Satindra (2011). ***Antioxidants and cutaneous T cell Lymphoma growth.*** Ph.D. Thesis. CSIR-IMTECH, Chandigarh/ Jawaharlal Nehru University, New Delhi: India.
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SUMMARY OF THE THESIS

Pentoxifylline (PTX), a xanthine-derived antioxidant and a non specific PDE inhibitor, has recently gain attention due to its anticancer property in different cancer cells. It has been shown to enhance the efficacy of various chemotherapeutic agents such as cisplatin, etoposide, thiotepa, fludarabin, andriamycin as well as sensitize tumor cells to ionizing irradiation (Ohsaki et al., 1996; Alas et al., 2000; Li et al., 1999; Lerma- Diaz et al., 2006; Bravo-Cuellar et al., 2010). PTX with α-tocopherol (Vit E) are now in phase II and III clinical trails for breast cancer lymphedema and non small cells lung cancer respectively. Recent, work of our laboratory also showed that, PTX, potently scavenges ROI in CTCL cell line, HuT-78 and enhances FasL mediated apoptosis by upregulating Fas surface expression (Rishi et al., 2009). PTX also tend to upregulates TRAIL mRNA expression in these cells. But its effect on TRAIL mediated apoptosis in CTCL cells was still unknown.

Therefore, we proceeded to see whether PTX has ability to enhance TRAIL mediated apoptosis in CTCL cells. First we checked the cytotoxic potential of PTX on HuT-78 cells (sézary syndrome) and MyLa cells (mycosis fungoides) by PI staining. PTX causes dose dependent cytotoxicity in both the cell types. Next, we explored the ability of PTX to enhance TRAIL mediated apoptosis in both the cell lines. Interestingly, PTX at 3 mg/ml with TRAIL 100 ng/ml causes maximum synergistic apoptosis. The apoptosis was completely abrogated in the presence of human neutralizing anti-TRAIL Ab, RIK-2. This was further confirmed by sub-G_1 peak analysis, annexin V staining and TUNEL assay where, PTX (3 mg/ml) potentiate the TRAIL (100 ng/ml) mediated apoptosis, synergistically. Next, apoptosis mechanism with combined treatment was elucidated which involved caspase-8, -3 and -9 activation with bid truncation, cytochrome *c* release and PARP cleavage. This suggested the link between extrinsic and intrinsic apoptotic pathways. Various anti-apoptotic proteins like cFLIP, Bcl-xL, cIAP-1, cIAP-2 and XIAP were also found to be downregulated with combined treatment as compared to alone.

We next explore the ability of PTX to enhance the TRAIL death receptors which are the main contributors for TRAIL induced signalling inside the cell. Interestingly, PTX significantly upregulated the surface expression of DR4 and DR5 without having any effect on decoy receptors (DcR1 and DcR2).

Many factors have been reported to regulate the expression of DR4 and DR5, such as NF-κB, AP-1, STAT3, p53, CHOP and MAP kinases. Therefore, next we aimed to explore the ability of PTX and TRAIL to induce NF-κB and AP-1. Our EMSA analysis, clearly revealed that PTX upregulated the DNA binding activity of AP-1 which did not increase further with the addition of TRAIL whereas NF-κB remains unaffected. We also checked the expression of MAP kinases, p38, ERK and JNK. PTX resulted in the upregulation of both phosphorylated and non-phosphorylated form, of JNK, whereas ERK and p38 activity did not alter significantly. Simultaneously, PTX also downregulated the STAT3 protein dose dependently. Besides this, PTX upregulated the expression of CHOP/GADD153 and leads to calcium release at 12 h, suggested the role of ER stress in PTX mediated apoptosis. However, several reports demonstrated the role of CHOP in the DR5 gene expression.

Because PTX significantly induces JNK activation, we also checked the expression of c-Jun, downstream substrate of JNK. As expected PTX not only caused the phosphorylation of c-Jun but it also increased the basal level of c-Jun protein which was reduced with the addition of JNK inhibitor, SP600125. Pre-treatment of JNK inhibitor for 1 h followed by PTX 3 mg/ml treatment abrogated the DR4 and DR5 expression significantly. We also studied the mechanism of cFLIP degradation with PTX, where, cFLIP inhibition by PTX was completely abrogated in the presence of JNK inhibitor. This clearly suggested that besides regulating the expression of DR4 and DR5, JNK also regulate the turnover of cFLIP.

Furthermore, to account for the role of death receptors in PTX induced TRAIL mediated apoptosis, DR4 and DR5 were knock down with siRNA either alone or together and apoptosis was examined after 24 h treatment with PTX and TRAIL. Interestingly, apoptosis was predominantly reduced with the combined silencing of DR4 and DR5 in comparison to alone silencing. However, DR4 was shown to have more pronounced effect on apoptosis than DR5. This suggested that upregulation of death receptors are essential for PTX and TRAIL induced apoptosis. We obtained similar results when we used neutralizing antibodies against DR4 and DR5. Schematic diagram showing the

overall signaling pathways induced by PTX and TRAIL in CTCL cells is mentioned in **Fig. 1**.

We also explore the cytotoxic potential of PTX along with different PDE inhibitors such as, IBMX (non-specific PDE inhibitor), rolipram (PDE 4 inhibitor) or BRL 50481 (PDE 7 and 8 inhibitors), but we could find synergistic apoptosis in any combinations. We further attempted to see the ability of above mentioned phosphodiesterse inhibitors to induce TRAIL mediated apoptosis in HuT-78 cells. Interestingly, IBMX (non specific PDE inhibitor) and rolipram (PDE4 specific inhibitor) synergistically enhance the TRAIL mediated apoptosis. However, rolipram was able to show more pronounced effect on apoptosis.

Rolipram has been described to modulate the balance between pro-and anti-apoptotic members of Bcl-2 family and induces caspase dependent apoptosis in B-CLL cells (Siegmund et al., 2001). As PTX, rolipram has an inhibitory effect on pro-inflammatory mediators (TNF-α) as well as Th1 (IFNγ) and Th2 (IL-4 and IL-5) cytokines. It has been shown to inhibit NF-κB and NF-AT activation and stimulates AP-1 and CREB transcription factors in T lymphocytes (Jimenez et al., 2001). Accumulating evidences suggested that rolipram as alone or in combination has apoptotic potential against various cancer cells. Therefore, further studies were carried out to explore the apoptotic potential of rolipram on HuT-78 cells. Rolipram caused dose dependent apoptosis in HuT-78 cells at 24 and 48 h. Apoptosis mechanism involved intrinsic mitochondrial pathway as indicated by mitochondrial hyperpolarisation with depolarised peak with increasing concentrations and subsequent activation of caspase-9 and -3 followed by PARP cleavage. Rolipram significantly downregulated the expression of cFLIP and other anti-apoptotic proteins such as Bcl-xL, cIAP-1 and cIAP-2. Moreover, rolipram (0.5 mM) was found to activate JNK with the suppression of p38 and ERK activity at 24 and 48 h.

Our results also revealed that rolipram induces TRAIL mediated apoptosis in HuT-78 cells. Rolipam induced TRAIL mediated apoptosis exhibit caspase 8,-9 and -3 activation with PARP cleavage. The combined treatment also suppressed cFLIP, Bcl-xL, cIAP-1 and cIAP-2 expression. Interestingly, XIAP was found to be cleaved to generate 29 kDa fragment with the combined treatment. In addition, rolipram did not exert significant

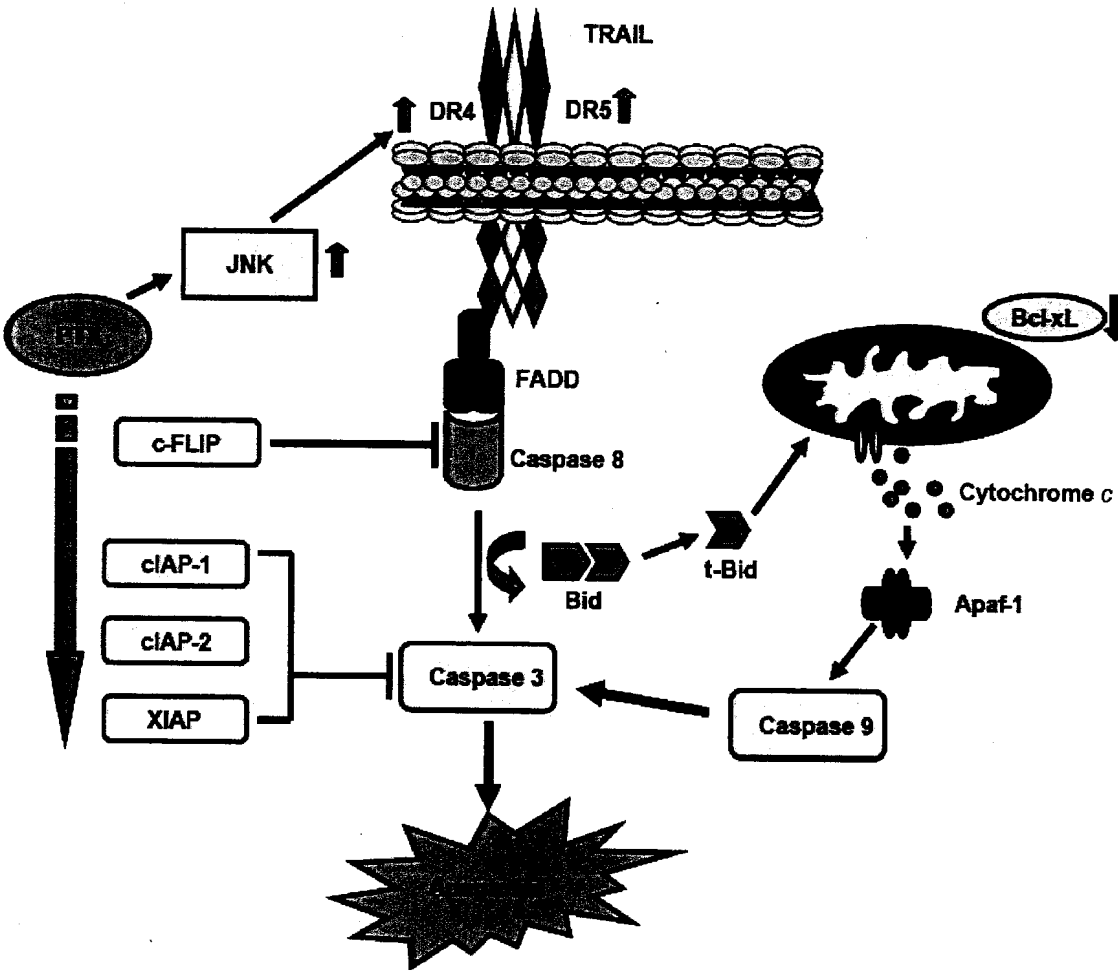

Fig.1. Schematic diagram showing apoptotic signaling pathway induced by PTX and TRAIL

alterations in the TRAIL death receptors. However, it suppressed the NF-κB and AP-1 activity dose dependently. Thus, next we were interested to see the effect of addition of PTX along with rolipram and TRAIL. PTX at 1.5 mg/ml did not induce TRAIL mediated apoptosis however it was able to enhance DR4 and DR5 very significantly. Surprisingly, PTX addition drastically enhanced the rolipram induced TRAIL mediated apoptosis in HuT-78 cells. The apoptosis was further substantiated by TUNEL assay. Next, we investigated the mechanism of apoptosis where we obtained caspase-8 and -3 activation followed by PARP cleavage with combine treatment of PTX, rolipram and TRAIL. Interestingly, the apoptosis was completely abrogated with the addition of z-VAD-Fmk, suggested the crucial role of caspases in apoptosis. Inhibition of caspase-8 by its inhibitor, z-IETD-Fmk also reverted the apoptosis, indicating major role of caspase-8 to activate downstream signalling or caspase cascade to induce apoptosis. Addition of PTX to rolipram further enhanced peak of depolarisation, indicating involvement of intrinsic mitochondrial apoptotic pathway.

Further combined treatment of PTX, rolipram and TRAIL, lead to the inhibition of cIAP-1, cIAP-2 and XIAP, the cellular inhibitors of apoptosis. In addition, XIAP was found to be cleaved with a fragment of 29 kDa which was partially reverted by caspase-8 inhibitor. We also obtained a 16 kDa cleaved fragment of BcL-xL with the combined treatment which was disappear with z-VAD-Fmk. Combined treatment further resulted in the appearance of $cFLIP_L$ processed form p43 whose processing occurs at DISC and require caspase-8 activity. This processing of $cFLIP_L$ was abrogated in the presence of z-VAD-Fmk, suggested the role of caspases in the processing of $cFLIP_L$, this finding further substantiate the caspase-8 activation with the combined treatment. Next, we examine the effect of combined treatment of PTX, rolipram and TRAIL on Hsp90 expression. Interestingly, combined treatment resulted into the cleavage of Hsp90 with approx. 50 kDa cleaved fragment. This cleavage was found to be caspase dependent with the major role of caspase-8.

We next investigated the STAT3 expression with the combine treatment. Our result clearly demonstrated the downregulation of STAT3 (Ser 63) with the combined treatment. Surprisingly, combined treatment synergistically enhances the JNK activity, whereas ERK and p38 were found to be downregulated with the PTX, rolipram and TRAIL

treatment. In order to further elucidate the mechanism of apoptosis with the addition of PTX 1.5 mg/ml to rolipram and TRAIL. Because PTX provides enhanced expression of DR4 and DR5 to rolipram and TRAIL mediated apoptosis, we next preferred to make out the role of these upregulated death receptors in the apoptosis. Interestingly, combined silencing of DR4 and DR5 by siRNAs abrogated the PTX, rolipram and TRAIL mediated apoptosis, suggested the role of TRAIL death receptors in the apoptosis. Collectively our results suggested that, CTCL cells can exerts TRAIL mediated sensitivity by downregulating cell survival proteins and upregulating death receptors. Here, PTX enhances the TRAIL death receptors expression and rolipram by manipulating anti-apoptotic proteins causing TRAIL mediated apoptosis in CTCL cells. Schematic diagram showing the overall signaling pathways induced by rolipram, PTX and TRAIL in CTCL cells is mentioned in **Fig. 2**.

In addition to PDE inhibitors with antioxidant properties, many natural antioxidants has been shown to enhance the TRAIL mediated sensitivity of many cancer cells. Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene), a natural dietary polyphenol with antioxidant property has demonstrated to affects all three discrete stages of carcinogenesis (initiation, promotion, and progression) by modulating signal transduction pathways that control cell division and growth, apoptosis, inflammation, angiogenesis, and metastasis, and hence is considered to be a promising anticancer therapy. Therefore, we next investigated the apoptotic potential of resveratrol and its underlie mechanism in CTCL cell line, HuT-78.

We found that resveratrol has cytotoxic potential against HuT-78 cells as revealed by increase in PI positive cells, sub-G_1 population and annexin V positive cells. Resveratrol acts as a potent ROI scavenger in HuT-78 cells. The apoptosis mechanism involved extrinsic as well as intrinsic apoptotic pathways. This was evident by the activation of caspase-8, -2 and caspase-3 followed by PARP cleavage. Next, we studied the involvement of intrinsic mitochondrial pathway where, resveratrol causes mitochondrial hyperpolarisation followed by the depolarisation peak in a dose dependent manner. This was followed by the release of cytochrome *c* and caspase-9 activation. Resveratrol also tend to cleave Bid into t-Bid, which clearly demonstrate the link between extrinsic and intrinsic apoptotic pathway. We obtained the suppression of cFLIP, cIAP-1, cIAP-2 and Bcl-xL protein expression. Moreover, resveratrol cleaved the XIAP protein in a caspase