CHAPTER 2

Review of Literature

Chapter 2: Review of Literature

2.1 Cancer progression

Cancer arises due to transformation of normal cells into tumor cells, where the cell undergoes a multi-step process that involves progression of a pre-cancerous lesion into a malignant tumor. In multicellular organisms, epithelial tissues are compartmentalized into regions which consist of cells that multiply to perform tasks specific for each organ. A small number of cells continuously divide and differentiate within each compartment. In a normal tissue, homeostatic regulation in each of the compartments ensure that cell numbers remain constant over time [88]. However, the loss in equilibrium of cell birth and death leading to shift towards uncontrolled proliferation results in cancer. Every time a cell divides, there is a chance for error during DNA replication, thus producing a mutant progeny cell. The mutation could provide a survival advantage by increasing the cell's proliferation rate and this could be advantageous for somatic selection [89]. Moreover, the mutation could also provide fitness disadvantages by impairing an important cellular function thereby decreasing proliferation which might be deleterious with respect to somatic selection. Thirdly, the mutation could have a neutral effect on somatic selection by ensuring stability in the cell's reproductive rate. The rate of accumulation of mutations is greatly influenced by the tissue architecture/compartment size and somatic selection.

The development of cancer is driven by mutations in the cancer-susceptibility genes namely, oncogenes, tumor-suppressor genes and genes that induces genetic instability:

2.1.1 Oncogene: Mutations activating oncogenes provide a survival advantage to the cell by enhancing its somatic fitness [89]. Cells with oncogene mutations are effectively contained when the tissue is arranged into smaller compartments [90]. The chances of accumulation of these mutations are decreased by utilizing a linear tissue architecture where only one/few stem cells are replenished in the compartment [91]. However, once a neoplasia is induced by one/several mutations, additional mutations accumulate faster due to increase in the population size.

2.1.2 Tumor-suppressor genes (TSGs): Inactivation of alleles of the TSG provides the cell with selective advantage and have an increased somatic fitness [92]. The inactivation process is governed by three kinetic principles, which involve the cell population, size and the frequency of mutation. For inactivation of TSG, two rate-limiting events are needed for small sized populations, whereas, intermediate and large populations require one and zero rate-limiting events, respectively. Mutations which inactivate the TSGs are contained in small compartments which tend to develop a neoplasia after accumulation of one/few mutations. However, inactivation of other TSGs and an increase in the frequency of mutation owing to genetic instability may be essential for a tumor to continue progressing.

2.1.3 Genetic instability: Genetic instability arises when genes which are involved in critical processes including repair of DNA and segregation of chromosomes acquire mutations [93]. Interruption of several pathways, such as, base-excision repair, mismatch repair, nucleotide-excision repair, recombinational repair, segregation of chromosomes and modulation of CpG-island methylation have been reported to contribute to genetic instability. The most common form of genetic instability, chromosomal instability (CIN), refers to the addition or deletion of whole or parts of chromosomes when the cell divides [94] [95]. This leads to abnormality in chromosome number (aneuploidy) and loss of heterozygosity, thus accelerating the rate of TSG inactivation. CIN mutations may arise during the initial stages of tumor formation [96]. Genetic-instability leads to decreased somatic fitness and tend to gather more quickly in smaller compartments.

2.2 Anti-cancer drug and cancer cell death

Anticancer drugs can induce cell death *via* three mechanisms namely, apoptosis, autophagy and necrosis [97] which have been discussed below:

2.2.1 Caspase dependent pathways: Apoptosis

The most essential aspect of the therapeutic effects induced by anticancer drugs involve the induction of cell death *via* apoptosis [98], and inhibition of apoptosis corresponds to development of drug resistance by the cells [99]. The mechanism includes upstream regulators which receives the death inducing signals, thus initiating apoptosis through either the extrinsic or intrinsic pathways. Whereas, the downstream effector components

comprises of caspases which executes the process of apoptosis [21]. The intrinsic and extrinsic pathways are controlled by B-cell lymphoma 2 (Bcl-2) family of regulatory proteins consisting of proapoptotic and antiapoptotic proteins. Apoptosis occurs when proapoptotic proteins like Bax, Bim, Puma, etc. are activated, while antiapoptotic proteins like Bcl-2, Bcl-xL, etc. are inhibited [15,100,101].

Intrinsic pathway involves non-receptor-mediated events that generate intracellular signals targeting the permeabilization of mitochondrial outer membrane that induces the intermembrane space to release pro-apoptotic proteins into the cytosol, thus initiating apoptosis [102,103]. Anticancer drugs activate c-Jun N-terminal kinase (JNK) with the help of mitogen-activated protein kinase kinase (MKK)4/7 which leads to phosphorylation of the 14-3-3 protein, thus releasing Bax [104-106]. Bax and Bak move across the outer membrane of mitochondria and stimulate the release of cytochrome c [101], which then leads to formation of protein complex apoptosome between apoptotic protease-activating factor-1(Apaf-1) and procaspase-9. The formation of apoptosome results in sequential induction of effector caspases, e.g. cleavage of procaspase-9 into the activated caspase-9, which ultimately induces the cleavage and activation of effector caspase-3 and caspase-7. Upon phosphorylation by JNK, Bid translocates to the mitochondria to release second mitochondria-derived activator of caspases (Smac)/high temperature requirement A2 (HtrA2) which leads to inhibition of X-linked inhibitor of apoptosis protein (XIAP) as well as cellular inhibitor of apoptosis protein (c-IAP) subsequently leading to induction of caspase cascade resulting in apoptosis [106]. JNK also inhibits Bcl-2 *via* phosphorylation.

Extrinsic pathway involves transmembrane receptor-mediated interactions and is mediated by death receptors (DRs) like Fas and DR4/5 [102,107]. Interaction between ligands and their corresponding death receptors, e.g. Fas ligand/Fas receptor and TNF α ligand/TNFR1 receptor binding interactions have been extensively investigated to study apoptotic cell death induced by anticancer drugs [101,108]. These drugs activate death receptors which recruits tumor necrosis factor (TNF) receptor-associated death domain (TRADD), the Fas-associated death domain (FADD) and procaspase-8 for generating a death inducing signaling complex [109]. This interaction leads to activation of procaspase-8 and eventual activation of effector caspase-3, caspase-6 and caspase-7, and generates truncated Bid (BH3 interacting-domain) death agonist which goes on to

activate Bax and Bak, and translocates to mitochondria and induces release of Cyt c and Smac/HrtA2, thus activating the intrinsic pathway.

JNK and p53 signaling pathways have been known to activate genes that promote apoptosis, namely, Bak, TNF-α, and Fas L, thus inducing the apoptotic cell death [110-112]. Activator protein 1 (AP-1) and p53 are the two crucial transcription factors involved in the induction of apoptotic cell death [113]. Activated JNK phosphorylates c-Jun which forms a heterodimer with the c-Fos family in the form of AP-1 which leads to activation of genes which induce DNA damage, such as growth arrest- and DNA damage-inducible gene 153 (GADD153) along with Bak, Bim and p53, thus promoting apoptosis [114]. The modulation or inhibition of signaling pathways involved in apoptosis mediated by transcription factor often leads to resistance to drugs by cancer cells [115].

2.2.2 Caspase independent pathways

***Autophagy-dependent cell death*:** Autophagy is the intracellular degradation process by which unwanted or non-functional proteins or cellular organelles are removed from the cells by wrapping them in autophagosomes which are then fused with lysosomes to from autophagolysosomes. The contents of the autophagolysosomes are degraded to achieve cryoprotection through cellular homeostasis and re-utilization of amino acids [116,117]. However, in a number of developmental and pathophysiological situations, autophagy may contribute to cell death. When apoptosis is ineffective, the cell may turn to excessive autophagy for cell death [116]. This is distinct from other situations where autophagy is associated with other types of regulated cell death (RCD) such as: (1) ferroptosis, autophagy contributes to ferroptosis through ferritinophagy *i.e.*, the autophagic degradation of ferritin [118]; (2) Fas-mediated extrinsic apoptosis, autophagy can enhance this form of apoptosis by degrading PTPN13 (also called FAP1), a phosphatase that acts as a regulatory checkpoint to suppress apoptosis [119]; (3) necroptosis, autophagy can promote necroptosis, which is a form of programmed necrosis, by acting as a scaffolding structure for the formation of a protein complex called the necrosome [120,121]. Further, autophagy promote necroptosis by also degrading c-IAPI1 and c-IAPI2, proteins that normally inhibit necroptosis [122].

***Necroptosis*:** Necroptosis or regulated necrosis is a type of RCD initiated by disturbances in the cellular microenviroment by ligands of specific receptors, such as, Fas and TNFR1 (also called death receptors) [123,124], or pathogen recognition receptors such as TLR3, TLR4 and Z-DNA binding protein-1 (ZBP-1) [125,126]. The activation of necroptosis requires kinase function mediated by the Receptor-interacting protein 1 (RIP1), and its inhibition by necrostatins may block necroptosis and allow cells to survive. The RIP1 kinase activates RIP3 and mixed-lineage kinase domain-like (MLKL). However, necroptosis is inhibited by caspase-8 and FADD-mediated apoptosis. Lack of caspase-8 and FADD may cause pathological conditions, such as, embryonic mortality, and degeneration of tissue and inflammation, which can be inhibited by targeting RIP1 and RIP3 [127].

2.2.3 Necrosis

Necrosis differs from apoptosis or autophagy-associated cell death without exhibiting any of the their typical features, and results in the disposal of cell debris in the absence of involvement of autophagosomes or lysosomes [128]. A type of necrosis called the Mitochondrial permeability transition (MPT)-led necrosis is a specific type of RCD initiated by disturbances of microenvironment of the cell, such as, excessive oxidative stress and overload of Ca^{2+} ions in the cytosol, and depends on the protein cyclophilin D (CYPD) [129,130]. The MPT-led necrosis results the inner mitochondrial membrane to abruptly lose its impermeability to small solutes due to which there is a dissipation of membrane potential and osmotic breakdown of both the mitochondrial membranes resulting in necrosis [131,132]. The MPT-led necrosis is proposed to follow a supramolecular complex at the junction between the inner- and the outer mitochondrial membrane, known as the permeability transition pore complex (PTPC). The composition and mechanism of action and its regulation is not yet fully described, however, the protein CYPD has been proven to be of critical requirement for the induction of MPT-driven necrosis [133]. The MPT-led necrosis is dependent on various mediators for the formation of PTPC, prominent among which includes: (1) apoptosis mediators of the Bcl-2 family, such as, Bax, Bak, BID, Bcl-2 and Bcl-X_L ([134-136]; (2) dynamin 1 (DNM1, also called DRP1), which promotes the opening of PTPC, as a result of the

stimulation of β-adrenergic receptors, resulting in its phosphorylation [137]; (3) p53, which physically interacts with CYPD to participate in MPT-led necrosis [138].

2.3 Metastasis and its regulation

The emergence of secondary tumors at distant areas which is far away from the site of primary tumor is called metastasis. It may develop during both early or later stages of cancer, where early metastatic cells contain truncal mutations, while the cells at later stages of metastasis carry sub-clonal mutations [139]. Metastasis occurs through a multi-step and organ-specific process, and the complexity of the process provides a huge challenge to effective therapy and for developing anti-metastatic drugs (called migrastatics) [140]. Gaining insights about the various mediators involved in tumor metastasis would help in the identification of major targets to develop relevant interventions. The key steps of metastasis are invasion, intravasation, survival and maintenance, extravasation, and colonization (outgrowth) [141-143]. The first step 'invasion', involves the escape of primary cancer cells which mostly occurs at the interface between tumor and stroma. Invasion can either be single-cell invasion, (monoclonal metastasis) where the cells alter their shape and attachment status, or collective invasion (polyclonal metastasis), which is commonly observed in breast, colon, prostate, thyroid, lung and glioblastoma where the multi-cellular strands maintain cell-to-cell attachments. Tumor metastatic cells undergo epithelial-mesenchymal transition and stem-cell like characteristics, and have acquired mechanisms to evade apoptosis and withstand therapy [144].

In the next step 'intravasation', the tumor cells enter the blood circulation, probably due to abnormal leaky vasculature, where the single cells are known as circulating tumor cells (CTC) and CTC clusters for collective cells [140,145]. This process involves various intrinsic (EMT and protease production) and extrinsic factors (pro-tumor N2 neutrophils, fibroblasts and M2 macrophages) [146]. The CTCs face numerous environmental stress, such a,s oxidative stress, shear force, and attack by the immune system in blood circulation due to which only a few cells (~0.01%) that stay alive can reach the secondary site, achieving metastasis. The metastatic cells which survive employ four types of strategies for their survival *i.e.*, rewiring metabolic demands, anoikis resistance, adapting flow mechanics, and immune suppression [140]. Factors

such as release of cytokines promote metastasis, and platelets form aggregates with CTCs to shield and protect CTCs from stress and immune response [147]. The CTCs extrude from the blood to their secondary site, where they are called disseminative tumor cells (DTCs), through a process called 'extravasation'. The extravasation of CTCs with the endothelium and secondary site is mediated by cell adhesion molecules. The DTCs after entering the secondary site normally undergo either dormancy or proliferation depending on a number of intracellular and extracellular factors, and signaling molecules from the bone marrow [33,148]. The DTCs are able to selectively target specific organs for metastatic colonization [149].

A number of promoters and suppressors play an important role in the regulation of metastasis. For instance, macrophages have two phenotypes, the classically activated M1 type is anti-tumor as they help to recruit T-cells and suppress the activity of pro-tumor M2 type (alternatively activated cells) [150]. The M2 type contribute in proliferation of cancer cells, angiogenesis, escape from immune cells, and metastasis [151]. Another important factor which promotes the development and regression of metastasis is inflammation. In a study by Rodrigues et al., the effect of cell migration-inducing and hyalurinan-binding protein (CEMIP) and associated inflammation on microglial cells resulted in metastasis of breast cancer cells in the brain [149]. In another study by Du et al., the activation of nuclear factor kappa B (NF-κB), which is a major regulator of inflammation in fibroblasts, resulted in intra-pulmonary metastasis of lung cancer cells [152]. Moreover, sometimes surgical removal of primary tumor may also enable metastasis by inducing the metastatic cells to escape dormancy [153].

2.4 Recent advances in anticancer drug development

The ever-increasing burden of cancer patients worldwide, along with inadequacies of currently available anticancer drugs, has made it necessary to develop newer anticancer drugs. In this context, computer-aided drug design (CADD) has emerged as an efficient and reliable approach in anticancer drug discovery. In recent years, CADD has aided scientists to simulate chemical microenvironments, predict 3D structures, develop and optimize new chemical compounds, and even analyze pharmaceutical compounds at the atomic level. It has brought forward numerous candidates for clinical trials, few of which

(eg. axitinib, crizotinib, gefitinib, and erlotinib) have also received approval by the United States Food and Drug Administration (US FDA). Some of the prominent strategies of CADD include structure-based approach (molecular docking), ligand-based approach (Quantitative structure-activity relationship modeling), molecular dynamics simulation, fragment based drug design, pharmacophore modeling and pharmacokinetics [154]. Recently, Artificial intelligence (AI) has been utilized to predict anticancer drug activity and also assist in the anticancer drug development. Machine learning models have successfully predicted sensitivity of drugs in patients of ovarian cancer, gastric cancer and endometrial cancer [155].

Increasing evidence on various mediators, which play critical role in development, maintenance and proliferation of cancer cells, have made them prominent targets in anticancer drug development. For instance, among the numerous post-transcriptional modifications that have been discovered in eukaryotes, the N6-methyladenosine (m6A) is the most common one, which is also responsible for development of multiple diseases including cancer. The m6A plays a critical role in stability, RNA processing, splicing, translation and nucleation, and they are regulated by writers (methylases) and erasers (demethylases) [156]. The dysregulation of m6A modification is prominently observed in various types of cancer and it affects proliferation, metastasis and drug resistance by controlling the expression of oncogenes and tumor-suppressor genes [157,158]. The m6A regulators have been identified as novel targets of anticancer drugs, and modern anticancer drug development platforms are attempting to develop m6A-specific drugs [156]. Another important target which has emerged for anticancer drug development is MicroRNAs (miRNAs), which are small endogenous non-coding RNAs controlling gene expression by modulating translation and stability of mRNA [159]. Abnormal expression of miRNAs are commonly observed in numerous cancers resulting in uncontrolled proliferation and evasion of apoptosis by cancer cells [160]. Restoration of abnormal miRNAs by utilizing miRNA mimics or antogomirs could normalize the gene expression and reverse the cancer phenotype, due to which miRNA are regarded as an interesting target for developing anticancer drugs [161]. For instance, TargomiRs, a micrRNA mimic, underwent phase I clinical trial in 2014 in patients with recurrent malignant pleural mesothelioma and non-small cell lung cancer. The results of phase I were

satisfactory with no side effects, and TargomiR is expected to undergo phase II clinical trials next [162].

2.5 Natural products and animal venom as source of anti-cancer leads

A natural product is a chemical compound which is obtained from living beings, be it plants, animals or microbes, which can contribute in developing novel therapeutics to treat various deadly diseases including cancer. As the need for developing effective chemotherapeutic and anticancer agents is increasing, it is an urgent need to develop new chemicals, drugs and drug leads from natural products [163]. It has been estimated that between 1940 to 2014 about 49% of the small molecules approved by the US FDA have been obtained from natural products [164]. Several commercialized anticancer drugs have been obtained from natural sources or synthesized based on natural compounds with modifications in their structure [165]. Novel natural products could provide a solution to the current issues related to chemotherapeutic compounds, such as, multidrug resistance, side-effects, high toxicity and non-specificity [166]. Therefore, the quest for anticancer drug development from natural sources, such as, microbes, phytochemicals and animal venom have been explored and are discussed below.

2.5.1 Microbial products

Most of the chemotherapeutic drugs have been developed from microbes, although the derivatives obtained from the parent drug have often exhibited better anticancer activity [167,168]. The availability of large microbial species provides an enormous supply of microbial compounds which is also cost-effective when compared to the chemical synthesis route [168]. It has been observed that microbial compounds have exhibited anticancer activity by regulation of immune response, inhibiting proliferation of cancer cells and inducing apoptosis [169]. The genus *Streptomyces*, a well-known representative of the phylum *Actinobacteria*, is the primary source of about 70% of commercially useful antibiotics, and has also been a source of large number of anticancer compounds [170,171]. Some of the novel anticancer drugs derived from natural compounds and their derivatives from different *Streptomyces* sp. include doxorubicin, daunorubicin, bleomycin, actinomycin D, distamycin A, geldamycin, chartreusin, dactinomycin, mitomycin C, deforolimus, idarubicin (chemical analog of daunorubicin) and epirubicin

(derivative of doxorubicin). Apart from *Streptomyces*, various anticancer drugs have also been obtained from other microbes, such as, calicheamicin (*Mincromonospora echinos*), epothilone B (*Sporangium cellulosum*), diazepinomycin (*Microsora* sp.), prodigiosin (*Serratia marcescens*), and ixabepilone (analog of epothilone B). The anticancer drugs obtained from microbial sources can be grouped into different classes, namely, anthracyclin, carbohydrates, polyketide macrlactone, glycopeptide, peptolide, oligopeptide macrocyclic polyketide, polyketide glycoside and alkaloids [163].

2.5.2 Phytochemicals

Plants are commonly utilized for treating numerous diseases since ancient times, and these natural products have acted as precursors to drugs and as templates for multiple synthetic drugs to treat various diseases including cancer. The phytochemicals obtained from plats are promising compounds to improve cancer treatment as they are biologically active compounds with antitumor potential. The phytochemicals may act by following alternative or overlapping pathways and help in scavenging free radicals, inhibit proliferation of malignant cells, and reduce tumor invasiveness and angiogenesis. They also exert a complex range of actions on transcription factors, miRNAs, cyclins, caspases, membrane receptors, kinases, tumor suppressor and activator proteins. Some of the phytochemicals and their class currently in the pre-clinical trials for their anti-cancer activity include 6-shogaol (phenylpropanoid), allicin (organosulfurs), alpinumisoflavone (pyranoisoflavone), andrographolide (diterpenoid), apigenin, baicalein and baicalin (flavonoids), curcumin (phytopolyphenol), decursin and decursinol (coumarin), capsaicin (capsaicinoid) and resveratrol (stilbenoid polyphenol) to name a few [57,172]. The phytochemicals currently in clinical trials for anticancer activity mainly focus on three aspects *i.e.*, improving the response of cancer cells to standard therapy, reducing side-effects, and identifying undesirable interations with standard therapy. Some of the phytochemicals in clinical trials of various cancers include berberine (colorectal cancer), curcumin (breast cancer), epigallocatechin (colorectal cancer), quercetin (prostate cancer), resveratrol (gastrointestinal neuroendocrine tumors) and sulforaphane (lung cancer). Currently, a number of plant-derived anticancer drugs are used which mainly belong to 4 classes: vinca alkaloids, camptothecin derivatives, taxane diterpenoids, and epipodophyllotoxin [172].

2.5.3 Animal venom

Venomous animals secrete venom, which is a mixture of diverse toxic chemicals, and used by the animal for the purpose of both offense and defense. Venom contain numerous biologically active compounds which have exhibited pharmaceutical properties such as treatment for diverse diseases like neurodegenerative, cardiovascular, autoimmune diseases and cancer [62]. Venom from animals, such as bee, scorpion, wasp, beetle, ant, spider, caterpillar and snakes have been explored to study their anticancer activity [58]. A toxin solenosin A isolated from ant (*Solenopsis invicta*) venom possess anti-angiogenic activity, and synthetic anti-antiogenic solenopsin A have exhibited strong inhibition of class-I phosphatidylinositol-3-kinase signaling and angiogenesis making it a potential lead for cancer therapy [173]. Similarly, bee venom has also been explored to neutralize the adverse effects of chemotherapy. For example, Melittin, which is the major compound in bee venom, have been explored as a compound for cancer chemotherapy [174]. Beetle (*Mylabris caragnae*) venom contains a compound named Cantharidin, which has exhibited inhibition of leukemic cells as well as DNA damage and apoptosis [175,176]. Cecropins isolated from the caterpillar *Hyalophora cecropia* have exhibited anticancer activity against different cell lines *via* formation of pores in the cell membrane [177].

Scorpion venom comprises of a wide range of biologically active compounds and around 250 such compounds have been discovered from about 1500 species [178]. For instance, Gonearrestide isolated from scorpion venom exhibited potent anticancer activity against colon cancer and growth of solid tumors by inhibiting cyclin-dependent kinase 4 and promoting cyclin D3, p27 and p21, thus, arresting cell cycle in G1 phase [63]. Spider venom also contains numerous biologically active compounds which have been explored for their anticancer potential. For instance, the peptide LAFr26 isolated from *Lachesanasp* sp. venom exhibited potent cytotoxic activity against lung cancer cell lines (LX22 and BEN) due to pore forming capacity of the venom compound [179]. Similarly, wasp venom, which produces extreme pain and serious tissue damage, also contains pore forming peptides which cause cell death by necrosis or apoptosis. For instance, Polybia MPI from *Polybia paulista* exhibited selective cytotoxicity against T-lymphocytes in leukemic cells [180]. Similar to the animal venom mentioned above, snake venom toxins

and their derivatives have also exhibited anticancer activity in numerous cancer types which have been discussed in the subsequent sections.

2.6 Snake venom

Snake venom is a complex blend of inorganic and organic components which is composed of several peptides, enzymes and proteins with defined chemical and biological functions, along with other non-protein components [181]. Proteins make up to approximately 95% of the dry weight of venom and play a key role in exerting major biological effects. Most of the proteins produce a wide range of pathophysiological effects encompassing localized tissue injury or systemic impacts in the envenomated patient [181,182]. Proteins are the prominent biomolecules present in snake venom and it may either be enzymatic or non-enzymatic. The snake venom proteins which have been reported for inducing severe pathophysiological consequences in envenomated patients include α-neurotoxins- non-enzymatic nAChRs binding proteins [183,184], β-neurotoxins, pre-synaptic phospholipase A_2 (PLA_2), [185,186], cytotoxins [74,86], non-neurotoxic PLA_2s [187], and Zn^{2+} dependent metalloproteinases [188].

Snake venom components could be classified as enzymatic and non-enzymatic components. The list of the venom components is given below in Table 2.1 [189].

Table 2.1: Enzymatic and non-enzymatic components of snake venom toxins.

Enzymatic components	Non enzymatic components
Phospholipase A_2 (PLA_2)	Cysteine-Rich Secretory Proteins (CRiSP)
Acetylcholinesterase (AChE)	Three-Finger Toxins (3FTx)
L-Amino Acid Oxidases (LAAO)	Disintegrins
Snake Venom Metalloprotease (SVMP)	Kunitz-type serine protease inhibitor (KSPI)
Phosphodiesterase (PDE)	C-type Lectins (CTL)
Snake Venom Serine Protease (SVSP)	Vascular Nerve Growth Factors (VNGF)
5'-Nucleotidases	Vascular Endothelial Growth Factors (VEGF)

Snake venom includes a wide range of bioactive components that performs a vast array of functions including pharmacological effects, and they have been categorized depending upon their mode of action and effects which are briefly described as follows:

a) Neurotoxins: These act within the central nervous system and their effects include respiratory difficulties and cardiac arrest. Neurotoxins disrupt the cholinergic neurons and inhibit the binding of acetylcholine, blocks movement of ions across the cellular membrane, thus blocking the transfer of signals between neurons [190]. Sea snakes, mambas, cobras, krait, and coral snakes are the few examples of snakes whose venom are reported to be neurotoxic.

b) Hemotoxins: These induce the disruption of the red blood cells mainly affecting the cardiovascular system, hematological functions, and muscular tissues (induces gangrene and scarring). Snakes belonging to Viperidae family namely, rattlesnake, cotton head and copperhead contain venom of hemotoxic nature. However, some snakes contain both hemotoxins as well as neurotoxins.

c) Cardiotoxins: These affect the heart muscle by binding to the cells of the heart and by blocking muscle contraction [191].

d) Cytotoxins: These target specific cellular sites by affecting the cell membrane or interfering with the transport of substances, or the transduction of signals across the membranes [192]. Cytotoxins are commonly found in the venom of cobras.

Even though snake venom has important toxicological effects, recent understanding of structure-function of venom compounds (proteins and peptides) points out that these substances could also be used as pharmaceutical agents [181,182,193].

2.7 Snake venom protein families with anti-cancer potential

The anticancer activity of snake venom has been described by alterations in the metabolism of cell, with a prominent impact on cancer cells in comparison to normal cells. The various protein/s from the venom of different snakes exhibiting anticancer activity have been listed in Table 2.2.

Crude venom and isolated proteins from snake venom reveal *in vitro* antitumor functions in initial assays, with definitive clinical responses in the advanced therapeutic phases.

Table 2.2: Protein/protein complex with cytotoxic activity from snake venoms

Crude venom/protein	Snake species	Cell lines	Effects	References
PLA2	*Bungarus fasciatus*	MCF 7, A549	PI3K/Akt pathway and MAP kinase pathway	Tran et. al., 2019
CT1, CT2, CT3	*Naja oxiana, Naja haje, Naja kaouthia*	A549, HL60	Lysosomal damage	Feofanov et.al., 2005
NKCT1	*Naja kaouthia*	U937, K562	Increase in Bax–Bcl2 ratio, decrease in HSP70, caspase-3 & caspase-9 dependent apoptosis	Debnath et. al., 2010
Fraction 1 (Disintegrin)	*Trimeresurus purpureomaculatus*	MCF 7	Cytotoxicity	Tan et. al., 2020
Fraction 21	*Pseudocerastes persicus*	A549, Hu02	Apoptosis	Shahbazi et. al., 2019
SVT	*Vipera lebentina turnica*	PA-1, SK-OV3	Inhibition of NF-kB and STAT3 signal, inhibition of p50 and p65 translocation	Song et. al., 2012
Cardiotoxin III (CTX III)	*Naja atra*	K562	Modulation of Bax, Bcl-XL and Endo G proteins, release of mitochondrial cytochrome c, caspase-9 and -3 dependent apoptosis	Yang et. al., 2005: 2006
CTX III	*Naja atra*	MDA-MB-231	Apoptosis *via* inactivation of JAK2, STAT3, PI3K, and Akt signaling pathways	Lin et. al., 2010
CTX III	*Naja atra*	MCF 7	Apoptosis *via* NF-κB downregulation	Chiu et. al., 2009
NN 32	*Naja naja*	EAC cells	Apoptogenic-antioxidant property	Das et. al., 2011
NN 32	*Naja naja*	U937	Apoptosis, cell cycle arrest, anti-angiogenesis activities.	Das et. al., 2013
NN 32	*Naja naja*	MCF 7, MDA-MB-231	Cytotoxicity	Attarde et. al., 2017
Crude venom	*Naja annulifera, Naja kaouthia, Ophiophagus hannah, Echis carinatus*	PaTu 8988t	Cytotoxicty, anti-metastatic and anti-angiogenesis	Kerkkamp et. al., 2018

The enzymes and proteins with promising anticancer activities include PLA_2 (cytotoxicity), L-amino acid oxidases (LAAO) (apoptosis), snake venom metalloproteinases (SVMP) (inhibitor of cell proliferation), peptides such as cardiotoxin III (antiangiogenic) and cytotoxin P4 (cytotoxicity), cytotoxins CT1, CT2 and CT3 (cytotoxicity), C-type lectins (CTLs) (cytotoxicity), disintegrins (anti-angiogenic), and snake venom serine proteases (SVSP) such as ancrod (inhibitor of tumor growth) [64,181]. The mechanism of action of these toxins include direct toxic action (PLA_2), free radical generation (LAAOs), apoptosis induction (PLA_2, SVMP and LAAOs), and anti-angiogenesis (disintegrins and CTLs) [194].

Some of the bioactive compounds of snake venom that have been reported have therapeutic properties which are anticancer in nature, and their mechanism of action has been investigated. Several studies have been conducted in various *in vitro*, *in vivo* and clinical set up for developing newer anticancer drugs following the cytotoxicity observed in different snake venom proteins [195,196] (Table 2.2). Moreover, some snake venom proteins have also demonstrated the inhibition of metastasis, which is a major reason for mortality in cancer patients. The metastatic activity depends on certain processes like cell-adhesion, migration, invading blood or lymph vessels, exiting the vessel (utilizing matrix metalloproteinases- MMPs), and ultimately interacting with the target tissues [197]. The integrins, an important cell surface receptor plays an important role in the cell–cell and cell–matrix interactions.

The initial studies on anticancer activity of snake venom on tumor cells were associated to the defibrination activity. Ancrod, a venom protein from *Agkistrodon rhodostoma*, when combined with cyclophosphamide, could induce defibrination, resulting in decrease of the tumor mass and reduced tumor spread [198]. *Pseudocerastes persicus* venom induced apoptosis in lung cancer cells A549. HPLC fraction 21 of the venom stimulated the release of Lactate dehydrogenase in normal fibroblast cells (Hu02) and increased the activity of caspase-3 and caspase-9 in A549 cells indicating apoptosis. Treatment with venom fraction 21 (10 μg/ml) resulted in 60% cell death [199]. *Vipera lebentina turnica* venom triggered apoptosis in ovarian cancer cells by inhibiting NF-κB and STAT3 signals as well as reduced the translocation of p50 and p65 into nucleus. Moreover, an increase in the expression of pro-apoptotic proteins, such as, Bax and

caspase-3 were also observed, whereas, the expression of anti-apoptotic protein like Bcl-2 was decreased [200].

There are numerous reports on different snake venom toxins families exhibiting an anticancer activity, some of which are discussed below:

2.7.1 Three-finger toxins

Three-finger toxins (3FTxs) are the major group of snake venom proteins which are present in almost all snake venoms, and 3FTxs from different snake venoms have exhibited anticancer activity in numerous studies. For instance, Cardiotoxin 3 (CTX 3) isolated from the Taiwanese cobra (*Naja atra*) inhibited metastasis in breast cancer cell (MDA-MB-231) by inhibiting signaling pathways like phosphatidylinositide (PI3K)/protein kinase B (Akt), and p38 mitogen-activated protein kinases (MAPK) [201]. Cytotoxins from *Naja* species (*N. oxiana* and *N. haje*) exhibited cytotoxicity against A549 (lung cancer) and HL-60 (promyelocytic leukemia) cells [86]. The inhibitory effects on proliferation of HL-60 were due to the activation of JNK-, mitochondrial apoptosis- and endoplasmic reticulum-pathways [202,203]. Cytotoxins accumulated in lysosomes suggested increased susceptibility of some tumor cells to cytotoxin-mediated toxicity [74,86]. CTX 3 isolated from *N. atra* venom also triggered apoptosis by increasing the expression of Bax and endonuclease G, and lowering Bcl-x levels in human erythroleukemia (K562) cells [204]. CTX 3 promoted apoptosis *via* caspase-12 activation and JNK pathway, and increased cytosolic Ca^{2+} concentration [205]. CTX 3 also induced apoptotic cell death in breast cancer cells (MDA-MB-231 and MCF-7) by inhibition of NF-κB pathway [206,207]. CTX 3 could also exhibit antimetastatic activity, since the expression of matrix metalloproteinase (MMP-9) was suppressed in a concentration dependent manner, *via* downregulation of PI3K/Akt signaling pathways and p38 MAPK, and decreased NF-κB activity which ultimately led to the inhibition of migration and invasion of cells [201].

2.7.2 Other toxins

Apart from 3FTxs, other toxins families of snake venom, including PLA_2, have also exhibited anticancer activities in various studies. For instance, Tran *et al*., reported that a

basic Asp49 PLA_2 from *Bungarus fasciatus* venom exhibited dose and time-dependent cytotoxicity towards MCF-7 and A549 cell lines but did not show toxic effect on normal HK2 cells [208]. Similarly, MCF-7 cells treated with fractionated *Pseudechis porphyriacus* venom demonstrated a range of cytotoxic effects, with fractions containing numerous proteins including acetylcholinesterase, PLA_2 and 3FTxs [209]. Anti-cancer potential of snake venom belonging to both Viperidae and Elapidae have been extensively studied in the past few decades [210-212]. For instance, a disintegrin named purpureomaculin (7.5 kDa) isolated from Malaysian *Trimerusurus purpureomaculatus* exhibited dose-dependent cytotoxicity on human breast cancer cells (MCF-7) [213]. Venom from *N. naja* and *D. russelii* which belong to the group of "Big-Four" snakes of India, as well as other medically important snake species are studied for their anticancer activity. Thangam *et al.* reported the *in vitro* anticancer effect of a disintegrin protein (64 kDa) derived from *N. naja* venom from Chennai, India against lung (A549), breast (MCF-7) and liver (HepG2) cancer cell lines [214]. In another study, Neema *et al*. described the anti-cytotoxic effect of L-amino acid oxidase from *N. naja* venom (from Haffkine Institute, Mumbai) against colon (HCT-116) and breast (MDA-MB-231) cancer cell lines [215]. Debnath *et al.* studied the anti-cancer role of *D. russelii* and *N. kaouthia* venom from Eastern India (Kolkata) which exhibited significant efficacy against leukemic cell lines (U937/K562), Echrlich ascites carcinoma (EAC) and sarcoma in mice models [212,216]. Furthermore, the purified *N. naja* venom toxin (NN-32) conjugated with gold nanoparticles (GNP-NN-32) exhibited apoptosis along with cell cycle arrest against MCF-7 and MDA-MB-231 cell lines [217].

Venom toxins belonging to Viperidae and Crotilidae family consists of SVMPs as the major toxin which demonstrated a wide range of biological activities, such as, blockade of platelet aggregation, activation of coagulation factors and fibrinolysis, and also anticancer effects like apoptosis and promotion of inflammatory responses [188]. It was reported that adhesion of cancer cell is inhibited by an SVMP (Jararhagin) isolated from *Bothrops jararaca via* the increased caspase-3 activity [218]. Another basic SVMP isolated from the venom of *T. stejnegeri* exerts alterations of cell morphology as well as prevents endothelial bladder carcinoma (ECV304) cells from proliferating [219].

Snake venom lectins which are proteins that bind to carbohydrate is another important venom component that can potentially inhibit cancer cell proliferation. C-type lectin, BJcuL, from the venom of *B. jararacussu* showed inhibitory effects on different cancer cell types including CFPAC-1 (pancreatic cancer), Caki-1 and A-498 (renal cancers) [220]. BJcuL also induced cytotoxicity against human breast carcinoma (MDA-MB-435), glioblastoma (A-172 and U87) and ovarian carcinoma (OVACAR-5) [221,222]. The cytotoxic effects of BJcuL like cell adhesion alteration and apoptosis was also seen in gastric cancer (MKN45 and AGS) cells [223]. Lebecetin, another C-type lectin isolated from *Macrovipera lebetina* venom, inhibited the adhesion, migration, and invasion of the tumor cells, thus showing the anti-integrin activity [224]. Also, snake C-type lectin-like proteins (Snaclecs), namely Lebecin, isolated from *Macrovipera lebetina* venom, inhibited the proliferation and migration of human breast cancer cells (MDA-MB-231) [225].

The LAAO isolated from the venom of *B. leucurus*, exhibited a time and dose-dependent cytotoxic effect on human adenocarcinoma (HUTU), stomach cancer (MKN-45), fibroblast (LL-24) and colorectal cancer cells (RKO) [226]. A Snake venom serine protease (SVSP) isolated from *Montivipera bulgardaghica* exhibited an inhibitory effect on human lung cancer cells (A549) [227]. Muller *et al* have reported that Crotoxin, a PLA_2 isolated from *Crotalus durissus terrificus* have exhibited pro-apoptotic effects in glioma (HCB151 and GAMG) and pancreatic cancer cells (PANC-1 and PSN-1) [228].

Snake venom contains disintegrins which inhibit integrin-dependent adhesion of cells and platelet aggregation [229,230]. Salmosin, a disintegrin purified from *Bothrops asper* venom was reported to induce apoptosis through direct interaction with integrin [231]. Similarly, contortrostatin (CN) that has been purified from *Agkistrodon contortrix* venom, showed strong binding ability with integrins in cancer and vascular endothelial cells that leads to anticancer effects. Another report suggested the liposomal delivery system for CN (LCN) which assemble at the tumor site and exert their influence affecting the growth and blood vessel formation, and suppress metastasis [232]. Yang *et al.* reported an integrin inhibitor which represents a critical category of receptors responsible for cell-to-cell and cell-matrix interactions on the cell surface [204]. The CTX 3 isolated from the venom of *N. atra* venom inhibits the MMP-9 expression as

observed from the downregulation of PI3K/Akt signaling pathways, and decreased p38 MAPK and NF-κB activities, thus suppressing metastasis in cancer cells [201].

Based on the above literature, it can be summarized that snake venom toxins trigger numerous cellular activities resulting in anticancer activity, which includes: inhibition of NF-κB, STAT3, and PI3K/Akt signaling pathways; increase in Ca^{2+} influx; ROS generation leading to increase in expression of pro-apoptotic proteins (Bax-Bcl2 ratio); cell cycle arrest; blocking of p50 and p65 movement into the nucleus (Figure 2.1). Hence, in this study the venom of *Naja kaouthia* was explored for its anticancer activity.

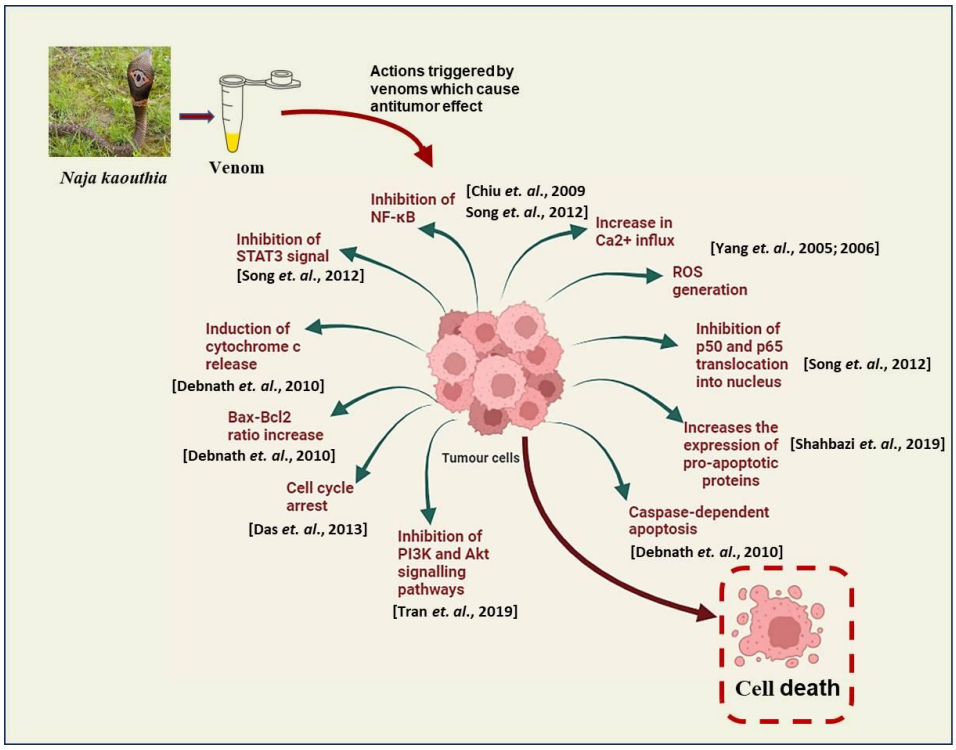

Figure 2.1: Actions triggered by snake venom toxins which leads to anticancer activity

2.8 *Naja kaouthia*

Naja kaouthia, which is commonly referred as the Monocellate cobra, or Monocled cobra, is a venomous snake which belongs to the Elapidae family and can be easily recognized due to the presence of a single 'O-shaped' or monocellate hood (Figure 2.2). The snake attains a length of 1.5 to 1.6 meters, with a maximum recorded length of 2.3 meters, hatchlings measure between 25 and 35 centimeters. The snakes's base color

varies from light brown and tan to black, and include two black spots on the lower part of the throat, and two or three broad black crossbars on the belly behind the hood. The midbody is covered by 21 (occasionally 19-23) rows of scales, with the vertebral scales being of the same size as the adjacent scales. The tail ends in a pointed tip, and the scales on the underside of the tail are divided [233].

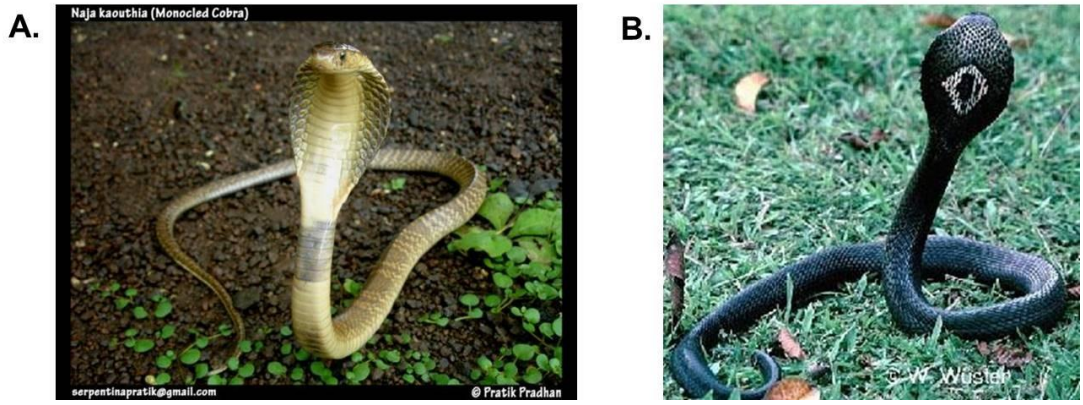

Figure 2.2: Photographs of *Naja kaouthia*. A. Frontal view (Photo Credits: Pratik Pradhan); B. Dorsal view (Photo Credits: W. Wuster). Prominent monocellate hood mark can be observed behind the head. Photo Source: www.reptile-database.org.

It is commonly distributed in South Asia (India, Bangladesh, Nepal, Myanmar and Bhutan), Southern China (Yunnan) and South East Asia (Malaysia, Thailand, Cambodia, Laos and Vietnam). In India, it is mostly found in the Eastern (Bihar, Odisha, West Bengal) and North-East states (Sikkim, Arunachal Pradesh, Assam, Tripura, Manipur, Meghalaya, Mizoram and Nagaland), and parts of Uttar Pradesh, Haryana and Delhi as well (Figure 2.3). The conservation status of the snake is listed as “Least Concern (LC)” since 2011 according to the International Union for Conservation of Nature (IUCN).

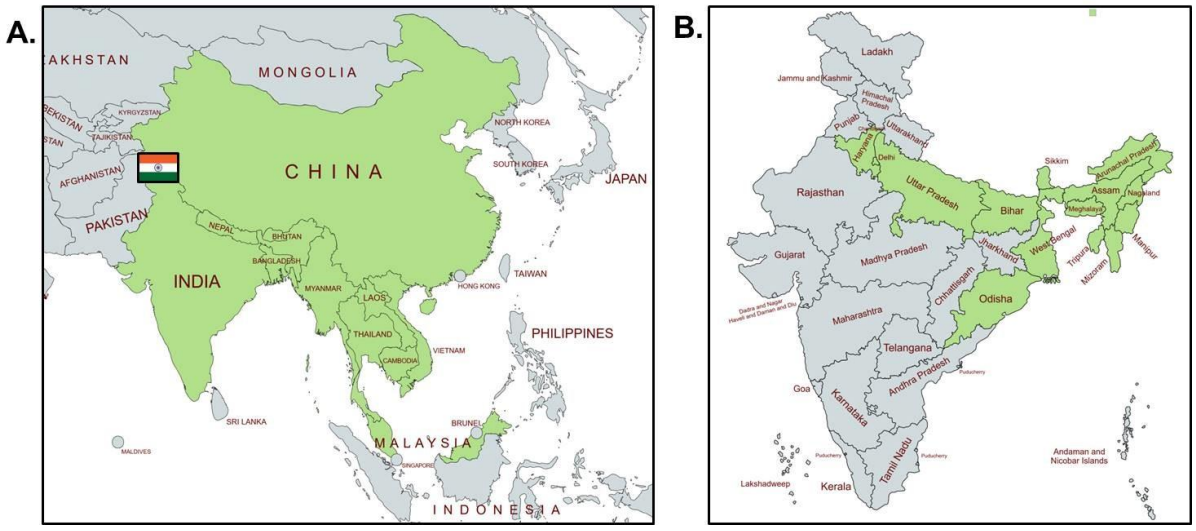

Figure 2.3: Distribution map of *Naja kaouthia* (highlighted in light green color): A. Distribution in Asia; B. Distribution in India. (Created in www.mapchart.net).

Naja kaouthia has been designated as a "Category 1" snake in India, Nepal, Bangladesh, Myanmar, Cambodia, Malaysia, Thailand and Vietnam by the WHO, which means that the snake is of highest medical importance in these countries, since it is responsible for large number of snakebites which results in numerous mortalities, morbidities and disabilities. Similarly, in China, Bhutan and Laos, it is designated as a "Category 2" snake by the WHO, which means it is of secondary medical importance in these countries, which implies that although the snake is capable of causing significant numbers of deaths and disabilities, there is a lack of sufficient data or these are less implicated in bites due to their behavior, habitat and activity cycles.

2.9 Proteomics of *Naja kaouthia* venom

Previously, proteomics study of *N. kaouthia* venom of North-East India origin has been carried out in our lab by Deka et. al [234]. In that study, *N. kaouthia* venom was fractionated using RP-HPLC in to 13 fractions. These fractions were then analyzed using LC-MS/MS approach and it was observed that a major fraction of the venom proteins belonged to 3FTxs. The 3FTx family consists of 5 sub-families, namely, short neurotoxins (SNTX), long neurotoxins (LTX), muscarinic toxin-like proteins (MTLP), weak toxins (WTX) and cytotoxins. Nine unique isoforms of SNTX were obtained from *N. kaouthia* venom in the study and the one obtained from fraction 2 exhibited sequence

homology to cobrotoxins from *N. kaouthia* and *N. naja* venoms. The WTX which belong to the non-conventional 3FTx family has been identified in fraction 3 and 5 which exhibits similarity to the WTX CM-9a from *N. kaouthia* venom. Moreover, muscarinic toxin-like proteins (MTLP) were identified from fraction 4, 5, 6 and 8 which were structurally similar to the MTLPs from mamba venom. The LTX were identified only in fraction 4, which also constituted majority (~33%) of the venom proteins, and were structurally isoforms of LTX 1 and alpha-cobrotoxin. Overall, the neurotoxins exhibited twenty isoforms from the *N. kaouthia* venom of North-East India origin. Cytotoxins were also identified in the venom fraction 7, 8, 9 and 10 and a total of 19 distinct isoforms were identified. The second major protein family identified was the PLA_2 superfamily, which has been reported from the venom of all *Naja* sp. The study reported both basic and acidic PLA_2s from *N. kaouthia* venom.

Other components of *N. kaouthia* venom included the Kunitz-type serine protease inhibitor (KSPI), which is the second most abundant non-enzymatic component of the venom after 3FTx. Proteins belonging to L-amino acid oxidase (LAAO) exhibited sequence similarity to those previously identified from *N. atra*, *Bungarus fasciatus* and *Oxyruranus scutellus*. The venom proteins identical to Natrin (*N. atra*) and Latisemin (*Laticauda semifasciata*) belonged to the Cysteine-rich secretory proteins (CRiSPs). Venom nerve growth factors (VNGFs) were also identified which exhibited sequence homology with a protein isoform from *N. sputatrix*. Similarly, Vespryns identified from the venom exhibited similarity with Thaicobrin and Ohanin from *Ophiophagus sp*. A low presence of proteases was observed in the *N. kaouthia* venom which indicates a low protease activity of the venom. Only one protein isoform of SVMP was identified which exhibited sequence homology with an isoform from *N. atra* [234].

Proteomics of *N. kaouthia* venom from other states of East India (West Bengal) and North-East India (Arunachal Pradesh and Mizoram) have also been reported [235]. Comparative donut-charts depicting the variations of snake venom protein families in the *N. kaouthia* venom from different locations, as well as with *N. naja* from Tamil Nadu (Irula) and Himachal Pradesh have been shown in (Figure 2.4). The non-enzymatic 3FTx family forms the majority (total ~64-87%) of the *Naja* venoms. For the convenience of

description, Cardiotoxins/Cytotoxins are depicted separately (~14.5-61.2%) in Figure 2.4, although they belong to 3FTx.

The PLA_2 superfamily forms the second largest (-6-30%) venom family present in the *Naja* venoms observed. Vespryn (1,67%) and the extracellular secretory protein Waprin (0.38%) have been reported only from the venom of *N. kaouthia* from Assam. The KSPI (KUN) proteins were observed in both the *N. naja* venoms (1.12-1.78%) and the *N. kaothia* venom from Assam (1.57%), however, it was absent from the venom from West Bengal, Arunachal Pradesh and Mizoram. The SVMP were present in all the *Naja* venom samples (0.49-5.07%), whereas, the CVF was present in all the *Naja* venoms (0.22-5.51%), except in *N. kaouthia* from Assam. 'Others' include venom families observed in minor or trace amounts, such as CRiSP, LAAO, VNGF, PDE, Peroxidase, 5´-Nucleotidase and Complement C3.

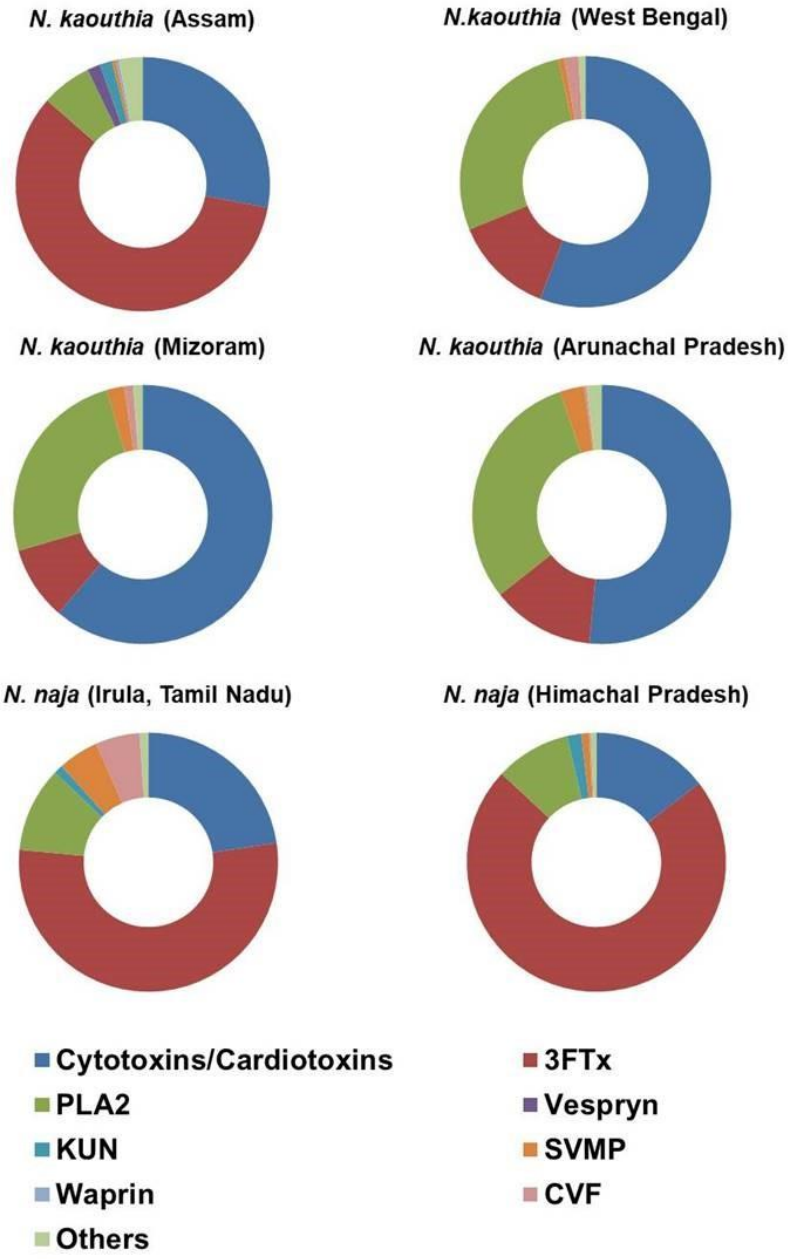

Figure 2.4: Venom protein families reported from *Naja kaouthia* and *Naja naja* from different locations of India (Adapted from Deka et al. 2019 [234] and Deka et al. 2023 [235]).

2.10 Toxicological and pharmacological effects of venom proteins present in *Naja kaouthia* venom

The venom of *N. kaouthia* causes blistering and necrosis at local bite site and elicits severe neurotoxic symptoms in envenomated victims like ptosis, dysphagia, broken neck syndrome, hypertension, and respiratory paralysis which may even lead to death if left untreated [236,237]. The various protein families identified in *N. kaouthia* is known for

various toxicological effects in envenomated victims. For instance, the WTX CM-9a from *N. kaouthia* is known to exhibit neurotoxicity in sciatic nerve tissue by lowering the compound action potential and velocity of nerve conduction [238]. Similarly, the α-neurotoxins exhibit severe neurotoxicity following envenomation, as they adhere and interact with nicotinic acetylcholine receptors (nAChRs) resulting in the post-synaptic blockade of neuro-muscular junction, leading to respiratory paralysis and even death in victims [239,240]. Cytotoxins commonly found in *N. kaouthia* venom are relatively less lethal as compared to alpha-neurotoxins, however, they are responsible for the disruption of cell membranes which lead to necrotic damage to tissues after envenomation [241,242]. The cytotoxins typically contain 59-62 amino acid residues and have two beta-folded loops with four disulfide bridges. Some of these cytotoxins are also cardiotoxins which increase the heart beat at low concentrations, whereas, they may cause cardiac arrest by modulation of ion channels at higher concentrations [86]. The high levels of cytotoxins/cardiotoxins point to the significant neurotoxic role these venom proteins play during prey capture or defense against predators of *Naja* species [243]. PLA_2s present in *N. kaouthia* venom form complexes with cytotoxins which may cause severe necrosis in envenomated victims [73,244]. Moreover, catalytically active PLA_2s are also known to induce edema in envenomated patients [245].

N. kaouthia venom (Assam) contains minor amounts of KSPI (~1.57%) which are peptides of low molecular mass (50-60 amino acids) with a conserved Kunitz motif typically observed in the bovine pancreatic trypsin inhibitor [246,247]. Most of these KSPI have typical alpha- or beta-fold structures rich in disulfide bridges and a conserved active site (p1 site) which specifically binds and inhibits serine proteases [248,249]. KSPI are reported to inhibit trypsin and chymotrypsin and are also involved in numerous biological activities like ion channel blockade, fibrinolysis, induction of inflammation and inhibition of blood coagulation [250]. KSPI may form non-covalent bonds with other protein complexes to enhance the overall toxicity of individual components. For instance, an oligomeric toxin (Taicatoxin) isolated from the venom of *Oxyuranus scutellatus scutellatus* consisted of three multimeric components, an alpha-neurotoxin of 8 kDa, a neurotoxic PLA_2 of 16 kDa, and a 7kDa KSPI linked by non-covalent bonds. The toxin was toxic to mice at a very low dose by blocking the high threshold calcium channels present at the excitable membranes of the heart [251]. Similarly, a protein

complex called the Rusvikunin complex comprising of two KSPI, were reported to be non-toxic individually in mice, however, the complex exhibited a prey-specific lethality in NSA mice at a dose of 5 mg/kg (*i.p*), which suggests that the non-toxic components may interact synergistically to enhance their overall toxicity [252,253]. Due to the presence of KSPI in small amounts in *N. naja* and *N. kaouthia* (Assam) venom, their pharmacological impact in envenomated patients is yet to be deciphered.

The venom of *N. kaouthia* (Assam) also contains a small amount of vespryns (~1.67%). The vespryn family is a group of neurotoxins which cause hyperlocomotion and hyperalgesia in experimental animals. For instance, Ohanin is a vespryn isolated from the venom of *Ophiophagus hannah* and affects the central nervous system in *in vivo* model, which indicates its role in predatory function, however, the exact mechanism of action is still unknown [254]. Another minor component of *N. kaouthia* venom is the SVMP (0.5-3.0%) also present in *N. naja* venom (Irula, Tamil Nadu) in modest amounts (~5.07%) [234,235]. Based on their structure and size these are classified into three classes, Class I (PI) of 30 kDa, Class II (PII) of 30-60 kDa, and Class III (PIII) of 60-100 kDa. The SVMPs are well-known for their hemolytic activity (PI<PII<PIII), however, they also have multiple functions, such as, fibrinogenolytic activity, prothrombin activation, factor X activator, apoptotic activity, platelet aggregation inhibition, inhibition of serine protease inhibitors and pro-inflammatory activity.

The LAAO protein family has been reported in minor quantity from *N. kaouthia* venom (0.10 to 1.07%) from Assam, Arunachal Pradesh and West Bengal, as well as from *N. naja* venom from Tamil Nadu and Himachal Pradesh [234,235]. The protein exhibits moderate lethality when injected intravenously with LD_{50} higher than the crude venom, due to which it is not considered a major lethal component owing to its low abundance (<5%) and lethality. However, they have been extensively studied in biomedical science due to their edema-inducing activity, hemorrhagic activity, anticoagulant effects, platelet aggregation, apoptosis-inducing effect, antibacterial activity, leishmanicidal activity as well as antiviral activity [255].

The CRiSP protein family also form a minor fraction of the *N. kaouthia* venom proteome (~0.90-1.46%) [234,235]. The protein families are ubiquitously found in all snake

families including Elapidae, Viperidae and Colubridae. They have been reported to block the L-type calcium channels and/or potassium channels [256]. Although some of the key signaling molecules involved in CRiSP signaling have been identified [257], however, the molecular mechanisms and their targets are still largely unknown. There is no report on acute toxicity related to this protein family, and as a result, there is currently no identifiable pathophysiological manifestation of this venom protein family in envenomated victims [258].

The pharmacological properties of cobra venoms, particularly *N. kaouthia* have been studied by few authors to understand its potential as a probable drug candidate. Kerkkamp et al. studied the *in vitro* cytotoxicity of crude *N. kaouthia* venom in pancreatic cancer cell line (PaTu 8988t) where the venom exhibited an IC_{50} of 1.42 ng/ml [259]. *N. kaouthia* venom from Eastern India have exhibited anticarcinogenic properties in previous studies. Debnath et al. have reported about 44% decrease in Ehrlich ascites carcinoma (EAC) cell count in mice treated with crude *N. kaouthia* venom (4.5 µg/kg day) for 10 days. In sarcoma mice models, the venom inhibited solid tumor growth by ~93% when treated with crude venom (1 µg/kg day) for 3 months. Moreover, *in vitro* treatment of leukemia cells (U937 and K562) with *N. kaouthia* venom exhibited a concentration dependent inhibition from 0.2 µg to 2 µg/ml after 24 h of incubation (IC_{50} 0.65 µg/ml) [212]. Another study reported a cardiotoxic-cytotoxic protein (NKCT1) of 6.76 kDa from *N. kaouthia* venom with 96% sequence similarity to Cytotoxin 3 from Indian cobra venom. NKCT1 had an LD_{50} of 2.5 mg/kg in BalbC male mice. NKCT1 also exhibited cytotoxicity against human leukemia cell lines U937 (IC_{50} = 3.5 µg/ml) and K562 (1.1 µg/ml). Morphological observations using confocal and SEM revealed fragmented nuclei and blebbed membranes with pores which suggested the formation of apoptotic bodies. Flow cytometry also revealed cells undergoing apoptosis in these cell lines and arrest of cell cycle at sub-G1 stage. Caspase-3 and caspase-9 were upregulated in a dose-dependent manner in both the cell lines after 48 hours of treatment which suggests activation of the intrinsic apoptotic pathway. Treatment with increasing dose of NKCT1 exhibited an elevation in Bax:Bcl ratio and PARP cleavage as well as downregulation of Heat-shock proteins (HSP70 and HSP90) [241].