CHAPTER 3

Study of apoptotic potential of nitric oxide & its mechanism
3.1. Introduction

Nitric oxide (NO), a highly diffusible free radical plays an important role as mediator of several pathophysiological responses such as vasodilation, platelet aggregation, immune and inflammatory diseases (D'Acquisto et al., 2001). In the biological systems NO has a short half-life on the order of seconds (Kim et al., 2001). NO is produced by a group of enzymes called nitric oxide synthases (NOS). There are three main isoforms of the NOS enzyme, endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). Among these forms, eNOS and nNOS generate NO in the picomolar range. However, iNOS allows high output quantities of NO at 100-1000 fold greater than its constitutive NOS counterparts. Till date the data have been presented which highlight both antitumor as well as pro-tumor properties associated with NO (Coulter et al., 2008). For instance, NO may stimulate apoptosis by changes in the expression of proapoptotic and antiapoptotic Bcl-2 family members, decrease in mitochondrial membrane potential, cytochrome c release, caspase-3 activation, PARP cleavage and DNA damage (Li and Wogan, 2005). However, its effect may depend on the timing, concentration and tissue type. Low concentrations of NO can stimulate cell growth and protect many cell types from apoptosis whereas high concentrations of NO can inhibit cell growth and induce apoptosis (Chung et al., 2001).

To study NO signal transduction irrespective of NOS involvement, NO-releasing compounds are valuable tools. NO donors preserve NO in their molecular structure and evoke biological activity after decomposition. These drugs exhibit considerable variation in their chemical structure, stability and biological activity (Brune et al., 1998). Nitric oxide-donating non-steroidal anti-inflammatory drugs (NO-NSAIDs) is an emerging class of compounds with chemopreventive properties against a variety of cancers cell lines including, colon, prostate, lung, pancreas, tonsil, breast cancer and leukemia (Rigas, 2007). NO-donating aspirin is the most extensively studied NO-NSAIDs. It inhibited colon carcinogenesis in preclinical animal models and a clinical trial for colon cancer prevention is underway (Gao et al., 2006). Using various NO-generating compounds viz. sodium nitroprusside (SNP), S-nitroso-N-acetyl-penicillamine (SNAP), S-nitroso-glutathione (GSNO), glyceryl trinitrate (GTN) it has been shown that NO can induce apoptosis in human promyelocytic leukemia cells (Wang et al., 2007), oral squamous carcinoma cells (Zhao et al., 2005), pheochromocytoma cells (Li et al., 2005), lung carcinoma cells (Chao et al., 2004), colon cancer cells (Liu et al., 2003; Millet et al., 2002). NO donors
DETA NONOate, SNP and GSNO are known to inhibit constitutive NF-κB activity in prostate carcinoma cells (Huerta-Yepez et al., 2004), murine cell line (D'Acquisto et al., 2001) and lymphoblastic leukemia cells (Santos-Silva et al., 2001). Numerous molecular mechanisms have been documented by which NO can inhibit NF-κB. For instance, NO can inhibit NF-κB activity by increasing mRNA expression of IκBα, stabilization of IκBα or directly inhibiting DNA binding activity of NF-κB by post translational modifications (Peng et al., 1995; Matthews et al., 1996; Katsuyama et al., 1998).

The effect of NO has not been explored in case of constitutive NF-κB producing CTCL cells. With this information, the main objective of the present study is to explore the apoptotic potential of NO on CTCL cell line, HuT-78. Further, the molecular mechanism underlying NO induced apoptosis will also be investigated. For evaluating NO effect, we made use of nitric oxide generating compound, sodium nitroprusside (SNP).

3.2. Results

3.2.1. SNP shows cytotoxic activity against HuT-78 cells

We first studied the cytotoxic potential of nitric oxide generating compound, SNP on human CTCL cell line, HuT-78 by MTT assay. MTT assay is a quantitative colorimetric assay based on the cleavage of the yellow water-soluble tetrazolium salt, MTT to form water-insoluble dark blue formazan crystals. MTT cleavage occurs only in living cells by the mitochondrial enzyme succinate dehydrogenase. For this purpose, HuT-78 cells were treated with different concentrations of SNP (0-2 mM) for 24 h. The cytotoxicity of SNP was increased in a dose dependent manner with 1 mM SNP inducing 50% cytotoxicity as tested by MTT assay. Thereafter, with increase in concentration of SNP percentage of living cells were further decreased (Fig. 3.1.A).

3.2.2. SNP increases nitrite level in dose and time dependent manner

To verify that killing by SNP is mediated by released nitric oxide, we measured nitrite level in the culture supernatants of HuT-78 cells treated with different concentrations of SNP for various time points (6, 12 and 24 h) by Griess reagent. Our results revealed that
Fig. 3.1. Effect of SNP on cell viability and nitrite production in SNP treated cells. (A) HuT-78 cells were treated with different concentrations of SNP (0-2 mM) for 24 h. The cytotoxicity was assessed by MTT assay. Data are expressed as percentage over untreated control. (B) Cells were treated with different concentrations of SNP for indicated time points. Nitrite production was measured in culture supernatant by Griess reagent. Values are expressed as mean ± S.D. (n = 3).
nitrite level increased in a dose as well as time dependent manner, with 1 mM of SNP releasing 32.21 ± 1.6, 41 ± 2.3 and 77 ± 12 μM (P<0.05) nitrite in culture supernatants after 6, 12 and 24 h of incubation respectively (Fig. 3.1.B).

3.2.3. SNP induces apoptosis in HuT-78

After confirmation of cytotoxic potential of SNP, it was evaluated for its apoptotic potential by various flow cytometric methods viz. determination of sub-G₁ fraction and TUNEL assay and fluorescence microscopy for investigation of cell death. First, we quantified the percentage of sub-G₁ population (hypoploidic cells) after 1 mM SNP treatment in HuT-78 cells by flow cytometry at 0, 6, 12 and 24 h. As seen in Fig. 3.2.A, a marked increase in percentage of cells in sub-G₁ fraction was observed in HuT-78 cells after 24 h of treatment with 1 mM SNP in compared to untreated cells (5.82% to 31.49%).

To determine if the sub-G₁ fraction affected by SNP was related to apoptosis, a morphological assay of cell death was investigated using AO/EB staining after treatment of cells with 1 mM SNP for 24 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. 3.2.B).

Apoptosis was also confirmed by TUNEL assay where HuT-78 cells were treated with 1 mM SNP for 24 and 48 h. We observed that HuT-78 cells showed time dependent increase in percentage of apoptotic cells as compared to untreated cells after treatment with 1 mM SNP from 1.44% to 9.75% at 24 h and 1.44% to 23.08% at 48 h (Fig. 3.3.).

3.2.4. SNP causes chromatin condensation and DNA fragmentation

We further examined the apoptosis by analysis of nuclear morphology using DAPI staining and examining the fragmentation of DNA. DAPI is a DNA–specific dye which forms a fluorescent complex by attaching in the minor groove of (preferentially) A-T rich sequence of DNA. DAPI staining was performed after treatment of cells with 1 mM SNP for 24 h. Our results showed that SNP treated HuT-78 cells showed chromatin condensation as compared to control untreated cells (Fig. 3.4.A). In addition to nuclear morphological evaluation, apoptosis induction by SNP was further studied by DNA fragmentation. The biochemical marker of apoptosis is DNA fragmentation which results due to activation of
Fig. 3.2. Effect of SNP on sub-G$_1$ peak and cell morphology. (A) Cells were treated with 1 mM SNP for indicated time points, stained with PI/RNase staining buffer and evaluated for DNA content by flow cytometry at FL-2 channel, where M1 represents sub-G$_1$ peak; M2 represents G$_0$/G$_1$ peak. Data represent results from one of the three similar experiments. (B) Changes in cell morphology were observed under fluorescent microscope by staining with AO/EB after 24 h of treatment with 1 mM SNP. The viable cells appeared green while apoptotic cells appeared orange.
Fig. 3.3. Induction of apoptosis in SNP treated HuT-78 cells. Apoptosis was studied by flow cytometry at FL-1 channel after 24 and 48 h of treatment with 1 mM SNP by TUNEL assay. Data represent the results from one of the three similar experiments. The M1 and M2 gates demarcate non-apoptotic and apoptotic populations, respectively.
Fig. 3.4. Effect of SNP on nuclear morphology and DNA fragmentation. (A) Changes in the nuclear morphology was observed under fluorescence by staining with DAPI after treatment with 1 mM SNP for 24 h. The arrows indicate apoptotic cells. (B) Cells were treated with 1 mM SNP and apoptosis was evaluated by DNA fragmentation after 24 h. M; 100 bp ladder.
endogenous endonucleases, which subsequently cleave chromatin DNA into internucleosomal fragments of 180 base pair and multiples thereof. DNA was isolated from 1mM SNP treated and untreated HuT-78 cells after 24 h and analyzed by agarose gel electrophoresis (Fig. 3.4.B). A characteristic laddering pattern was observed in SNP treated cells as compared to control cells.

3.2.5. SNP induces loss of mitochondrial membrane potential

It has been shown that integrity of mitochondria plays an important role for programmed cell death and disruption of mitochondrial membrane potential enhances apoptosis (Wang, 1999; Kaufmann and Earnshaw, 2000). In order to understand the mechanism of SNP mediated apoptosis, cells were treated with 1 mM SNP for different time points (0, 6, 12 and 24 h) and stained with JC-1, a potentially sensitive fluorescent dye that detects polarized mitochondria giving red fluorescence. The drop in red fluorescence, indicative of change in mitochondrial membrane potential was measured by flow cytometry. SNP induced loss of membrane potential in time dependent manner. Mitochondrial function was assessed as JC-1 green (FL-1 channel) versus JC-1 red (FL-2 channel) fluorescence. As seen in Fig. 3.5.A, JC-1 staining of HuT-78 cells showed that the percentage of cells exhibiting red fluorescence decreased from 99.98% to 15.10%, 8.43% and 8.23% after 6, 12 and 24 h of treatment with 1 mM SNP respectively, as determined by quadrant analysis. A corresponding and matching increase in green fluorescence was also observed. This result was further substantiated by confocal fluorescence microscopy where 1 mM SNP treated cells showed less red fluorescence as compared to control cells after 24 h of treatment (Fig. 3.5.B).

3.2.6. SNP causes cytochrome c release in HuT-78 cells

To determine whether alteration in mitochondrial membrane potential leads to cytochrome c release into the cytosol from mitochondria, the subcellular localization of cytochrome c was determined by confocal fluorescence microscopy using anti-cytochrome c monoclonal antibody in combination with a specific mitochondrial fluorescent dye, MitoTracker Red. As seen in Fig. 3.6, cytochrome c specific fluorescence (green) was
Fig. 3.5. SNP induces alteration in mitochondrial membrane potential. (A) Cells treated with 1 mM SNP for 0, 6, 12 and 24 h were incubated with JC-1 dye and changes in mitochondrial membrane potential was measured by flow cytometry. Data presents green versus red fluorescence, where in red fluorescence represents intact mitochondrial potential and green fluorescence representing breakdown of mitochondrial potential. (B) Changes in mitochondrial membrane potential with JC-1 dye after 24 h of treatment with 1 mM SNP by confocal microscopy. Data represent the results from one of the three similar experiments.
Fig. 3.6. SNP induces cytochrome c release in HuT-78 cells. Cells treated for 8 h with 1 mM SNP were stained with 100 nM MitoTracker Red and monoclonal antibody against cytochrome c. Immunofluorescence analysis of cytochrome c release in HuT-78 cells was performed using confocal fluorescence microscope. The arrows indicate the subcellular localization of cytochrome c.
colocalized with MitoTracker Red staining (red) in healthy cells whereas 1 mM SNP induced cytochrome c release into cytosol as indicated by diffusive staining pattern (green).

**3.2.7. SNP induces caspase 3 and 9 activation**

Caspases are cysteiny1 aspartate specific proteases that play an important role in apoptosis pathway. Normally they remain in inactive form in cytosol and are categorized into initiator or effector caspases. Caspase-8 and caspase-9 are characterized among initiator caspases while caspase-3 is characterized among effector caspases and is known to be a key executioner of apoptosis (Elmore, 2007). HuT-78 cells were treated with 1 mM SNP for 24 h and analyzed for caspase-8 activation in whole cell lysate by western blotting. Our results showed that SNP failed to activate caspase-8 as compared to control cells (Fig. 3.7.A). In contrast, SNP activates caspase-9 by 2.57 folds in compared to control cells after 24 h of treatment as evident by luminescent caspase-Glo® 9 assay. Moreover, both curcumin (25 μM) and actinomycin D (2 μg/ml) treated cells for 24 h, which served as positive controls, activated caspase-9 by nearly 2.8 folds as compared to untreated cells (Fig. 3.7.B). Caspase-3 activation was checked after 0, 6, 12 and 24 h of treatment of cells with 1 mM SNP by colorimetric CaspACE assay. Our results showed that enhanced apoptosis of SNP treated cells also correlated with the increase in caspase-3 activity of HuT-78 cells in a time dependent manner (Fig. 3.8.A).

**3.2.8. SNP causes PARP cleavage in HuT-78 cells**

PARP is a substrate for caspase-3 and its cleavage is an indicator of apoptosis. Since our results showed that SNP activates caspase-3, therefore next we studied PARP cleavage. For PARP cleavage, whole cell lysates were prepared after treatment with 1 mM SNP for 0, 6, 12 and 24 h. PARP cleavage was detected by immunoblotting using anti-PARP antibody which recognizes both 116 kDa intact and 85 kDa cleaved forms of PARP. Our results revealed that HuT-78 showed PARP cleavage at 24 h time point after 1 mM SNP treatment (Fig. 3.8.B).

**3.2.9. SNP suppresses constitutive NF-κB**
Fig. 3.7. Effect of SNP on caspase-8 and -9 activation in HuT-78 cells. (A) Cells were treated with 1 mM SNP for 24 h and analyzed for caspase-8 activation by western blotting. Procaspase-8 band intensity was normalized to actin. (B) Cells were treated with 1 mM SNP, 25 μM curcumin or 2 μg/ml actinomycin D (Act. D) for 24 h and analyzed for caspase-9 activity by luminescent caspase-Glo®9 assay.
Fig. 3.8. Induction of caspase-3 activation and PARP cleavage in HuT-78 cells. (A) Cells were treated with 1 mM SNP for indicated time points and caspase-3 activity was determined colorimetrically as described in Materials and methods section. Similar results were obtained in two separate experiments. (B) Cells were treated with indicated time points with 1 mM SNP and detected for PARP cleavage using Western blot analysis.
Study of apoptotic potential of nitric oxide and its mechanism

One of the features found in CTCL cells is basal high level of constitutively active NF-κB (Izban et al., 2000), therefore, next we studied the effect of SNP on constitutive NF-κB expression in HuT-78 cells. For this purpose, HuT-78 cells were treated either with different concentrations of SNP (0.5, 1 and 1.5 mM) or curcumin (12.5, 25 and 50 μM, used as a NF-κB inhibitor) for 8 h. We examined the cells for NF-κB in the nucleus by EMSA. A constitutively basal level of NF-κB activity was detected in HuT-78 cells. Dose dependent inhibition of NF-κB was observed with SNP as well as curcumin, where curcumin showed more inhibition than SNP. We found that 50 μM curcumin was sufficient to fully suppress the activation of constitutive NF-κB in HuT-78 cells (Fig. 3.9.).

To investigate whether inhibitory effect of SNP on NF-κB is mediated through alteration of IκBα protein expression as well as IκBα phosphorylation, cytoplasmic extracts were subjected to Western blot analysis. Interestingly, our results showed that IκBα protein level increased in cytoplasmic fraction by SNP as compared to control untreated cells. On the other hand, NF-κB inhibitor curcumin decreased IκBα protein level dose dependently as compared to control untreated cells. Untreated HuT-78 cells constitutively express phosphorylated IκBα. SNP did not show any effect on phospho-IκBα level but curcumin suppressed constitutive phosphorylated IκBα level in a dose dependent manner (Fig. 3.10.A). Moreover, treatment with proteasome inhibitor, MG132 and SNP increased cytosolic IκBα phosphorylated proteins in compared to SNP alone treated cells. This result support the data of enhancement in IκBα protein level in SNP treated cells (Fig. 3.10.B).

3.2.10. SNP inhibits nuclear translocation of p65

Here, we tested the effect of 1 mM SNP on nuclear translocation of p65 by immunofluorescence after 8 h of treatment. Fig. 3.11. showed that 1 mM SNP decreased translocation of p65 subunit of NF-κB (green fluorescence) to nucleus in HuT-78 cells.

3.2.11. NF-κB inhibitor curcumin induces apoptosis in HuT-78 cells
Fig. 3.9. Inhibition of NF-κB activation by SNP and curcumin. HuT-78 cells were treated with SNP (0, 0.5, 1 and 1.5 mM) or curcumin (0, 12.5, 25 and 50 μM) for 8 h. Nuclear extracts prepared from treated cells were assayed for NF-κB by EMSA as described in Materials and methods section.
Fig. 3.10. Effect of SNP on expression and phosphorylation of IκBα. (A) HuT-78 cells were treated with different concentrations of SNP (0, 0.5, 1 and 1.5 mM) or curcumin (0, 12.5, 25 and 50 μM) for 8 h. Cytosolic fractions were subjected to Western blot analysis for expression and phosphorylation of IκBα. Equal loading of protein was confirmed by actin. In SNP treated cells, IκBα band intensity was normalized to phospho-IκBα. In curcumin treated cells, IκBα and phospho-IκBα bands intensities were normalized to untreated cells. (B) HuT-78 cells were treated with 1 mM SNP in the presence or absence of proteasome inhibitor, MG132 (10 μM) for 8 h. Cytoplasmic extracts were fractionated and then subjected to Western blot analysis using phospho-specific anti-IκBα antibody. Ub-pIκBα; Ubiquitinated- p IκBα.
Fig. 3.11. SNP inhibits nuclear translocation of p65 in HuT-78 cells. Cells were processed for immunofluorescence staining after 8 h of treatment with 1 mM SNP as described in Materials and methods section. Green stain indicates the localization of p65 and blue stain (DAPI) indicates the nucleus.
In order to determine whether suppression of NF-κB leads to apoptosis, cells were treated with various concentrations of curcumin (12.5, 25 and 50 μM) or SNP (0.5, 1 and 1.5 mM) for 48 h and analyzed for apoptosis by TUNEL assay. Both curcumin and SNP showed a dose dependent increase in the percent of apoptotic cells as indicated by increase in FITC staining (FL-1). Maximum apoptotic effect was observed with curcumin (Fig. 3.12.). Thus, our results demonstrated the downregulation of NF-κB and induction of apoptosis in SNP treated HuT-78 cells.

3.2.12. SNP downregulates antiapoptotic protein Bcl-xl

Here, we examined the mRNA expression of several Bcl-2 family members by RPA using hAPO-2b set (BD Pharmingen, San Diego, CA). For this purpose, HuT-78 cells were treated either with 1 mM SNP or 12.5 μM curcumin (taken as control) and total RNA was isolated after 6 h of treatment. Our results showed that HuT-78 cells express high level of Bcl-xl mRNA. The addition of SNP downregulated Bcl-xl mRNA expression approximately two fold. In contrast, no significant effect in Bcl-xl mRNA expression was observed with the addition of 12.5 μM curcumin (Figs. 3.13.A and B).

The decrease in Bcl-xl expression was also examined at protein level. Western blot analysis of cell lysates prepared at different time points (0, 6 and 12 h) after treatment with 1 mM SNP showed time dependent inhibition of Bcl-xl protein (Fig. 3.14.A).

NF-κB dependent antiapoptotic cIAPs are endogenously produced proteins that inhibit apoptosis by inactivating caspases (Jin and Lee, 2006). In order to investigate whether SNP has any effect on the expression of cIAP protein in HuT-78 cells, further experiments were performed at different time points (2, 4, 12 and 24 h). Western blot analysis of 1 mM SNP treated or untreated cell lysates revealed that SNP had no effect on cIAP expression until 12 h treatment as compared to untreated cells whereas at 24 h time point inhibition was observed (Fig. 3.14.B).

3.2.13. HuT-78 cells are resistant to apoptosis by exogeneous TNF-α

It is reported that TNF-α, a pleiotropic cytokine acts as an autocrine growth factor for HuT-78 cells, since proliferation of HuT-78 cells can be inhibited by lowering TNF-α
Fig. 3.12. Dose dependent induction of apoptosis by SNP and curcumin in HuT-78 cells. Cells were treated with various concentrations of curcumin (12.5, 25 and 50 μM) or SNP (0.5, 1 and 1.5 mM) for 48 h. Apoptosis was studied by TUNEL assay by flow cytometry at FL-1 channel.
Fig. 3.13. Downregulation of Bcl-xL mRNA expression by SNP in HuT-78 cells. (A) Cells were treated with 1 mM SNP or 12.5 μM curcumin for 6 h. Total RNA was isolated and analyzed by RPA as described in Materials and methods section. Similar results were obtained in two independent experiments. (B) Result of RPA with a graphical representation is shown for Bcl-xL mRNA expression after normalization to GAPDH mRNA expression for two different experiments. Values are expressed as mean ± S. D.
Fig. 3.14. Effect of SNP on Bcl-xl and cIAP protein expression. Cells were treated with 1 mM SNP for indicated time points. Protein extracts were fractioned and then subjected to Western blot analysis using antibodies either against (A) Bcl-xl or (B) cIAP. Equal loading of protein was confirmed by actin. M, cells incubated in medium only; S, cells treated with SNP.
level using anti-TNF antibody (O'Connell et al., 1995; Giri and Aggarwal, 1998). Numerous studies have demonstrated that NF-κB plays a critical role in inhibiting TNF-α mediated apoptosis (Beg and Baltimore, 1996; Van Antwerp et al., 1996). Moreover, our studies indicated that SNP inhibits NF-κB. Therefore, we made an attempt to study whether NF-κB inhibition by SNP could make HuT-78 cells sensitive to TNF-α mediated apoptosis. For this purpose, HuT-78 cells were treated either alone or together with SNP (1 mM) and human recombinant TNF-α (250, 500 and 1000 pg/ml) for 24 h and analyzed for the percentage of sub-G1 fraction by flow cytometry. Our results showed that SNP alone increased the percentage of sub-G1 fraction from 4.53% to 28%. However, no significant change with respect to control in sub-G1 fraction was observed after treatment of cells with different concentrations of TNF-α. Furthermore, the percentage of sub-G1 fraction in combined treated cells was similar to SNP alone treated cells (Fig. 3.15.). Thus, our results suggest that NF-κB inhibition by SNP did not make HuT-78 cells susceptible to TNF-α mediated apoptosis.

We also analyzed the mRNA expression of several molecules those play important role in apoptosis by RPA using hAP0-3 multiprobe template set (BD PharMingen, San Diego, CA). For this purpose, cells were treated with various concentrations of SNP (0, 1 and 1.5 mM) and total RNA was isolated after 8 h of treatment. Our results showed that SNP did not significantly change the expression of molecules (caspase-8, FAS, FADD, FAF, TRAIL and TNFRp55) in hAP0-3 template at 8 h of treatment (Fig. 3.16.).

### 3.3. Discussion

Apoptosis is a type of programmed cell death, involving a series of biochemical events which lead to a variety of morphological changes, including membrane blebbing, loss of membrane integrity, cell shrinkage and nuclear fragmentation. Apoptosis occurs normally during development and aging and as a homeostatic mechanism to maintain cell population in tissues. In addition to its importance as a biological phenomenon, defective apoptotic processes have been implicated in an extensive variety of diseases. Excessive apoptosis causes hypotrophy, such as in ischemic damage, whereas an insufficient amount results in uncontrolled cell proliferation, such as cancer (Elmore, 2007). In addition, proper
Fig. 3.15. Effect of SNP on TNF-α mediated killing. Cells were treated with different concentrations of TNF-α in the presence or absence of 1 mM SNP for 24 h and analyzed for sub-G₁ peak by flow cytometry.
Fig. 3.16. Expression of molecules in hAPO-3 template in SNP treated HuT-78 cells. Cells were treated with varying concentrations of SNP (0, 1 and 1.5 mM) for 8 h. Total RNA was isolated and analyzed by RPA as described in Materials and methods. Results of RPA is presented as percent activation of mRNA expression of various molecules in hAPO-3 template after normalization to GAPDH. Similar results were obtained in two separate experiments.
functioning of the apoptotic machinery is critical for tumor susceptibility to treatment (Zhivotovsky and Orrenius, 2006).

Apoptosis can be triggered by a variety of stimuli, one such inducer of apoptosis which has been well recognized is NO. In the present chapter, we aimed to study the apoptotic potential of NO on CTCL cells. For this purpose, all experiments were carried out using SNP, a NO-generating compound on HuT-78 cells, a CTCL cell line.

We found that SNP possesses cytotoxic activity against HuT-78 cells and this cytotoxicity is related to the release of NO by SNP as evident by time and dose dependent increase in nitrite level. Thereafter, our results demonstrated that SNP initiates a series of events leading to apoptosis in HuT-78 cells. Evidence for this important function is based on several observations. First, SNP treated cells showed increase in the percentage of cells in sub-G1 fraction. A time dependent increase in the percentage of apoptotic cells in SNP treated cells was further revealed by TUNEL assay. Second, SNP treatment induced chromatin condensation and DNA fragmentation. Third, SNP treated cells showed a drastic drop in MMP followed by cytochrome c release into the cytosol. Fourth, caspase-9 and -3 activation were observed in treated cells. Lastly, PARP cleavage was also detected in these cells.

Next, we studied the effect of SNP on NF-κB activation in HuT-78 cells, which express a basal high level of NF-κB. Our results demonstrated a dose dependent inhibition of constitutive NF-κB by SNP and curcumin (used as a positive control) as evident by EMSA. This result was further supported by immunofluorescence, where SNP treated cells showed a decrease in p65 (a subunit of NF-κB) nuclear translocation. Furthermore, our results also revealed a dose dependent increase in apoptosis by SNP and curcumin, thus suggesting a correlation between downregulation of NF-κB and induction of apoptosis in these cells.

Thereafter, we examined whether the mechanism of NF-κB inhibition by SNP and curcumin involves the alteration of phospho IκBα and IκBα protein expression. Our results showed a dose dependent inhibition in expression and phosphorylation of IκBα protein by curcumin, this result is consistent with curcumin studies in case of other cell types (Bharti et al., 2003; Shishodia et al., 2005). Interestingly, SNP increased IκBα protein expression, on the other hand, it failed to show any effect on IκBα phosphorylation. The enhancement of IκBα protein was further verified by
examining the effect of SNP on proteasome mediated IκBα degradation. Our result indicated an accumulation of phospho-IκBα in SNP and MG-132 combined treated cells. This accumulation probably resulted due to an increase in IκBα protein level followed by its phosphorylation and thereafter inhibition of proteasome mediated degradation.

NF-κB regulates the expression of several genes whose products inhibit apoptosis (Sethi et al., 2008). It has been reported that HuT-78 cells constitutively express Bcl-xl, an antiapoptotic member of Bcl-2 family, regulated by NF-κB (Zhang et al., 2003). As mentioned above, our results demonstrated an inhibition of NF-κB level by SNP, therefore, we next investigated the effect of SNP on Bcl-2 family members. We observed a basal high level of Bcl-xl at mRNA and protein level, which decreased with SNP treatment. We also investigated the effect of SNP on cIAP, a NF-κB regulated gene product. We did not find any significant change in the cIAP expression till 12 h treatment. Although, SNP inhibited cIAP expression at 24 h time point.

TNF-α is a pleiotropic cytokine that regulates multiple cellular responses including inflammation and cell survival. Ligation of TNF-α to its receptor activates NF-κB, which mediates cellular activation, gene transcription and survival. However, binding of TNF to its receptor can also promote a death signal (Ashkenazi and Dixit, 1998). Furthermore, cells resistant to TNF-α induced apoptosis may become sensitive following inhibition of NF-κB and appears cell type specific (Beg and Baltimore, 1996; Van Antwerp et al., 1996; Liu et al., 2004). Our results showed that HuT-78 cells are resistant to TNF-α induced apoptosis, these results are consistent with reports from other laboratories (O’Connell et al., 1995; Giri and Aggarwal, 1998). Although, SNP inhibited NF-κB, but suppression of NF-κB failed to make these cells sensitive to TNF-α induced apoptosis.

In conclusion, our study for the first time demonstrated the apoptotic potential of NO generating compound, SNP on CTCL cell line, HuT-78. The mechanism involving the induction of apoptosis includes inhibition of constitutive NF-κB and Bcl-xl expression. Therefore, NO generating agents could be an effective approach in the treatment of CTCL cells.