Mahanine synergistically inhibits stat3 in combination with cisplatin in cervical cancer
Signal Transducer and Activator of Transcription 3 (STAT3) play an important role in different cellular processes such as survival, proliferation, and differentiation. Constitutively activated STAT3 is found in wide variety of human cancers such as breast, prostate, lung, ovarian, head and neck and cervical cancers as well as leukemia and lymphoma [Chen et al., 2007]. Upstream activators of STAT3 include epidermal growth factor receptor (EGFR), IL-6 cytokine receptors or non-receptor tyrosine kinases, Src or Janus kinase (JAK). Phosphorylation at tyrosine (Tyr) residue 705 activates STAT3 resulting in its dimerization, nuclear translocation, DNA binding, and transcriptional activation of target genes. Activated STAT3 acts as a proto-oncogene by regulating proliferation and apoptosis associated proteins such as cyclin D1, c-Myc, survivin, Bcl-xL, Bcl-2, Mcl-1 and Fas. STAT3 also increases invasion and metastasis by inducing matrix metalloproteinases (MMPs) and angiogenesis by the activation of VEGF and HIF-1α [Huang et al., 2007]. It has recently been shown that STAT3 is overexpressed and thus confer resistance to cisplatin-induced apoptosis in human cancers [Stewart et al., 2007]. Thus, inhibition of STAT3 activation could sensitize cancer cells to cisplatin-induced apoptosis [Yao et al., 2011].

Therefore, we focussed our study to investigate the mode of action of mahanine in cervical cancer alone or in combination with cisplatin.

5.1 Objective of the study

i. Decode mahanine-mediated signaling pathways in cervical cancer.

ii. Demonstrate the mechanistic insight of mahanine in combination with cisplatin.

5.2 Materials and Methods (see Appendix I)

5.3 Results

5.3.1 Mahanine suppressed STAT3 activation in cervical cancer

Constitutive activation of STAT3 signalling is reported to be found in cervical carcinoma and inhibition of this signalling may take a role in cancer interference [Stewart et al., 2007; Yao et al., 2011]. In this context, we investigated whether mahanine could inhibit the STAT3 activation in HeLa and SiHa cells. Mahanine significantly inhibited phosphorylation of STAT3 at Tyr705 in a concentration-dependent manner in both the cells after 24 h. Besides, the total protein levels of STAT3 were almost unchanged and decreased only at 16 µM of mahanine [Fig. 5.1a].
Src and JAK tyrosine kinases contribute in the activation of STAT3. To further elucidate mahanine-mediated suppression of p-STAT3, expression of total and phosphorylated of Src and JAK1 proteins were checked. Mahanine after 24 h treatment resulted in a reduction of phosphorylation of Src and JAK1 in concentration-dependent in both the cells. The expressions of total Src were also decreased after mahanine treatment. Though, no significant inhibitions were observed in total JAK1 [Fig. 5.1a].

Tyr705 phosphorylation activated STAT3, promoted its dimerization and nuclear localization. Consequently, we examined the expression levels of p-STAT3 and total STAT3 in nuclear portions of HeLa and SiHa cells after 24 h mahanine treatment. We found that mahanine led to decrease in the levels of STAT3 and inhibition of its phosphorylation at Tyr705 in nucleus [Fig. 5.1b]. Therefore, we suggested that dephosphorylation of STAT3 indicates mahanine-induced inhibition of STAT3 signalling in cervical cancer.

**Fig. 5.1: Mahanine suppressed STAT3 activation** a) HeLa and SiHa cells were exposed to increasing concentrations of mahanine (0-16 µM) for 24 h. After treatment cells were sonicated, cell lysates were electrophoresed. Concentration-dependent deactivation of STAT3 was detected by immunoblot analysis using anti-STAT3, p-STAT3 (Tyr705), Src, p-Src, JAK1, p-JAK1 antibodies. b) Equal amount of proteins (40 µg) of cytosolic and nuclear fractions of mahanine (0-16 µM)-treated cells after 24 h were separated by SDS-PAGE and proteins were analysed by immunoblotting with anti-STAT3 and p-STAT3 antibodies. Nuclear accumulation of p-STAT3 was blocked in HeLa and SiHa cells. In cytosolic and nuclear fraction, β-actin and HDAC3 served as the loading control respectively.
5.3.2 Mahanine promoted ubiquitin-dependent proteasome-mediated degradation of p-STAT3 (Tyr705)

Turnover of p-STAT3 is reported to be regulated by the ubiquitin-dependent proteasomal degradation [Selvendiran et al., 2006]. Therefore, next we investigated any involvement of ubiquitin-dependent degradation in the marked decrease in p-STAT3 at Tyr705. Pre-incubation of HeLa and SiHa cells with MG132, a proteasome inhibitor for 1 h followed by 24 h mahanine treatment restored both phosphorylated and total STAT3 [Fig. 5.2a]. Furthermore, enhanced polyubiquitination of p-STAT3 at Tyr705 was observed in the presence of MG132 in SiHa cells [Fig. 5.2b]. These findings suggested that mahanine mediates ubiquitination of p-STAT3 leading to its reduction in cervical cancer cells.

Fig. 5.2: Mahanine promoted ubiquitin-dependent proteasomal degradation of p-STAT3 Tyr 705

a) Mahanine-induced STAT3 deactivation was proteasome-mediated. HeLa and SiHa cells were briefly exposed for 1 h in presence and absence to MG132 (10 µM), a proteasome inhibitor. Next they were kept for 24 h in presence and absence of mahanine (12 µM). Cell lysates were prepared, electrophoresed and immunoblotted with anti-p-STAT3 (Tyr705) and anti-STAT3 antibodies. b) Mahanine suppressed STAT3 activation and promoted ubiquitin-dependent proteasomal degradation of p-STAT3 Tyr705. In presence of MG132, mahanine induced polyubiquitinated p-Stat3 was immunoprecipitated after 24 h of treatment.
5.3.3 Cisplatin and mahanine combination enhanced deactivation of STAT3 and its downstream proteins

Over expression of STAT3 is reported to confer resistance in cisplatin-induced apoptosis. By now, we demonstrated that mahanine suppressed STAT3 activation. Therefore, here we wanted to check combination-induced STAT3 regulation in cervical cancer. Cisplatin (3 µM) alone increased the level of total as well as phosphorylated STAT3 in HeLa cells after 24 h whereas in SiHa, total STAT3 and p-STAT3 status remained almost unaffected. As shown in Fig 5.1a, mahanine (12 µM) dephosphorylated STAT3 in both the cells. Interestingly, combination of cisplatin (3 µM) and mahanine (12 µM) treatment resulted in a drastic decrease in phosphorylation of STAT3 at Tyr705 in both the cells compared to single agent alone [Fig. 5.3a]. Additionally, phosphorylation of Src and JAK1 were repressed more in combination-treated cells. Combined treatment also reduced the expressions of total Src and JAK1 proteins. Moreover, combination of mahanine and cisplatin blocked nuclear translocation of p-STAT3 more than when used them individually [Fig. 5.3b]. Inhibition of both Src and JAK exhibited similar down regulation of p-STAT3 level as mahanine in combination with cisplatin suggesting their role in this event under the experimental condition [Fig. 5.4a-b].
Fig. 5.3: Mahanine in combination with cisplatin induced enhanced deactivation of STAT3. Cells were exposed to either cisplatin (3 µM) or mahanine (12 µM) alone or their combination at 1:4 (cisplatin: mahanine) for 24 h. a) Cell lysates of treated cells were electrophoresed and analysed by Western blot with anti-STAT3, p-STAT3 (Tyr705), Src, p-Src, JAK1, p-JAK1 antibodies. b) Treated cells were separated into cytosol and nuclear portions and equal amount of proteins (40 µg) of both fractions were analysed by Western blot with anti-p-STAT3 (Tyr705) and anti-STAT3 antibodies.

5.3.4 Cisplatin and mahanine combination deactivate STAT3 downstream proteins more

STAT3 activation has been shown to promote tumorigenesis by regulating the expression of various gene products involved in cell survival, proliferation and angiogenesis [Huang et al., 2007]. Accordingly, we wanted to ensure the status of a few downstream proteins of STAT3 after the combination treatment. Our results showed that after 24 h exposure of cisplatin and mahanine (1:4 ratio) decreased phosphorylation of c-myc and c-Jun more in HeLa and SiHa cells than in presence of single agent. Total c-myc and c-Jun levels were also reduced in combination-treated cells. In contrast, c-myc and c-Jun were almost unchanged in cisplatin or mahanine treated cells [Fig. 5.5a]. Next, we verified expression of the proteins associated with apoptosis of cancer cell. In the case of combination, we observed the down regulation of anti-apoptotic proteins Bcl-xl and Bcl-2 and increased level of Fas, apoptotic protein [Fig. 5.5b] in both the cells. Moreover, among the angiogenic factors regulated by STAT3, we checked VEGF expression which was also decreased significantly after combination treatment [Fig. 5.5b]. Taken together, our results indicated that mahanine enhances cisplatin-induced apoptosis of cervical cancer cells only in-combination.
5.3.5 Combination of mahanine and cisplatin decreased more cell motility in cervical cancer

Matrix metalloproteinases (MMPs) play a pivotal role in cancer cell motility and invasion among which, MMP-9 is the transcriptional targets of STAT3 [Huang et al., 2007]. In this perspective, we examined the status of MMP-9 after treated HeLa and SiHa cells with either cisplatin (3 μM) or
mahanine (12 µM) or their combination. We demonstrated more decreased MMP-9 expression in the combination-treated cells [Fig. 5.6a]. Furthermore, results from scratch wound assay showed more decreased motility of both HeLa and SiHa cells after exposure of cisplatin and mahanine in-combination as indicated by area of wound. The wound area after both mahanine and cisplatin exposure was greater than the area after treatment with single agent alone [Fig. 5.6b]. These data assured that cervical cancer cell motility is inhibited more by cisplatin when it was combined with mahanine.
5.4 Discussion

The constitutive phosphorylation at Tyr705 of STAT3 mainly confers drug resistance to several cancers [Barré et al., 2007]. Activated STAT3 is also linked with cisplatin-resistance in head and neck squamous cell carcinoma [Gu et al., 2010]. These make STAT3 an effective target for cancer therapy [Wang et al., 2012]. Here, we observed the ability of mahanine to deactivate STAT3 by reducing its Tyr705 phosphorylation and subsequent decreasing its nuclear translocation. Thus it could be hypothesised that mahanine may enhance chemo-sensitivity of cervical cancer cells to cisplatin by reducing STAT3 activity. In this perspective, we combined mahanine and cisplatin at fixed ratio and observed enhanced accumulation of cytosolic p-STAT3 and inhibition of nuclear translocation, although cisplatin alone had little effect on STAT3 deactivation under this condition. We already demonstrated that mahanine mediates ubiquitination of p-STAT3 leading to its reduction in SiHa cells. It might be possible that combination treatment resulted in ubiquitination of p-STAT3 in advance as compared to the individual treatments due to enhancement of their cytotoxic effect. This could be the reason that the robust enhancement of p-STAT3 does not occur after combined exposure in cytosolic portion of SiHa cells in comparison to single agent alone.

Furthermore, cisplatin, at lower concentration, was unable to decrease expressions of cell proliferation-related proteins (c-myc, c-Jun). However, at the same dose, in combination with mahanine, cisplatin showed inhibition of all these proteins. It is reported that STAT3 downregulates tumour-suppressor pathway proteins in melanoma and colon cancer [Niu et al., 2005] and previously we reported mahanine-mediated activation of p53 in colon cancer [Chapter IV, Das et al., 2013]. Therefore, it may be envisaged a possible link between involvement of STAT3 inactivation and activation of p53 in mahanine-treated cancer cells.
ROS generation is one of the key mechanisms of cisplatin-induced apoptosis [31]. Mahanine-induced ROS [Bhattacharya et al., 2010] played an important role in Hsp90 inhibition in pancreatic cancer [Sarkar et al., 2013], PTEN activation in colon cancer [Das et al., 2013] and G0-G1 phase arrest in glioblastoma multiforme [Bhattacharya et al., 2014]. However, the role of ROS in mahanine-mediated STAT3 deactivation is yet to be investigated.

Natural compounds are reported to suppress STAT3 activation either through direct binding to its SH2 domain or by inhibition of upstream tyrosine kinases JAK1 and Src and/or up regulation of SHP-1 and SHP-2 [Shin et al., 2009; Li et al., 2010]. Mahanine-mediated suppression of JAK1 and Src might play a significant role in the inhibition of phosphorylation of STAT3. Inhibition of both Src and JAK exhibited similar down regulation of p-STAT3 level as mahanine in combination with cisplatin suggesting their role in this event under the experimental condition. Ubiquitin-dependent proteasomal degradation of STAT3 is involved as negative regulator of this pathway in hepatocellular carcinoma [Selvendiran et al., 2006]. Here, we also observed that mahanine promotes ubiquitin-dependent proteasome-mediated degradation of p-STAT3. Therefore, our data convincingly demonstrated that mahanine induces apoptosis of cervical cancer cells through inhibition of tyrosine kinases followed by reduction of p-STAT3 leading to its proteasome-dependent degradation. Moreover, combination treatment resulted in enhanced suppression of upstream regulators and subsequent inhibition of p-STAT3. PTEN is reported to act as a negative controller of STAT3 activation [Sun et al., 2003]. Mahanine-mediated PTEN activation in colon cancer [Das et al., 2013] suggested that PTEN may also be involved in the inhibition of STAT3 in treated cervical cancer cells.

Furthermore, down regulation of MMP9 after combination-treatment confirmed the involvement of mahanine-mediated STAT3 deactivation and further verified its anti-metastatic activity [Sarkar et al., 2013]. This is corroborated by enhanced inhibition of cell migration after combination treatment.

Taken together, our findings highlight another major application of mahanine in reducing the concentration of cisplatin for its wider use.
**Highlights**

i. Mahanine potentially deactivated STAT3 by suppressing its phosphorylation.

ii. The decreased expression of JAK1 and Src might play important role in deactivation of STAT3.

iii. Mahanine also promoted ubiquitin-dependent proteasome-mediated p-STAT3 degradation.

iv. In combination with cisplatin, mahanine enhanced further inhibition of STAT3 phosphorylation, its upstream JAK1 and Src, cancer cell migration and increased apoptosis of cervical cancer cells compared to single agent.

- *This work was published in Cancer Lett. 351:81-90 [doi: 10.1016/j.canlet.2014.05.005]*.