Summary

Melanoma is a malignancy with increasing rate of incidences worldwide especially in white population. A growing body of researches have suggested that alteration in apoptotic pathways and activation of various signalling cascade due to the presence of mutations like NRAS, BRAF in melanoma makes it resistant to the present anti-melanoma therapies (de Snoo and Hayward, 2005; Lee et al., 2009). Despite the great advancement in treatment approaches for metastatic melanoma, the overall survival rate remains almost less than 5% and hence, still there is a need to explore for new strategies to combat melanoma. PTX belongs to the group of xanthine derivatives and is used for the cure of peripheral artery disease. Emerging evidences have revealed its potential as an anticancer agent (Barancik et al., 2012; Bravo-Cuellar et al., 2013). Previously, we have reported that PTX triggers apoptosis in Cutaneous T cell lymphoma (Gahlot et al., 2010). Although studies have shown that PTX shows anti-metastatic and anti-angiogenic activity against A375 (BRAF-mutant) melanoma cells, however its effect on other melanoma cells (BRAF-wild type) and the molecular mechanism of cell death remains to be explored (Kamran and Gude, 2012, 2013). The present study was mainly focused to study the effect of PTX on apoptosis, autophagy and ER stress response in human melanoma cells. Further, it aims to study the combined effect of PTX and TRAIL in melanoma cells.

In this study we have demonstrated that PTX shows cytotoxicity against both BRAF mutant (A375 and A2058) as well as BRAF wild type (MeWo) melanoma cells. However, the PTX showed greater effect on viability at longer time points (48 h). Moreover, PTX also decreased the colony forming ability of both A375 and MeWo cells after treatment. This observation suggests that PTX is effective in inhibiting the long term survival of melanoma cells. Further, our annexinV/7-AAD analysis revealed that PTX induces apoptosis in melanoma cells in a concentration dependent manner. The increase in TUNEL positive population and increase in nuclear condensation in melanoma cells further support the induction of apoptosis after PTX treatment.

Next, we have examined the activation of Bax, which is a pro-apoptotic protein known to alter mitochondrial permeability and thus facilitating cytochrome c release. Our results revealed a time dependent activation Bax after PTX treatment. Further, a time-dependent alteration in mitochondrial membrane potential, increase in cytochrome *c* release and activation of caspase-9 suggests the involvement of mitochondrial or intrinsic apoptoic

pathway. Caspase-9 ultimately causes activation of caspase-3 which is an executioner caspase. We observed that PTX causes activation of caspase-3 that subsequently results in PARP cleavage. Next, to check the participation of caspases in PTX mediated cell death, we monitored apoptosis in the presence of broad range caspase inhibitor z-VAD-fmk and observed that apoptotic population was effectively reduced in the presence of inhibitor. Moreover, the PTX mediated PARP cleavage was also blocked in the presence of broad range caspase inhibitor. All these observation lead us to conclude that PTX induces apoptosis in melanoma cells and activation of caspase plays a major role.

A large number of studies have shown that cancer cells activate autophagy in response to various chemotherapeutic agents which provide resistance to apoptosis caused by these agents (Sui et al., 2013). In our study, we have observed that PTX induces apoptosis effectively at longer time point, however, a little effect was observed at earlier time points. So, we thought whether PTX can induce autophagy which may restrict PTX to show its effect at earlier time points. Moreover, in melanoma, autophagy deregulation has been reported to be one of the major contributors of chemoresistance. Further, the effect and role of PTX on autophagy in cancer is still unknown. Therefore next, we checked the effect of PTX on autophagy and observed a concentration dependent increase in the LC3-II level in both BRAF mutated and BRAF wild type cell lines. A time dependent study revealed that PTX increased autophagy till 24 h in A375 cells. Increase in the vacuolar structure containing cytoplasmic materials by TEM analysis, increase in the GFP-LC3 punctae and increase in the number of acidic vacuoles in PTX treated cells further confirms the induction of autophagy. PTX induces autophagy by increasing the autophagosomes biosynthesis as confirmed by increased accumulation of GFP-LC3 punctae and increased level of LC3-II in the presence of lysosomal inhibitors. Our immunohistochemistry results revealed that PTX also induces autophagy in *in vivo* conditions. Collectively, all these observations clearly suggest that PTX induces autophagy in melanoma cells.

Autophagy is a very coordinated process and regulated by various autophagy related genes like Atg5, Beclin-1 and Atg7 etc. (Ravikumar et al., 2010). We observed an increase in the expression of Atg5 both at mRNA as well as at protein level. However, no change in the expression of Beclin-1 was observed after PTX treatment. The importance of Atg5 and Beclin-1 in PTX induced autophagy was confirmed by silencing their expression with siRNA. Our results revealed that Atg5 is playing a crucial role in PTX mediated autophagy as

silencing of Atg5 effectively blocked PTX induced autophagy. However, silencing of Beclin-1 did not show any effect. Further, the phosphorylation of mTOR and its downstream molecule 4E-BP was inhibited by PTX. These results suggest that PTX regulates autophagy at two steps: first at initiation step by inhibiting mTOR phosphorylation and at an elongation step by increasing the expression of Atg5.

Interestingly, one important observation of our study is the upregulation of p62 on PTX treatment. p62 is an adapter protein which carries cargo proteins to autophagosomes and get degraded there. In the literature, it is known that the level of p62 get decreased with increase in autophagy (Lippai and Low, 2014) but we observed an increase in p62 level. The increase in expression of p62 in the presence of bafilomycin-A1 and increased co-localization with LC3, confirm its involvement autophagy process. Further, the effect of silencing p62 on cell death was studied. The increase in PTX mediated cell death in the absence of p62 suggests its anti-apoptotic functions in melanoma cells.

Next, the functional outcome of autophagy was examined by inhibiting autophagy by either siRNA or by using pharmacological inhibitor chloroquine (CQ). Inhibition of PTX induced autophagy resulted in increased cell death as evident by annexinV/PI analysis, caspase-3 activation and PARP cleavage. Further, the effect of inhibition of autophagy on tumor growth was also studied. The marked decrease in the mean tumor volume and increased TUNEL positive population in mice group treated with PTX along with CQ compared to other treatment groups further confirms the survival role played by PTX induced autophagy. All these results lead us to conclude that PTX induces cell survival autophagy response that provides resistance to PTX mediated apoptosis in melanoma.

In the recent years, a strong relationship has been established between autophagy and ER stress (Verfaillie et al., 2010). Studies have shown that ER stress response may activate autophagy as an alternative degradation mechanism to relieve ER from accumulated misfolded proteins. So, next the effect of PTX on ER stress response was studied by analysing the expression of ER stress marker proteins viz. GRP-78, CHOP and a sensor protein IRE-1α. We observed a concentration dependent increase in the expression of GRP-78, CHOP and IRE-1α in all the three cell lines. A time dependent increase in GRP-78 and IRE-1α was observed but expression of CHOP was only upregulated upto 24 h. Splicing of XBP1 in the presence of PTX further confirms the induction of ER stress response.

ER stress is generally associated with disturbances in calcium homeostasis. The level of intracellular calcium was observed to be increased after PTX treatment in a time dependent manner. The intracellular calcium level was effectively reduced on addition of intracellular calcium chelator BAPTA-AM in presence of PTX. This further confirms that PTX treatment results in the increase in intracellular calcium level. Moreover, the expression of GRP78 and CHOP was markedly inhibited in the cells treated with PTX in the presence of BAPTA-AM suggesting that PTX mediated increase in intracellular calcium level is playing a crucial role in regulating ER stress response.

Studies have revealed that intracellular calcium plays an important role in regulating calcium mobilising agents mediated autophagy (Hoyer-Hansen and Jaattela, 2007). We also observed that blocking of intracellular calcium by BAPTA-AM resulted in the inhibition of PTX induced autophagy. Since calcium is regulating ER stress response and it also regulates the PTX induced autophagy, it means PTX induced autophagy and ER stress are linked to each other. Further, inhibition of autophagy on silencing the expression of CHOP confirms that PTX induces autophagy via ER stress response.

CHOP is known to regulate autophagy by inhibiting mTOR or by regulating the expression of various autophagy related genes (Li et al., 2013b; Rouschop et al., 2010). Our results revealed that CHOP regulates autophagy by increasing the expression of Atg5 as silencing of CHOP effectively inhibited the expression of Atg5 in the presence of PTX in both A375 and MeWo cells. However, no significant difference in the mTOR phosphorylation was observed in the A375 cells treated with PTX alone or in the CHOP silenced cells.

Further, the functional significance of PTX induced ER stress response on cell survival was studied by silencing the expression of IRE-1α (sensor protein) and CHOP. We observed that cell death was enhanced as evident by increase in apoptotic population, increased caspase-3 and PARP cleavage when the ER stress response was abrogated either by inhibiting the expression of one of the sensor arm or by silencing the stress induced transcription factor CHOP. These observations suggests that PTX induced ER stress response is also playing as a survival mechanism which is providing resistance to PTX mediated apoptosis.

As per our observations, calcium acts a central player which regulates PTX induced ER stress response and autophagy and also, it is known to play an important role in the process of apoptosis. Interestingly, on chelating the intracellular calcium we observed an enhancement

in PTX mediated cell death. The increase in cell-death was confirmed by annexinV/PI staining, increased activation of caspase-3 and PARP cleavage. Further, while deciphering the mechanism of PTX/BAPTA-AM mediated increase in cell death, we observed that blocking of calcium by BAPTA-AM in presence of PTX activates intrinsic apoptotic pathway as evident by Bax activation, increased cytochrome *c* release and activation of caspase-9. Activation of caspases play important role in PTX/BAPTA-AM mediated apoptosis as addition of broad range caspase inhibitor reverts the cell death.

Next, it is known that upregulation of Mcl-1 provides resistance to ER stress mediated apoptosis by interfering with the activation of pro-apoptotic proteins Bak and Bax (Jiang et al., 2007). Our results indicated that PTX itself is able to downregulate the expression Mcl-1 at protein level which was further decreased in the presence of calcium chelator. These results suggest that inhibition of Mcl-1 also helps in sensitizing melanoma cell toward cell death.

Conclusively, the first part of our study revealed that PTX besides inducing apoptosis in melanoma cells it also activates ER stress response and autophagy as secondary mechanisms that provides resistance to PTX induced apoptosis. Autophagy was activated as downstream effecter arm of ER stress response and PTX induced rise in intracellular calcium levels plays a critical role in regulating these processes. Further, we have provided evidences that abrogating ER stress response as well as autophagy can improve the therapeutic benefits of PTX against BRAF mutated as well as in melanoma cells.

In the next part of our study we have examined the effect of PTX in combination with TRAIL in TRAIL sensitive (A375) and TRAIL resistant (MeWo) melanoma cell lines. TRAIL is a member of TNF super family and is gaining much importance for cancer treatments because it selectively targets cancerous cells without showing any effect on normal cells (Walczak et al., 1999). In this study, we have demonstrated that PTX in combination with TRAIL augments cell death in both TRAIL sensitive as well as in TRAIL resistant cell lines.

Our results revealed that PTX in combination with TRAIL decrease the viability of A375 and MeWo cells. The decrease in viability was associated with apoptosis as evident by increase in the apoptotic population after annexinV/7-AAD staining and increase in nuclear condensation in the combined treated cells.

TRAIL initiates its apoptotic signal by activating caspase-8 after interacting with the TRAIL receptors. Results from the previous part of our study revealed that PTX alone is not able to activate caspase-8. So, we have examined the expression of pro-caspase-8 and observed that activation of caspase-8 was increased after PTX/TRAIL treatment. Caspase-8 plays an important role in TRAIL mediated killing by connecting extrinsic pathway of apoptosis to intrinsic pathway through Bid truncation. Ours results also revealed that combined treatment of PTX and TRAIL causes Bid-truncation, alters MMP, increases cytochrome *c* release and finally activates caspase-9, suggesting the activation intrinsic pathway of apoptosis.

As extrinsic and intrinsic pathway both converges at a single point that is activation of executioner caspases. Therefore, we examined the expression of pro-caspase-3 and observed that combined treatment increases caspase-3 activation and subsequently the cleavage of its substrate PARP. Hence, all these observation suggests that PTX in combination with TRAIL augments cell death in melanoma cells by activating both arms of apoptosis.

TRAIL triggers its apoptotic signals after interacting with the receptors DR5 and DR4 present on the cell surface. We observed upregulation of TRAIL receptor DR5 in both the cell line while the expression of DR4 was slightly increased in A375 cells. When we blocked the expression of DR5 by siRNA, we observed a partial reversion of PTX/TRAIL mediated cell death. The possible reason behind the incomplete inhibition of cell death may be incomplete inhibition of DR5 by siRNA due to high basal expression of DR5 in melanoma cells.

The susceptibility of melanoma cells to TRAIL mediated killing mainly depends on the expression of various anti-apoptotic proteins. Overexpression of various proteins like c-FLIP, Bcl-xL and XIAP has been reported to provide intrinsic as well as acquired resistance in melanoma (Quast et al., 2014). The expression of all these proteins was found to be inhibited in the combination treatment with PTX and TRAIL. We also observed that PTX only inhibited the expression of XIAP in MeWo cells. This observation suggests that PTX could sensitize TRAIL resistant MeWo cells to TRAIL mediated killing. Further the activation of Nf-κB was inhibited in both A375 and MeWo cell line on combination treatment.

Earlier, we have shown that PTX induces protective autophagy and ER stress response in melanoma cells. There are evidences that TRAIL can disrupt the protective autophagic machinery by degrading various autophagy related genes. In our study, we observed that PTX induced autophagy was effectively inhibited in the presence of TRAIL. Further, the

expression of Atg5 was also inhibited. In addition to this, PTX induced ER stress response was abrogated as evident by inhibition of CHOP expression in the combination treatment. Therefore, these results suggest that TRAIL augments the PTX induced apoptosis by disrupting the cell survival machinery.

Taken together, this part of study revealed that PTX and TRAIL both works in a mutually exclusive way to augment cell death in melanoma cells. On the one hand PTX sensitizes melanoma cells to TRAIL mediated killing by upregulating the expression of DR5 receptors and on the other hand TRAIL disrupts the cell survival autophagy response and ER stress response, thus helping each other to enhance cell death in the melanoma. Further, inhibition of various anti-apoptotic proteins in the combination increases its effectiveness. In conclusion, we have provided evidence that the treatment strategy involving combination of PTX with TRAIL proves to be beneficial *in vitro* conditions, however, further *in vivo* studies will be needed to make the study more conclusive.

Collectively, in this study we have showed that PTX activates intrinsic apoptotic pathway in melanoma cells irrespective of their mutational status. However, like many other chemotherapeutic drugs the efficacy of PTX is limited by the induction of cell survival autophagy activated as a downstream arm of ER stress response. Targeting of ER stress response or autophagy by their specific inhibitors effectively enhances the PTX mediated cytotoxicity in melanoma cells. Apart from autophagy and ER stress response, the efficacy of PTX is restricted because of its inability to activate the extrinsic arm of apoptosis. In this direction when we used the TRAIL which exerts its effect through extrinsic apoptotic cascade by activating caspase-8, the cytotoxic effects of PTX were enhanced. The combined treatment of PTX and TRAIL not only activated caspase-8 but also inhibited the cell survival ER stress response and autophagy. So we believe that the combination therapy of PTX and TRAIL can be the important therapeutic strategies for melanoma treatment but animal experiments are needed to further validate these studies under *in vivo* conditions.