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Review Article

Antitumoral Potential of Tunisian Snake Venoms Secreted Phospholipases A₂

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Phospholipases type A₂ (PLA₂s) are the most abundant proteins found in Viperidae snake venom. They are quite fascinating from both a biological and structural point of view. Despite similarity in their structures and common catalytic properties, they exhibit a wide spectrum of pharmacological activities. Besides being hydrolases, secreted phospholipases A₂ (sPLA₂) are an important group of toxins, whose action at the molecular level is still a matter of debate. These proteins can display toxic effects by different mechanisms. In addition to neurotoxicity, myotoxicity, hemolytic activity, antibacterial, anticoagulant, and antiplatelet effects, some venom PLA₂s show antitumor and antiangiogenic activities by mechanisms independent of their enzymatic activity. This paper aims to discuss original finding against anti-tumor and anti-angiogenic activities of sPLA₂ isolated from Tunisian vipers: *Cerastes cerastes* and *Macrovipera lebetina*, representing new tools to target specific integrins, mainly, α5β1 and αv integrins.

1. Introduction

Snake venom is a natural biological resource, containing several neurotoxic, cardiotoxic, cytotoxic, and many other different active compounds [1, 2]. Due to this broad range of biological functions, these biomolecules have been the subject of hundreds of scientific articles in different research fields, including biochemistry, biophysics, pharmacology, toxicology, and medicine [2–5]. Viperidae snake venoms contain class II PLA₂s, which share structural features with secreted PLA₂ (sPLA₂) of the class II-A present in inflammatory exudates in mammals. A number of venom PLA₂s have been shown to induce a variety of pharmacological effects although comprehensive studies of the actions of

venom PLA₂s in the various events of toxicity are scarce [6, 7].

2. Viperidae Snake Venom Phospholipase A₂ Enzymes: Secreted Phospholipases A₂

Secreted PLA₂ constitute a large superfamily of enzymes that are widely distributed in living organisms. The sPLA₂ from Viperidae snake venoms fall under group II. They are generally Ca²⁺-dependant enzymes that catalyze the hydrolysis of the sn-2 fatty acid bond of phospholipids to release free fatty acids and lysophospholipids [7]. These enzymes are small proteins (~13-14 kDa), containing 120–125 aminoacid residues, 7 disulfide bridges, and have a partially conserved

structure that define the PLA₂ fold [8]. Group II snake venom PLA₂ enzymes can also be divided into different subgroups on the basis of the aminoacid residue in the forty-ninth position. Asp49 plays an important role in catalysis and it is conserved in most snake venom PLA₂ enzymes, and hence these are identified as D49 enzymes [9]. However, in some of the group IIA PLA₂ enzymes this aminoacid residue is replaced by lysine, serine, asparagine, or arginine and they are identified as K49 [10], S49 [11], N49 [12], or R49 [13] enzymes, respectively. Substitution of Asp in the forty-ninth position interrupts the binding of cofactor Ca²⁺ to the Ca²⁺-binding loop, and hence “mutants” show low or no hydrolytic activity [10, 14, 15]. In addition, there are several substitutions in the Ca²⁺-binding loops of these mutant enzymes.

Secreted phospholipases A₂ constitute major components of snake venoms and have been extensively investigated not only because they are very abundant in these venoms but mainly because they display a variety of relevant toxic actions such as neurotoxicity, myotoxicity, cytotoxicity, cardiotoxicity, edema-inducing, artificial membrane disrupting convulsant, hypotensive and proinflammatory effects [7, 16–19]. Besides, they exert a wide range of biological effects, including anticoagulant, platelet aggregation inhibiting [7, 20, 21], bactericidal [22], anti-HIV [23], antimalarial and anti-parasitic [24], antitumor [21, 25, 26], and recently anti-angiogenic effect [27–29]. Due to this functional diversity, these structurally similar proteins aroused the interest of many researchers as molecular models for study of structure-function relationships. One of the main experimental strategies used for the study of myotoxic PLA₂s is the traditional chemical modification of specific aminoacid residues and examination of the consequent effects upon the enzymatic, toxic, and pharmacological activities. Furthermore, some venom sPLA₂ have no catalytic activity while they exert various toxic and pharmacological effects [17, 21, 26]. The absence of direct correlation between catalytic activity and pharmacological effects has led to the hypothesis that specific actions of sPLA₂ are due to the presence of pharmacological sites on the sPLA₂ surface overlapping or distinct from the catalytic site. These pharmacological sites would allow the sPLA₂ to bind specifically to soluble or membrane-bound proteins that participate to the sPLA₂ mechanism of action [30].

Since this hypothesis was proposed, a collection of binding proteins have been identified using several toxic snake venom sPLA₂ [31]. Besides β -bungarotoxin [32, 33], early studies with the neurotoxic sPLA₂ OS2 from Australian Taipan snake *Oxyuranus scutellatus scutellatus* have led to the identification of two families of binding proteins called N- and M-type receptors [31, 34, 35]. The N-type receptors are present in mammalian brain and other tissues. Neurotoxic sPLA₂, such as OS2, bind with N-type receptors with high affinity, while nontoxic sPLA₂ including OS1 bind with much lower affinity, suggesting that these receptors are involved in neurotoxicity.

Conversely, the M-type receptors bind with high affinity both toxic and nontoxic sPLA₂ including OS1 and OS2 [31].

Importantly, the M-type receptors also bind with several mammalian sPLA₂ [31, 36], suggesting that these proteins are the endogenous ligands for these receptors, and possibly for the collection of binding proteins initially identified with venom sPLA₂.

3. Tunisian Viperidae Snake Venom Proteins

Snake venom is a natural source for molecules known as modulators of integrin-mediated functions [37]. Pharmacological study of snake venoms reveals structural and functional polymorphisms of proteins they contain. In our laboratory in Pasteur Institute of Tunis, we are interested in studying different pharmacological effects of Tunisian Viperidae venoms, mainly, the horned viper, *Cerastes cerastes*, *Macrovipera lebetina transmediterranea*, and *Cerastes vipera* [38]. Baza'a et al. showed that these venoms contain proteins belonging to a few protein families. However, each venom showed distinct degree of protein composition complexity. The three venoms shared a number of protein classes though the relative occurrence of these toxins was different in each snake species. On the other hand, the venoms of the *Cerastes* species and *Macrovipera lebetina* each contained unique components [38]. The comparative proteomic analysis of Tunisian snake venoms provides a comprehensible catalogue of secreted proteins, which may contribute to a deeper understanding of the biological effects of the venoms and may also serve as a starting point for studying structure-function correlations of individual toxins.

Thereby, disintegrins and C-type lectins (CLPs) are among the most studied proteins proved to be components of medical and biotechnological value [39–42]. Indeed, they are potent and specific antagonists of several integrins, such as $\alpha v\beta 3$ and $\alpha 5\beta 1$ [43, 44] and can thus act in many biological processes including platelet aggregation, angiogenesis, tumor invasion, and bone destruction [39, 45–47]. On the other hand, CLPs were first described as modulators of platelet before their antiadhesive activity was highlighted [48–50]. CLPs are thus able to inhibit integrin-dependent proliferation, migration, invasion, and angiogenesis [26, 44, 51, 52]. Sarray and coworkers have isolated lebecetin and lebecetin, two C-type lectins, from *Macrovipera lebetina* snake venom inhibiting $\alpha 5\beta 1$ - and αv -containing integrins [43, 44]. Since their initial characterization, snake venom disintegrins have been extensively studied [39, 46], they are potent inhibitors of integrin-ligand interactions. The integrin inhibitory profile of disintegrins primarily depends on the sequence of a tripeptide located at the apex of a mobile loop and constrained in its active conformation by the appropriate pairing of disulfide bonds. So, CC5 and CC8 have been previously characterized as *Cerastes cerastes* dimeric disintegrins targeting $\alpha_{11b}\beta_3$, $\alpha v\beta 3$, and $\alpha 5\beta 1$ integrins [53]. In addition to dimeric disintegrins, *Macrovipera lebetina* venom includes short disintegrin, namely, lebestatin which targets $\alpha 1\beta 1$ integrin [46].

Recently, phospholipases A₂ (PLA₂s, EC 3.1.1.4) have been demonstrated to modulate integrins which are essential

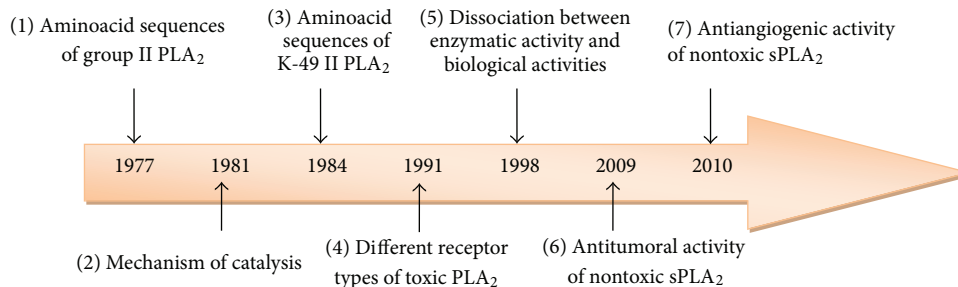


FIGURE 1: Milestones in PLA₂ enzyme research. (1) Henrikson et al. [81], (2) Lambeau et al. [56], (3) Maraganore et al. [10], (4) Lambeau et al. [82], (5) Landucci et al. [83], (6) Zouari-Kessenti et al. [21], Baza'a et al. [27], and (7) [27, 28].

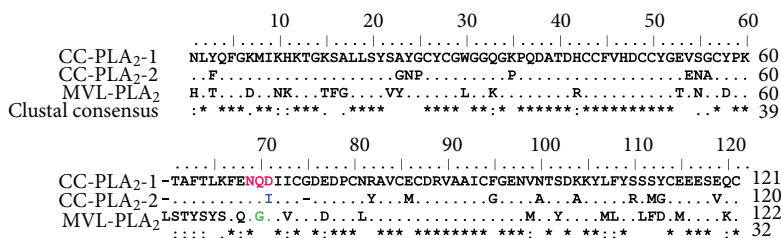


FIGURE 2: Sequence alignment of Tunisian Viperidae sPLA₂: CC-PLA₂-1 (ACO92622) [28], CC-PLA₂-2 (ACO92623) [28], and MVL-PLA₂ (CAR40186) [26]. Gaps (—) have been introduced to optimize alignment.

protagonists of the complex multistep process of angiogenesis, the major target for the development of anticancer therapies [21, 27, 28] (Figure 1).

4. Secreted Phospholipases A₂ from Tunisian Vipers

Three acidic, nontoxic, Asp49 phospholipases A₂ have been isolated from Tunisian vipers: CC-PLA₂-1 and CC-PLA₂-2 from *Cerastes cerastes*, and MVL-PLA₂ from *Macrovipera vipera*. They have a molecular weight of 13737.52, 13705.63, and 13626.64 Da, respectively. They contain, respectively, 121, 120, and 122 aminoacids, including 14 cysteines each [21, 26]. The sequences alignment shows similarity as high as 50% (Figure 2). Furthermore, none of the three PLA₂s is cytotoxic up to 2 μM.

CC-PLA₂-1 and CC-PLA₂-2 present a high enzymatic activity [21], while MVL-PLA₂ shows a low one. Although they differ greatly in their catalytic properties, these shared many pharmacological activities proving the lack of correlation between enzymatic and pharmacological activities.

5. Pharmacological Activities of sPLA₂ from Tunisian Vipers

CCPLA₂-1, CC-PLA₂-2, and MVL-PLA₂ show many pharmacological effects [21, 26]. The most interesting are the anti-tumor and antiangiogenic activities which involve integrins [27, 28].

5.1. Tunisian Viperidae sPLA₂ Effects on Haemostatic System. Snake venom toxins are now regularly used in laboratories for assaying haemostatic parameters and as coagulation reagents [54, 55]. PLA₂ enzymes are known to inhibit blood coagulation. Depending on the dose required to inhibit coagulation, they are classified into strong, weak, and nonanticoagulant enzymes [56, 57]. Strong anticoagulant PLA₂ enzymes inhibit the activation of FX to FXa by both enzymatic and nonenzymatic mechanisms and inhibit the activation of prothrombin to thrombin by nonenzymatic mechanism [58, 59]. In our case, 0.14 μM of both CC-PLA₂s completely inhibited plasma coagulation. Thus, CC-PLA₂s could be considered among the most anticoagulant yet described for PLA₂s snake venom [21]. Lizaro and coworkers showed that myotoxin II, a basic PLA₂ from *Bothrops nummifer*, was unable to inhibit coagulation of the platelet-poor plasma until 3.57 μM [60]. Moreover, it has been shown that BaspPLA(2)-II, an acidic, Asp49 PLA₂ from *Bothrops asper* venom lacks anticoagulant activity [61].

Platelet aggregation plays a role in clot retraction and wound healing. Any alteration in platelet aggregation could lead to debilitation or death. CC-PLA₂-1 and CC-PLA₂-2 showed high antiplatelet aggregation activities induced by arachidonic acid or ADP [21], contrary to b/D-PLA₂ which displays high enzymatic and anticoagulant activities but has no platelet aggregation [62]. Moreover, Kashima and coworkers reported that BthA-I-PLA₂, a nontoxic acidic PLA₂ from *Bothrops jararacussu* snake venom, inhibited ADP-induced platelet aggregation with moderate effect [63].

While, OHVA-PLA₂, an acidic PLA₂ from *Ophiophagus hannah*, strongly inhibited platelet aggregation in the presence of ADP or arachidonic acid [64]. It thus appears that PLA₂ platelet activity is not directly due to its acidic nature or its anticoagulation activity.

5.2. Tunisian Viperidae sPLA₂ Effects on Tumor Cell Behavior. Snake venom sPLA₂ present a wide range of pharmacological effects [7], including cytotoxicity on tumor cells [7, 63, 65]. Concerning CC-PLA₂-1, CC-PLA₂-2, and MVL-PLA₂, concentrations up to 2 μM during 4 days did not induce detectable cytotoxicity on human cell lines IGR39 (melanoma) and HT1080 (fibrosarcoma) [21, 26].

Adhesion and cell migration are two fundamental steps in numerous diseases, like cancer. CC-PLA₂-1, CC-PLA₂-2, and MVL-PLA₂ inhibit adhesion and migration of human HT1080 and IGR39 cells to fibrinogen and fibronectin. This effect persists even after complete blockage of the catalytic activity suggesting that, contrary to Bth-A-I-PLA₂ whose antitumoral effect appears to be linked to enzymatic site [63], the inhibitory and enzymatic activities are supported by different sites. RVV-7, a cytotoxic basic PLA₂ from *Russell's viper* venom, inhibits also tumor development [65]. On the contrary, b/D-PLA₂ represents the exception of these enzymes as it stimulates tumor growth [62]. Since Tunisian phospholipases A₂ are not cytotoxic, it seems that their antitumoral activity is exerted by a different mechanism. Using different assays, such as a solid-phase binding assay and a panel of immobilized antibodies, we have proved that CC-PLA₂-1, CC-PLA₂-2, and MVL-PLA₂ inhibit cell adhesion and migration by interacting directly with αv and α5β1 integrins [26, 28].

5.3. Tunisian Viperidae sPLA₂ Effects on Angiogenesis. Angiogenesis is fundamental to normal healing, reproduction, and embryonic development. However, this process is also important in the pathogenesis of a broad range of disorders such as arthritis and cancer [66]. Angiogenesis is thus required to sustain malignant cells with nutrients and oxygen for tumors to grow beyond a microscopic size. Thus, the microvascular endothelial cell recruited by a tumor is an important target in cancer therapy and has the advantage of being genetically stable. Therefore, treating both the cancer cell and the endothelial cell in a tumor may be more effective than treating the cancer cell alone.

The role of αvβ3 integrin in the angiogenic process is well documented [67]. In the last decade, several clinical trials evaluating the efficacy of αvβ3 blockers have led to encouraging results in cancer therapy and diagnosis. Similarly, α5β1 integrin is involved in angiogenesis and more precisely in growing vessels, but its expression disappears in mature vessels [68]. Thereby, when tested *in vitro*, the two CC-PLA₂ and MVL-PLA₂ impaired adhesion and migration of HBMEC (human brain microvascular endothelial cells) and HMEC-1 (human microvascular endothelial cell), respectively, by interfering with integrin function. Moreover, using the CAM assay, an *ex vivo* model, these sPLA₂ strongly reduced vasculature development. The treatment reduced the

number of new capillaries and branching, without affecting the mature blood vessels, suggesting once again the implication of α5β1 integrin. Interestingly, CC-PLA₂-1 and CC-PLA₂-2 inhibit spontaneous angiogenesis as well as angiogenesis induced by growth factors such as VEGF or bFGF [28]. The antiangiogenic effect of PLA₂ can be due partly to the blockage of the αvβ3 and α5β1 integrins functions. However, inhibition of angiogenesis can also result from blockage of VEGF or its receptor. Thus, it has been reported that inactive PLA₂ homologues, such as KDR-bp isolated from *Eastern cottonmouth* venom, are common antagonists of KDR, a VEGF receptor [69].

Focal adhesions are specialized sites of attachment of cells where integrins receptors, such as αvβ3, link the extracellular matrix to the actin cytoskeleton, allowing migration [70]. Cell migration is a complex cellular behavior that results from the coordinated changes in the actin cytoskeleton and the controlled formation and dispersal of cell-substrate adhesion sites. While the actin cytoskeleton provides the driving force at the cell front, the microtubule network assumes a regulatory function in coordinating rear retraction. The polarity within migrating cells is further highlighted by the stationary behavior of focal adhesions in the front and their sliding in trailing ends [71].

Treatment of HMEC-1 cells with MVL-PLA₂ induced important changes in cell morphology. Treated cells have a circular shape and actin stress fibers are thinner or absent, with the actin mainly located at the cell periphery. Moreover, MVL-PLA₂ leads to drastic reduction in the size of focal adhesions and their distribution all over the ventral surface of cells, consistent with a decrease in αvβ3 integrin clustering and its absence from lamellipodia [27]. Therefore, it appears that the inhibition of migration is associated with important reorganization of the actin cytoskeleton and focal adhesions. Again, there is a clear dissociation between the anti-angiogenic effect and the catalytic activity.

Furthermore, MVL-PLA₂ strongly increased MT dynamics in HMEC-1 cells. Because the microtubule cytoskeleton is essential in the orchestration of endothelial cell motility [72, 73], microtubule-targeting agents are known to have antiangiogenic effects through the modulation of cytoskeleton dynamics [27]. Thus, microtubule-binding drugs are widely used in cancer chemotherapy and also have clinically relevant antiangiogenic and vascular-disrupting properties [74].

6. Importance of the Identification of Pharmacological Sites

The pharmacological sites of PLA₂ enzymes determine the affinity between the PLA₂ and target proteins. The identification of pharmacological sites helps in (1) understanding the structure-function relationships of PLA₂ enzymes, (2) developing strategies to neutralize the toxicity and pharmacological effects by targeting these sites, and (3) developing prototypes of novel research tools and pharmaceutical drugs [7, 8].

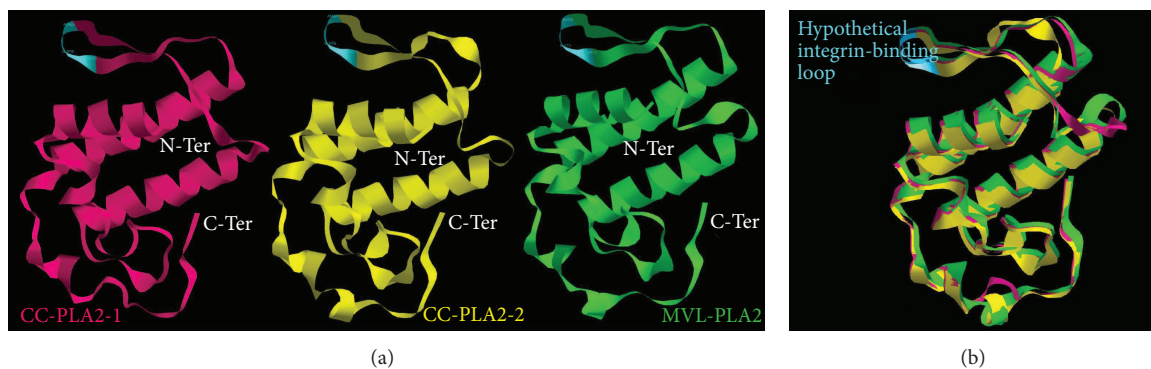


FIGURE 3: (a) Tertiary model structure of CC-PLA₂-1 (pink), CC-PLA₂-2 (yellow), and MVL-PLA₂ (green). (b) Superimposition of the structural models of CC-PLA₂-1, CC-PLA₂-2, and MVL-PLA₂. The hypothetical integrin-binding loop (blue) is presented in each PLA₂.

In our studies, we showed that CC-PLA₂-1, CC-PLA₂-2, and MVL-PLA₂ target the $\alpha 5\beta 1$ and αv integrins, particularly $\alpha v\beta 3$. Moreover, angiogenesis involves expression of the later, which binds to RGD-containing components of the interstitial matrix [75].

To further understand the mechanism of action, we report that endothelial cells are able to adhere on immobilized MVL-PLA₂ and that this adhesion is impaired by RGD peptides [27]. This suggests that interaction between MVL-PLA₂, CC-PLA₂-1, or CC-PLA₂-2 and integrins involves RGD-like sequence which may be responsible for the inhibition of integrin function. This hypothesis is supported by Ramos and coworkers' study, showing that general folding of electrostatic potential is the main intervening of disintegrin-integrin interaction [76].

When MVL-PLA₂ contains a NGD sequence, which could be considered as an RGD-like motif, CC-PLA₂-1 and CC-PLA₂-2 present NQD and NQI, respectively, that may also be responsible for the inhibition of integrin function.

Therefore, bioinformatics study and structural criteria that would allow identifying biologically active RGD-sites on the base of a protein's spatial structure may become a helpful tool for analysis of cellular function of proteins [77]. Furthermore, conformation of the integrin-binding loop in a protein is defined not only by physicochemical properties and conformation of the sequence itself, but also by its structural environment and therefore of the potential biological activity. Besides the RGD-like sequence site should be placed on a loop or a beta-turn to be well exposed. We can cite disintegrin, like applied model, in which we can note a loop accessible stabilized by disulfide bridges [78].

7. Molecular Modeling of CC-PLA₂-1, CC-PLA₂-2, and MVL-PLA₂

In order to examine the site of the suspected RGD-like sequence, using the SWISS-MODEL Workspace (<http://swissmodel.expasy.org/>), we have determined the three-dimensional models of CC-PLA₂-1, CC-PLA₂-2, and MVL-PLA₂.

Firstly, as shown in Figure 3(a), the three models are very similar. Interestingly, we can note the presence of very-well-exposed loop containing the suspected RGD-like motif. This loop is very similar to that of the disintegrins.

In the case of MVL-PLA₂, we find the NGD motif, while for CC-PLA₂-1 and CC-PLA₂-2 there is NQD and NQI, respectively. According to our hypothesis, the residue R in RGD motif is replaced by the N which is hydrophilic and polar residue, it is even more hydrophilic than R residue, this leads to higher affinity towards the $\alpha v\beta 3$ integrin [79]. Besides, the D residue favors recognition of $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins [79]. In addition, in CC-PLA₂-1 and CC-PLA₂-2 the RGD-like motif is flanked by two E residues, highly polarized which could enhance the inhibitory effect towards integrins that bind to ligands through RGD sites, including the fibronectin receptor, mainly, the $\alpha 5\beta 1$ integrin [80].

On the other side, based on the study of disintegrins, it is known that integrin-binding ability is apparently more related to the Cys-rich domain. Similarly, CC-PLA₂-1, CC-PLA₂-2, and MVL-PLA₂ present 14 Cys forming 7 disulfide bridges. We can postulate that disulfide bonds, especially Cys50–Cys86 and Cys57–Cys79, stabilized the hypothetical integrin-binding loop. The superimposition of the structural models of CC-PLA₂-1, CC-PLA₂-2, and MVL-PLA₂ shows that they share similar conformational features (Figure 3(b)).

Nevertheless, further structure-function relationships study must be carried to verify this hypothesis.

8. Conclusion

Secreted phospholipase A₂ enzymes, especially from *Viperidae* Snake venom, exhibit a wide variety of pharmacological effects despite their structure similarity. These enzymes provide a great challenge to protein chemists as subtle and complex puzzles in structure-function relationship. A better understanding will contribute to our knowledge of protein-protein interactions, protein targeting, and protein engineering, and to the development of better-targeted delivery systems. Further research in identifying target proteins will bring details on the mechanisms of the pharmacological effects at the cellular and molecular levels. Studies in these

areas will result in new, exciting, and innovative opportunities in the future, both in finding answers to the toxicity of PLA₂ enzymes and could bring useful tools for developing proteins with novel functions.

Interestingly, we have demonstrated that two isoforms of PLA₂ (CC-PLA₂-1 and -2), from horned Tunisian viper *Cerastes cerastes* and another from *Macrovipera lebetina* MVL-PLA₂ target integrins, a large and very important family of adhesion molecules that promote stable interactions between cells and their environment [26, 28]. Indeed, these sPLA₂ exhibit a potent antitumor and antiangiogenic activities. We showed that their effect is likely due to the inhibition of $\alpha 5\beta 1$ - and αv -containing integrins [26, 28].

These nontoxic secreted phospholipase A₂ could be new tools to disrupt different steps of tumor and angiogenesis progression through integrins. It is noteworthy that this effect is independent of the enzymatic activity. This finding may serve, on the one hand, as a mean to discuss the molecular regions involved in recognition of tissue targets and, on the other hand, as starting point structure-function relationship studies leading to design a new generation of anticancer drugs.

Abbreviations

sPLA₂: Secreted phospholipase A₂
 CLP: C-type lectin protein
 VEGF: Vascular endothelial growth factor
 bFGF: Basic fibroblast growth factor
 CAM: Chick chorioallantoic membrane.

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