CHAPTER 6

Conclusion and Future Prospects

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6.1 Conclusion

In this study, we explored the cytotoxic activity of crude venom and a purified protein (Cytotoxin 10) from *N. kaouthia* venom of North-East India origin. The cytotoxic activity of the crude *N. kaouthia* venom was determined against normal and cancer cell lines and it was observed that the crude venom could induce cytotoxicity against breast and lung cancer cell lines in a dose and time-dependent manner suggesting the presence of cytotoxic protein/s. Therefore, to identify the bioactive compound responsible for its cytotoxicity, the crude *N. kaouthia* venom was subjected to fractionation using RP-HPLC and the peaks were collected and screened for cytotoxicity against cancer cell lines. Further, the peak which exhibited the highest activity was identified as Cytotoxin 10 by ESI-LC-MS/MS analysis. This protein belongs to the 3FTx family of snake venom proteins. Pairwise Sequence Alignment of Cytotoxin 10 revealed Proline-30 at the phospholipid-binding site confirming the protein to be a P-type cytotoxin. The presence of Proline-30 residue might be responsible for interaction of Cytotoxin 10 with the phospholipid bilayer leading to internalization of the protein into the cancer cells which may trigger the signaling pathways involved in cancer cell death.

Tertiary structure of Cytotoxin 10 was also predicted using Swiss-Model EXPASY tool. Further, accuracy of the prediction was validated using Ramachandran plot from PROCHECK Saves v.6.0 tool. Elapid venoms are rich in low molecular weight toxins but have poor abundance of high molecular weight proteins. Cytotoxin 10 exhibited the maximum cytotoxic effect against lung cancer cell line (A549), whereas, it was least cytotoxic towards the normal HEK-293T cell line.

To check if Cytotoxin 10 could induce apoptosis in cancer cells flow cytometry analysis was performed. Treatment with Cytotoxin 10 induced dose-dependent increase in apoptotic cells in breast and lung cancer cells. In MCF-7 cells, dual staining with Annexin V (Alexa Fluor 488)/ PI showed increase in early apoptotic cells when treated with low dose of Cytotoxin 10 and increase in treatment dosage led to increase in late apoptotic/necrotic cells. However, Cytotoxin 10 treatment of A549 cells suggested a dose-dependent increase in late apoptotic cells. Furthermore, to understand the signaling

mechanism leading to venom-induced apoptosis in both the cell lines, expression level of apoptosis regulating proteins like Bax, Bcl-2, PARP, and caspase-7 was assessed and studied by western blotting. Treatment of cancer cells with Cytotoxin 10 demonstrated significant increase in the expression of pro-apoptotic protein Bax and inhibited expression of Bcl-2 leading to increase in Bax/Bcl-2 ratio in both the cell lines, a marker that indicates induction of apoptosis. The expression of caspase-7 along with its cleavage increased steadily in a concentration dependent manner. PARP cleavage with two bands corresponding to 116 kDa and 89 kDa was also observed after Cytotoxin 10 treatment. Tumor suppressor protein, p53 is expressed to regulate uncontrolled cell division/proliferation of cells. Cytotoxin 10 treatment of MCF-7 cells led to a dose-dependent increase in expression of p53 protein in MCF-7 cells. Thus, suggesting that Cytotoxin 10 induced caspase-7 dependent apoptotic cell death in cancer cell lines.

To understand if Cytotoxin 10 affects the metastatic behaviour of cancer cells, migration and adhesion assays were performed. The wound width was significantly decreased in untreated cancer cells. On the other hand, the wound width in cells treated with Cytotoxin 10 up to 48 hours remained high, suggesting Cytotoxin 10 could inhibit migration of cells. Further, pre-treatment of the cells with various doses of Cytotoxin 10 led to inhibition of adhesion in MCF-7 and A549 cells. In MCF-7 cells, the percentage of adhered cells decreased dose-dependently. Similar observation was noted when A549 cells were treated with different doses of Cytotoxin 10.

Thus, our study suggested that Cytotoxin 10 isolated from *N. kaouthia* venom exhibits more cytotoxicity towards cancer cells as compared to normal cell lines and the potential involvement of apoptotic cell death implied that Cytotoxin 10 may be explored as an anti-cancer agent for breast and lung cancer. However, considering the toxic effect of 3FTx family, structure-function relationship of Cytotoxin 10 needs to be evaluated to identify the residues involved in the cytotoxic effects. Further, this can be validated in animal models (*in vivo*) for development of potent cytotoxic agent that discriminates cancer cell from normal cells. Thus, the current findings extended our understanding of *in vitro* cytotoxic effect of snake venom protein against human breast and lung cancer cells.

6.2 Future prospects

i. The *in vivo* anticancer effects of Cytotoxin 10 on animal models of cancer may be studied to understand the efficacy of the venom protein, and to evaluate its safety and toxicity in complex biological environment.

ii. The anticancer property of Cytotoxin 10 against other cancer cells types (such as skin cancer, colon cancer, prostate cancer, etc.) may be explored, since, different cancers may share common molecular and cellular pathways, and it would also help to determine the broad therapeutic potential of Cytotoxin 10.

iii. Pharmacokinetics and Pharmacodynamics studies of Cytotoxin 10 may help to optimize the toxin-based drug prototypes by understanding their absorption, distribution, metabolism and excretion (ADME) as well as their bioavailability.

iv. Multi-drug resistance (MDR) is a major challenge which hampers effective anticancer therapy thereby limiting their interaction to the molecular targets. Novel anticancer snake venom toxins isolated from venomous snakes, such as, Cytotoxin 10 from *N. kaouthia*, can be explored to study their potential to inhibit MDR in different cancer cells.

6.3 Critical Discussion

Snake venom toxins, including cytotoxins, have exhibited anticancer properties in different studies through the induction of various effects, such as, induction of cytotoxicity through apoptosis, autophagy or necrosis, and inhibition of cancer cell proliferation, metastasis and angiogenesis. Hence, further investigation is required to understand the underlying mechanisms which affect the anticancer property, and thus develop effective anticancer drugs based on snake venom toxins or their derivatives [369]. However, there are some challenges associated to these efforts which limit the potential clinical use of snake venom toxins, and these challenges need to be addressed to achieve breakthrough in toxin-base anticancer drug production. Some these challenges and means to overcome them are briefly outlined below [370].

6.3.1 Availability of adequate snake venom toxins for anticancer drug development

Development of effective anticancer drug would require extensive structure-function studies of the venom protein which requires the isolation of pure proteins from snake proteins which can be challenging at times. Moreover, the yield of various snake venom toxins may be relatively low which makes it difficult to assess its anticancer activity. However, the development of high-throughput systems (eg. HPLC, FPLC) have made significant progress and made it relatively easier to obtain various venom toxins. Moreover, the development of recombinant expression systems has allowed the production of adequate amounts of toxin analogs, with structure and function similar to their corresponding wild type toxins, and is also considered a cost-effective alternative [370,371]. For instance, a recombinant Cytotoxin 1 (*N. oxiana*) was produced with an associated N-terminal Met-residues (rCTX-1) in *E. coli* expression system [372]. Similarly, chimeric recombinant toxins have been studied in pre-clinical research, such as chimeric recombinant disintegrins, which may retain parental properties of the toxins or can have new properties based on addition of specific sequences, may also be explored [373].

6.3.2 Optimization of candidate toxin-based anticancer drug

Although oral administration is the most preferred method, however, oral bioavailability of anticancer venom-based anticancer drugs may be poor due to their degradation in the gastrointestinal tract and permeation inefficiency owing to their large size. Hence, most of these drugs candidates are administered through intravenous or subcutaneous injection which is generally not preferred by the patients. These issues can be addressed by chemical modification of tertiary structures or by coating toxin-based drug candidates with permeation enhancers, mucoadhesive polymers or enzyme inhibitors. Similarly, anticancer drugs are also conjugated with hydrogels, lipids, macromolecular scaffolds or inorganic carriers to optimize their delivery to the targeted site [371,374].

6.3.3 Development of targeted drug-delivery systems

Various properties of snake venom toxins such as molecular weight, tertiary structure and physicochemical properties may affect their selectivity and interaction with cancer cells which may result in off-target side-effects. In order to resolve these issues two

different approaches are being extensively studied, the first is the use of monoclonal antibodies, which specifically detect and bind to epitopes of cancer cells [370]. For instance, cobra venom factor conjugated to murine monoclonal antibody exhibited selective cytotoxicity to human nasophyrngeal cells (CNE2) in the presence of fresh human serum [375]. The second approach is the combination of snake venom toxin to nanoparticles including polymeric nanoparticles, liposomes or micelles which have exhibited promising results in different studies [374]. For instance, Bhowmik et al. have exhibited that GNP-NKCT1 *i.e.,* a gold nanoparticle (GNP) conjugated with cytotoxin 1 (NKCT1) from *N. kaouthia* venom, exhibited a dose and time-dependent cytotoxicity against leukemic cell lines (U937 and K562) through cell cycle arrest and apoptosis [376].

Overcoming these challenges associated with anticancer drug development from snake venom toxins, and advances in target-specific drug delivery may help to provide safe and effective alternatives to currently available chemotherapeutic drugs.