of three similar experiments. (*$P < 0.05$ vs. control.)

Apaf-1 was also observed with combined treatment of PTX and TRAIL as compare to alone treatment (Fig. 3B). These results clearly indicating that mitochondrial pathway is associated with PTX-induced TRAIL-mediated apoptosis.

It is known that cytochrome *c* released from mitochondria into the cytosol binds to the apoptotic protease activating factor (Apaf) complex and triggered the activation of pro-caspase-9 to the active caspase-9 [28,29]. In Fig. 3C, PTX and TRAIL combined treatment significantly enhanced the caspase-9 activities in both the cell types as compared to PTX or TRAIL alone at 24 h time point as evident by luminescence assay. Therefore, PTX and TRAIL treatment induces apoptosis in HuT-78 and MyLa cells through extrinsic as well as intrinsic pathway.

3.4. PTX enhances TRAIL-mediated apoptosis through caspase-3 activation and PARP cleavage

Colorimetric assays were performed to see whether PTX treatment can enhance TRAIL-mediated caspase-3 activity in HuT-78 and MyLa cells. Our result revealed that combined treatment significantly enhanced caspase-3 activation as compared to PTX or TRAIL alone treatment at 24 h in both the cell lines (Fig. 4A). The specificity of caspase-3 activity was detected by using pan caspase inhibitor, Z-VAD-FMK (data not shown).

Poly-ADP Ribose Polymerase (PARP) is a substrate for caspase-3 and its cleavage is an indicator of apoptosis. For studying PARP cleavage in HuT-78 cells, whole cell lysates were prepared after 24 h treatment with 3 mg/mL PTX and 100 ng/mL TRAIL alone or together. PARP cleavage was detected by immunoblotting using anti-PARP antibody which recognizes both 116 kDa intact and 85 kDa cleaved forms of PARP (Fig. 4B). Interestingly, a marked cleavage of PARP with an 85 kDa cleaved product was found in combined treatment as compared to alone treatment.

We found that combination of PTX and TRAIL resulted in activation of initiator caspases, caspase-8, caspase-9 and effector caspases such as caspase-3. Therefore, to further substantiate the role of caspases in PTX-induced TRAIL-mediated apoptosis, we determined apoptosis in the presence of pan caspase inhibitor Z-VAD-FMK. Our result indicated that apoptosis was reduced from 27.1% to 5.1% with Z-VAD-FMK in HuT-78 cells as evident by sub-G_1 peak analysis (Fig. 4C). Taken together, these results demonstrated that caspase activation was involved in augmented induction of apoptosis by PTX and TRAIL.

3.5. TRAIL-induced augmentation of apoptosis by PTX affects Bcl-xl, c-FLIP and inhibitors of apoptosis cIAP-1, cIAP-2 and XIAP protein levels

We also examined the combined effects of PTX and TRAIL on the expression of different antiapoptotic proteins, c-FLIP, Bcl-xl, cIAP-1, cIAP-2 and XIAP in both the cell lines, HuT-78 and MyLa by immunoblotting at 24 h time point. Interestingly, we found that combined treatment of PTX and TRAIL effectively downregulated the expression of c-$FLIP_L$ and c-$FLIP_S$ forms as compared to PTX and TRAIL alone treatment in both the cell lines (Fig. 5A). In Fig. 5B, PTX and TRAIL in combination also markedly inhibited the expression of Bcl-xl, cIAP-1, cIAP-2 and XIAP, in both the cell lines.

TNF superfamily molecules stimulation results in clustering of the receptor, which in turn leads to recruitment of the adaptor

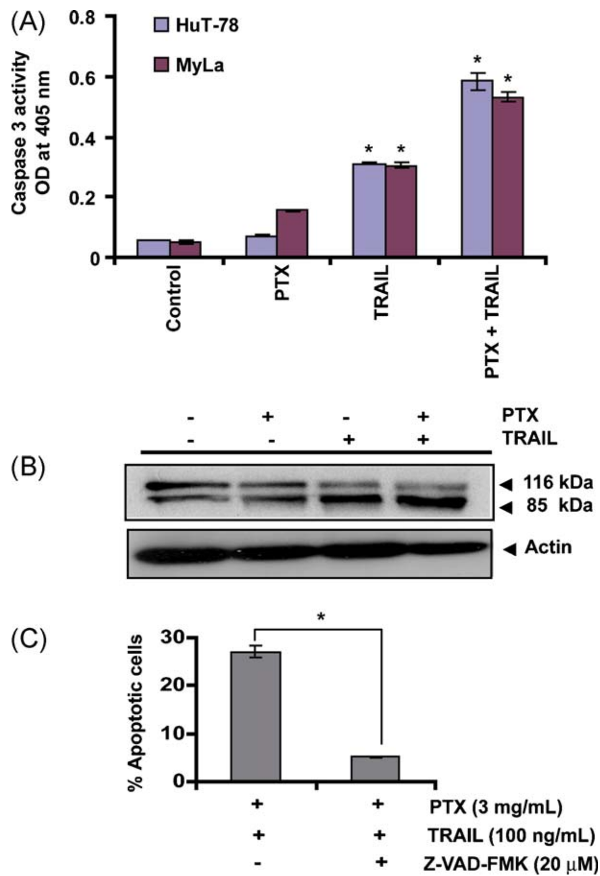

Fig. 4. PTX and TRAIL treatment induces caspase-3 activation and PARP cleavage. (A) Cell lysates were prepared from HuT-78 and MyLa cells after 24 h treatment with PTX (3 mg/mL) or TRAIL (100 ng/mL) either alone or together and analyzed for caspase-3 activation by colorimetric assay as described in Section 2. Values are expressed as mean ± S.D. of three similar experiments. (* $P < 0.05$ vs. control.) (B) PARP cleavage was detected in HuT-78 cells by Western blotting using mouse monoclonal anti-PARP antibody. (C) HuT-78 cells were pre-treated with Z-VAD-FMK (20 μM) for 1 h and then subjected to PTX (3 mg/mL) and TRAIL (100 ng/mL) combined treatment for 24 h. Apoptosis was determined by sub-G_1 peak analysis through flow cytometry. Values are expressed as mean ± S.D. of three similar experiments. (* $P < 0.05$ vs. control.)

molecules like Fas-associated death domain (FADD), which further activate caspase cascade, resulting in the execution phase of apoptosis [30]. Therefore, we further investigated the change in the expression of FADD in HuT-78 cells. PTX and TRAIL combined as well as alone treatment did not alter the expression of FADD (Fig. 5C).

3.6. PTX induces upregulation of TRAIL death receptors (DRs) TRAIL-R1/DR4 and TRAIL-R2/DR5 through MAPK/JNK pathway

It is known that upregulation of death receptor on the cell surface may enhance the biological activity of TRAIL [31]. Since, PTX enhances the apoptosis-inducing potential of TRAIL, we sought to examine the effect of PTX on death receptors expression in HuT-78 and MyLa cells. As shown in Fig. 6A, mean fluorescence intensity (MFI) value indicated the increase in surface expression of TRAIL-R1/DR4 and TRAIL-R2/DR5 with 3 mg/mL PTX in comparison to control untreated cells at 12 h time point. However, we did not find any change in the expression of DcR1 and DcR2 with PTX treatment (Fig. 6A, left panel). We also obtained similar effect of PTX in MyLa cells, where 3 mg/mL PTX upregulated DR4 and DR5 expression in comparison to control untreated cells at 12 h time point (Fig. 6A, right panel). Next, we were interested to evaluate the effect of PTX on DR4 and DR5 at mRNA level after 12 h treatment in HuT-78 cells. Our RT-PCR analysis showed that PTX treatment enhanced DR4 mRNA expression more than that of DR5 expression in comparison to control untreated cells as shown in Fig. 6B. However, 100 ng/mL TRAIL did not change the expression of DR4 and DR5 at mRNA level.

Studies from different laboratories have shown that upregulation of TRAIL receptors as the key player in TRAIL-induced apoptosis is mediated by the members of the mitogen-activated protein kinase (MAPK) family [32–35]. Therefore, in order to see whether this pathway is involved for the expression of death receptors by PTX in HuT-78 cells, we first treated HuT-78 cells with PTX (0, 1.5 and 3 mg/mL) for 12 h and whole cell extracts were used to see the activity of different MAPKs (ERK, p38 and JNK) by immunoblotting. Fig. 7A, showed that PTX treatment upregulated the level of p-JNK/SAPK and JNK1/JNK2 compared to phosphorylated and non-phosphorylated form of either p38 or ERK. As shown in Fig. 7B, 3 mg/mL PTX not only enhanced the level of p-c-Jun but also enhanced total c-Jun level. Addition of SP600125 (20 μM) inhibited the PTX-induced increase of p-c-Jun. Correspondingly, the elevated c-Jun level induced by PTX (3 mg/mL), was effectively suppressed by JNK inhibitor, SP600125 (Fig. 7B).

Next, we examined the effect of JNK inhibitor on DR4 and DR5 induction by PTX in HuT-78 cells. To address this issue, we performed immunoblot analysis for the expression of DR4 and DR5 in the presence or absence of JNK inhibitor. Interestingly, we found that, 3 mg/mL PTX upregulated the protein expression of DR4 by 7.3-fold and DR5 by 1.9-fold in comparison to control untreated cells, which were downregulated by JNK specific inhibitor, SP600125 (Fig. 7C). We also tested the effect of JNK inhibitor on PTX-induced DR4 and DR5 expression in MyLa cells. Addition of SP600125 downregulated the expression of both the death receptors (data not shown).

Further, we attempted to see the effect of combined treatment of PTX and TRAIL on the DNA binding activity of AP-1 and NF-κB in HuT-78 and MyLa cells. In Fig. 7D, upregulation of AP-1 DNA binding activity was observed with PTX treatment only which did not enhanced further in combination with TRAIL. However, NF-κB remains unaffected with combined as well as alone treatment in both the cell lines.

3.7. Death receptors are required for PTX-induced TRAIL-mediated killing in HuT-78 cells

To examine the role of enhanced DR4 and DR5 expression by PTX in TRAIL-induced apoptosis, we used siRNA specific to DR4 and DR5 to downregulate the expression of both the receptors. Transfection of HuT-78 cells with siRNA targeting the receptors (DR4 or DR5) but not with the control siRNA (scrambled) resulted in a marked inhibition of PTX-mediated DR4 and DR5 upregulation at cell surface (Fig. 8 A). Next, we examine, whether the suppression of DR4 or DR5 by siRNA either alone or together could inhibit the sensitizing effects of PTX on TRAIL-mediated killing. Our results clearly revealed that the effect of PTX on TRAIL-mediated killing was effectively abolished in cells transfected with both DR4 and DR5 siRNAs, whereas the treatment with control (scrambled) siRNA had no effect (Fig. 8B). Silencing of DR4 had more effect on TRAIL-induced apoptosis than that of DR5. Thus, our data suggested that both DR4 and DR5 playing major role in PTX-induced TRAIL-mediated killing.

4. Discussion

The TRAIL is regarded as a potential anticancer agent against many cancer cells [36]. Human CTCL cell lines, HuT-78 (SS) and MyLa (MF) are less sensitive to TRAIL. Experimental evidences

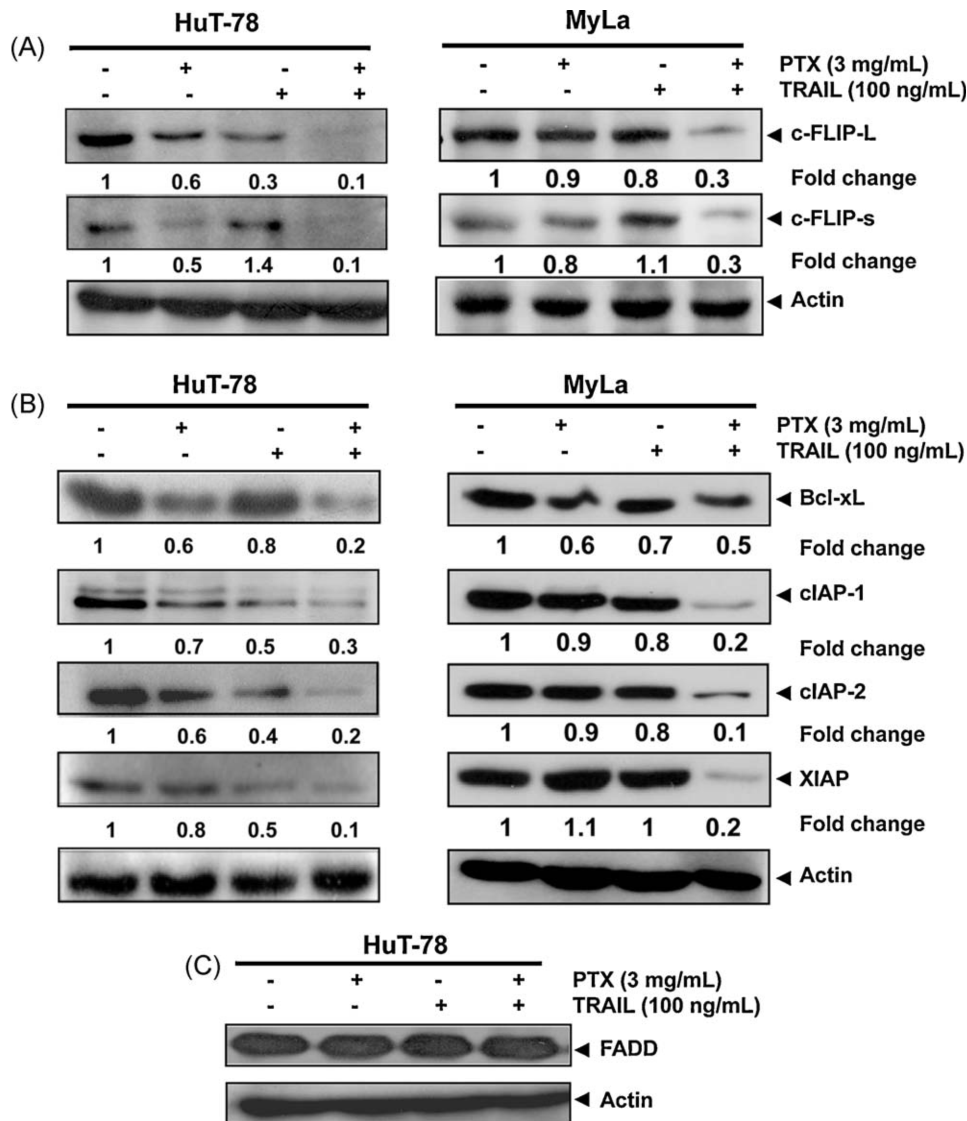

Fig. 5. PTX and TRAIL combined treatment inhibits expression of antiapoptotic proteins. (A–C) Whole cell lysates prepared from HuT-78 and MyLa cells after 24 h treatment with PTX (3 mg/mL) or TRAIL (100 ng/mL) either alone or together were resolved on 12% SDS-PAGE and immunoblotting was performed: (A) c-FLIP$_L$ and c-FLIP$_S$ (B) Bcl-xl, cIAP-1, cIAP-2 and XIAP (C) FADD. Band intensity of individual protein was normalized to actin.

provided in this report clearly indicate that treatment with PTX significantly enhances TRAIL-induced apoptosis in both the cell lines.

TRAIL sensitivity can be modulated by extrinsic death receptor pathway as well as intrinsic mitochondrial dependent pathway [37]. Although, PTX alone cannot activate caspase-8 but it enhances TRAIL-mediated activation of caspase-8. Caspase-8 activation is a critical step for the initiation of the extrinsic apoptotic pathway whereas caspase-9 activation is required for intrinsic mitochondrial dependent pathway [38]. The cross talk between these two pathways is mediated by the truncated pro-apoptotic protein, Bid [6]. Our results clearly indicate that combined treatment with PTX and TRAIL significantly enhance the level of truncated Bid. We also found that combined treatment not only activates caspase-9 but also induces cytochrome *c* release. The pro-apoptotic protein Bax remains unchanged with combined treatment (data not shown), in contrast, Apaf-1 expression increases with the treatment, suggesting more apoptosome formation with the association of cytochrome *c* and pro-caspase-9 which resulted in the cleavage of pro-caspase-9 to active caspase-9. Further, PTX and TRAIL in combination activates downstream effector caspase, caspase-3, which in turn cleaves its substrate PARP and finally dismantle the cell. Moreover, inhibition of apoptosis in the presence of Z-VAD-FMK suggests the involvement of caspases.

Presence of intracellular apoptosis inhibitory substances (Bcl-xl, c-FLIP, cIAP etc.) are also known to be responsible for TRAIL resistance in different cancer cells [39–41]. HuT-78 and MyLa cells constitutively express high amount of these antiapoptotic proteins [2]. Interestingly, combined treatment of PTX and TRAIL down-regulate the expression of both short and long form of c-FLIP in both the cell lines. c-FLIP is the major protein that prevents caspase-8 activation by death receptors through binding to FADD and caspase-8 at the DISC [42]. Therefore, downregulation of c-FLIP by PTX plus TRAIL probably explained caspase-8 mediated apoptosis of HuT-78 and MyLa cells.

Downregulation of XIAP, cIAP-1 and cIAP-2 were observed when cells were treated with PTX and TRAIL together. It has been reported that in prostate cancer cells IAP proteins inhibit caspases, making the cells resistant to TRAIL [43]. FADD is reported to be the universal adaptor used by death receptors to recruit and activate the initiator caspase-8 to execute apoptosis [44]. HuT-78 cells

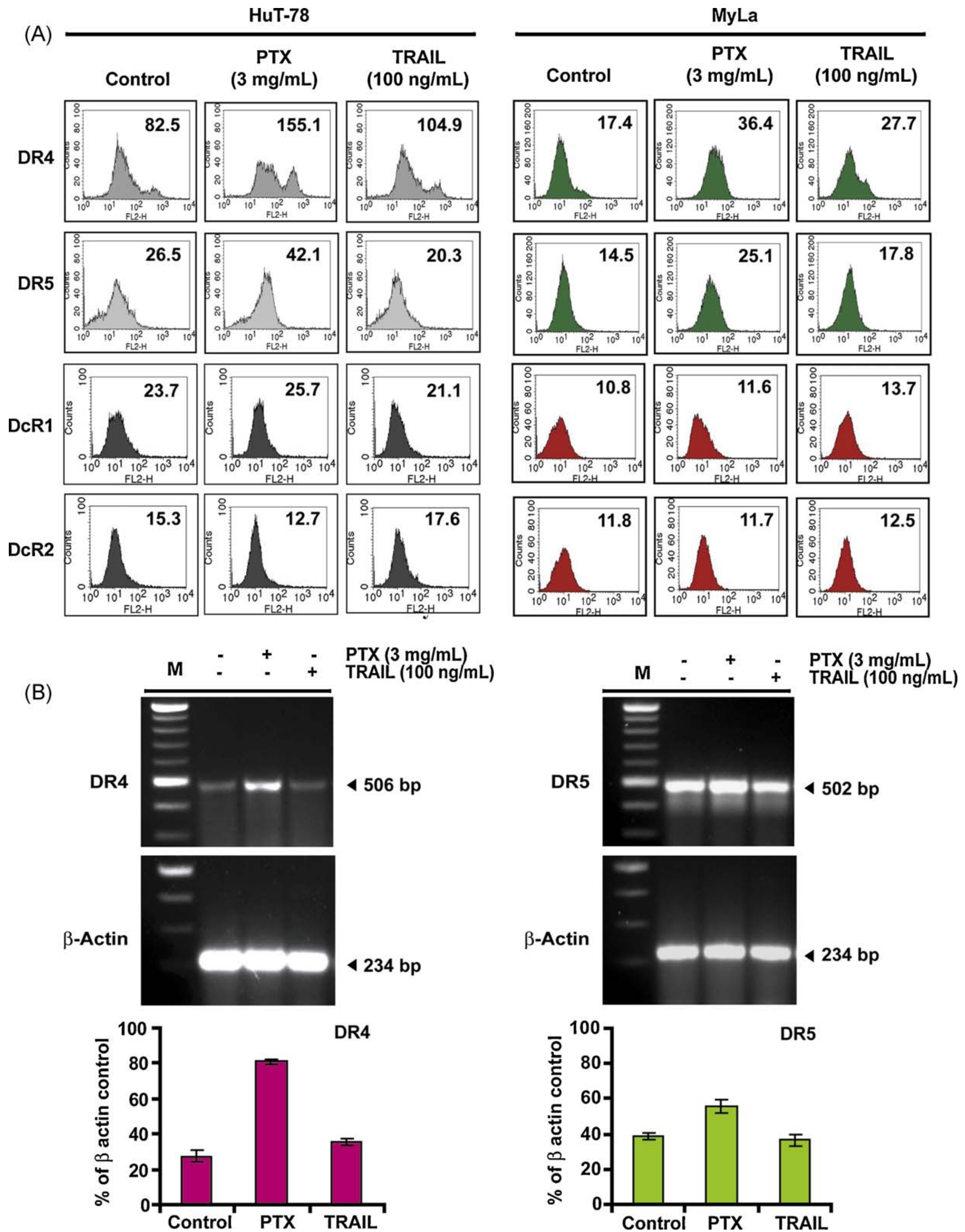

Fig. 6. PTX upregulates DR4 and DR5 expression. (A) HuT-78 and MyLa cells were treated with PTX (3 mg/mL) or TRAIL (100 ng/mL) for 12 h time point, surface stained for TRAIL-R1/DR4, TRAIL-R2/DR5, TRAIL-R3/DcR1, TRAIL-R4/DcR2 expression and analyzed through flow cytometry as described in Section 2. Values shown in the parentheses are the MFI scores. Data represent the results from one of the three similar experiments. (B) HuT-78 cells were treated with PTX (3 mg/mL) or TRAIL (100 ng/mL) for 12 h and total RNA was extracted and examined for expression of DR4 and DR5 by RT-PCR. Relative quantity of DR4 and DR5 was determined by normalizing β-actin mRNA for three separate experiments. Values are expressed as mean ± S.D. of three similar experiments.

express high amount of FADD at the basal level. PTX or TRAIL alone or in combination cannot upregulate FADD expression. Thus, it seems that pro-caspase 8 may not be able to recruit at FADD due to competition between pro-caspase 8 and c-FLIP for the binding to FADD.

Sensitivity of cells to TRAIL-mediated apoptosis has been shown to be a function of the ratio of TRAIL death receptors to decoy receptors [7]. When we studied the effect of PTX on surface expression of TRAIL death receptors and decoy receptors, interestingly, we found that PTX enhances the expression of

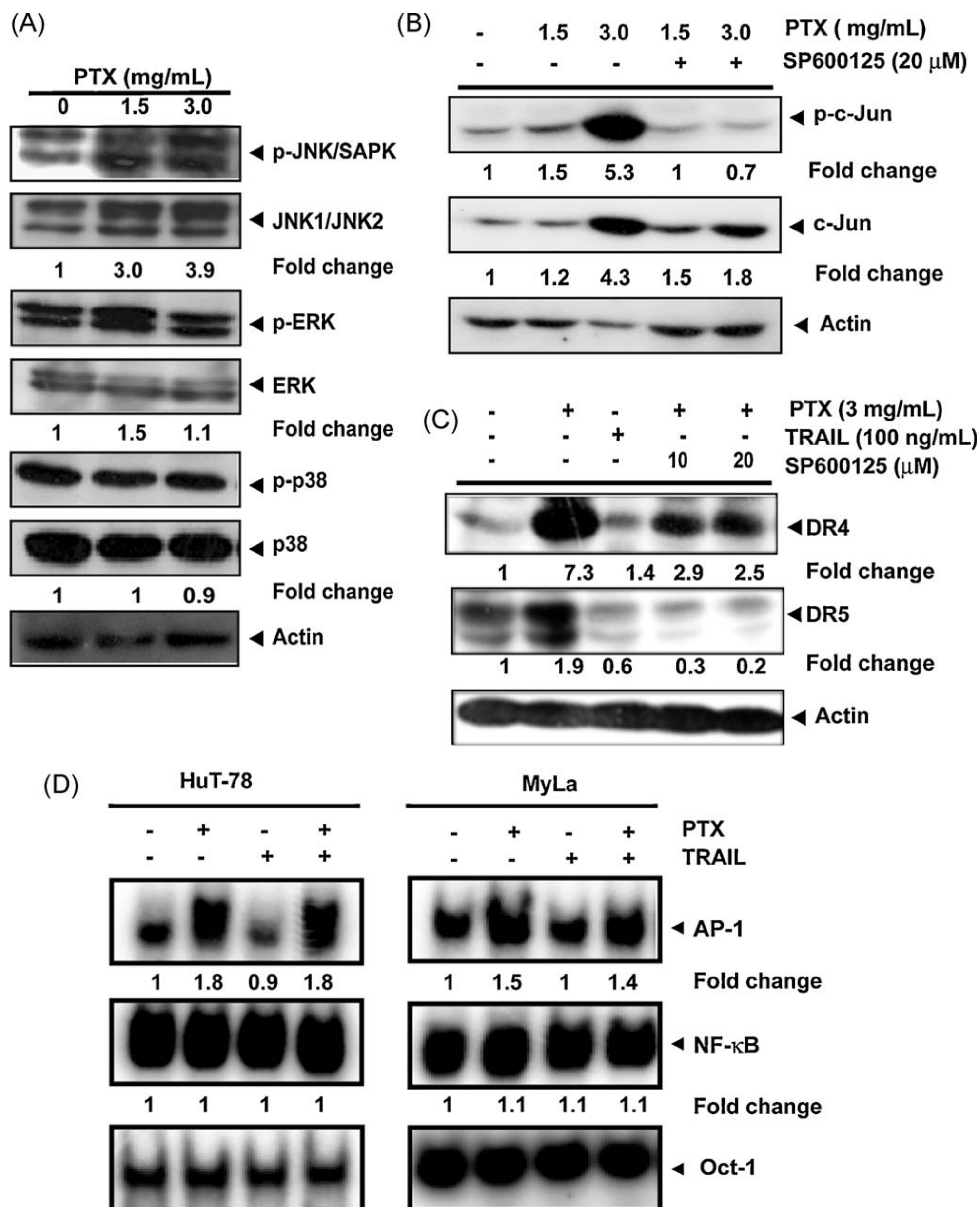

Fig. 7. PTX upregulates DR4 and DR5 expression through JNK/MAPK pathway. (A) Whole cell lysates were prepared from PTX (0-3 mg/mL) treated cells after 12 h. Lysates obtained were resolved on 12% SDS-PAGE and immunoblotted for p-JNK/SAPK, JNK1/JNK2, p-ERK, ERK, p-p38 and p-38 activity by using their specific antibodies. Fold increase was calculated after normalization to non-phosphorylated forms. (B) HuT-78 cells were pre-treated with JNK inhibitor, SP600125 (20 μM) for 1 h and then treated with PTX (1.5 and 3 mg/mL) for 12 h. Whole cell lysates were prepared and then analyzed for the expression of p-c-Jun and c-Jun by Western blotting. Fold increase was calculated after normalization to actin. (C) Cells were pre-treated with SP600125 (10 and 20 μM) for 1 h and then treated with PTX (3 mg/mL) or TRAIL (100 ng/mL) for 12 h. Whole cell extracts were prepared and analyzed by immunoblotting using anti-human DR4 and DR5 antibodies. Equal loading of protein was evaluated by actin. (D) HuT-78 and MyLa cells were treated with PTX (3 mg/mL) or TRAIL (100 ng/mL) either alone or together for 12 h. Nuclear extracts were prepared and assayed for NF-κB and AP-1 by EMSA.

DR4 and DR5 on the cell surface of both the cell lines. We did not find any change in the expression of decoy receptors DcR1 and DcR2. PTX upregulated the expression of DR4 and DR5 at mRNA level also in HuT-78 cells. Next, we investigated the role of death receptors in PTX-induced TRAIL-mediated apoptosis. We found that, combined silencing of DR4 and DR5 by siRNA drastically reduces the TRAIL-mediated killing in comparison to individual silencing of DR4 or DR5 which clearly showed the involvement of both the receptors in apoptosis.

Further, we were interested to investigate the mechanism of upregulation of TRAIL death receptors. Several studies have suggested the involvement of MAPK family members in the regulation of TRAIL death receptors in different cancers [32–35]. In addition, the role of NF-κB, AP-1 and STAT3 has also been reported in the regulation of TRAIL and its receptors promoter activity in melanomas [45–47]. In this study, we found that PTX treatment did not significantly enhance the activity of p38 and ERK1/2 of MAPK family, in contrast it significantly enhance JNK activity. Addition of JNK inhibitor, SP600125 downregulated the expression of PTX-induced DR4 and DR5 which suggested that the induction of TRAIL death receptors was regulated through JNK/MAPK pathway.

PTX enhances AP-1 DNA binding activity in HuT-78 and MyLa cells without affecting NF-κB activity. The two well-known substrates of JNK are p-c-Jun and p-ATF2 [33]. PTX not only enhance the p-c-Jun level but also total c-Jun level. Activation of JNK has been shown to induce the E3 ubiquitin ligase ITCH to

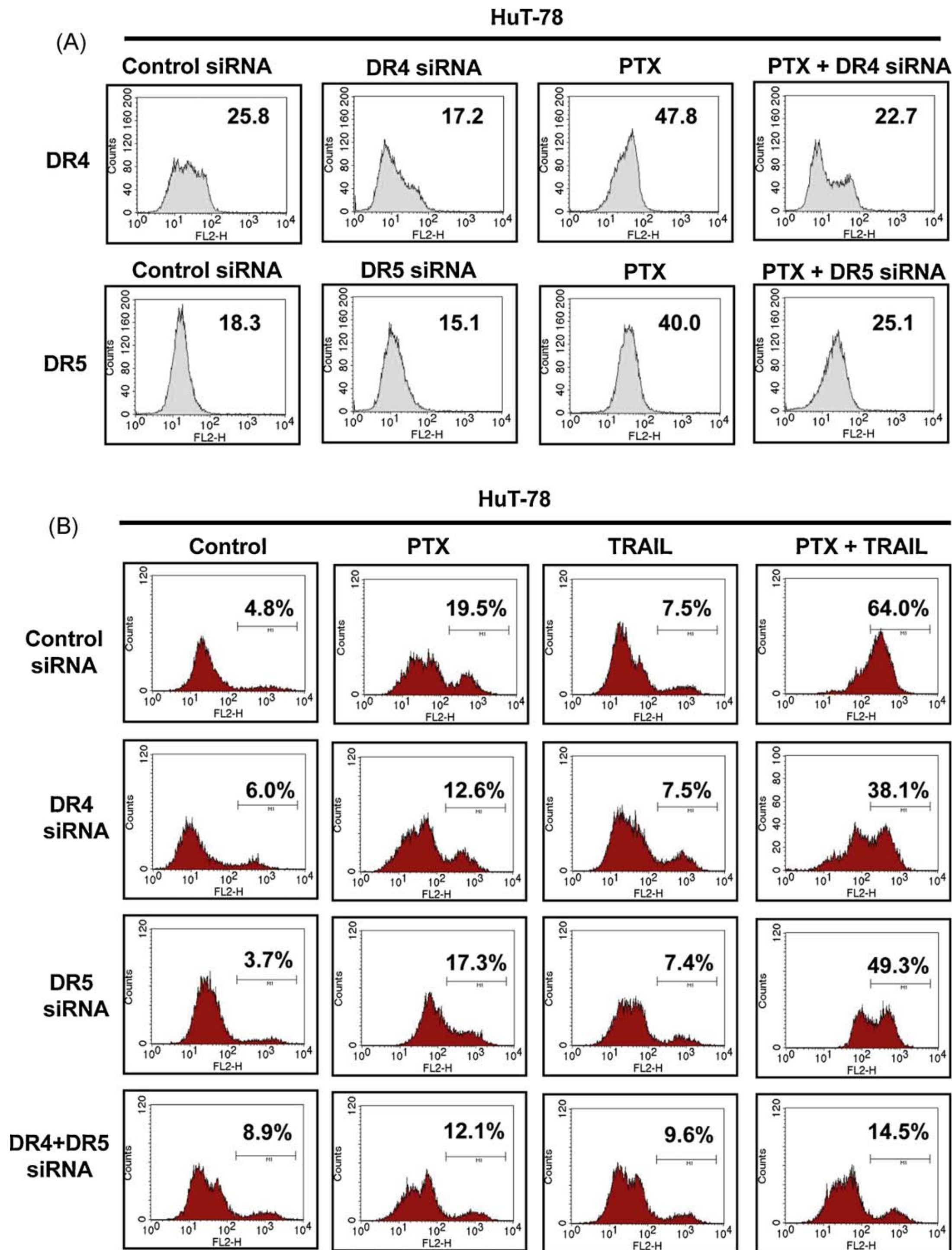

Fig. 8. Effects of silencing of DRs on PTX-induced sensitization of TRAIL. (A) HuT-78 cells were transfected with DR4 siRNA, DR5 siRNA and control siRNA alone or combined. After 48 h, cells were treated with PTX (3 mg/mL) for 12 h, surface stained for DR4 and DR5 and analyzed through flow cytometry. (B) HuT-78 cells were transfected with siRNAs, treated with PTX (3 mg/mL) or TRAIL (100 ng/mL) either alone or together for 24 h and then examined for cell viability by PI exclusion method, where M1 represent % dead cells. Similar results were obtained in two separate experiments.

ubiquitinate c-FLIP which leads to its degradation [48]. Therefore, upregulation of JNK activity may be one of the possible mechanisms for c-FLIP downregulation in HuT-78 and MyLa cells. It will be interesting to know how this JNK activation and AP-1 upregulation by PTX is playing role in TRAIL-mediated apoptosis in CTCL cells.

Overall, our study provided strong evidences that PTX could potentiate TRAIL-mediated apoptosis through downregulation of cell survival gene products and upregulation of death receptors. Thus, in terms of clinical perspective PTX in combination with TRAIL may be a novel strategy for the treatment of CTCL patients.However, therapeutic use of any anticancer agent depends on its selectivity against a particular cancer cell type and its overall toxicity. Reports indicate that dose of 400 mg PTX is being administered twice or three times daily to patients suffering from various diseases like haematological disorder and cancer [10]. Further study is needed to consider PTX and TRAIL combination as potential therapeutic agents for treating CTCL cancer.

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