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Review

Insights into the antiviral activity of phospholipases A_2 (PLA_2s) from snake venoms

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ABSTRACT

Viruses are associated with several human diseases that infect a large number of individuals, hence directly affecting global health and economy. Owing to the lack of efficient vaccines, antiviral therapy and emerging resistance strains, many viruses are considered as a potential threat to public health. Therefore, researches have been developed to identify new drug candidates for future treatments. Among them, antiviral research based on natural molecules is a promising approach. Phospholipases A_2 (PLA_2s) isolated from snake venom have shown significant antiviral activity against some viruses such as Dengue virus, Human Immunodeficiency virus, Hepatitis C virus and Yellow fever virus, and have emerged as an attractive alternative strategy for the development of novel antiviral therapy. Thus, this review provides an overview of remarkable findings involving PLA_2s from snake venom that possess antiviral activity, and discusses the mechanisms of action mediated by PLA_2s against different stages of virus replication cycle. Additionally, molecular docking simulations were performed by interacting between phospholipids from Dengue virus envelope and PLA_2s from *Bothrops asper* snake venom. Studies on snake venom PLA_2s highlight the potential use of these proteins for the development of broad-spectrum antiviral drugs.

Contents

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1. Viral diseases: a public health problem

Viruses are associated to several endemic diseases, including Enterovirus [1], HPV (Human papillomavirus) [2], HIV (Human immunodeficiency virus) [3] and HSV (Herpes simplex virus) [4], as well as in outbreaks as Ebola virus, ZIKV (Zika virus), Influenza virus, YFV (Yellow Fever virus), DENV (Dengue virus) and, currently, the SARS-CoV-2 (Severe Acute Respiratory Coronavirus 2) [5–9]. Most of the reported outbreaks since 1980 were related to virus infections [10], which are still a global burden for public health and economy. In addition, due to their genetic diversity, viruses are able to infect a wide range of hosts that can result in host jumps after zoonotic contacts [11,12].

Pandemics caused by viruses are usually severe and can claim up to million lives, as shown during the pandemic of H1N1 in 1918 [13], H1N1 'swine flu' in 2009 [14] and Coronavirus Disease 2019 (COVID-19) [15], that infected 4,993,470 people and caused 327,738 deaths until May 22, 2020 in worldwide according to World Health Organization. Furthermore, the global incidence of dengue has grown dramatically in recent decades. It is estimated that 100 to 400 million cases of dengue occur annually worldwide [16].

Viral infection depends on the successful replication into the host cells [17,18]. In general, the replicative cycle starts by the viral particle attaching to specific receptors in the surface of host cells that triggers the viral entry by endocytosis (non-enveloped or enveloped virus), membrane fusion (enveloped virus) and direct penetration [19–21]. After internalization, the capsid is released into the cytoplasm, allowing viral genome uncoating [22], which is replicated to produce copies of the genome and translated to viral proteins. In the endoplasmic reticulum (ER) and Golgi complex, the viral structure is assembled, matured and then forwarded to host cell membrane, where the progeny of virus particles is released [23].

Currently, specific antiviral drugs and vaccines are not sufficient to control emerging and reemerging viral diseases [24,25]. Thus, the discovery of novel antiviral drugs is mandatory. In general, antiviral therapy is the only approach to specifically treat viral infections, abrogating viral replicative cycle [26]. However, due to the high genetic variability, viruses can rapidly acquire resistance to antiviral treatment, especially RNA viruses [27–29]. Furthermore, antiviral therapy and the prolonged treatment can cause several adverse effects, including gastrointestinal effects, fatigue, headache, neuropathy and liver toxicity [30–32]. In addition, there are no antivirals to all diseases and the only course is supportive therapy and, thereby, numerous innovative drugs have been developed from natural prototypes such as aspirin (anti-inflammatory) and morphine (analgesic) [33].

In this way, a diversity of compounds isolated from natural sources has set grounds for further advances in drug development against various diseases [34]. Among them, many drugs based on snake venoms were approved by the FDA or are involved in preclinical or clinical trials for a variety of therapeutic applications [35–39]. The development of snake venom-derived drugs gained a significant improve since the discovery of bradykinin-potentiating peptides (BPP) isolated from the Brazilian arrowhead viper (*Bothrops jararaca*) venom, which allowed the development of captopril, an inhibitor of the angiotensin-converting enzyme that is widely used against hypertensive process [40,41]. Besides that, other snake venom-derived drugs have been found in clinical use, such as tirofiban and eptifibatide (antiplatelet agents) [42,43], batroxobin, moojenin and vivostat (anticoagulant agents) [44–47]. Other drugs which comprise molecules from snake venom as scaffolds are also being explored in preclinical studies [48].

Due to this therapeutic potential, snake venom toxins have been widely explored for the discovery of new bioactive compounds and stand out as an alternative source for therapeutics for a variety of diseases, including life-threatening viral illnesses [49–52].

2. Phospholipases A_2 from snake venom

Phospholipases (EC 3.1.) family is widely distributed in nature and includes hydrolase enzymes, which are essential for phospholipid metabolism and for the regulation of membrane lipids, membrane composition, signaling, digestion, and inflammation [53]. These proteins are classified into four major families (A, B, C and D) based on the site cleaved in the phospholipid molecule [54,55].

Among phospholipases family, the Phospholipases A_2 (PLA_2s) are the most studied group [53,54]. These enzymes hydrolyze 2-acyl ester bond to 2-sn phospholipids, releasing free fatty acid and lysophospholipids [56,57]. The free fatty acids (arachidonic acid) can be converted into eicosanoids (prostaglandins, thromboxanes, prostacyclins and leukotrienes), which are associated to a range of physiological and pathological effects, such as inflammation and platelet activation. In addition, the lysophospholipids are also related to a variety of physiological roles in cell signaling [53,58].

PLA_2s are classified into six groups: cytosolic ($cPLA_2$), $Ca^{(2+)}$-independent ($iPLA_2$), platelet-activating factor acetylhydrolase (PAF-AH), lysosomal PLA_2 ($LyPLA_2$), adipose specific PLA_2 ($AdPLA_2$) and secretory PLA2 ($sPLA_2$) [53]. In addition, the $sPLA_2$s are divided into the following groups: IA, IB, IIA, IIB, IIC, IID, IIE, IIF, III, V, IX, X, XIA, XIB, XII, XIII and XIV [53,54]. PLA_2s from snake venom belong to the group of secreted type of enzymes ($sPLA_2$s) and can be classified into the structural group IB (in Elapidae snake venoms), which exhibits homology to the mammalian pancreatic juice PLA_2, and also into the group IIA (in Viperidae snake venoms), that is homologous to the mammalian 'inflammatory' PLA_2 [59]. Although the PLA_2s family is more frequent in snake venom, recent proteome studies have demonstrated that Phospholipases B (PLB) can also be found in snake venom [60–62].

$sPLA_2$s are proteins with molecular mass of about 14 kDa, pH optimum at 7 and share a conserved catalytic mechanism based on a His/Asp dyad using Ca^{2+} as an essential cofactor for the catalytic activity. Group II of $sPLA_2$s presents an extended C-terminal segment (5–7 amino acids) [63,64] and is subdivided into two main subgroups, depending on the amino acid residue at position 49 in the protein primary structure. Aspartate (Asp49 or D49) $sPLA_2$s are enzymatically active, while lysine (Lys49 or K49) $sPLA_2$s present no enzymatic activity [65–67]. However, there are further variants, as the serine (Ser49), asparagine (Asn49) or arginine (Arg49) [68–70].

Lys49 PLA_2s are devoid of catalytic activity due to their inability to bind Ca^{2+}, a key cofactor for PLA_2 activity. Although the lack of enzymatic activity, the Lys49 PLA_2 homologues have shown to display toxicity, especially myotoxicity [67]. The toxicity of Lys49 proteins can be related to a cluster of cationic and hydrophobic/aromatic amino acid residues located at the C-terminal region of these toxins [71,72].

Therefore, the cytotoxicity of $sPLA_2$ is probably mediated by the interaction between the C-terminal region and the plasma membrane [73,74]. Moreover, the PLA_2 effects can be mediated through the integrins and other receptors, such as vascular endothelial growth factor receptor-2 (VEGFR-2), and M–Type receptors [75–77]. Recently, it was demonstrated that the cell surface nucleolin interacts with and internalizes the PLA_2-like *Bothrops asper* myotoxin-II, which is responsible to mediate its toxic activity [78].

$sPLA_2$s from snake venom can act on cell membranes of specific tissues inducing several pharmacological actions such as myotoxicity, neurotoxicity, cardiotoxicity, platelet aggregation activation or inhibition, hypotension, edema among others [57,79]. In this scenario, these proteins have emerged as a potential therapeutic model, since numerous studies have focused on their microbicidal [80], antitumor [81–83], antiangiogenesis [84], antiparasitic [85–87] and antiviral activities [51].

The development of efficient antiviral therapies has become a global health emergency. In this sense, several researches have demonstrated

the antiviral activity of $sPLA_2$s from snake venom against human viruses, including DENV, YFV, HCV and others [88–92]. Hence, the present review aimed to summarize the $sPLA_2$s from snake venom that were previously described to possess antiviral activity, highlighting the mechanisms of action of $sPLA_2$s against different stages of virus replication cycle (Table 1).

Table 1
$sPLA_2$s from snake venom with antiviral effects.

Source/species	Protein name	EC50/used dosage	Virus	Proposed action mechanism (inhibition)	Reference
Bothrops asper	Mt–I	50 μg/mL	DENV-1, 2, 3	Entry	[92]
		50 μg/mL	YFV	(virucidal activity)	
		1.5 ng/mL (EC50)	DENV-2		
	Mt–II	50 μg/mL	DENV-1, 2, 3		
		50 μg/mL	YFV		
		2768 ng/mL (EC50)	DENV-2		
Bothrops jararacussu	BthTX-I	4.8 ng/μL (EC50)	DENV-2	Entry	[88]
		7.063 ng/μL (EC50)	YFV	(virucidal activity)	
		57.3 ng/μL (EC50)	DENV-2	Entry	
		25.0 ng/μL (EC50)	YFV	(interfering in adsorption)	
		69.0 ng/μL (EC50)	DENV-2	Entry	
		23.4 ng/μL (EC50)	YFV	(interfering in internalization)	
Bothrops leucurus	BlK-PLA_2	20 μg/mL	DENV-1, 2 3	Replication	[91]
	BlD-PLA_2	20 μg/mL		(interfering in host cell components)	
Crotalus durissus terrificus	Crotoxin	0.001 ng/μL (EC50)	DENV-2	Entry	[88–90,97]
		0.00045 ng/μL (EC50)	YFV	(virucidal activity)	
		0.0046 ng/μL (EC50)	ROCV		
		0.0036 ng/μL (EC50)	MAYV		
		0.0054 ng/μL (EC50)	OROV		
		–	HIV-1,2		
		10 μg/mL	HCV		
		0.018 ng/μL (EC50)	DENV-2	Entry	
		0.0365 ng/μL (EC50)	YFV	(interfering in adsorption)	
		34.4 ng/μL (EC50)	DENV-2	Entry	
		13.7 ng/μL (EC50)	YFV	(interfering in internalization)	
		0.05 ng/μL (EC50)	DENV-2	Replication	
		0.04 ng/μL (EC50)	YFV	(interfering in host cell components)	
		10 μg/mL	HCV	Release	
	PLA_2-CB (subunit of crotoxin)	0.00003 ng/μL (EC50)	DENV-2	Entry	[88–90]
		0.0037 ng/μL (EC50)	YFV	(virucidal activity)	
		0.021 ng/μL (EC50)	ROCV		
		0.066 ng/μL (EC50)	MAYV		
		0.0067 ng/μL (EC50)	OROV		
		10 μg/mL	HCV		
		0.044 ng/μL (EC50)	DENV-2	Entry	
		0.01647 ng/μL (EC50)	YFV	(interfering in adsorption)	
		17.2 ng/μL (EC50)	DENV-2	Entry	
		3.3 ng/μL (EC50)	YFV	(interfering in internalization)	
		10 μg/mL	HCV		
		10 μg/mL	HCV	Entry	
				(interfering in host cell components)	
		0.06 ng/μL (EC50)	DENV-2	Replication	
		0.26 ng/μL (EC50)	YFV	(interfering in host cell components)	
		6.08 μg/mL (EC50)	HCV		
	PLA_2-IC	0.0137 ng/μL (EC50)	DENV-2	Entry	[88]
		0.0054 ng/μL (EC50)	YFV	(virucidal activity)	
		0.133 ng/μL (EC50)	DENV-2	Entry	
		0.268 ng/μL (EC50)	YFV	(interfering in adsorption)	
		21.6 ng/μL (EC50)	DENV-2	Entry (interfering in internalization)	
		0.775 ng/μL (EC50)	DENV-2	Replication	
		1.30 ng/μL (EC50)	YFV	(interfering in host cell components)	
Naja mossambica mossambica	CM-II-$sPLA_2$	0.036 ng/mL (EC50)	HCV	Entry	[114]
		0.031 ng/mL (EC50)	DENV	(virucidal activity)	
		1.34 ng/mL (EC50)	JEV		
		10,000 ng/mL (EC50)	MERS-CoV		
		>10,000 ng/mL (EC50)	SINV		
		>10,000 ng/mL (EC50)	FLUAV		
		>10,000 ng/mL (EC50)	SeV		
		2300 ng/mL (EC50)	VSNJV		
		5.4 ng/mL (EC50)	HIV-1		
		>10,000 ng/mL (EC50)	HSV-1		
		>10,000 ng/mL (EC50)	CV-B3		
		>10,000 ng/mL (EC50)	EMCV		
	Nmm_{CMIII}	0.4 nM (EC50)	HIV-1 isolates	Entry	[107]
Naja nigricollis	Nigexine	0.4 nM (EC50)	HIV-1 isolates	(interfering in host cell components)	
Oxyuranus scutellatus	Taipoxin	0.8 nM (EC50)	HIV-1 isolates		

CV-B3 (Coxsackievirus B3; Picornaviridae); DENV (Dengue virus); EMCV (Encephalomyocarditis virus; Picornaviridae); FLUAV (Influenza A virus); HCV (Hepatitis C virus); HIV (Human immunodeficiency virus); HSV (Herpes simplex virus); JEV (Japanese encephalitis virus); MAYV (Mayaro virus); MERS-CoV (Middle East respiratory syndrome coronavirus); OROV (Oropouche virus); ROCV (Rocio virus); SeV (Sendai virus); SINV (Sindbis virus); VSNJV (Vesicular stomatitis New Jersey virus); YFV (Yellow Fever virus).

3. sPLA$_2$s from snake venom with antiviral effects

3.1. Crotoxin, PLA$_2$-CB (basic chain of crotoxin) and PLA$_2$-IC from Crotalus durissus terrificus venom

The venom of *Crotalus durissus terrificus* (*C. d. terrificus*), a South American rattlesnake, is composed by a large number of molecules with biological activities, such as crotoxin, crotamin, PLA$_2$ "inter-cro" (PLA$_2$-IC), convulxin and gyroxin [93,94]. Crotoxin, which comprehends more than a half of the dry weight of *C. d. terrificus* venom, is a heterodimeric compound composed by the PLA$_2$-CB (a basic phospholipase component) and crotapotin (an acidic nontoxic catalytically inactive protein) [95,96]. Villarrubia and coworkers [97] reported that crotoxin has anti-HIV (HIV-1, 2) effect by a direct interaction with Gag p24 glycoprotein on the viral surface, which appears to abrogate the HIV anchoring to host cell.

Furthermore, Muller and colleagues [88] working with diverse sPLA$_2$s isolated from *C. d. terrificus* venom explored different approaches to unveil the potent antiviral activity mediated by crotoxin, PLA$_2$-CB and PLA$_2$-IC against DENV-2 and YFV (enveloped virus). The authors demonstrated that all investigated sPLA$_2$s promoted a significant inhibition of DENV-2 and YFV entry into VERO E6 cells by a direct action on the viral particles (virucidal activity), and by interfering in the adsorption and internalization steps (early stages of the viral replication cycle) [88]. Besides that, cell pretreatment with three sPLA$_2$s was able to protect host cell against flaviviruses infection after 7 days by the reduction in the number of plaque formation. Interestingly, sPLA$_2$s treatment after viral infection promoted an enhancement of load viral, indicating that antiviral effect occurs in the early stages of viral infection [88]. In addition, the researchers gained insights into the role of catalytic sites of the tested sPLA$_2$s, proposing the use of a sPLA$_2$ without catalytic activity (BthTX-I) isolated from *Bothrops jararacussu* [98].

BthTX-I revealed antiviral activity against YFV and DENV-2 in the virucidal, adsorption and internalization assays. Interestingly, as shown to other catalytically-active sPLA$_2$s at 100 ng/μL, BthTX-I at the same concentration was also able to inhibit YFV entry by virucidal activity (100%), interfering in adsorption (77%) and internalization (78%) [88]. Although BthTX-I showed antiviral activity, the effective concentration 50% (EC50) values obtained for this toxin were extremely higher when compared to the catalytically-active sPLA$_2$s. For example, for the half-maximum virucidal activity against YFV, this toxin required 7.063 ng/μL, while crotoxin, PLA$_2$-CB and PLA$_2$-IC demanded 0.00045, 0.0037 and 0.0054 ng/μL, respectively. In a similar way against DENV-2, BthTX-I acted at 4.8 ng/μL, in contrast to the crotoxin, PLA$_2$-CB and PLA$_2$-IC that required 0.001, 0.00003 and 0.0137 ng/μL, respectively. As shown, the huge differences of EC50 values between BthTX-I and enzymatically active proteins reflect that the enzymatic activity is an important factor for the antiviral activity of sPLA$_2$s [88].

In a further study, PLA$_2$-CB and crotoxin inhibited virus entry by virucidal activity against other enveloped viruses, such as Rocio virus (ROCV; Flaviviridae family), Oropouche virus (OROV; Bunyaviridae family), and Mayaro virus (MAYV; Togaviridae family). However, these compounds did not show virucidal effect against Coxsackie B5 virus (CV-B5; Picornaviridae family; non-enveloped virus), hence suggesting that the possible antiviral action occurs upon the lipid bilayer viral envelope [89]. To corroborate these findings, it was demonstrated that preincubating DENV-2 with PLA$_2$-CB or crotoxin resulted in an increase of exposure and degradation of viral RNA [89]. Also, Russo and collaborators [99] expressed two recombinant PLA$_2$-CB isoforms through a prokaryotic system and noted that both rPLA$_2$-CB1 and rPLA$_2$-CB2 maintained the viral inhibitory activity against CHIKV, DENV-2, YFV and ZIKV when compared to the native sPLA$_2$-CB. Additionally, Muller and colleagues [88,89] suggested that the mechanism of action of PLA$_2$-CB isolated from *C. t. terrificus* against DENV can occur through an interaction with components on the host cell surface or mainly due to the glycerophospholipid cleavage on the virus envelope, destabilizing viral E proteins and resulting in the viral envelope disruption and RNA viral exposure before the infection of host cells.

In order to gain insights into the antiviral mechanism of sPLA$_2$s obtained from *C. t. terrificus*, Shimizu and colleagues [90] showed that PLA$_2$-CB inhibited HCVcc JFH-1 virus strain entry and replication in Huh 7.5 cells, and crotoxin blocked virus entry and release, suggesting that these proteins possess multiple antiviral effects against HCV. Moreover, the authors also reported that PLA$_2$-CB significantly decrease the levels of lipid droplets, which are essential for the HCV replication complex, and reduced the levels of HCV NS5A protein due to the replication inhibition, evidencing that besides the action on virus entry, PLA$_2$-CB is able to disrupt HCV replication probably by an interference in lipid metabolism of host cell [90,100,101].

3.2. BlK-PLA$_2$ and BlD-PLA$_2$ from Bothrops leucurus venom

Both BlK-PLA$_2$ (Lys49 sPLA$_2$s) and BlD-PLA$_2$ (Asp49 sPLA$_2$s) are two basic sPLA$_2$s isolated from *Bothrops leucurus* venom, a pit viper (white-tailed-jararaca) commonly found in the northeast of Brazil [102]. Cecilio and coworkers [91] showed that the pretreatment of LLC-MK2 cells (Rhesus Monkey Kidney Epithelial cells) with each isoform of Bl-PLA$_2$ followed by viral infection was able to inhibit DENV infectivity (serotypes 1, 2 and 3), measured by qRT-PCR quantification of the DENV viral load in the cell supernatants after virus infection. On the other hand, Bl-PLA$_2$s treatment after viral entry was not capable of inhibiting viral replication, then suggesting that the antiviral effect occurs upon components on the surface of the host cell membrane. The authors did not assess the potential virucidal mechanism of Bl-PLA$_2$s against DENV. However, they suggested that the possible mechanism of action of Bl-PLA$_2$s does not depend exclusively on their catalytic site. The Lys49-BlK-PLA$_2$ treatment was able to interfere in the viral load, indicating that the functional effect mediated by Bl-PLA$_2$s also may occur due to the presence of pharmacological domains on the enzyme surface that would allow the interaction with host cell proteins, as well as the enzymatic activity [91]. The authors hypothesized that the DENV RNA level reduction is mediated by the intracellular action of Bl-PLA$_2$s due to the higher penetrability capacity of basic sPLA$_2$s, in comparison to neutral and acidic enzymes [91,103].

3.3. Mt-I and Mt-II from Bothrops asper venom

Bothrops asper is a viperid specie found in Central America and its venom contains significant concentrations of acid and basic sPLA$_2$ enzymes [104]. The *B. asper* venom has both the basic enzymatically-active sPLA$_2$ (Mt-I) and the catalytically-inactive sPLA$_2$-like protein (Mt-II) [74].

Brenes and collaborators [92] investigated the antiviral potential triggered by both Mt-I and Mt-II isoforms isolated from *B. asper* venom. The authors showed that these sPLA$_2$s at concentration of 50 μg/mL completely blocked virus entry by a virucidal action against members of Flaviviridae family, such as DENV and YFV, while exhibited moderate to negligible effects against other enveloped viruses (HSV-1, HSV-2, Influenza A H3N2 and Vesicular stomatitis VSV) or non-enveloped viruses (Sabin Poliovirus 1, 2 and 3). Interestingly, for the half-maximum virucidal activity against DENV-2, Mt-I required 1.5 ng/mL, while Mt-II acted at 2768 ng/mL, revealing that Mt-I is extremely more potent than Mt-II [92]. Investigating the role of the enzymatic activity in the inhibitory effect upon DENV-2, it was promoted the inactivation of the catalytic activity of Mt-I with *p*-bromophenacyl bromide (*p*BPB). The data showed that the chemical inactivation of Mt-I resulted in a reduction of the virucidal potency, indicating the relevant role of the enzymatic action against viral infection [92]. Even without enzymatic activity, the C-terminal region of Mt-II, which encompasses the amino acid residues 115–129, is responsible for the membrane-permeabilizing effect caused in many cellular types [67], as well as its bactericidal activity [105]. Notwithstanding that, the authors

demonstrated that, even at high concentrations, the synthetic peptide "p115" corresponding to the C- terminal region of Mt-II (amino acid residues 115–129) did not inhibit DENV-2 [92]. Thus, the authors speculate that the weak virucidal effect of Mt-II may be intrinsic or more possible related to a trace contamination with Mt-I, where the total chromatographic separation for these toxins is hardly achieved [92].

In addition, it was suggested that Mt-I acts by a direct virucidal mechanism that depends on its enzymatic activity, which may hydrolyze viral envelope phospholipids and disrupt the viral envelope of flaviviruses leading to the impairment of the infection. Also, the mode of action of Mt-I and Mt-II is not related to an effect on host cell, since cell treatment after infection did not interfere in viral replication [92]. Furthermore, in a pretreatment assay, it was demonstrated a partial reduction of viral plaques, that may be explained by a slight cytotoxic action of Mt-I on cells [92]. Finally, the higher antiviral activity of Mt-I against Flaviviridae viruses in comparison to other enveloped virus families may be related to the specific structural organization, physicochemical composition, curvature and fluidity of viral envelope from flaviviruses, which may positively affect the catalytic activity of Mt-I against this family [106].

3.4. Taipoxin (*Oxyuranus scutellatus*), nigexine (*Naja nigricollis*) and Nmm_{CMIII} (*Naja mossambica mossambica*)

In a previous study, Fenard and colleagues [107] demonstrated anti-HIV-1 effects of different $sPLA_2$s from snake venom, such as taipoxin (*Oxyuranus scutellatus* venom), Nmm_{CMIII} (*Naja mossambica mossambica* venom) [108,109] and nigexine (*Naja nigricollis* venom) [110]. Investigating the possible mode of action of some of these $sPLA_2$s, it was observed that despite their enzymatic activity, Nmm_{CMIII} and taipoxin did not show virucidal effects against HIV-1, but promoted an efficient inhibition of HIV-1 entry by preventing the intracellular release of HIV-1 Gap p24 proteins from the viral capsid [107].

The blockage of HIV entry appears to not depend exclusively on $sPLA_2$s catalytically active, which was confirmed through two manners: i) the use of inhibitors of $sPLA_2$s activity, such as phenacyl bromide, aristolochic acid or oleoyloxyethylphosphocholine, that were not able to interfere in the blockage of virus entry mediated by $sPLA_2$s; ii) the use of cleavage products of $sPLA_2$s, such as arachidonic acid, lysophosphatidylethanolamine, lysophosphatidic acid, oleoyl-lysophosphatidylcholine and palmitoyl-lysophosphatidylcholine, which were also not able to inhibit virus entry [107]. In addition, competition binding assays between $sPLA_2$s and host cells showed extremely low dissociation constant (K) values for Nmm_{CMIII}, taipoxin and nigexine, suggesting that the inhibition of HIV-1 entry triggered by $sPLA_2$s is more probably linked to $sPLA_2$s binding membrane receptors of host cells than their enzymatic activity [107,111].

3.5. CM-II-$sPLA_2$ from *Naja mossambica mossambica* venom

CM-II-$sPLA_2$ is a secreted PLA_2 isoform isolated from *Naja mossambica mossambica* venom [112,113]. Recently, Chen and coworkers [114] reported that this $sPLA_2$ possesses a potent dose-dependent virucidal activity that impairs the entry of enveloped viruses from budding through the endoplasmic reticulum, such as HCV, DENV and JEV (Japanese encephalitis virus) belonging to the Flaviviridae family. In contrast, CM-II-$sPLA_2$ demonstrated a low antiviral activity against other enveloped viruses by: i) budding through the plasma membrane, as observed for SINV (Sindbis virus; Togaviridae), SeV (Sendai virus; Paramyxoviridae), FLUAV (Influenza A virus; Orthomyxoviridae), VSNJV (Vesicular stomatitis New Jersey virus; Rhabdoviridae) and HIV-1 (Retroviridae); ii) budding through the trans-Golgi network, as seen for HSV- 1 (Herpes simplex virus type 1; Herpesviridae); iii) budding through the ER-Golgi intermediate compartment, as for MERS-CoV (Middle East respiratory syndrome coronavirus; Coronaviridae). Additionally, the slight effect was also observed against non-enveloped viruses, such as EMCV (Encephalomyocarditis virus; Picornaviridae) and CV-B3 (Coxsackievirus B3; Picornaviridae) [114].

The disruption of viral envelope by CM-II-$sPLA_2$ appears to be directly related to its enzymatic activity, which was confirmed by the use of manoalide (a specific $sPLA_2$ inhibitor) that inhibited the virucidal activity of CM-II-$sPLA_2$ against HCV and DENV [114]. Moreover, the selectivity of CM-II-$sPLA_2$ for virus buds through endoplasmic reticulum may be related to the differences in the phospholipid contents and physicochemical characteristics (thickness and sturdiness) that can differ among the different routes of viral budding, which would enhance the sensitivity to CM-II-$sPLA_2$ mediated by hydrolysis against HCV, DENV and JEV [114–117].

4. Proposed antiviral mechanism of $sPLA_2$s from snake venom

Findings from the current literature about the antiviral activity of toxins (Table 1) are heterogeneous, since authors developed a variety of assays/models using different $sPLA_2$s and viruses. The virucidal model corresponds to the strategy in which the toxins act directly on the virus particles before infecting the cell monolayer; in the pre-infection model, the uninfected monolayers are previously treated with toxins before viral infection; and in the post-infection model, cell monolayers are adsorbed with the virus followed by toxin treatment. In this sense, many studies raised the following questions: in which stages of virus replication are the $sPLA_2$s able to interfere? Does the antiviral action of $sPLA_2$s depend on their catalytic activity? Based on this, we summarize a possible model of antiviral action mediated by $sPLA_2$s from snake venom.

As discussed above, $sPLA_2$s have demonstrated to be potent antiviral inhibitors by interfering in different stages of virus replicative cycle as entry steps, replication and release (Fig. 1). Current studies have reported that the antiviral action of $sPLA_2$s on steps of viral cycle can occur through a direct action upon viral particle and/or by an interaction with virus or host cell components.

Regarding the virucidal activity, studies have shown that both catalytically-active $sPLA_2$s (crotoxin, PLA_2-IC, PLA_2-CB, Mt-I and CM-II-$sPLA_2$) and catalytically-inactive $sPLA_2$s (Mt-II and BthTX-I) indicated virucidal activity preferentially against enveloped viruses, such as DENV (serotypes 1, 2 and 3), YFV, ROCV, OROV, MAYV, HCV, JEV, MERS-CoV, SINV, FLUAV, SeV, VSNJV, HIV-1 and HSV-1 [88–90,92,114].

It is proposed that the potent virucidal activity of $sPLA_2$s against enveloped viruses is likely associated with the ability that catalytically-active $sPLA_2$s have to cleave glycerophospholipids in the virus lipid envelope, and it is reasonable to propose that $sPLA_2$s also present domains that are capable to interact with viral envelope components, which could lead to viral envelope disruption, hence resulting in exposure of the viral content (viral inactivation) and compromising the early stages of viral replication. Additionally, Muller and colleagues [88,89], through a steric and electrostatic analysis of the interaction of PLA_2-CB with the DENV envelope lipid bilayer, showed that PLA_2-CB probably accesses the DENV lipid bilayer through the pores found on each of the twenty 3-fold vertices in the E protein shell on the DENV surface, which would allow the glycerophospholipid cleavage on the virus envelope and destabilization of the E proteins. Interestingly, it has been demonstrated that the structural organization and lipid composition of viral envelope may influence the antiviral efficiency of some $sPLA_2$s, suggesting that the virucidal mechanism mediated by $sPLA_2$s is specific [92].

Independent studies have revealed that $sPLA_2$s such as crotoxin, PLA_2-IC, PLA_2-CB, Bl-PLA_2 and BthTX-I are also able to dramatically impact the entry, replication and release of viruses by targeting host cell components [88–91]. To gain insights into these viral cycle stages, it was demonstrated that Nmm_{CMIII}, taipoxin and nigexine prevented the intracellular release of HIV-1 Gap p24 proteins from the viral capsid (inhibition virus entry) by a direct binding to membrane receptors of

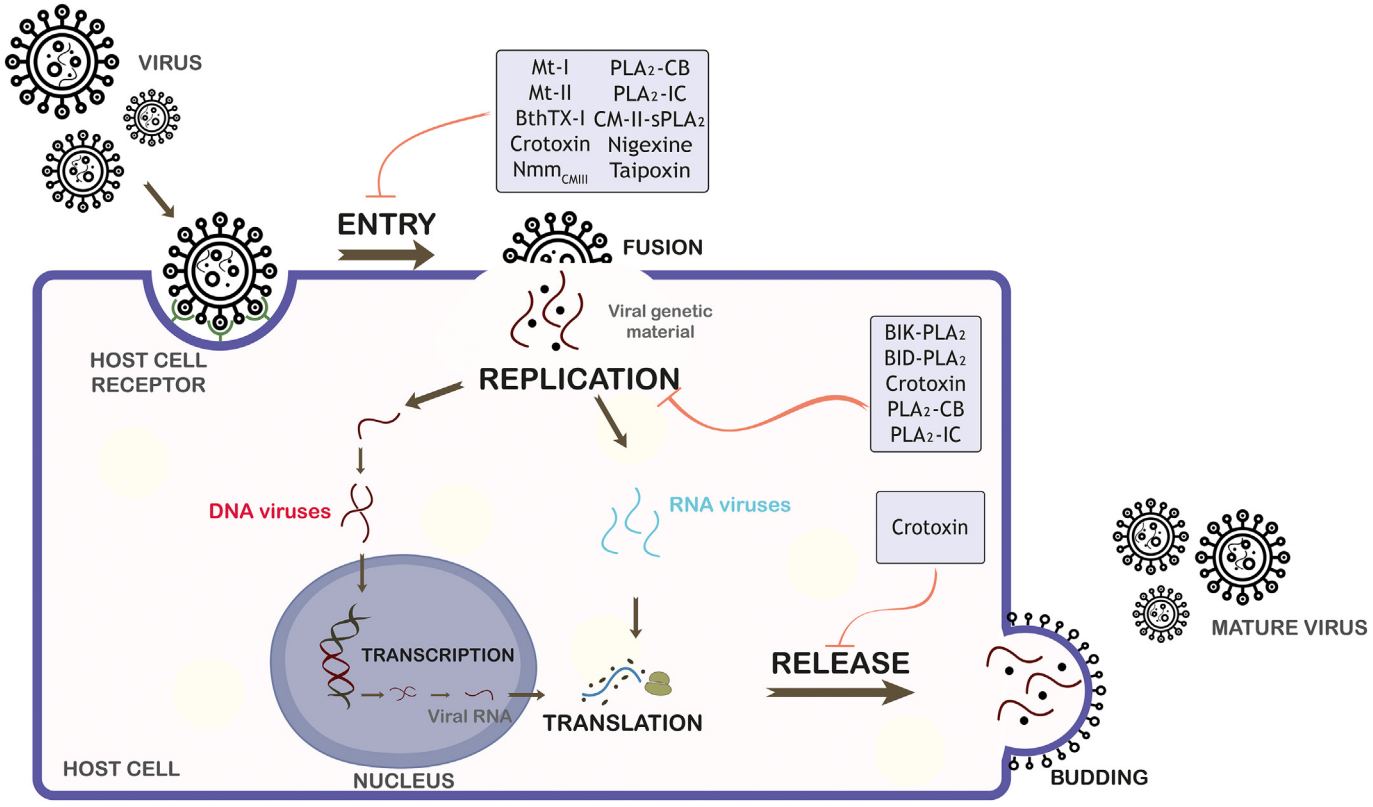

Fig. 1. Schematic representation of the mechanisms of action of sPLA$_2$s from snake venom on viral replicative cycle. sPLA$_2$s, which possess antiviral activity, are indicated in early and/or late stages of the viral life cycle: entry, replication and release. Mt-I and Mt-II (*Bothrops asper*), BthTX-I (*Bothrops jararacussu*), crotoxin, PLA$_2$-CB and PLA$_2$-IC (*Crotalus durissus terrificus*), CM-II-sPLA$_2$ and Nmm$_{CMIII}$ (*Naja mossambica mossambica*), nigexine (*Naja nigricollis*), taipoxin (*Oxyuranus scutellatus*), BlK-PLA$_2$ and BlD-PLA$_2$ (*Bothrops leucurus*) are demonstrated.

host cells [107]. In addition, PLA$_2$-CB was able to disrupt HCV replication probably by an interference in lipid metabolism of host cell [90].

It was demonstrated through the use of both specific sPLA$_2$ inhibitors and the catalytically-inactive sPLA$_2$s that the antiviral effect of the major tested catalytically-active sPLA$_2$s, such as crotoxin, PLA$_2$-IC, PLA$_2$-CB, BlD-PLA$_2$, Mt-I and CM-II-sPLA$_2$ is significantly higher when presented their functional catalytic site to sPLA$_2$s with no enzymatic activity (BthTX-I, BlK-PLA$_2$ and Mt-II) [88,91,92,114].

In order to corroborate with the data from the current literature, we performed docking simulations between the sPLA$_2$s from *Bothrops asper* venom and three phospholipids found in the DENV envelope, which are 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC), 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE) and 1-Palmitoyl-2-oleoylphosphatidylserine (POPS) [118]. The molecular docking simulations were done by using the 3D crystal structure of Mt-I (PDB: 5TFV) and Mt-II (PDB ID 4YV5) retrieved from Protein Database (https://www.rcsb.org/).

We simulated the interaction in the enzymatic site of sPLA$_2$s with palmitoyl phospholipids (head or complete structure) using AutoDock Vina software [119]. The predicted affinity between sPLA$_2$s and palmitoyl phospholipids (head or complete) was similar (Table 2).

Table 2
Docking simulations between Mt-I and Mt-II with palmitoyl phospholipids (head or complete structure) from DENV envelope.

PLA$_2$s affinity (kcal/mol)	Phospholipids					
	POPC		POPE		POPS	
	Head	Complete	Head	Complete	Head	Complete
Mt-I	−5.1	−5.4	−4.7	−5.1	−5.0	−5.4
Mt-II	−4.3	−4.5	−4.3	−4.7	−4.3	−4.8

POPC - 1-palmitoyl-2-oleoylphosphatidylcholine; POPE - 1-palmitoyl-2-oleoylphosphatidylethanolamine; POPS - 1-palmitoyl-2-oleoylphosphatidylserine.

Concerning to the interaction with the phospholipids head, Mt-I showed a higher affinity to POPC, POPE and POPS (−5.1, −4.7 and −5.0 kcal/mol, respectively) related to Mt-II (Table 2). When we simulated the docking with the complete palmitoyl phospholipid molecule, Mt-I also has a higher affinity than Mt-II (Table 2).

Although we do not observe a strong difference in the affinity between Mt-I and Mt-II for palmitoyl phospholipids, it is possible to note structural variation in the enzymatic site of these two toxins. Compared to Mt-II, the enzymatic site of Mt-I (Fig. 2A) is more suitable due to a smaller aspartic acid radical group. The van der Waals radii volume of aspartic acid is 91 and hence it is more prominent, while the lysine has a volume of 135, and this results in less space in enzymatic site entrance in Mt-II (Fig. 2B). This difference could create an enzymatic site more restricted to palmitoyl phospholipid entrance/binding and be partially responsible for the absence of enzymatic activity in Mt-II [120]. In addition, the enzymatic activity of Mt-I can be attributed to highly conserved catalytic site formed by the amino acid residues His48, Asp49, Tyr52 and Asp99. Asp49 coordinates the hydrolysis reaction of phospholipids together with the residues of the Ca2+ binding loop, essential in the catalytic activity of PLA$_2$s. The substitution of lysine residue at the same position affects the ability of this protein to bind to Ca^{2+}, resulting in the absence of catalytic activity [92].

Despite the stronger antiviral activity is associated with the enzymatic activity, the antiviral mechanism of sPLA$_2$s does not depend exclusively on their catalytic site, since Lys49 sPLA$_2$s and inhibited catalytically-active sPLA$_2$s were also able to show antiviral effects, suggesting that sPLA$_2$s may possess different mechanisms of action. However, additional studies with different Lys49 from snake venom are required to better characterize the antiviral potential of this protein class.

Functional and structural studies have described that the activity of Lys49 PLA$_2$s from snake venom toward cell membranes in myotoxic mechanism involves an allosteric transition, and the participation of

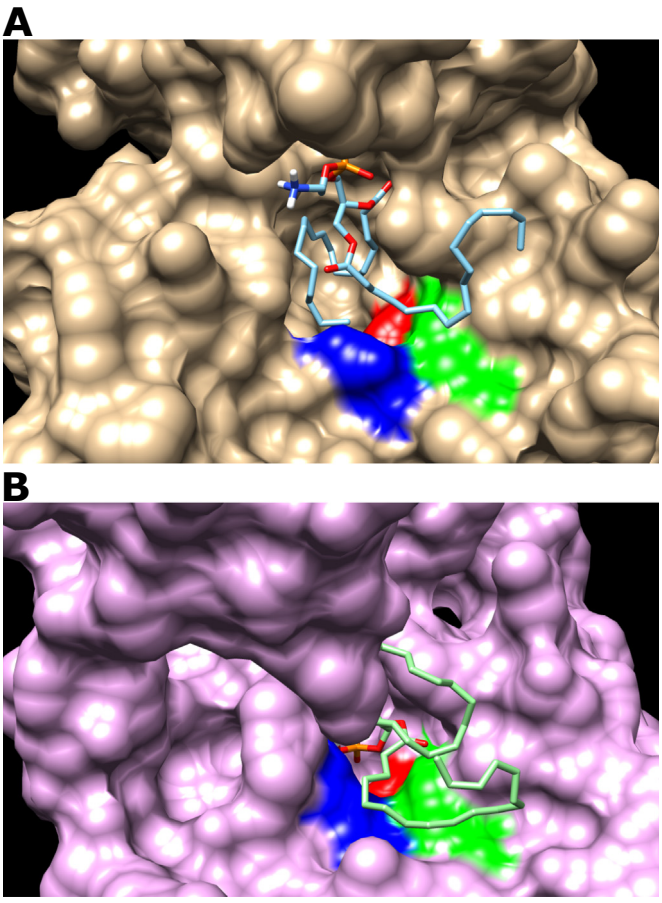

Fig. 2. Docking simulations between $sPLA_2$s and 1-palmitoyl-2-oleoylphosphatidylcholine (POPC). Mt-I (A) and Mt-II (B) are showed as surface and the enzymatic site is colored. The amino acids from the enzymatic site are H48 in red, D49 (Mt-I) or K49 (Mt-II) in blue, and Y52 in green. The POPC is showed as wire structure. The aspartic acid has a smaller volume than lysine, which may result in a less open entrance in Mt-II (panel B) compared with Mt-I (panel A). H: histidine; D: aspartate; K: lysine; Y: tyrosine.

two independent interaction sites with the target membrane [67,72,121–123]. The action of Lys49 PLA_2s is related to a cluster of cationic and hydrophobic/aromatic amino acid residues located at the C-terminal region of this toxin. These two conserved regions in most Lys49-PLA_2s are designed by "cationic membrane-docking site" (MDoS), which are formed by the strictly conserved C-terminal residues (Lys115 and Arg118), eventually aided by other cationic and exposed residues such as Lys20, Lys80, Lys122 and Lys127; and the "hydrophobic membrane-disruption site" (MDiS) formed by residues of Leu121 and Phe125. The key step for protein activation is the binding of a fatty acid at the hydrophobic channel, which leads to allosteric transition and structure stabilization exposing MDoS and MDiS to the membrane, following by the insertion of the MDiS region from both monomers into the target membrane. This penetration disrupts the lipid bilayer, causing alterations in the membrane permeability, highlighted by a prominent influx of ions (*i.e.*, Ca^{2+} and Na^{+}), and eventually, irreversible intracellular alterations and cell death [123].

According to myotoxic mechanism of Lys49 PLA_2s from viperid snake venoms, it is proposed that the fatty acids which are important to protein activation may come from membrane phospholipid hydrolysis by catalytic PLA_2s (Asp49), highlighting the synergism between Asp49 PLA_2s and Lys49 PLA_2 in snake envenomation [124]. In this way, the antiviral effects of the Lys49 PLA_2s from snake venom, showed in this review, may be associated to fatty acids from the catalytic activity of cytosolic PLA_2 ($cPLA_2$) from virus lipid envelope, once it was demonstrated that enzymatic activity of the $cPLA_2$ is required for replication of various virus [125–127]. Muller and colleagues [126] showed that the pharmacological inhibition of a cellular phospholipase, $cPLA_2$, using a specific small-molecule inhibitor, drastically reduces coronavirus RNA synthesis and, as a consequence, protein accumulation and the production of infectious virus progeny. In addition, $cPLA_2$ activity was shown to be critically involved in the production of infectious progeny of HCV and DENV [128].

5. Concluding remarks: $sPLA_2$s as a possible useful tool for the development of antiviral compounds

The present review highlighted that PLA_2s from snake venom have become valuable as pharmacological tools and/or therapeutic approaches due to their extremely high specificity and potent activity against microbial infection. Regarding to antiviral properties, we highlighted the following remarks: (i) the antiviral effects of $sPLA_2$s can be mediated by either a dependent or independent catalytic mechanism; (ii) $sPLA_2$s-antiviral effects are more evident against enveloped virus; (iii) $sPLA_2$s promoted the blockage of viral entry into host cells by the direct action on the viral particle, resulting in glycerophospholipids cleavage and destabilization of viral envelope proteins; (iv) the structural organization, physicochemical composition, and the curvature and fluidity of viral envelope may influence in the antiviral efficiency of some $sPLA_2$s; (v) $sPLA_2$s promoted the blockage of entry, replication and release of virus probably by the interference on the host cell components.

The structure and function of $sPLA_2$s from snake venom have been widely explored. Homology studies with $sPLA_2$s have demonstrated highly conserved regions in these proteins capable of disrupting the integrity of membranes and provoking many pharmacological effects. Despite extensive studies on $sPLA_2$s in over decades, there is few of them focusing on mechanistic aspects of the antiviral activities and to date are limited to *in vitro* and *in silico* models. It is important to note that these $sPLA_2$s showed damage effects *in vivo*, such as myotoxicity and inflammation. Thus, further *in vivo* studies for attesting antiviral effects of $sPLA_2$s need to be addressed to investigate their safety, toxicity and pharmacokinetics. Taken together, new structural and functional studies with $sPLA_2$s are essential to discover new relevant motifs responsible for the antiviral activities that would allow the future use of these proteins or peptides for the design of antiviral drugs, capable of ensuring more stability and targeting the specific site of action.

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