CHAPTER 5

Summary
Nitric oxide (NO) is a diffusible gas molecule and can act as an immunomodulator owing to its ability to sensitize tumor cells to Fas (Garban and Bonavida, 1999), TNF-α (Garban and Bonavida, 2001) and TRAIL (Huerta-Yepez at al., 2004) mediated apoptosis. Moreover, NO delivered by NO donors can also modulate the toxicity of other agents including doxorubicin (Evig et al., 2004), taxol (Jia et al., 2003), cisplatin (Wink et al., 1997) and alkylating agent such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (Laval and Wink, 1994). Nitric oxide (NO) plays both pro and antiapoptotic function and these effects are dose dependent and cell type specific (Li and Wogan, 2005; Chung et al., 2001). NO can induce nitrosative stress, which is linked to apoptosis (Marshall and Stamler, 2002). NO is known to inhibit NF-κB in both constitutively active and inducible NF-κB system, however the mechanism of NO mediated inhibition of NF-κB differs from cell type to cell type (Katsuyama et al., 1998; Marshall et al., 2004; D’Acquisto et al., 2001). Present work was undertaken to investigate the effect of NO generating compound, sodium nitroprusside (SNP) on HuT-78 cells which expresses NF-κB constitutively.

Our results revealed apoptotic capability of SNP as manifested by increase in percentage of cells in sub-G₁ fraction, increase in percentage of apoptotic cells, chromatin condensation and DNA fragmentation. This apoptosis followed mitochondrial dependent pathway as it decreased MMP, released cytochrome c into the cytosol and activated caspase-9. Thereafter, SNP activated caspase-3 which is succeeded by PARP cleavage.

Next, we studied the effect of SNP on constitutive NF-κB in HuT-78 cells. Inhibition of NF-κB and decrease in p65 nuclear translocation were observed in these cells. SNP inhibited antiapoptotic molecule, Bcl-xl at protein as well as RNA level as evident by western blotting and RNase protection assay respectively. The ability of SNP to inhibit NF-κB in these cells is a good explanation for the observed apoptotic features and inhibition of antiapoptotic molecule, Bcl-xl which is known to be regulated by NF-κB.

In order to see that inhibition of NF-κB leads to apoptosis in HuT-78 cells, curcumin, a known NF-κB inhibitor was used. Dose dependent induction of apoptosis in HuT-78 cells by curcumin correlated with its ability to inhibit NF-κB in a concentration dependent manner. SNP induced less apoptosis than curcumin under similar condition.
Thereafter, we investigated the mechanism of NF-κB inhibition in HuT-78 cells by SNP and curcumin. Since phosphorylation and subsequent degradation of IκBα protein activates NF-κB (Kim et al., 2005), we studied IκBα protein level and phosphorylation level of IκBα in HuT-78 cells treated with either SNP or curcumin. Curcumin dose dependently inhibited both the expression as well as the phosphorylation of IκBα, in accordance with findings in case of other cell types (Bharti et al., 2003; Shishodia et al., 2005). On the contrary, SNP enhanced IκBα protein expression but failed to show any effect on IκBα phosphorylation. To account for the enhancement of IκBα protein expression in SNP treated cells, we further investigated the effect of SNP on proteasome mediated IκBα degradation. For this purpose, cells were treated with SNP for 8 h in presence or absence of MG132. SNP alone did not affect phosphorylation of IκBα. Interestingly, IκBα phospho-form accumulated in SNP plus MG132 treated cells, which probably resulted due to enhanced IκBα protein formation, subsequent phosphorylation and thereafter inhibition of proteasome mediated degradation. Thus, SNP had no effect on ubiquitin mediated degradation of IκBα. However, inhibition of NF-κB by SNP failed to make these cells sensitive to TNF-α mediated apoptosis, as inhibition of NF-κB is known to make cells susceptible to TNF-α mediated killing (Beg and Baltimore, 1996; Van Antwerp et al., 1996; Liu et al., 2004).

Incidentally, both SNP and curcumin inhibit constitutive NF-κB in HuT-78 cells leading to apoptosis but mechanism of inhibition differs in case of these two compounds. It is well known that curcumin inhibits constitutively active IκB kinase (IKK) in many cells, as a result, phosphorylation of IκBα is inhibited, which ultimately inhibits NF-κB activity. It is reported that IκBα protein expression is regulated by NF-κB (Bharti et al., 2003; Shishodia et al., 2005). Thus, inhibition of NF-κB activity by curcumin lowered IκBα protein expression. SNP is unlikely to inhibit IKK in HuT-78 cells as no alteration in phosphorylation of IκBα was observed. Inhibition of NF-κB binding activity by SNP probably resulted from the occurrence of enhanced level of IκBα protein. Earlier in murine cells it was reported that NO prevents constitutive NF-κB activity by increasing IκBα protein level (D'Acquisto et al., 2001). Although the possibility of the direct modification of NF-κB DNA binding activity cannot be ruled out (Matthews et al., 1996; Marshall et al., 2004; Park et al., 2005).
HuT-78 cells constitutively express NF-κB and tumor necrosis factor (TNF-α). TNF-α, pleiotropic cytokine is an autocrine growth factor for HuT-78 cells. Proliferation of HuT-78 cells can be inhibited by lowering TNF-α level using anti-TNF antibody (O'Connell et al., 1995; Giri and Aggarwal, 1998). Pentoxifylline (PTX, a xanthine derived antioxidant which is known to inhibit inducible and constitutive NF-κB (Bellas et al., 1995; Wang et al., 1997; Jimenez et al., 2001). Moreover PTX is also known to lower TNF-α in many cells (Marques et al., 1999; Lima et al., 2005). PTX is generally used as a therapeutic drug for the treatment of hematological disorders. In addition, it has been documented that PTX enhances antitumor activity of many chemotherapeutic agents and sensitizes tumor cells to radiotherapy (Rauko et al., 1998, Alas et al., 2000; Lerma-Diaz et al., 2006; Waldeck et al., 2007). The effect of PTX on HuT-78 cells constitutively expressing NF-κB and TNF-α has not been explored.

Interestingly, our results unraveled the cytotoxic potential of PTX on HuT-78 cells. Further experiments were carried out to enquire apoptotic potential of PTX. These experiments pave the way for apoptotic potential of PTX. This promising potential of PTX is apparent by dose dependent as well as time dependent increase in sub-G₁ peak, concentration dependent increase in percentage of apoptotic cells, chromatin condensation as well as DNA fragmentation. Apoptosis induced by PTX involves alterations in MMP which is chased by cytochrome c release. Ultimately, PTX activated caspase-3 and lead to PARP cleavage.

Surprisingly, PTX induced apoptosis in HuT-78 cells without affecting NF-κB. The intensity of NF-κB band in PTX treated cells at different concentrations as well as different time points was always nearly equivalent to untreated control. Although, the inhibition of NF-κB by PTX has been reported by various researchers (Bellas et al., 1995; Wang et al., 1997; Jimenez et al., 2001).

Our results also bring into light the downregulation of antiapoptotic proteins: Bcl-xl and cFLIP by PTX in HuT-78 cells, these cells are known to express basal high levels of Bcl-xl and c-FLIP (Rishi et al., 2007; Braun et al., 2007). Downregulation of Bcl-xl protein by PTX probably explained mitochondrial dysfunction caused by PTX in HuT-78 cells. Since PTX did not change NF-κB expression, therefore, the regulation of these molecules by transcription factors other than NF-κB cannot be ruled out. Although, it is known that other than NF-κB, transcription factors like signal transducers and activators of...
transcription (STAT), Ets and AP-1 can also regulate Bcl-xl expression (Jazirehi et al., 2004). Besides NF-kB, we also tested the effect of PTX on transcription factor, AP-1. The results displayed enhancement in AP-1 activity by PTX in these cells. Inspite of the fact that AP-1 can participate in survival as well as apoptosis of cell (Shaulian and Karin, 2002). Moreover, the mechanism of apoptosis induced by PTX in HuT-78 cells did not involve cAMP. This result is evident from the flow cytometry experiments where cAMP analogue: cAMP analogue; dibutyryl cAMP (Bt2cAMP) and 3-isobutyl-1-methylxanthine (IBMX) failed to show any cytotoxic effect on HuT-78 cells.

RNase protection assay gave a new insight into 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 (Chung et al., 2003). It is possible that reexpression of GADD45 after treatment with PTX may induce 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. This result is in agreement with Tolomeo et al. who reported that HuT-78 cells do not express p53 protein (Tolomeo et al., 1998).

Our results displayed antioxidant nature of PTX as discernible by dose dependent decrease in basal ROI level. Our results also showed that ROI has very little effect on NF-kB activation as inhibition of ROI by PTX failed to show any effect on NF-kB level. Thus, our results emphasize on ROI independent activation of NF-kB in HuT-78 cells, although Giri and Aggarwal had demonstrated that ROI plays a major role in maintaining constitutive NF-kB level in HuT-78 cells which ultimately prevents apoptosis (Giri and Aggarwal, 1998).

Next, we studied the effect of PTX on mRNA level of various molecules involved in death receptor mediated apoptosis. Our results elucidated that low concentration of PTX that did not induce apoptosis could significantly upregulate 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 (Zhang et al., 2007). CTCL cells are generally resistant to TRAIL mediated killing (Braun et al., 2007). It will be interesting to know whether HuT-78 cells can be sensitized to TRAIL mediated killing by downregulating c-FLIP expression without affecting constitutive NF-κB. Besides mRNA expression, surface Fas expression also enhanced upon PTX treatment as testified by flow cytometry. On the contrary, SNP did not upregulate significantly Fas or TRAIL expression.

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 (Kang and Choi, 2001). Many cytotoxic agents known to increase cellular oxidative stress downregulate c-FLIP expression and sensitize cells to Fas induced apoptosis in a ROI dependent manner (Kinoshita et al., 2000). In contrast, cytotoxic agent PTX scavenged ROI, increased surface Fas expression and augmented FasL mediated killing and apoptosis.

Taken together, our results point towards the involvement of two pathways in apoptosis of HuT-78 cells: NF-κB dependent and NF-κB independent. The results indicated towards NF-κB dependent pathway followed by SNP and NF-κB independent pathway followed by PTX. It seems probable that there is no cross talk between these two pathways, at a time, only one pathway is operative. The existence of this probability is drawn as a conclusion from SNP and PTX combined treated cytotoxic experiment, where treatment of HuT-78 cells with SNP and PTX resulted in additive effect.

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, Denileukin diftitox, a recombinant fusion protein and extracorporeal photochemotherapy (Mann et al., 2007; Zhang et al., 2002; Senturk and Sahin, 2003).

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 (Ni et al., 2005). 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 (Contassot et al., 2008). Furthermore, as mentioned, our studies highlighted the increase in cell surface Fas expression and enhancement in FasL mediated killing by PTX.

In the past the application of NO donors as cancer therapeutics has not been appreciated owing to it’s primarily use in the treatment of blood vessel-related diseases and
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other non-cancer related applications. The demonstration of NO mediated cytotoxicity directly on cancer cells presents new challenges for its optimal use in chemotherapy (Bonavida et al., 2006). Our results expect the application of NO-donor, SNP in cancer therapy against CTCL cells. Furthermore, the outcome of this study may also hold PTX as a potential chemotherapeutic agent for treatment of CTCL patients. In addition, our results propose the existence of NF-κB dependent and independent pathways in HuT-78 cells owing to the ability of SNP and PTX to induce apoptosis in these cells with and without involvement of NF-κB inhibition, respectively.