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The myth of cobra venom cytotoxin: More than just direct cytolytic actions

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ABSTRACT

Cobra venom cytotoxin (CTX) is a non-enzymatic three-finger toxin that constitutes 40–60% of cobra venom. Thus, it plays an important role in the pathophysiology of cobra envenomation, especially in local dermonecrosis. The three-finger hydrophobic loops of CTX determine the cytotoxicity. Nevertheless, the actual mechanisms of cytotoxicity are not fully elucidated as they involve not only cytolytic actions but also intracellular signalling-mediated cell death pathways. Furthermore, the possible transition cell death pattern remains to be explored. The actual molecular mechanisms require further studies to unveil the relationship between different CTXs from different cobra species and cell types which may result in differential cell death patterns. Here, we discuss the biophysical interaction of CTX with the cell membrane involving four binding modes: electrostatic interaction, hydrophobic partitioning, isotropic phase, and oligomerisation. Oligomerisation of CTX causes pore formation in the membrane lipid bilayer. Additionally, the CTX-induced apoptotic pathway can be executed via death receptor-mediated extrinsic pathways and mitochondrial-mediated intrinsic pathways. We also discuss lysosomal-mediated necrosis and the occurrence of necroptosis following CTX action. Collectively, we provided an insight into concentration-dependent transition of cell death pattern which involves different mechanistic actions. This contributes a new direction for further investigation of cytotoxic pathways activated by the CTXs for future development of biotherapeutics targeting pathological effects caused by CTX.

Author contributions

Conceptualisation – Yap, M.K.K.; Writing of first draft – Hiu, J.J.; Review and editing of first draft – Hiu, J.J. and Yap, M.K.K.; Revision of final draft – Yap, M.K.K.; Approval and validation of final draft – Hiu, J. J. and Yap, M.K.K.

1. Introduction

Cytotoxin (CTX) is a basic polypeptide that constitutes an average of 40%–60% of the cobra venom's proteome (Feofanov et al., 2004; Yap et al., 2014). It consists of 59–62 amino acids with an approximate molecular weight ranging from 6 to 9 kDa (Hodges et al., 1987). The three-dimensional structure of CTX is characterised by five antiparallel β-pleated sheets that form three hydrophobic functional loops, as illustrated in Fig. 1. Thus, it is classified as a member of the three-finger toxin (3FTx) family (Forouhar et al., 2003; Kini, 2002; Munawar et al., 2018). Its name implies toxicity to a variety of cells. It is also known as cardiotoxin due to its direct toxicity to cardiomyocytes (Fletcher and Jiang, 1993). It is noteworthy that the structure of CTX remains conserved across different cobra species, particularly the central hydrophobic core formed by the three functional loops flanked by basic Lys and Arg residues (Gasanov et al., 2014). Unlike neurotoxin, CTX is an amphipathic protein with an overall positive charge based on the ratio of acidic Asp and Glu residues and basic Lys and Arg residues (Dubovskii and Utkin, 2014). Therefore, CTX exhibits more non-specific binding to cell membranes.

There are two major categories of CTX, namely P- and S- types owing to the presence of the amino acid residues Pro-31 and Ser-29, respectively, near the tip of the loop within the putative phospholipid binding site (Chien et al., 1994). The interaction of P-type CTX with the anionic phospholipid membranes is more prominent than the S-type due to enhanced hydrophobicity by a continuous hydrophobic patch present in P-type CTX (Dubovskii et al., 2005). Thus, P-type CTX is more cytotoxic than S-type CTX (Gasanov et al., 2015). In contrast, the presence of the polar Ser-29 residue causes the S-type CTX to exhibit hydrophilic properties and strong hydrogen bonding forces. The CTX-membrane interaction induces structural defects in the lipid membrane, which

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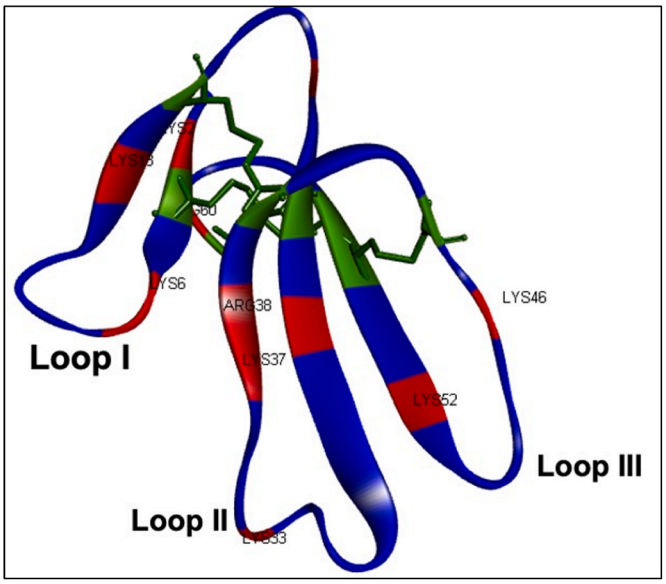

Fig. 1. The authors constructed the structure of cytotoxin (CTX) using MODELLER v9.20. The basic residues are annotated in red colour, while the disulphide bridges are annotated as green colour stick formation. It is a highly basic polypeptide consisting of 60–62 amino acid residues and it is stabilised by disulphide bonds. Like other three-finger toxins, its secondary structure comprises anti-parallel β-pleated sheets that form three hydrophobic loops, with asymmetric distribution of non-polar and polar amino acid residues. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

will further lead to downstream pathological and subsequent cytotoxic effects (Dubovskii et al., 2014; Forouhar et al., 2003; Konshina et al., 2011). Cytotoxin is found to destabilise anionic phospholipid membranes (Dubovskii et al., 2005, 2014; Konshina et al., 2011) upon interaction with the cardiolipin components of the membrane. Penetration of the functional loops of cytotoxin into the membrane is often associated with structural changes in the toxin (Dubovskii et al., 2017).

Local tissue damage is a major clinical macroscopic observation that results from cobra envenoming. This is often attributed to the various active components present in cobra venom (Ciszowski and Hartwich, 2004; Kularatne et al., 2009; Rivel et al., 2016; Wong et al., 2010), including the high abundance of CTX in the cobra venom. The toxin contributes to cytotoxicity which eventually causes tissue necrosis (Gasanov et al., 2014). Moreover, CTX exhibits relatively lower bioavailability compared to other cobra toxins (Yap et al., 2014) indicates a substantial unabsorbed amount due to high binding affinity at the biting site (Guo et al., 1993), suggesting severe pathological outcomes that often leaving victims permanently disfigured.

The exact molecular mechanisms of cytotoxicity remain inconclusive because few competing hypotheses have been proposed: not only physical interaction with the cell membrane but also activation of intracellular signalling cascades (Dubovski and Utkin, 2015; Wang et al., 2006). Although the clinical observations resulting from snakebite envenomation are mostly dermonecrosis, most laboratory findings have reported CTX-induced apoptosis (Chen et al., 2008; Chong et al., 2020; Körper et al., 2003; Wu et al., 2013). Moreover, CTX-induced necroptosis has recently been introduced as another mode of cell death in addition to apoptosis and necrosis (Hiu and Yap, 2021). Recently, researchers have focused on natural resources, particularly animal venoms, for the development of new anticarcinogenic drugs (Chaisakul et al., 2016; Moga et al., 2018). Given that CTX exhibits antiproliferative activity against cancer cell lines, it has been suggested as a potential candidate for anticancer therapy (Gasanov et al., 2014). Extensive studies have revealed varying cytotoxic effects exerted by CTXs on different normal and cancer cell lines (Tables 1 and 2). It can be deduced that, the sequence variations in CTXs from different cobra species result in differential cytotoxicity and cell death pathways (Feofanov et al., 2004). In addition, geographical variation can also affect the venomic profiles, and thus the cytotoxicity of the same cobra species (Tan et al., 2015a). The proportion of CTXs in different cobra venom in different cobra venom from diverse geographical locations is summarised in Fig. 2.

2. Biophysical interactions of cytotoxin with membrane

Although the exact mechanism of the CTX-membrane interaction has yet to be established, it is suggested that the binding of CTX to membranes involves three-fingered structural loops. Membrane permeabilisation of CTX requires structural stability upon some auxiliary interactions with the membranes (Dubovskii et al., 2017; Levtsova et al., 2009). The hydrophobicity of the functional loops of CTX allows its penetration into hydrocarbon regions of the cellular membrane which consists of various lipid compositions (Dufton and Hider, 1988). The harrow conformation of CTX plays a crucial role during the penetration into the lipid bilayer membrane system, whereby only loops II and III penetrate the lipid membrane bilayer, as demonstrated by coarse-grained molecular dynamics (CGMD) simulation (Su and Wang, 2011). Furthermore, the initial membrane binding of CTX involves electrostatic interaction, whereas the hydrophobic insertion of the CTX molecule is prevented because of the higher pressure of insertion exerted at the upper leaflet of the membrane lipid bilayer (Dubovskii et al., 2014). At lower CTX concentrations, the CTX-lipid interaction relies on the degree of saturation of fatty acids in hydrophobic parts of the membranes (Dyba et al., 2021). Subsequently, hydrophobic partitioning occurs when CTX penetrates the upper membrane leaflet in an edgewise manner through the incorporation of the tips of the CTX structural loops. As CTX binds to membrane lipids stoichiometrically, an isotropic phase is formed, resulting in an overall neutral CTX-lipid complex system (Dubovskii et al., 2014). The transition from the electrostatic interaction

Table 1
Cytotoxicity of various cytotoxins from different cobra species against different normal cell lines.

Species	CTX type	Cell types	IC_{50}/LD_{50}	References
Naja atra	CTX III	H9C2 rat cardiomyocyte cells	2 μM	Stevens-Truss et al., 1996
Naja oxiana	NA	MDCK normal dog kidney cells	47.1 μg/mL	Ebrahim et al. (2016)
		L929 normal mouse fibroblast cells	NA	Strizhkov et al. (1994)
Naja sumatrana	P-type CTX	RWPE-1 prostate epithelial cells	0.35 ± 0.08 μg/mL	Chong et al. (2020)
		184B5 breast epithelial cells	6.21 ± 0.37 μg/mL	
		NL20 lung epithelial cells	1.91 ± 0.52 μg/mL	
Naja kaouthia	S-type CTX	RWPE-1 prostate epithelial cells	0.65 ± 0.20 μg/mL	
		184B5 breast epithelial cells	2.83 ± 0.34	
		NL20 lung epithelial cells	2.76 ± 0.49	
Naja nigricollis	CTX-1N	mouse red blood cells (RBC)	>90 μM	Conlon et al. (2020)
	CTX-2N	mouse red blood cells (RBC)	45 ± 3 μM	
	CTX-3N	mouse red blood cells (RBC)	NA	
	CTX-4N	mouse red blood cells (RBC)	>90 μM	

Table 2
Cytotoxicity of various cytotoxins from different cobra species against different cancer cell lines.

Species	CTX type	Cell types	IC_{50}/LD_{50}	References
Naja atra	CTX III	K562 human leukaemia cells	1.7 μg/mL	Yang et al. (2005)
		Human leukemic T-lymphocytes	2 μM	Stevens-Truss et al., 1996
		CAL27 oral squamous carcinoma cells	0.28 μM	Chien et al. (2010)
		SAS human tongue carcinoma cells	0.35 μM	
		Ca9-22 oral squamous carcinoma cells	0.15 μM	
Naja haje	CTX I	A549 human lung adenocarcinoma cells	132 ± 9 μg/mL	Feofanov et al. (2005)
		HL60 promyelocytic leukaemia cells	2.6 ± 0.1 μg/mL	
	CTX II	A549 human lung adenocarcinoma cells	116 ± 6 μg/mL	
		HL60 promyelocytic leukaemia cells	1.9 ± 0.1 μg/mL	
Naja naja	CTX III	K562 human leukaemia cells	2.63 μg/mL	Chen et al. (2009)
		MDA-MB-231 human breast cancer cell line	NA	Tsai et al. (2016)
	NN-32	U937 cell human leukaemia cells	NA	Das et al. (2013)
Naja oxiana	CTX I	A549 human lung adenocarcinoma cells	16.6 ± 0.6 μg/mL	Feofanov et al. (2005)
		HL60 promyelocytic leukaemia cells	0.58 ± 0.03 μg/mL	
	CTX II	A549 human lung adenocarcinoma cells	1.7 ± 0.1 μg/mL	
		HL60 promyelocytic leukaemia cells	0.33 ± 0.02 μg/mL	
		K562 human leukaemia cells	NA	Strizhkov et al. (1994)
	CTX	HepG2 human hepatocellular carcinoma cells	26.59 μg/mL	Ebrahim et al. (2016)
		MCF-7 human breast cancer cells	28.85 μg/mL	
		DU145 human prostate carcinoma cells	21.17 μg/mL	
Naja sumatrana	P-type CTX	A549 lung cancer epithelial cells	0.88 ± 0.06 μg/mL	Chong et al. (2020)
		PC-3 prostate epithelial cells	3.13 ± 0.58 μg/mL	
	*suma*CTX	MCF-7 breast cancer cells	3.89 ± 0.39 μg/mL	Teoh and Yap (2020)
Naja kaouthia	S-type CTX	A549 lung cancer epithelial cells	1.22 ± 0.09 μg/mL	Chong et al. (2020)
		PC-3 prostate epithelial cells	4.46 ± 0.36 μg/mL	
		MCF-7 breast cancer cells	12.23 ± 0.74 μg/mL	
		HL60 promyelocytic leukaemia cells	0.18 ± 0.01 μg/mL	Feofanov et al. (2005)
	CTX I	U937 human leukaemia cells	3.5 μg/mL	Debnath et al. (2010)
		K562 human leukaemia cells	1.1 μg/mL	
Naja nigricollis	CTX-1N	A549 non-small cell lung adenocarcinoma cells	0.8 ± 0.2 μM	Conlon et al. (2020)
		MDA-MB-231 breast adenocarcinoma cells	7 ± 1 μM	
		HT-29 colorectal adenocarcinoma cells	9 ± 1 μM	
		HUVEC human umbilical vein endothelial cells	7 ± 1 μM	
	CTX-2N	A549 non-small cell lung adenocarcinoma cells	1.4 ± 0.2 μM	
		MDA-MB-231 breast adenocarcinoma cells	6 ± 1 μM	
		HT-29 colorectal adenocarcinoma cells	8 ± 1 μM	
		human umbilical vein endothelial HUVEC cells	7 ± 1 μM	
	CTX-3N	A549 non-small cell lung adenocarcinoma cells	7 ± 1 μM	
		MDA-MB-231 breast adenocarcinoma cells	>30 μM	
		HT-29 colorectal adenocarcinoma cells	>30 μM	
		HUVEC human umbilical vein endothelial cells	22 ± 2 μM	
	CTX-4N	A549 non-small cell lung adenocarcinoma cells	0.9 ± 0.2 μM	
		MDA-MB-231 breast adenocarcinoma cells	8 ± 1 μM	
		HT-29 colorectal adenocarcinoma cells	25 ± 3 μM	
		HUVEC human umbilical vein endothelial cells	2 ± 0.2 μM	
Naja ashei	CTX	Human histiocytic lymphoma U-937 cells	126.80 ± 2.94 mg/L/1 × 10^6 cells	Dyba et al. (2021)
		HL60 promyelocytic leukaemia cells	121.29 ± 1.42 mg/L/1 × 10^6 cells	

(non-insertion mode) between the CTX and membrane lipid to the hydrophobic insertion (insertion mode) has been shown to involve the orientation and localisation of the hydrophobic tips of all three loops (Dubovskii et al., 2005; Ma et al., 2002). This allows the embedding of CTX in the membrane interior. The rigidity of the hydrophobic loops is enhanced by the formation of a salt bridge between the Asp 57 and Lys 2 side chains (Kao et al., 2009; Lo et al., 1998). The insertion of CTX into the membrane results in lipid dehydration of the local spots which consequently induces vesicle fusion (KholodovaIu, 1981; Gasanov et al., 1990a,b). The extent of vesicle fusion depends on the lipid composition and is hypothesised to be directly proportional to the anionic lipid content of membranes (Dubovskii et al., 2014). This is followed by CTX oligomerisation and pore formation which ultimately contribute to the leakage of intracellular contents (Chen et al., 2007; Dufourcq et al., 1982; Forouhar et al., 2003).

Moreover, CTX-membrane insertion and internalisation are stepwise lipid-dependent mechanisms (Wang et al., 2006). The pore formation process is glycosphingolipid (SGC)-dependent which requires a series of coupling interactions: (1) begins with binding of CTX monomer to the SGC molecule, (2) followed by dimerization of the CTX, and (3) alteration of the conformation of SGC that ultimately induces CTX oligomerisation. In addition, CTX is also shown to be internalised into the cells and co-localised with mitochondria. The membrane-damaging activity of CTX is highly dependent on the phosphatidylserine (PS) lipid present on the membranes (Konshina et al., 2011). In addition, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) has also been demonstrated to be involved in complex formation with CTX from *Naja atra* and *Naja naja* venoms (Gorai et al., 2016). Moreover, CTX exhibits favourable interactions with membrane cholesterol to facilitating its endocytosis into cells (Lee et al., 2014; Wang et al., 2006). It can be concluded that CTX interacts with various anionic/zwitterionic lipids in the membrane which promotes their ability to induce membrane deterioration.

The differential binding affinity of CTX is determined by the distinct distribution of hydrophobic amino acid residues and the spatial configuration of the three-fingered loops (Dubovskii et al., 2005). The binding domains of CTX are formed by residues 5–11, 46–50, and 24–29, 31–37 which mostly consist of hydrophobic amino acids encompassing the tips of loops I to III (Dubovskii et al., 2001). Notably, the Lys residues at the penetrating domain also allow the anchoring of the CTX as its polar group is found near the micelle/water interface while the non-polar residues can extend to the hydrophobic core of the membrane bilayer (Dubovskii et al., 2001). Moreover, the degree of membrane-damaging activity is also highly influenced by different lipid

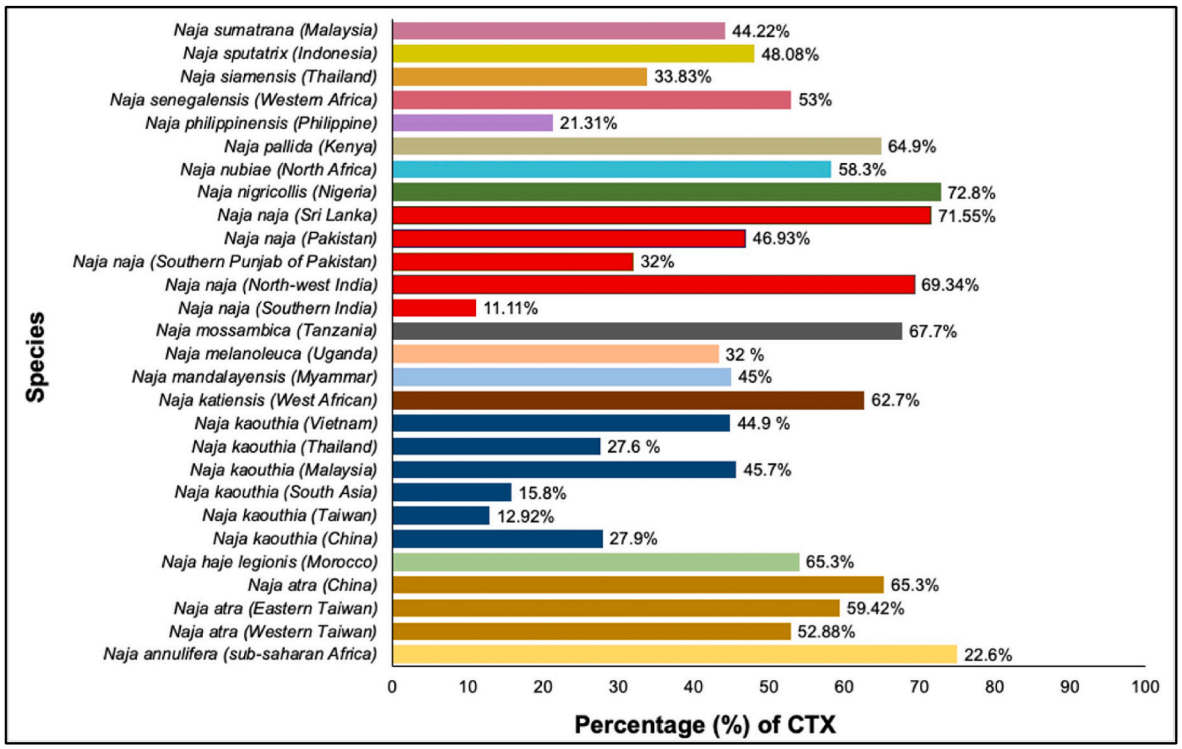

Fig. 2. The percentage of cytotoxin in different cobra venom from diverse geographical locations. On average, 47% of cobra venom's dry weight consists of cytotoxin. The percentage of cytotoxin is acquired from the venomics profile of different cobra species (Asad et al., 2019; Beraldo et al., 2021; Chanda et al., 2018; Choudhury et al., 2017; Huang et al., 2015; Lauridsen et al., 2017; Liu et al., 2017; Malih et al., 2014; Petras et al., 2011; Shan et al., 2016; Sintiprungrat et al., 2016; Tan et al., 2015b; Tan et al., 2017, 2019, 2020; Wong et al., 2018, 2021; Xu et al., 2017; Yap et al., 2014).

compositions of the membranes which contributes to the charge differences of the lipid polar parts (Dyba et al., 2021). In addition to pore formation, CTX can interact with membrane acceptors to form oligomeric transmembrane channels that lead to ion fluxes (Dufton and Hider 1988; Ksenzhek et al., 1978). The formation of ion channels induces osmotic fragility and eventually cytolysis (Dufton and Hider 1988; Thelespam and Möllby, 1979). In brief, extensive evidence demonstrates that the CTX-membrane interaction is highly attributed to the hydrophobic three-finger loops of CTX. The penetration of CTX into the hydrocarbon region of the membrane is a plausible explanation for CTX-induced compromised membrane integrity.

3. Activation of cell death pathways

3.1. Apoptosis

Most studies have shown CTX-induced cell death by activating different apoptotic pathways. Apoptosis, commonly known as programmed cell death, is a regulated cell elimination event resulting from the activation of cysteine-aspartic proteases (caspases) via extrinsic death receptors and/or intrinsic mitochondrial signalling pathways (Feinstein-Rotkopf and Arama, 2009; Hengartner, 2000; Thornberry and Lazebnik 1998).

Cytotoxin has been reported to induce calcium (Ca^{2+}) influx which causes elevated Ca^{2+} levels in the cytosol (Langone et al., 2014). Cytotoxin from *Naja atra* venom induces apoptosis via the Ca^{2+}/protein phosphatase 2 A (PP2A)/5′ adenosine monophosphate-activated protein kinase (AMPK) axis in U937 human myeloid leukaemia cell line at concentrations of 150 nM (Chiou et al., 2019). The Ca^{2+}/PP2A/AMPK signalling pathway is initiated by the CTX-mediated Ca^{2+} influx that leads to PP2A degradation, followed by phosphorylation of AMPK to trigger mitochondrial fragmentation, lysosomal disruption, phosphorylation of p53, and the activation of mitochondrial apoptotic pathway (Baumann et al., 2007; Langone et al., 2014; Sekar et al., 2018; Toyama et al., 2016).

Moreover, CTX has been shown to trigger reactive oxygen species (ROS) generation which leads to apoptosis (Chen et al., 2008; Chiou et al., 2021). Cytotoxin-induced ROS production is associated with elevated intracellular Ca^{2+} levels, followed by the overexpression of NADPH oxidase 4 (NOX4), a major contributor to intracellular ROS (Chiou et al., 2021). The downstream events involve the activation of p38 MAPK/c-Jun-mediated Fas and p38 MAPK/ATF-2-mediated FasL cell death signalling pathways. On the other hand, upregulation of the Fas gene has also been observed after CTX injection in mouse skeletal muscle cells (Hirata et al., 2003). Fas/FasL-mediated apoptosis is commonly characterised as the death receptor (DR) signalling pathway which involves the recruitment and activation of caspase-8 and caspase-10 (Green and Llambi, 2015; Wang et al., 2001; Yamada et al., 2017).

Extrinsic apoptosis pathways also involve phosphorylation of c-Jun N-terminal kinase (JNK), a mitogen-activated protein kinase (MAPK) mediated pathway. For example, CTX-VI from *Agkistrodon acutus* (Zhang and Cui 2007) was found to trigger JNK phosphorylation to stimulate the receptor-mediated extrinsic apoptosis pathway (Park et al., 2012a, 2012b), which also involves the overexpression of Fas-FasL (Zhang and Cui, 2007). FAS-associated death domains (FADDs) are important for the execution of DR-extrinsic apoptosis which is crucial for the activation of caspase-8. Therefore, the extrinsic DR pathway may be one of the mechanisms that explain the CTX-induced apoptosis.

In addition to the extrinsic DR signalling pathways, CTX also triggers intrinsic mitochondrial-mediated apoptosis (Chien et al., 2008; Yang et al., 2005, 2007). It has been suggested that, after internalisation, CTX is co-localised with mitochondria and disrupts the mitochondrial network that leading to mitochondrial fragmentation (Gasanov et al., 2015; Zhang et al., 2019). This is attributed to an interaction between CTX and cardiolipin components of the mitochondrial membrane

(Aripov et al., 1989; Zhang et al., 2019). Arg, Lys and Leu are the major amino acid residues that facilitate the binding of CTX to the outer mitochondrial membrane (OMM) (Li et al., 2020). Apart from the direct membrane-interacting action, the CTX-activated AMPK pathway can also mediate mitochondrial fragmentation via phosphorylation of the mitochondrial fission factor (Toyama et al., 2016). Outer mitochondrial membrane permeabilisation is often coupled with an alteration of mitochondrial membrane potential ($\Delta\Psi_m$) and subsequent activation of apoptotic pathways (Körper et al., 2003). Teoh and Yap (2020) have also demonstrated a dose-dependent change in $\Delta\Psi_m$ with an initial mitochondrial hyperpolarisation at low CTX concentration (approximately 2 μg/mL) followed by mitochondrial membrane depolarisation when the CTX levels increase. A notable alteration in mitochondrial membrane potential was also observed in human breast adenocarcinoma cells after exposure to CTX-II from *Naja oxiana* (Ebrahim et al., 2014). Following the impairment of $\Delta\Psi_m$, cytochrome *c* is released from mitochondria. The mitochondria-released cytochrome *c* binds to apoptotic protease-activating factor 1 (APAF 1) and promotes its oligomerisation (Zou et al., 1997). Subsequently, the initiator caspase-9 is recruited to the oligomer to form an apoptosome (Yuan et al., 2010). After its processing, the activated caspase-9 cleaves and activates the executioner caspase-3 which directs the cell to undergo apoptosis (Ebrahim et al., 2014; El Hakim et al., 2011; Green and Llambi, 2015; Wang and Wu, 2005). Cytotoxin from *Naja oxiana* triggers a time-dependent increase in caspase-3 activity in human promyelocytic leukaemia, hepatocellular and prostate carcinoma (Ebrahim et al., 2015). Similarly, CTX from *Naja sumatrana* venom could also activate caspase-3 and -7 at its IC_{50} (4 μg/mL), following deprivation of $\Delta\Psi_m$ (Teoh and Yap, 2020).

Furthermore, several studies have also indicated that CTX-induced apoptosis is accompanied by the upregulation of proapoptotic proteins including Bax, Bad, and endonuclease G, as well as the downregulation of anti-apoptotic proteins such as Bcl-2, Bcl-XL, Mcl-1, X-linked inhibitor of apoptosis protein (XIAP), and survivin (Chien et al., 2010; Lin et al., 2010; Su et al., 2010; Tsai et al., 2006; Yang et al., 2006). Together with the modulation of apoptotic proteins, CTX induces apoptosis via the concomitant inactivation of epidermal growth factor receptor (EFGR), phosphatidylinositol 3-kinase (Pl3K)/Akt, and Janus tyrosine kinase 2 (JAK)/signal transducer and activator of transcription 3 (STAT3) apoptotic signalling pathways (Chien et al., 2010; Lin et al., 2010; Su et al., 2010; Tsai et al., 2016). Altogether, these findings suggest that cobra venom CTX induces both extrinsic and intrinsic apoptotic pathways.

3.2. Cell cycle arrest

In addition to apoptotic cell death, cell cycle arrest has been reported in MCF-7 and K562 cells with the treatments of 4 μg/mL and 0.3 μM of CTX, respectively (Ebrahim et al., 2014; Yang et al., 2007). This is likely due to the inhibition of protein kinase C (PKC) (Chiou et al., 1993), whereby PKC plays a significant role in all stages of the cell cycle (Black and Black, 2013). Cytotoxin-induced intrinsic apoptosis is associated with cell cycle arrest at the sub-G1 stage and an increase in hypoploid DNA content (Debnath et al., 2010). This is corroborated by the findings reported by Chien et al. (2008) who reported an increase in DNA fragmentation and poly (ADP-ribose) polymerase (PARP) cleavage upon CTX treatment in HL-60 cells. PARP cleavage is caused by an elevated Bax/Bcl-2 ratio which initiates downstream signalling cascades for nuclear DNA fragmentation, and eventually apoptosis. Under normal physiological conditions, the cell cycle is divided into 4 phases: Gap 1 (G1) phase, S phase, Gap 2 (G2) phase, and M phase (Hunt et al., 2011). The G2/M phase in the cell cycle is terminated by CTX as evidenced by the downregulation of G2/M regulatory proteins including cyclin A, cyclin B1, cyclin-dependent kinase 2 (Cdk 2), and the cell division cycle 25C (Cdc25C) (Yang et al., 2007). In addition, CTX induces apoptosis through S-phase arrest and the inactivation of proto-oncogene tyrosine-protein kinase (Src) in a time- and dose-dependent manner (Chien et al., 2010). A remarkable decline in cell cycle regulatory proteins which including cyclin A, cyclin B, and CDK1 is also observed following S-phase arrest (Chien et al., 2010). Cyclins and Cdks are positive regulators of cell cycle progression (McGowan, 2003). Additionally, the progression of the cell cycle is also regulated by Src via the PI3K, STAT3, and Akt signalling pathways (Liu et al., 2013). As CTX exerts cytotoxicity by diminishing these cell cycle regulators, it is convincing that intrinsic apoptosis is a CTX-mediated cell death pathway.

3.3. Necrosis and necroptosis

Necrosis is an uncontrolled mode of cell death that occurs due to extensive damage or severe stress that often includes mechanical stress or perturbations of both extracellular and intracellular environments (Green and Llambi, 2015). The hallmarks of cellular necrosis are characterised by cell swelling and rupture of plasma membranes which causes cytoplasmic leakage (Yuan and Kroemer, 2010). Although necroptosis exhibits similar cellular morphological changes, it is a caspase independent regulated cell death (Dhuriya and Sharma, 2018) which involves the death receptors Fas and tumour necrosis factor receptor 1 (TNFR1) (Galluzzi et al., 2014; Vercammen et al., 1997). Sequential cascade events of necroptosis are regulated by receptor-interacting protein (RIP) kinase (Degterev et al., 2005; Zhang et al., 2009).

Given the ability of CTX to interact with phospholipid membranes, the lysosomal membrane could be another target for the internalised CTX to execute its cytotoxicity (Li et al., 2020). This is supported by Feofanov et al. (2005) who demonstrated a remarkable accumulation of CTX in lysosomes. Cytotoxin-induced lysosomal cell death leads to the release of cathepsin B protease, a lysosomal cysteine enzyme that causes cell death, compromising lysosomal membrane integrity (Aits and Jäättelä, 2013; Liu et al., 2019; Wu et al., 2013). Cathepsin B elicits various physiological functions in the digestive system, circulatory system, cell proliferation, and cell death mechanisms (Patel et al., 2018). It is noteworthy that the cathepsin-mediated cell death pathways have been extensively studied including apoptosis, necrosis, and autophagy (Foghsgaard et al., 2001; Kaminskyy and Zhivotovsky, 2012; Patel et al., 2018; Turk and Stoka 2007). The occurrence of necrosis depends on the amount of cathepsin released, which correlates with the degree of lysosomal rupture (Turk and Stoka, 2007). A moderate level of cathepsin activates apoptosis whereas higher levels of cathepsin trigger necrotic cell death (Ebrahim et al., 2014). Lysosomal-mediated apoptosis is also associated with the mitochondrial-dependent intrinsic pathway. This is because cathepsin also promotes mitochondrial membrane permeabilisation, thus elevating pro-apoptotic proteins' levels and releases cytochrome *c* (Aits and Jäättelä, 2013).

Loss of lysosomal integrity appears to be correlated with the exposure levels of CTX, as observed in *N. oxiana* CTX-II treated human leukaemia, breast, prostate, and liver cancer cells (Ebrahim et al., 2015). Similar observations have been reported for CTX-I from *N. atra* venom which induces lysosomal membrane permeabilisation that leads to the release of cathepsin B (Aits and Jäättelä, 2013; Liu et al., 2019; Wu et al., 2013). Pathological dermonecrosis is also observed when a minimal necrosis dose of *N. atra* venom CTX was administered intradermally into a mouse model (Liu et al., 2020), further explaining CTX-induced necrosis. Similar in vivo necrosis was observed in the skeletal muscle cells of mice following intramuscular injection. (Ownby et al., 1993).

Thus far, necroptosis has only been reported in a few CTX from different *Naja* venoms. For example, CTX1 from *N. atra* venom has been reported to induce necroptosis in HL-60 and KG1a leukaemia cells (Liu et al., 2019), as the survival of these cells is rescued after addition of necroptosis inhibitor necrostatin-1, indicating that necroptotic cell death is executed by CTX1. On the other hand, high concentration of CTX (29.8 μg/mL) from *N. sumatrana* venom has also been found to activate necroptosis in MCF-7 breast cancer cells through elevation of HSP90AA1, HSP90AB1 and peptidyl prolyl isomerase in the cells (Hiu and Yap, 2021).

It is noteworthy that the CTX exhibits selectivity towards different cell lines, resulting in various degrees of cytotoxicity. On average, 47% of cobra venom's dry weight is CTX (Fig. 2), which appears to predominant toxin in the venom. Thus, it contributes significantly to the clinical manifestation of dermonecrosis involving cutaneous, muscular, and connective tissues (Iddon et al., 1987; Rivel et al., 2016; Wong et al., 2010). In a pilot clinical study of N. atra envenomation (Lin et al., 2022), only 14.2–1541.4 ng/mL of CTX were detected in the patients' excised necrotic tissues, which was substantially lower than the IC_{50} reported in laboratory findings (21.93 μg/mL, Huang et al., 2019). Besides, *N. atra* venom contains approximately 54.17% of CTX which could induce necrotic lesions in the in vivo model upon administration of a dose of 30 μg (Liu et al., 2020). The pathological effects of CTX in causing significant dermonecrosis are likely attributed to its relative abundances and concentrations at local envenomed sites (MOH Malaysia, 2017; Rivel et al., 2016; Liu et al., 2020). This further supports the clinical observation of dermonecrosis caused by CTX.

The mechanistic actions of CTX are summarised in Fig. 3.

Abbreviation

FAS/FASL, Fas and Fas Ligand; TNFR1, tumour necrosis factor receptor 1; PP2A, protein phosphatase 2; AMPK, AMP-activated protein kinase; Cyt C, cytochrome *c*; APAF 1, apoptotic protease-activating factor 1; CASP-3, caspase-3; CASP-8, caspase-8; CASP-10, caspase-10; MAPK, mitogen-activated protein kinase; EFGR, epidermal growth factor receptor; PI3K, phosphatidylinositol 3-kinase; Akt, RAC-alpha serine/threonine-protein; JAK, Janus tyrosine kinase 2; STAT, signal transducer and activator of transcription 3; ROS, reactive oxygen species; NOX4, NADPH oxidase 4; RIPK, receptor interacting protein kinases; HSP90AA1, heat shock protein 90 alpha family class A member 1; HSP90AB1, heat shock protein 90 alpha family class B member 1; PPIA, peptidyl prolyl isomerase.

4. Concentration-dependent transition of cell death pattern

A few hypotheses have been proposed to explain the molecular mechanisms elicited by CTX; not only biophysical alteration of cell membranes causes cytolytic action, but also involves activation of apoptotic pathways and cell cycle arrest, as discussed above. In contrast, CTX can produce necrotic features in dead cells such as membrane permeabilisation. It has also been found that CTX readily accumulates in lysosomes without disrupting the plasma membrane, resulting in necrosis. Differential cell death patterns exerted by CTX are likely to be attributed to the concentration of CTX whereby, the apoptotic effects may only be observed in a limited range of toxin concentrations (Ebrahim et al., 2015); the mode of cell death may rapidly switch from apoptosis to hypothetical necrosis when the concentration increases. This phenomenon was observed in *N. oxiana* venom CTX-I and CTX-II (Ebrahim et al., 2015). The percentage of necrotic cells significantly increased beyond a specific concentration, in different cell lines: MCF-7 (CTX-I, 10.24 μg/mL; CTX-II, 5.85 μg/mL), HepG2 (CTX-I, 41.33 μg/mL; CTX-II, 28.98 μg/mL), DU-145 (CTX-I, 26.14 μg/mL; CTX-II, 4.26 μg/mL), and HL-60 (CTX-I, 28.14 μg/mL; 14.87 μg/mL).

Both CTX-I and CTX-II caused membrane perturbation as demonstrated by a surge in extracellular lactate dehydrogenase (LDH) activity. Furthermore, CTX-I and CTX-II induced concentration and time-dependent apoptosis via the cathepsins-mediated lysosomal pathway. Apoptotic effects were observed only in a limited range of toxin concentrations (<2 μg/mL). The cell death patterns changed rapidly from apoptosis to necrosis when toxin levels increased beyond 8 μg/mL in

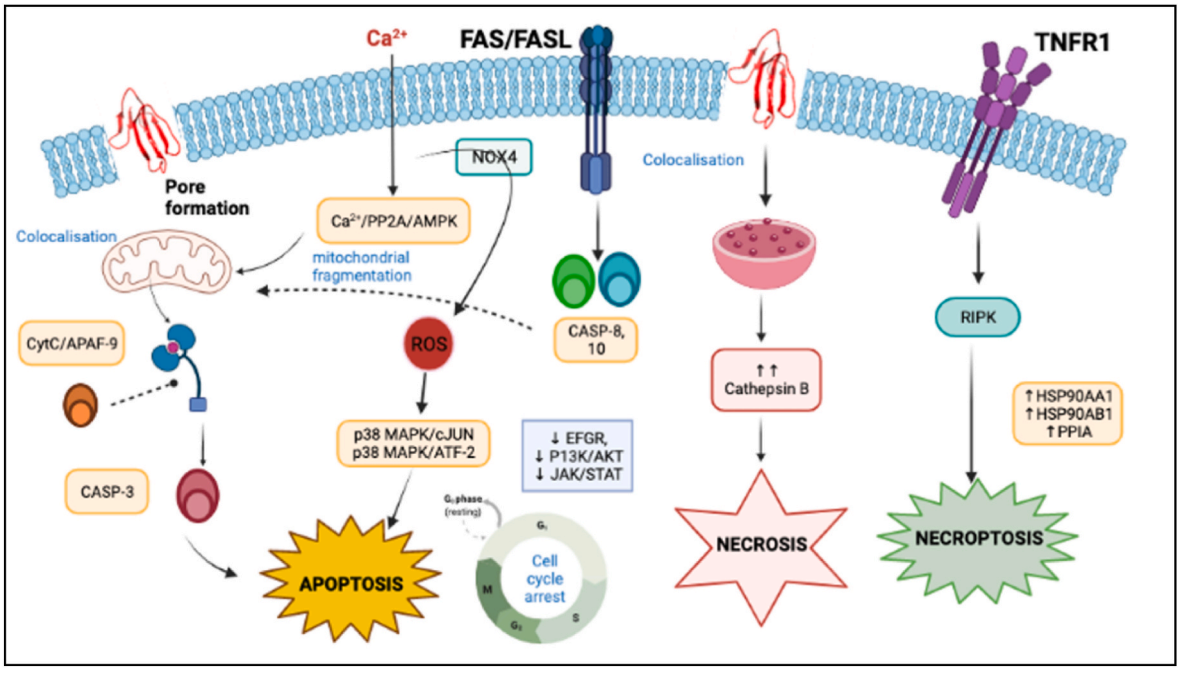

Fig. 3. A summary of the mechanistic actions of cobra venom cytotoxin (CTX) was created using BioRender.com. The basic hydrophobic loops of CTX interact with the phospholipid bilayer and destabilise cell membranes for cytolysis. Nevertheless, various intracellular cell death signalling pathways have been targeted by CTX. Cytotoxin triggers Ca^{2+} influx and activation of the Ca^{2+}/PP2A/AMPK pathway. This causes mitochondrial fragmentation. Furthermore, the internalised CTX following membrane permeabilisation could also co-localise at mitochondria to stimulate intrinsic mitochondrial-mediated apoptosis. Cytochrome *c* is then released following deprivation of mitochondrial membrane integrity and bind to APAF 1, which then recruits caspase-9 to form an apoptosome. The apoptosome activates the executioner caspase-3 for intrinsic apoptosis. Apoptosis is also associated with upregulation of proapoptotic proteins and inactivation of EFGR, Pl3K/Akt, and JAK/STAT3 pathways. In addition, CTX also induces extrinsic apoptosis involving Fas receptors. In addition, intracellular accumulation of Ca^{2+} activates NOX4 expression, a major contributor to oxidative stress which triggers p38 MAPK/cJUN/ATF-2 apoptosis pathways. Cell cycle arrest is a downstream cytotoxic effect of CTX. On the other hand, lysosomal-associated necrosis has also been reported for CTX-induced cell death, attributed to highly elevated cathepsin B following lysosomal membrane permeabilisation. Besides necrosis, CTX also induces caspase-independent, regulated necroptosis involving activation of TNFR1-RIPK signalling cascades, which is also associated with upregulation of antiapoptotic proteins such as HSP90AA1, HSP90AB1 and PPIA.

MCF-7 cells (Ebrahim et al., 2014). Furthermore, the time-dependent manner of CTX-induced cell death has also been reported in MCF-7 cells, whereby a significant elevation of apoptotic cells was detected with prolonged exposure time at 24 h (Ebrahim et al., 2014).

Similarly, a concentration-dependent transition mechanism of cytotoxicity was also reported in the *N. sumatrana* CTX, namely *suma*CTX (Hiu and Yap, 2021; Teoh and Yap, 2020). *Suma*CTX displayed the highest selectivity for cytotoxic effects in MCF-7 cell lines, when compared to human bronchial epithelial cells. The cytotoxicity of *suma*CTX might occur before 24 h. *Suma*CTX triggers caspase −3/7 activity at its IC_{50} (4 μg/mL) concentration. In addition, mitochondrial hyperpolarisation was observed in cells treated with lower toxin levels, which is presumably a prerequisite and sensitisation event in the early stages of apoptosis. As the toxin concentrations increased, mitochondrial membrane integrity was compromised, leading to typical apoptosis-associated depolarisation. Nevertheless, time-dependent patterns of apoptosis were not observed at higher toxin concentrations, thus, prolonged exposure to *suma*CTX in MCF-7 cells did not promote the progression of apoptosis, as demonstrated by caspase activation and alteration of mitochondrial membrane potentials. On the other hand, there were accountable levels (8–24%) of the PI^+ population at high levels of *suma*CTX treated cells, as revealed by annexin-V/propidium iodide double staining cytometry which was indicative of hypothetical necrosis. To further ascertain whether necrosis occurred, the free form of necrosis marker, high mobility group box protein 1 (HMGB1) was measured following cellular exposure to high levels of *suma*CTX. However, the absence of HMGB1 refuted the occurrence of necrosis in MCF-7 cells treated with high levels of *suma*CTX. *Suma*CTX appeared to trigger caspase-dependent mitochondrial-mediated apoptosis without transitioning to primary necrosis when toxin levels increased. Since a considerable percentage of the PI^+ cell population was observed and it did not indicate the occurrence of necrosis at high levels of *suma*CTX, it posed a question of underlying mechanistic action at high toxin levels. It was found that increasing *suma*CTX concentrations promoted membrane permeabilisation as reflected by an elevated extracellular LDH activity and calcein-AM fluorescence intensity (Hiu and Yap, 2021). Label-free quantitative (LFQ) secretome analyses showed that *suma*CTX caused stress response, inflammation, metabolic deprivation, and necroptosis, without apoptotic proteins in high *suma*CTX treated MCF-7 cells. These findings concluded that *suma*CTX triggered a concentration-dependent transition of apoptosis to necroptosis together with membrane permeabilisation when the toxin levels increased.

Therefore, the activation of either necrosis or necroptosis by CTX is highly dependent on the toxin's concentration, whereby low toxin levels promote apoptosis. This observation is crucial to comprehend concentration-dependent molecular cytotoxicity of CTX.

5. Synergistic effects between cytotoxin and other venom components

Synergism is known to potentiate venom toxicity, particularly between CTX and phospholipase A_2 (PLA_2). This was first observed in a significant haemolytic effect exerted by a combination of *N. naja* venom PLA_2 and CTX (Condrea et al., 1964). In contrast, each venom toxin did not exhibit any haemolytic effect. Subsequent studies also show a significant increase of haemolytic activity when CTX and PLA_2 coexisted following a drastic reduction of cell survival time and lowered activation energy (Louw and Visser 1978; Bougis et al., 1987). The enhanced cytotoxicity after the co-administration of CTX and PLA_2 is hypothesised to occur through supramolecular synergism resulting from the formation of CTX-PLA_2 hetero-oligomers (Pucca et al., 2020). Although the mechanism remains understudied, the synergism presumably depends on the structural and biochemical properties of the toxins (Pucca et al., 2020). There are three possible strategies to achieve synergistic effects, which are 1) the production of the same final effects through distinct pathways; 2) recognising the targets involved in the same or correlating biological pathway(s); 3) chaperoning of one toxin to another toxin (Xiong and Huang 2018). In addition, remarkable synergism in cytotoxicity has also been observed in *N. kaouthia* venom toxins, which is attributed to the formation of a non-covalent heteromeric complex between cytotoxic kaouthiotoxin (KTX) with PLA_2, without altering the biochemical properties of PLA_2 (Mukherjee, 2010). Altogether, synergistic actions of CTX and PLA_2 enhance the cytotoxic effects of CTX.

6. Conclusion

Cytotoxin is abundant in the cobra venom and undeniably plays a major role in causing local necrotic lesions at the wound site upon snakebite. Cytotoxin exhibits differential cytotoxic effects in different cells. One of the prominent cell death mechanisms exerted by CTX is direct cytolysis, which is attributed to its hydrophobicity and direct interaction with membrane phospholipids. The oligomerisation of CTX is responsible for pore formation which causes leakage of intracellular components. Nevertheless, CTX exhibits more than just direct cytolytic effects, it has also been found to activate different apoptotic signalling pathways and cell cycle arrest. Cytotoxin-mediated lysosomal damage in necrotic cell death is a plausible mechanism for the clinical observation of dermonecrotic effects of snakebite envenomation. It is also noteworthy that synergistic effects may present in real-life snakebite cases in which other venom components can potentiate the CTX-induced cytotoxicity. The synergism could also account for the enhanced necrotic effect even with lower CTX doses used in laboratory settings. Recent findings have also demonstrated the occurrence of necroptosis in CTX-treated cells. However, extensive research is required to establish possible mechanistic actions underlying necroptosis in real envenomation cases, especially using human skin models. Although the mechanism of CTX-induced necroptosis remains unclear, future studies involving -omics and bioinformatics can be performed at the molecular level to decipher the molecular mechanisms associated with the transition of cell death pattern. These findings have implications for the discovery of biomarkers of CTX-induced cell death, especially in the context of dermonecrosis. This certainly contributes to the development of toxin-directed biotherapeutics targeting CTX and its pathological effects.

Ethical statement

The authors confirm that this manuscript "The myth of cobra venom cytotoxin: more than just direct cytolytic actions" was prepared according to standard publishing ethics for scientific articles.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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