Toxins **2013**, *5*, 203-223; doi:10.3390/toxins5020203

OPEN ACCESS

toxins

ISSN 2072-6651
www.mdpi.com/journal/toxins

Article

Interactions of PLA_2-s from *Vipera lebetina*, *Vipera berus berus* and *Naja naja oxiana* Venom with Platelets, Bacterial and Cancer Cells

Mari Samel, Heiki Vija, Imbi Kurvet, Kai Künnis-Beres, Katrin Trummal, Juhan Subbi, Anne Kahru and Jüri Siigur *

National Institute of Chemical Physics and Biophysics, Tallinn 12618, Estonia;
E-Mails: mari.samel@kbfi.ee (M.S.); heiki.vija@kbfi.ee (H.V.); imbi.kurvet@kbfi.ee (I.K.); kkunnis@gmail.com (K.K.-B.); katrin.trummal@kbfi.ee (K.T.); juhan.subbi@kbfi.ee (J.S.); anne.kahru@kbfi.ee (A.K.)

* Author to whom correspondence should be addressed; E-Mail: juri.siigur@kbfi.ee; Tel.: +372-639-8360; Fax: +372-670-3662.

Received: 7 December 2012; in revised form: 16 January 2013 / Accepted: 17 January 2013 / Published: 24 January 2013

Abstract: Secretory phospholipasesA_2 ($sPLA_2$s) form a large family of structurally related enzymes widespread in nature. Herein, we studied the inhibitory effects of $sPLA_2$s from *Vipera lebetina* ($VLPLA_2$), *Vipera berus berus* ($VBBPLA_2$), and *Naja naja oxiana* ($NNOPLA_2$) venoms on (i) human platelets, (ii) four different bacterial strains (gram-negative *Escherichia coli* and *Vibrio fischeri*; gram-positive *Staphylococcus aureus* and *Bacillus subtilis*) and (iii) five types of cancer cells (PC-3, LNCaP, MCF-7, K-562 and B16-F10) *in vitro*. $sPLA_2$s inhibited collagen-induced platelet aggregation: $VBBPLA_2$ IC_{50} = 0.054, $VLPLA_2$ IC_{50} = 0.072, $NNOPLA_2$ IC_{50} = 0.814 μM. *p*-Bromophenacylbromide-inhibited $sPLA_2$ had no inhibitory action on platelets. 36.17 μM $VBBPLA_2$ completely inhibited the growth of gram-positive *Bacillus subtilis* whereas no growth inhibition was observed towards gram-negative *Escherichia coli*. The inhibitory action of $sPLA_2$s (~0.7 μM and ~7 μM) towards cancer cells depended on both venom and cell type. $VBBPLA_2$ (7.2 μM) inhibited significantly the viability of K-562 cells and the cell death appeared apoptotic. The $sPLA_2$s exhibited no inhibitory effect towards LNCaP cells and some effect (8%–20%) towards other cells. Thus, already sub-μM concentrations of $sPLA_2$s inhibited collagen-induced platelet aggregation and from the current suite of studied $svPLA_2$s and test cells, $VBBPLA_2$ was the most growth inhibitory towards *Bacillus subtilis* and K-562 cells.

Keywords: snake venom; *Vipera lebetina*; *Vipera berus berus*; *Naja naja oxiana*; phospholipase A_2; human platelet aggregation inhibition; antibacterial; bioluminescent bacteria; cancer cells

Abbreviations

svPLA$_2$, snake venom PLA$_2$; sPLA$_2$, secretory PLA$_2$; VLPLA$_2$, *Vipera lebetina* phospholipase A$_2$; VBBPLA$_2$, *Vipera berus berus* phospholipase A$_2$; NNOPLA$_2$, *Naja naja oxiana* phospholipase A$_2$; *p*-BPB, *p*-bromophenacylbromide; MALDI-TOF MS, matrix assisted laser desorption ionization time-of-flight mass spectrometry; lysoPC, lysophosphatidylcholine; PRP, platelet-rich plasma.

1. Introduction

Phospholipases A_2 (E.C. 3.1.1.4) are enzymes that catalyze the hydrolysis of the *sn-2* fatty acyl ester bond of *sn-3* phosphoglycerides, liberating free fatty acids, and lysophospholipids. Phospholipases A_2 (PLA$_2$s) are a large family of proteins found in various mammalian tissues: arthropods, as well as in the venoms of snakes, scorpions and bees. Based on their source, catalytic activity, amino acid sequence, chain length and disulfide bond patterns, PLA$_2$s are divided into 16 groups [1] including 10 groups of secretory PLA$_2$s (sPLA$_2$s) [2,3]. The variability of the structure of the conserved domains of sPLA$_2$s from bacteria to mammals was recently investigated by Nevalainen *et al.* [4].

The sPLA$_2$s are small-molecular-mass proteins (13–15 kDa) that require the presence of Ca^{2+} for their catalytic activity. In snake venoms, only two groups of sPLA$_2$s (GI and GII) have been identified. Group I (GIA) includes the svPLA$_2$s from *Elapinae* and *Hydrophiinae* venoms with 115–120 amino acid residues and these svPLA$_2$s are homologous to mammalian pancreatic GIB sPLA$_2$. Group II (GIIA and GIIB) comprises the svPLA$_2$s from *Crotalinae* and *Viperinae* venoms with 120–125 amino acid residues and homologous to mammalian non-pancreatic Group II-A sPLA$_2$ [3]. Group II PLA$_2$s are in turn divided into different subgroups on the basis of amino acid residue in the 49th position: catalytically active D49 enzymes, catalytically inactive or with low activity K49, S49, N49 or R49 forms [5,6]. The above described subgroups exhibit a wide variety of physiological and pathological effects. In addition to their possible role in the digestion of prey, snake venom sPLA$_2$s exhibit a wide spectrum of pharmacological effects such as neurotoxicity, cardiotoxicity, myotoxicity, anticoagulant, anticancer effects *etc.* [3,5–12].

Numerous snake venom sPLA$_2$s that modulate platelet function have been characterized [13–19] and different mechanisms of action shown [6,15,20–26]. The sPLA$_2$s effect on platelet aggregation can be independent or dependent on their catalytic activity. However, the mechanism of action of snake sPLA$_2$s on platelet aggregation is not fully elucidated.

In addition, an increasing number of sPLA$_2$s with antibacterial properties has been reported [27–36]. For example, sPLA$_2$s have been shown to be inhibitory (bacteriostatic) or killing (bactericidal) to gram-positive bacteria *Staphylococcus aureus* [37]. In case of svPLA$_2$ from *Crotalus durissus collilineatus* venom the bactericidal effect was entirely dependent on its enzymatic activity [38]. The effect of

sPLA$_2$s towards gram-positive and gram-negative bacteria and their role in the host defence against bacterial infections has been reviewed by Nevalainen *et al.* [39].

Different types of sPLA$_2$s and synthetic peptides derived from sPLA$_2$ homologues have been shown to possess antitumor and antiangiogenic activity against different cancer cells *in vitro*. The antitumor activities have been detected for the acidic BthA-I-PLA$_2$ from *Bothrops jararacussu* venom [40], for RVV-7, a basic 7 kDa toxin from Russell's viper venom [41], for two sPLA$_2$s from *Cerastes cerastes* venom [42], for sPLA$_2$ from *Naja naja atra* venom [43], for a Lys^{49} sPLA$_2$ from *Protobothrops flavoviridis* venom [44], for a Drs-PLA$_2$ from *Daboia russelii siamensis* venom [45]. Recent studies have shown that MVL-PLA$_2$ from *Macrovipera lebetina transmediterranea* venom inhibited cell adhesion and migration of melanoma IGR39 cells and fibrosarcoma HT1080 cells *in vitro* [46,47]. Antitumor properties of different snake venom phospholipases A$_2$ have been reviewed by Rodrigues *et al.* [12].

In the current study sPLAs from *Vipera berus berus* (common viper), *Vipera lebetina* (Levantine viper) and *Naja naja oxiana* (Middle-Asian cobra) venoms were studied for their biological effects using (i) human platelets, (ii) different gram-negative (*Vibrio fischeri*, *Escherichia coli*) and gram-positive (*Bacillus subtilis*, *Staphylococcus aureus*) bacterial strains and (iii) five different cancer cells lines (prostate cancer cell lines PC-3, LNCaP, breast cancer cell line MCF-7, chronic myeloid leukemic cell line K-562 and mouse melanoma cell line B16-F10).

2. Results

2.1. Purification and Characterization of sPLA$_2$s

VLPLA$_2$ (*Vipera lebetina* sPLA$_2$) was purified as described by Vija *et al.* [18] and VBBPLA$_2$ (*Vipera berus berus* sPLA$_2$) according to Križaj *et al.* [48]. In the case of NNOPLA$_2$ (*Naja naja oxiana* sPLA$_2$), a new two-step purification scheme involving Sephadex G-50 sf and pentylagarose chromatography was used resulting in homogeneous sample.

The relative activity of studied svPLA$_2$s was comparatively high: VLPLA$_2$—882 μmol/min mg; VBBPLA$_2$—1900 μmol/min mg and NNOPLA$_2$—1200 μmol/min mg. The molecular masses of PLA$_2$s after reduction with 2-mercaptoethanol detected by SDS-PAGE were about 14,000 Da. VLPLA$_2$ had pI value in the acidic region (4.3), VBBPLA$_2$ in the basic region (9.3) and NNOPLA$_2$ in the neutral region (6.7). The activity of svPLA$_2$ after isoelectric focusing in the gel was detected using egg-yolk overlay-technique (data not shown).

MALDI-TOF MS analysis confirmed the molecular masses estimates of native PLA$_2$s revealing single peaks for enzymes with the actual molecular masses of 13,683 Da for VLPLA$_2$, 13,824 Da for VBBPLA$_2$ and 13,229 Da for NNOPLA$_2$. To distinguish between the possible isoforms, PLA$_2$s of different venoms were subjected to trypsinolysis and the masses of the resulting peptides were analysed by MALDI-TOF MS. The peptide mass fingerprinting results confirmed that VBBPLA$_2$ was a close match with enzyme formerly sequenced by Križaj *et al.* [48], VLPLA$_2$ matched with sequence (EU421953) [18] and NNOPLA$_2$ with enzyme isoform 3 formerly sequenced by Ovchinnikov *et al.* [49] (Figure 1). MALDI-TOF analysis of tryptic peptides derived from NNOPLA$_2$ is provided in Figure S1.

Figure 1. Alignment of *V. lebetina* VLPLA$_2$ (EU421953) [18], VBBPLA$_2$ *V. berus berus* (P31854) [48] and NNOPLA$_2$ isozyme E from *N. naja oxiana* (P25498) [49]. The alignment was performed using the program CLUSTAL W (1.83) multiple sequence alignment. "*" indicates positions which have a single, fully conserved residue; ":" indicates that one of the "strong" amino acid groups is fully conserved; "." indicates that one of the "weaker" groups is fully conserved. Trypsin cleavage sites in NNOPLA$_2$ are indicated as ↑. Cysteine residues are on red background, conserved catalytic network formed by four amino acid residues His48, Asp49, Tyr52 and Asp99 are on blue background.

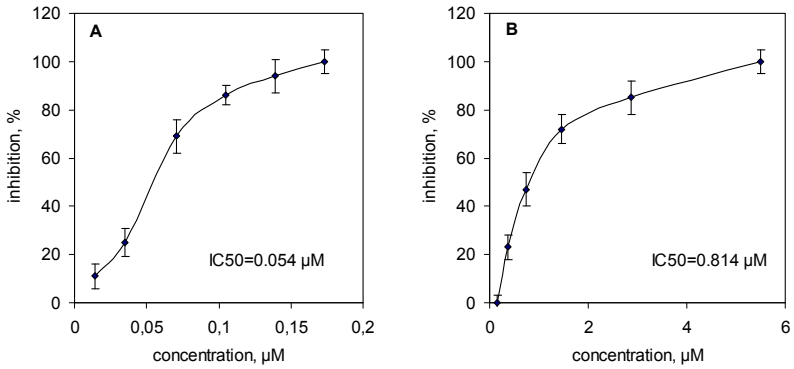
```
VLPLA2   1  DLSQFGDMINKKTGTFGLFSYIYYGCYCGWGGKGKPQDATDRCCFVHDCCY---GSVNGC 57
VBBPLA2  1  NLFQFGNMINHMVGKHAVWSYLSYGCYCGWGGQGKPQDATDRCCFVHDCCY---GRANGC 57
NNOPLA2  1 _NLYQFKNMIKCTVPSRSWLDFANYGCYCGRGGSGTPVDDLDRCCQIHDNCYNEAGKISGC 60
                ↑   ↑    ↑           ↑          ↑           ↑
             :* ** :**:  . . .  .:  ****** **.*.* *  **** :** **   *  .**


VLPLA2   58 DPKLSTYSYSFQNGDIVC-GDDDPCLRAVCECDRVAAICSGENMNTYDKKYMLYSLFDCK 116
VBBPLA2  58 DPKLSTYSYNFQNGNIVC-GNKYGCLRHICECDRVAAICFQKNMNTYNKKYKNYSSSNCQ 116
NNOPLA2  61 WPYFKTYSYECSQGTLTCKGDNNSCAASVCDCDRLAAICFAG-APYNNDNYNINLKARCQ 119
               ↑           ↑            ↑                   ↑
             * :.****. .:* :.* *:.  *   :*:***:****       :.:*       *:


                              Identity       pI      Da
VLPLA2    117    EESEKC 122   100%           4.3     13683
VBBPLA2   117    ENSNKC 122    71%           9.3     13824
NNOPLA2          ------        46%           6.7     13229
```

2.2. Inhibition of Human Platelet Aggregation in Vitro

sPLA$_2$s from all three venoms inhibited collagen-induced platelet aggregation in platelet-rich plasma in a concentration-dependent manner: the IC_{50} = 0.054 μM for VBBPLA$_2$ (Figure 2A); IC_{50} = 0.072 μM for VLPLA$_2$ [18] and IC_{50} = 0.814 μM for NNOPLA$_2$ (Figure 2B).

In order to explore if the inhibitory effects of sPLA$_2$s on platelet aggregation were related to their enzymatic activities, the native sPLA$_2$s were treated by *p*-bromophenacylbromide (*p*-BPB) that modifies the histidine in the active center causing the inhibition of the catalytic activity. The *p*-BPB-treated enzymes were tested in the same conditions as the native vPLA$_2$s. The treatment of all three svPLA$_2$s by *p*-BPB resulted in complete loss of their catalytic activity that was accompanied by the loss of their inhibitory effect on collagen-induced platelet aggregation.

Figure 2. Inhibitory effects of svPLA$_2$s on collagen-induced human platelet aggregation. (**A**) human platelet rich plasma (PRP) samples were stirred for 2 min at 37 °C with VBBPLA$_2$s (0.014–0.173 μM) and then 2 μg/mL of collagen (final concentration in the test) was added to induce platelet aggregation; (**B**) The PRP samples were preincubated with NNOPLA$_2$s (0.148–5.510 μM) under the same conditions. Results are reported as means ± SD (n = 3).



2.3. Inhibitory Effect of Snake Venoms and Their sPLA$_2$s on Bacteria

2.3.1. Acute Toxicity to *Vibrio fischeri*

For the evaluation of the acute toxicity of studied enzyme preparations, naturally luminescent gram-negative bacteria *V. fischeri* were used. In these bacteria, the exposure to toxicants causes rapid decrease of their bioluminescence whereas the effect is dose-dependent [50]. In the current study, in addition to svPLA$_2$s also the effect of the whole venom was evaluated. As a toxicity endpoint, inhibition of bacterial bioluminescence after 15 min of exposure to the whole venom or sPLA$_2$s was used. In general, the venoms and sPLA$_2$s were not acutely toxic to *V. fischeri*. Also, the sPLA$_2$s were not acutely toxic: only enzyme from *V. lebetina* inhibited the luminescence of bacteria at <100 μg/mL (<7.31 μM) level, the 15-min EC$_{50}$ was 58 μg/mL, *i.e.*, 4.24 μM; Table 1).

Table 1. Acute toxicity (15-min EC$_{50}$, μg/mL) of venoms and sPLA$_2$s from different snakes to bacteria *Vibrio fischeri*. As a toxicity endpoint, inhibition of the bacterial bioluminescence was used.

Tested item	**Acute toxicity (15-min EC$_{50}$, μg/mL)**			
	3,5-DCP *	*V. b. berus*	*V. lebetina*	*N. n. oxiana*
Venom	3–4	370	944	>1315
PLA$_2$	3–4	>909 (>65.76 μM)	58 (4.24 μM)	>606 (>45.81 μM)

* 3,5-dichlorophenol (a positive control).

2.3.2. Inhibitory Effect of the Snake Venom PLA$_2$s on Bacterial Growth

The inhibitory effect of svPLA$_2$s on bacterial growth (a chronic toxicity) was evaluated at 500 μg/mL (36.2 μM for VBBPLA$_2$; 37.8 μM for NNOPLA$_2$; 36.5 μM for VLPLA$_2$) level of the enzymes. The effect of VBBPLA$_2$ on the growth of gram-positive bacterial strains was studied in parallel for the native enzymes and *p*-bromophenacylbromide-inactivated VBBPLA$_2$s. The results are

shown in Table 2 and Figure 3. Although the tested concentration was relatively high, none of the svPLA$_2$s inhibited the growth of gram-negative bacteria *Escherichia coli* but there were inhibitory effects in case of some enzyme preparations on gram-positive bacterial strains (Figure 3A–C). Specifically, the *V. berus berus* PLA$_2$ was most potent and totally (100%) inhibited the growth of *B. subtilis* (Figure 3A). The total growth inhibition of *B. subtilis* was also observed in case of *p*-BPB-inactivated VBBPLA$_2$ (Figure 3B) whereas the effect was dose-dependent (Figure 3C). PLA$_2$ from *V. lebetina* showed also some inhibitory effect (13%) towards *B. subtilis* but this inhibitory effect was not observed in case of *p*-BPB-inactivated enzyme (Figure 3A). Intact VBBPLA$_2$ preparations (Table 2) had no inhibitory effect on gram-positive bacteria *S. aureus* but there was some inhibitory effect in case of inactivated enzyme (Figure 3B; Table 2). The *N. naja oxiana* PLA$_2$ was inhibitory (42%) towards *S. aureus* (Table 2).

Table 2. Inhibition of the bacterial growth (incubation time 6 h) in LB medium at 30 °C supplemented by svPLA$_2$s (500 μg/mL, *i.e.*, 36.2 μM for VBBPLA$_2$; 37.8 μM for NNOPLA$_2$; 36.5 μM for VLPLA$_2$) from three different snakes.

		Inhibition of the bacterial growth, % (*t* = 6 h)				
Bacteria(Gram staining)		**svPLA$_2$**				
		V. b. berus	***V. b. berus ****	***V. lebetina***	***V. lebetina ****	***N. n. oxiana***
Escherichia coli	Gram (−)	No effect	not tested	No effect	No effect	No effect
Bacillus subtilis	Gram (+)	100% **	99% **	13%	No effect	Slight effect (6.5%)
Staphylococcus aureus	Gram (+)	No effect	29%	No effect	No effect	42%

* histidine in PLA$_2$ was modified by *p*-bromophenacylbromide, to inactivate its catalytic activity; ** growth was inhibited by 100% but the viability of bacteria remained unchanged (*i.e.*, after the 6 h exposure to enzyme preparation, bacteria were able to grow on agarized LB-medium; data not shown).

Figure 3. The effect of different snake venom sPLA$_2$s on the growth of bacteria in LB medium at 30 °C. (**A**) The effect of different snake venom sPLA$_2$s (500 μg/mL, *i.e.*, 36.2 μM for VBBPLA$_2$; 37.8 μM for NNOPLA$_2$; 36.5 μM for VLPLA$_2$) on the growth of gram-positive bacteria *Bacillus subtilis* BR151. The different svPLA$_2$s are indicated as data labels; (**B**) The effect of *p*-BPB-treated *V. berus berus* sPLA$_2$ (500 μg/mL = 36.17 μM) on the growth of gram-positive bacteria *Bacillus subtilis* BR151 and *Staphylococcus aureus*. Growth of not treated bacteria is shown as data labels; (**C**) The effect of different concentrations of *p*-BPB-treated *V. berus berus* venom sPLA$_2$ on the growth of *Bacillus subtilis* BR151; concentrations (μM) are shown as data labels. Results are reported as means ± SD (*n* = 3).

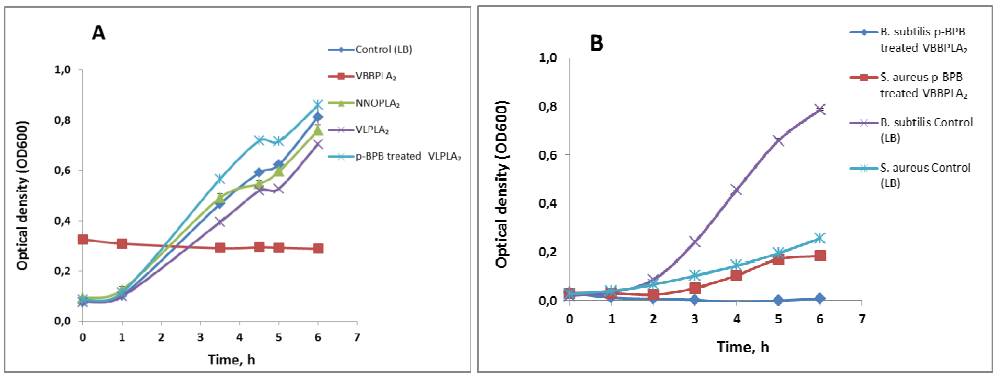
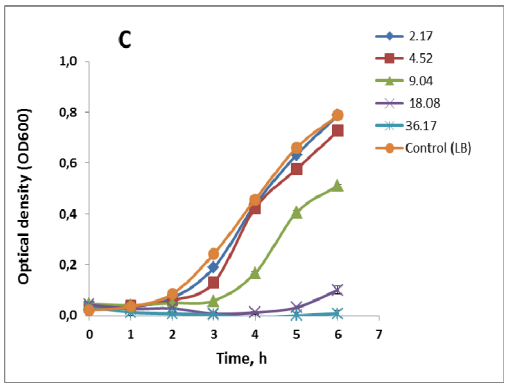


Figure 3. *Cont.*



2.4. Effects of Snake Venom PLA$_2$s on Cancer Cells Viability

Cancer cell lines (PC-3, LNCaP, MCF-7, B10-F16 and K-562) were exposed to PLA$_2$s from *V. lebetina*, *V. berus berus* and *N. naja oxiana* at concentrations of 10 and 100 μg/mL (~0.7 and ~7 μM). The results are shown in Figure 4. There was no inhibitory effect of studied PLA$_2$ preparations towards LNCaP cells in this concentration range (Figure 4A–C). The viability of PC-3 cells was not changed after treating with 7.31 μM of VLPLA$_2$ (Figure 4B). NNOPLA$_2$ had no cytotoxic effect on MCF-7 cells (Figure 4C), VBBPLA$_2$ and VLPLA$_2$ only slightly reduced the viability of MCF-7 cells (Figure 4A,B). VLPLA$_2$ and NNOPLA$_2$ decreased viability of B16-F10 cells about 17% (Figure 4B,C), VBBPLA$_2$ had no effect (Figure 4A). All three enzymes inhibited the viability of K-562 cells (Figure 4A–C), although VLPLA$_2$ had only slight effect (Figure 4B). The most potent inhibitory effect was observed in case of VBBPLA$_2$. After 48 h treatment of K-562 cells with 7.23 μM of VBBPLA$_2$, the cellular viability reduced to 20% (Figure 4D). *p*-BPB-treated VBBPLA$_2$ inhibited the viability of K-562 cells by 27%. VBBPLA$_2$ reduced the viability of K-562 cells in time- and dose-dependent manner.

Figure 4. Effect of svPLA$_2$s on viability of PC-3, LNCaP, K-562, MCF-7 and B16-F10 cells *in vitro*. (**A**–**C**) Cells were seeded in 96-well plates at a density 10^5 cells/mL and incubated at 37 °C for at least 24 h. After treatment with snake venom PLA$_2$s (~0.7 and ~7 μM) for 24 h, the viability of the cells was determined by MTT assay (PC-3) or by water-soluble tetrazolium salt WST-1 assay (LNCaP, K-562, MCF-7 and B16-F10); (**D**) K-562 cells were treated with VBBPLA$_2$ (0.72 μM and 7.23μM) for 24 and 48 h. Data are means (±SD) from two independent experiments performed in triplicate.

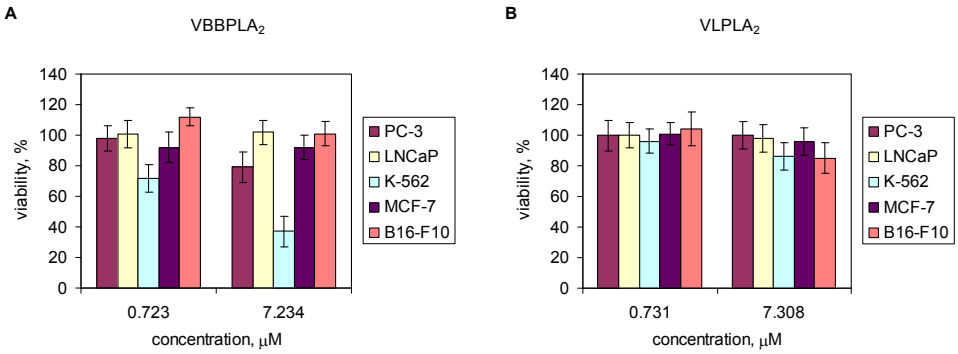
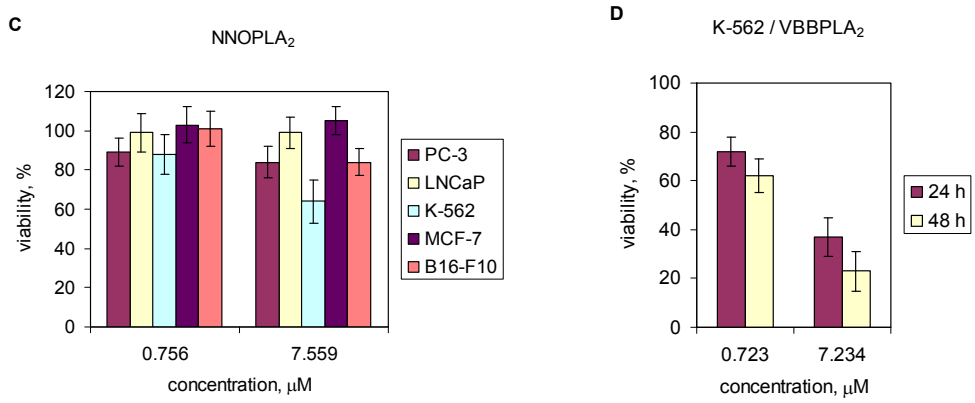


Figure 4. *Cont.*



To evaluate whether the cytotoxicity effect of $VBBPLA_2$ on K-562 cells (Figure 4D) was necrotic or apoptotic, the treated cells were stained with Annexin-V-FITC and propidium iodide (PI) (Figure 5). One characteristic feature of apoptosis is the externalisation of the lipid phosphatidyl serine (PS) from the inner to the outer plasma membrane. Annexin-V is a calcium-dependent phospholipid-binding protein that specifically binds PS and hence stains apoptotic cells. When used in conjunction with a live/dead cell discriminator such as propidium iodide, which measures membrane integrity, the bright green early apoptotic cells (Annexin-V positive) can be distinguished from the red colored late apoptotic/necrotic cells (PI positive). PI stains the cells with ruptured plasma membrane as cells with intact membranes are not permeable to PI. Thus, PI stains both, the cells in the late stage of apoptosis and the cells in necrosis. The treatment of K-562 cells with 0.36 μM $VBBPLA_2$ caused the loss of cell membrane's asymmetry which is a sign of early apoptosis (Figure 5A).

Figure 5. Epifluorescence micrographs of human K-562 cells after incubation with different concentrations of $VBBPLA_2$. After the exposure, the cells were stained with both Annexin V-FITC and propidium iodide, to visualize the early and late stage of apoptosis and/or necrosis of the cells, respectively. (**A**) 0.36 μM (expose 24 h)—early apoptotic cells with intact membranes—green; (**B**) 0.36 μM (expose 28 h)—mixture of early apoptotic cells (green) and cells which have already lost their membrane integrity (orange); (**C**) 0.72 μM (expose 24 h)—late apoptotic cells (orange to red) with blebbes (white arrows) and green membrane fragments; (**D**) 7.23 μM (expose 24 h)—totally destroyed necrotic (red) cells with membrane blebbes (white arrows).

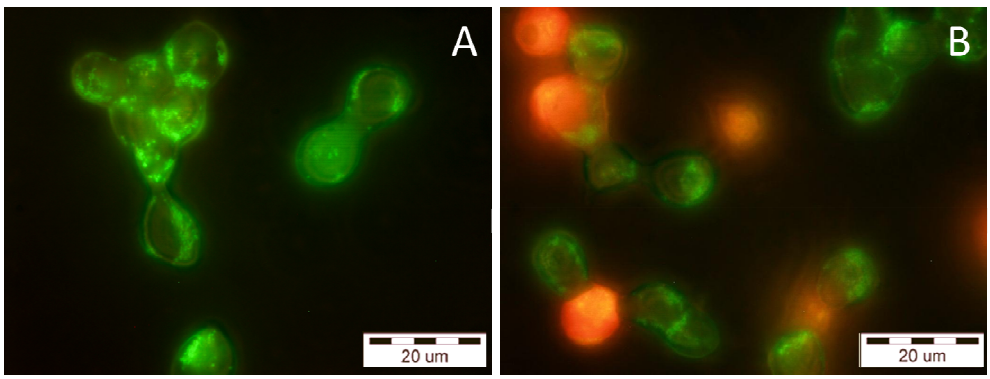
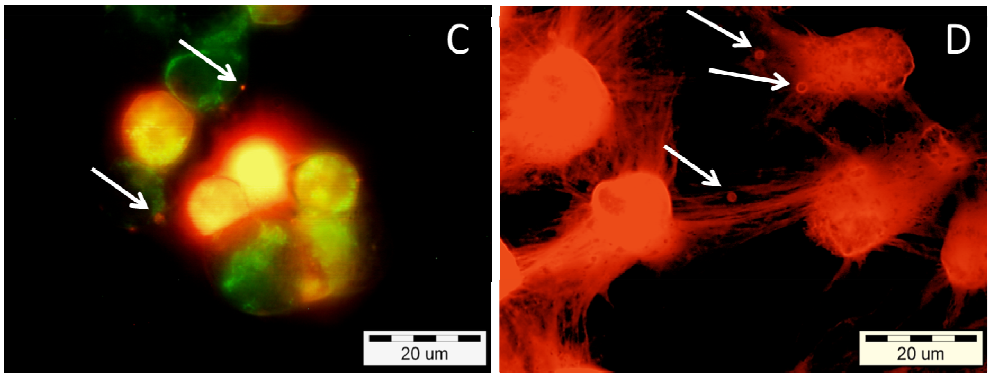


Figure 5. *Cont.*



The transition from apoptosis to necrosis is a loosely defined continuum that necessitates recognition of the various stages of the process. Therefore, we performed a time course experiment (the cells were photographed after 24 h and 28 h of incubation) to prove that the cells were traversing through early apoptosis before reaching the late apoptosis/necrosis (Figure 5A,B). In our study the bright green cells (Annexin-V positive early apoptotic cells) turned to orange (Annexin-V and PI positive late apoptotic cells) when $VBBPLA_2$ concentration was increased from 0.36 μM (Figure 5A) to 0.72 μM (Figure 5C) but also in case of lower $VBBPLA_2$ concentration (0.36 μM) if the incubation time was prolonged to 28 h (Figure 5B). The cells treated with 7.23 μM $VBBPLA_2$ appeared totally destroyed, but it was still possible to detect the characteristic sign of apoptosis—membrane blebbing (Figure 5D, white arrows).

3. Discussion

Snake venom $sPLA_2$s exhibit a large variety of pharmacological effects. In this work we compared the effects of $sPLA_2$s originating from the venoms of three different snakes on human platelets, different bacteria and five types of cancer cells *in vitro*. *Naja naja oxiana* PLA_2 belongs to PLA_2 from old world snakes (group I) and has different disulfide bond pattern than PLA_2s from new world's snakes such as $VBBPLA_2$ and $VLPLA_2$ (group II).

Kini and Evans [15] divided snake venom PLA_2s based on their effects on platelet function into three classes: class A involves PLA_2s which initiate platelet aggregation, class B PLA_2s cause only the inhibition of platelet aggregation induced by several physiological agonists such as collagen and class C involves PLA_2s that have dual activity acting as inducer and inhibitor, depending of conditions. Classes B and C are both subdivided into two subgroups. Inhibitory activity of class B1 PLA_2s (but not class B2) is dependent on their catalytic activity. Results of the current study show that $VBBPLA_2$ and $NNOPLA_2$ belong to class B1. In class B1 the inhibitory effects against platelets aggregation have been explained by hydrolysis of phospholipids from the plasma and/or from lipoproteins and the formation of lysophosphatidylcholine (lysoPC) [21,22,51]. The platelet aggregation inhibitory effects of PLA_2s have shown to be dependent on plasma factor for several snake venom PLA_2s, including $VLPLA_2$ [18], the antiplatelet PLA_2 purified from the venoms of *Austrelaps superba* [51], *Lachesis muta* [21,52], and *Micropechis ikaheka* [53]. Yuan *et al.* [51] showed that the formation of lysoPC after incubation with snake venom PLA_2 correlated with the inhibition of platelet aggregation.

The isoelectric point values of snake venom PLA_2s vary and therefore PLA_2s are classified as acidic, neutral or basic. This property may affect the binding affinity and specificity of PLA_2s to phospholipid membranes. However, pI values of PLA_2s are not predictive for their effect on platelet aggregation: the acidic $VLPLA_2$ [18], acidic PLA_2s from the venoms of *Trimeresurus gramineus* [13] and *Agkistrodon acutus* [14] and basic PLA_2s from *V. berus berus* venom (this work), from *Acanthopis praelongus* venom [16] and acanthins from *Acanthopis antarcticus* venom [22] are all potent platelet inhibitors. On the contrary, bothropstoxin-II (Bthtx-II), a basic Asp^{49} phospholipase A_2 isolated from *Bothrops jararacussu* snake venom was able to induce platelet aggregation in a concentration-dependent manner [17]. $NNOPLA_2$ with almost neutral pI (6.7) inhibited collagen induced platelet aggregation more slowly than $VBBPLA_2$ and $VLPLA_2$ (Figure 2).

Although only PLA_2 from *V. lebetina* but not the PLA_2s from *V. berus berus* and *N. naja oxiana* showed acute toxic effect on *Vibrio fischeri* at 4.24 μM level (Table 1), many snake venom phospholipases A_2 have been shown antibacterial and antiparasitic properties. For example, the Lys^{49} protein from *Bothrops asper* venom showed bactericidal activity on both, gram-positive and gram-negative bacteria [27]. Contrarily, the Lys^{49} $BmarPLA_2$ from *Bothrops marajoensis* showed no antibacterial and antiparasitic effects [36]. Two myotoxic Asp^{49} PLA_2s from *Bothrops neuwiedi pauloensis* venom were bactericidal towards *Escherichia coli* and *Staphylococcus aureus* [31]. Myotoxin I Lys^{49} PLA_2 from *Bothrops atrox* venom was weakly bactericidal against *E. coli* [30]. Myotoxin I Lys^{49} PLA_2 and myotoxin II Asp^{49} $PLA_2$from *Bothrops jararacussu* venom showed antibacterial effect against gram-negative bacteria *Xanthomonas* [54]. Myotoxic Asp^{49} PLA_2 MTX-I and Lys^{49} PLA_2 MTX-II isolated from *Botrops brazili* venom and cationic synthetic peptides derived from their 115–129 *C*-terminal region displayed toxic effects against *E. coli*, *Candida albicans* and *Leishmania sp*. and human T-cell leukemia (JURKAT) cell lines [55].

In the current study, the 36.17 μM $VBBPLA_2$ totally inhibited the growth of gram-positive bacteria *Bacillus subtilis* (Table 2, Figure 3A) but did not inhibit the growth of other bacterial strains analyzed (Table 2). $VBBPLA_2$ has highly cationic nature as it contains numerous positively charged Arg and Lys residues that may promote its binding to negatively-charged outer surface of bacteria. The majority of antimicrobial peptides are positively charged at physiological pH, and prevailing view is that their selectivity stems from electrostatic attraction of the cationic peptide to the anionic bacterial membranes [56]. However, to another gram-positive bacterium, *Staphylococcus aureus*, native $VBBPLA_2$ had no inhibitory effect (Table 2).

The activity and expression of several PLA_2 isoforms are increased in several human cancers, including breast, pancreatic and prostate cancers, suggesting that these enzymes may have a central role in both tumor development and progression and thus can be targets for anticancer drugs [12,57]. On the other hand, some snake venom PLA_2s may have antitumoral activity [12]. Crotoxin, a noncovalent complex (formed by two nonidentical subunits: a basic PLA_2 crotoxinB and a nonenzymatic acidic crotoxinA) isolated from the venom of *Crotalus durissus terrificus,* exhibits a preferential cytotoxic activity against various types of tumor cells including K-562 cells [58], MCF-7 cells [59] and lung adenocarcinoma A549 cells. Treatment of A549 cells with crotoxin significantly inhibited the cell growth in a dose-dependent manner and displayed anti-angiogenic effects *in vitro* [60]. Crotoxin has been used in the treatment of different advanced carcinomas [61]. It has been shown that *bl*D- PLA_2 from *Bothrops leucurus* snake venom reduced K-562 cellular viability in a

dose-dependent manner causing disruption of cellular membrane integrity [62]. Several secreted PLA_2s were found to play role in apoptosis [63]. PLA_2 from *Naja naja atra* venom induced apoptotic cell death of K-562 cells [43]. A Lys^{49} phospholipase A_2 from *Protobothrops flavoviridis* venom induced caspase-independent apoptotic cell death accompanied by rapid plasma-membrane rupture in human leukaemia cells. However, Asp^{49} PLA_2 from the same venom failed to induce death of JURKAT cells [1].

In this study, different cancer cell lines (PC-3, LNCaP, K-562, MCF-7, B10-F16) were exposed to different PLA_2s from *V. lebetina*, *V. berus berus* and *N. naja oxiana*. At the highest concentration tested (~7 μM), there was no inhibitory effect of studied PLA_2 preparations towards LNCaP cells (Figure 4A–C). This is coherent with the data of Sved *et al.* [64] on the consistent and dose-dependent stimulatory effect of human recombinant $sPLA_2$-IIA on LNCaP cell growth. In the current study, the most potent inhibitory effect of studied $svPLA_2$s was observed for $VBBPLA_2$ towards human chronic myeloid leukemic cell line K-562 (Figure 4D). In addition, *p*-BPB-treated inactive $VBBPLA_2$ yielded 27% loss of viability in K-562 cells. Thus, $VBBPLA_2$-induced cell death is dependent not only of enzymatic activity.

4. Materials and Methods

4.1. Materials

The venoms of *V. lebetina* and *N. n. oxiana* were commercial preparations from Tashkent Integrated Zoo Plant (Uzbekistan), *V. b. berus* venom was obtained from Khimki Serpentarium (Moscow, Russia). Sephadex G-100 (superfine) was product of Pharmacia (Uppsala, Sweden). 2,5-dihydroxybenzoic acid (DHB), 3,5-dichlorophenol, bovine serum albumin (BSA), ovalbumin, carboanhydrase, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), soybean trypsin inhibitor, Substance P, Cytochrome C, insulin B chain, *p*-bromophenacylbromide (*p*-BPB) and camptothecin were from Sigma (St. Louis, MO, USA), trypsin (Promega, Madison, WI, USA). WST-1 was from Roche Diagnostics, collagen from Chronolog. Annexin V/Dead Cell Apoptosis Kit with FITC annexin V and propidium iodide (PI) were from Invitrogen, Eugene, OR, USA. All other reagents used were of analytical grade.

4.2. Purification of Enzymes

Vipera lebetina PLA_2 was purified according to Vija *et al.* [18], *Vipera berus berus* PLA_2 ($VBBPLA_2$) was separated from the venom as described by Križaj *et al* [48]. *Naja naja oxiana* venom PLA_2 ($NNOPLA_2$) was purified by gel filtration on Sephadex G-50 sf. and hydrophobic chromatography on pentylagarose. Purity and molecular masses of enzymes were detected by SDS-PAGE and MALDI-TOF MS (see 4.6.).

4.3. PLA_2 Assay

Phospholipase A_2 activity was assayed by titrimetric method using egg yolk phosphatidylcholine as a substrate [65]. Briefly, one egg yolk was added to 100 mL of bidistilled water and aqueous emulsion was prepared by homogenisation. Per assay, 1.5 mL of the egg yolk emulsion was diluted with 3 mL

of Triton X-100 and $CaCl_2$ being 0.75% and 0.15 mM, respectively. The pH was set at 8.0; 10 μL (0.1 mg/mL) of PLA_2 sample was added and the fatty acids released were titrated with 10 mM KOH using a pH-stat (TTT80/pHM84/ABU80, Radiometer, Copenhagen, Denmark) at 25 °C.

4.4. PLA_2 Activity Inhibition with p-bromophenacylbromide (p-BPB)

PLA_2s (0.4 mg) were dissolved in 0.4 mL of 0.1 M ammonium acetate (pH 7.4) containing 0.4 mM of *p*-BPB and incubated for 24 h at room temperature. Excess of the reagents was removed by ultrafiltration through the microspin filter (cut-off 5000 MW, Cole-Parmer, Vernon Hills, IL, USA), the protein fraction was washed with 0.1 M ammonium acetate (pH 7.4) and lyophilized.

4.5. Protein Quantification

Protein concentrations were determined using the Pierce micro BCA kit. Bovine serum albumin was used as a standard. During the process of column chromatography, the elution profile of proteins was followed by the absorbance at 280 nm.

4.6. Molecular Mass Detection and Isoelectric Focusing of Proteins

The molecular masses of the purified proteins were determined by SDS-PAGE on 12.5% polyacrylamide gels using the method of Laemmli [66]. Molecular mass standards for SDS-PAGE were albumin—66 kDa, ovalbumin—45 kDa, carboanhydrase—29 kDa, soybean trypsin inhibitor—20 kDa, cytochrome C—12.3 kDa.

The molecular masses of the fractions were also determined using a home-built matrix-assisted laser desorption/ionization-time of flight mass spectrometer (MALDI-TOF MS) (National Institute of Chemical Physics and Biophysics, Tallinn, Estonia). Before the analysis the freeze-dried samples of protein fractions were dissolved in 5 μL of 50% acetonitrile containing 0.1% trifluoroacetic acid. Aliquots of 0.5 μL were applied onto the target, allowed to air dry and 0.5 μL of the matrix solution (2,5-dihydroxybenzoic acid) was applied to the target and allowed to dry in air. The mass calibration standards were cytochrome C, insulin B chain. A nitrogen 337 nm laser (4 ns pulse) was used and at least 30–40 shots were summarized.

Analytical isoelectric focusing was performed on 5% polyacrylamide gel plates according to the method of Vesterberg [67] in Multiphor 2117 (LKB, Bromma, Sweden) apparatus in the pH range of 3.6–9.3. Isoelectric focusing markers were amyloglucosidase (pI 3.60), soybean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.20), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase B (pI 6.55), horse myoglobin-acidic band (pI 6.85), horse myoglobin-basic band (pI 7.35) lentil lectin-acidic band (pI 8.15), lentil lectin-middle band (pI 8.45), lentil lectin-basic band (pI 8.65) and trypsinogen (pI 9.30). The gels were stained for proteins with Coomassie Brilliant Blue R250.

4.7. In-Gel Tryptic Digestion and Mass Fingerprinting of Proteins

After visualization with Coomassie Blue the gel-electrophoresis bands of protein in interest (native or reduced) were excised from SDS-PAGE gels, each gel slice cut into small pieces (1 mm^2), placed into eppendorf tubes and treated as described earlier [68]. Equal volumes (0.5 μL) of the peptide

mixture and the matrix (2,5-dihydroxybenzoic acid, or α-cyano-4-hydroxycinnamic acid) were mixed on the MALDI-TOF plate. The mass calibration standards were substance P and angiotensin II.

4.8. Preparation of Human Platelet Suspension and Collagen-Induced Platelet Aggregation Assay

Collagen-induced platelet aggregation assays were performed in human platelet-rich plasma (PRP). Blood was collected from healthy adult volunteers who had not taken any medication for at least two weeks prior to sampling. The blood was collected according to the permissions LO2354 (14.12.2010) and LO2513 (21.07.2011).

In order to obtain PRP the blood was dispensed into polystyrene tubes containing 0.129 M sodium citrate (9:1 *v*/*v*) as anticoagulant and after centrifugation at 180 × *g* at room temperature for 10 min platelet suspensions were prepared according to the previously described protocol [69]. Platelet aggregation was measured photometrically in a Whole-Blood aggregometer (Chronolog Corporation, Havertown, PA, USA) under continuous stirring at 900 rpm at 37 °C. Control experiments were done using collagen (platelet agonist) alone.

4.9. Antibacterial Activity

4.9.1. Bacterial Strains

Altogether, four different bacterial strains were used. Naturally luminescent *Vibrio fischeri* NRRL-B-11177 was purchased from Aboatox (Turku, Finland). Constitutively luminescent *Escherichia coli* MC1061(pSLlux) and *Staphylococcus aureus* RN4220(p602/22lux) were constructed earlier by Ivask *et al.* [70]. *Bacillus subtilis* BR151 was obtained from Turku University (Finland). Two former strains are gram-negative and two latter ones gram-positive bacteria.

4.9.2. Analysis of Antibacterial Activity of PLA_2s

Antibacterial activity of $sPLA_2$s was analyzed using two different methods: (i) inhibition of the luminescence of naturally luminescent gram-negative bacterium *Vibrio fischeri* after 15 minutes of exposure and (ii) inhibition of the growth of gram-negative bacteria *Escherichia coli* and *Staphylococcus aureus* and gram-positive bacteria *Bacillus subtilis* upon 6 hour exposure to PLA_2s of various snakes.

4.9.2.1. Bioluminescence Inhibition Assay Using *Vibrio fischeri*

The *Vibrio fischeri* test bacteria were prepared as described in Kurvet *et al.* [71]. Briefly, *V. fischeri* bacterial suspension was obtained by rehydration of freeze-dried *V. fischeri* Reagent (Aboatox, Turku, Finland) using 2% NaCl, stabilized for 40 min at 4 °C and then at 20 °C for 40 min and then used for testing. 2% NaCl served as a test diluent and as a negative control. 3,5-dichlorophenol was used as a positive control. The assay was performed at 20 °C instead of 15 °C recommended by standard operational procedure of Microtox™ (AZUR Environmental, Carlsbad, CA, USA) as most luminometers do not allow the temperature adjustment to 15 °C.

Testing was performed essentially as described in Kahru [50] using 1253 Luminometer and respective software for the data reduction (both BioOrbit, Turku, Finland). Toxicity (15-min EC_{50}), *i.e.*, the concentration of $svPLA_2$ causing a 50% reduction in light output of bacteria after 15-min contact time, was determined from respective concentration-effect curves.

4.9.2.2. Bacterial Growth Inhibition Assays

E. coli, *S. aureus* and *B. subtilis* were maintained in LB agar plates (LabM, Lancashire, UK) supplemented with respective antibiotics (see below) at +4 °C. For the toxicity tests, bacteria were cultivated (on a shaker at 200 rpm, 30 °C) overnight in 3 mL of LB medium. As a test medium for the growth inhibition assays and as a diluent for $svPLA_2$s LB medium without NaCl was used. Ampicillin (100 μg/mL) in case of *E. coli* and kanamycin (50 μg/mL) in case of *S. aureus* were added to LB medium. No antibiotics were added to *B. subtilis* culture medium. For the assay, overnight bacterial culture was diluted 1:25 in LB medium containing respective antibiotics (see above). Then, 100 μL of test bacteria was added to 100 μL of the $svPLA_2$ dilution. Each $svPLA_2$ was tested in following concentrations: 500, 250, 125, 62.5 and 31.25 μg/mL. Each $svPLA_2$ concentration was tested in three and the controls in ten replicates. 96-well polystyrene microplates with transparent bottoms and not-transparent sides of the wells (Greiner Bio-One, Frickenhausen, Germany) were used. Optical density of the bacterial suspensions at 600 nm (OD_{600}) was measured using Multiscan Spectrum spectrophotometer (Thermo Scientific, Vantaa, Finland). The measurements were performed in 1 h intervals till 6 h and then also 24 h data were registered. Between the measurements till 6 h the plates were incubated at 30 °C on a shaker (Heidolph Titramax 1000, Schwabach, Germany) at 750 rpm and then statically overnight in the incubator at 30 °C. The inhibition of the growth of bacteria was calculated as percentage of the non-exposed control.

To evaluate the ability of $svPLA_2$-exposed bacteria (after 6 h and 24 h incubation) to grow on solid media, 1 μL of bacterial suspension was streaked onto Petri dishes with LB agar containing no antibiotics. The growth of bacteria was visually checked after incubation of Petri plates at 30 °C for 48 h.

4.10. Human Cell Lines and Toxicity Testing of $sPLA_2$s

The human prostate cancer cell lines PC-3, LNCaP, human chronic myeloid leukemic cell line K-562, breast cancer cell line MCF-7 and mouse melanoma cell line B16-F10 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). PC-3 cells were maintained in DMEM/F-12 medium (Gibco, Grand Island, NY, USA), LNCaP, K-562, MCF-7 and B16-F10 cells in RPMI 1640 medium (Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (Gibco) and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) at 37 °C and 5% CO_2 in a fully humidified atmosphere.

4.10.1. Analysis of the Viability of the Cells

The viability was determined by the MTT assay (PC-3 cells) and WST-1 assay (LNCaP, K-562, MCF-7 and B16-F10) based on the reduction of MTT or WST-1 by viable cells, respectively.

4.10.1.1. MTT Assay

Human prostate cancer PC-3 cells were seeded in 96-well plates (Sarstedt, Germany) at a density of 1–2 × 10^5 cells/ml. After 24 h of incubation 37 °C the cells were incubated with svPLA$_2$s diluted with medium and added to the wells at final concentrations of 10 and 100 μg/mL. The cells not treated with sPLA$_2$ served as a control. After certain time intervals, MTT solution was added to each well at a final concentration of 0.5 mg/mL and the plates were incubated at 37 °C for 4 h. The MTT formazan product was dissolved by addition of 110 μL acidified 2-propanol (in 0.04 N HCl) to each well. The absorbance was detected in micro-plate reader (Multiskan Spectrum, Thermo, Vantaa, Finland) at 540 nm. Cell survival rate was calculated as (absorbance of the treated wells)/(absorbance of the control wells) × 100%.

4.10.1.2. WST-1 Assay

Human LNCaP, K-562, MCF-7 and B16-F10 cells were seeded in 96-well plates at a density of 1–2 × 10^5 cells/ml. After 24 h of growth cells were incubated with svPLA$_2$s diluted with medium and added to the wells at the desired final concentrations (10 and 100 μg/mL). The cells that were not treated with protein served as control cells. After various time intervals 10 μL/well WST-1 solution was added to each well and the plates were incubated for 1–2 h at 37 °C and 5% CO_2. The absorbance of the WST-1 formazan salt was detected in micro-plate reader at 450 nm. Cell survival rate was calculated as (absorbance of the treated wells)/(absorbance of the control wells) × 100%.

4.10.2. Apoptosis Detection Using Annexin V-FITC and Propidium Iodide (PI)

The detection of K-562 cells apoptosis was performed according to the instructions of FITC Annexin-V/Dead Cell Apoptosis Kit with FITC Annexin-V and PI (Invitrogen, Eugene, OR, USA). The suspension of K-562 cells was seeded into 24-well plates (2 × 10^5 cells/well) on round cover slips and incubated at 37 °C with 5% CO_2 for 24 h. After this period, the cells were treated with VBBPLA$_2$ (0.36, 0.72 and 7.23 μM) for 24 h. In case of 0.36 μM the treatment was prolonged to up to 28 h. 4 μM camptothecin-treated cells (4 h) were used as a positive control for apoptosis. The cells were washed twice with cold phosphate-buffered saline (PBS) and 200 μL of Annexin-V binding buffer, 10 μL of Annexin-V-FITC and 10 μL of PI working solution were added. After incubation in the dark for 15 min at room temperature the reaction mixture was removed and the cells were washed with Annexin-V binding buffer. Then, the cover slips with cells were taken out from the wells and the mounted preparations were made. The viability of the treated and non-treated (control) K-562 cells was observed under an epifluorescence microscope Olympus CX41 with a 100× oil immersion objective lens and fluorescence optics (excitation at 488 nm, >515 nm emission for Annexin V-FITC (green) and for propidium iodide (red)). The pictures were taken using an Olympus U-CMAD3 real time colour digital DP71 camera (Tokyo, Japan) using the CellB Software (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

5. Conclusions

The adverse effects of PLA_2s from *Vipera lebetina*, *Vipera berus berus* and *Naja naja oxiana* venom depended on venom (snake) as well as on target cells (platelets, different cancer cell types and bacteria). As a rule, the observed biological effects on platelets were observed already at 1 μg/mL level (<0.1 μM) and all three PLA_2s were dose-dependently inhibiting the collagen-induced platelet aggregation. The chemical modification of histidine in studied PLA_2s by *p*-bromophenacylbromide resulted in complete loss of their catalytic activity and inhibitory action on collagen-induced platelet aggregation. $VBBPLA_2$ (but not the PLA_2s from *V. lebetina* and *N. naja oxiana*) was totally inhibiting the growth of gram-positive *Bacillus subtilis* at 500 μg/mL (36.2 μM) whereas the inhibitory effect was not due to its catalytic activity but to other properties of the protein. To another gram-positive bacterium, *S. aureus*, native $sPLA_2$ from *N. naja oxiana* inhibited the growth of bacteria by 42% but caused only slight inhibition of growth of *B. subtilis*. None of the studied $svPLA_2$s was inhibitory to the growth of gram-negative bacteria *E. coli* even at 500 μg/mL (~37 μM) level.

The viability of the most sensitive cancer cell type (K-562) was reduced upon exposure of the cells to 7.2 μM $VBBPLA_2$ and to some extent also by PLA_2s from *V. lebetina* and *N. naja oxiana*. There was no inhibitory effect of all studied $svPLA_2$ preparations towards LNCaP cells and low inhibitory effect (8%–20%) towards the PC-3, MCF-7 and B10-F16 cells. Thus, from the current suite of studied $svPLA_2$s and test cells, $VBBPLA_2$ was most growth inhibitory towards gram positive bacteria *B. subtilis* and K-562 cells *in vitro*.

Acknowledgements

The work is financially supported by Estonian Science Foundation Grant No. 8899 and by the Estonian Ministry of Education and Research Target Financing Grant No.SF0690063s08.

Conflict of interest

The authors have no conflict of interest.

References

1. Murakami, M.; Taketomi, Y.; Miki, Y.; Sato, H.; Hirabayashi, T.; Yamamoto, K. Recent progress in phospholipase A2 research: From cells to animals to humans. *Progr. Lipid Res.* **2011**, *50*, 152–192.
2. Schaloske, R.H.; Dennis, E.A. The phospholipase A_2 superfamily and its group numbering system. *Biochim. Biophys. Acta* **2006**, *1761*, 1246–1259.
3. Burke, J.E.; Dennis, E.A. Phospholipase A_2 structure/function, mechanism, and signaling. *J. Lipid Res.* **2009**, *59*, S237–S242.
4. Nevalainen, T.J.; Cardoso, J.C.; Riikonen, P.T. Conserved domains and evolution of secreted phospholipases A(2). *FEBS J.* **2012**, *279*, 636–649.
5. Lomonte, B.; Angulo, Y.; Sasa, M.; Gutiérrez, J.M. The phospholipase A_2 homologues of snake venoms: Biological activities and their possible adaptive roles. *Protein Pept. Lett.* **2009**, *16*, 860–876.

6. Doley, R.; Zhou, X.; Kini, R.M. Snake Venom Phospholipase A_2 Enzymes. In *Handbook of Venoms and Toxins of Reptiles*; Mackessy, S.P., Ed.; CRC Press: Boca Raton, FL, USA, 2010; pp. 173–205.
7. Kini, R.M. Excitement ahead: Structure, function and mechanism of snake venom phospholipase A_2 enzymes. *Toxicon* **2003**, *42*, 827–840.
8. Kini, R.M. Structure-function relationships and mechanism of anticoagulant phospholipase A_2 enzymes from snake venoms. *Toxicon* **2005**, *45*, 1147–1161.
9. Lambeau, G.; Lazdunski, M. Receptors for a growing family of secreted phospholipases A_2. *Trends Pharmacol. Sci.* **1999**, *20*, 162–170.
10. Soares, A.M.; Giglio, J.R. Chemical modifications of phospholipases A_2 from snake venoms: Effects on catalytic and pharmacological properties *Toxicon* **2003**, *42*, 855–868.
11. Montecucco, C.; Gutiérrez, J.M.; Lomonte, B. Cellular pathology induced by snake venom phospholipase A_2 myotoxins and neurotoxins: Common aspects of their mechanism of action. *Cell. Mol. Life Sci*. **2008**, *65*, 2897–2912.
12. Rodrigues, R.S.; Izidoro, L.F.; de Oliveira, R.J., Jr.; Sampaio, S.V.; Soares, A.M.; Rodrigues, V.M. Snake venom phospholipases A_2: A new class of antitumor agents. *Protein Pept. Lett*. **2009**, *16*, 894–898.
13. Ouyang, C.; Huang, T.F. Potent platelet aggregation inhibitor from *Trimeresurus gramineus* snake venom. *Biochim. Biophys. Acta* **1983**, *757*, 332–341.
14. Chen, R.H.; Chen, Y.C. Isolation of an acidic phospholipase A_2 from the venom of *Agkistrodon acutus* (five pace snake) and its effect on platelet aggregation. *Toxicon* **1989**, 27, 675–682.
15. Kini, R.M.; Evans, H.J. Effects of Phospholipase A_2 Rnzymes on Platelet Sggregation. In *Venom phospholipase A_2 Rnzymes: Dtructure*, *Gunction and Mechanism*; Kini, R.M., Ed.; John Wiley & Sons: Chichester, UK, 1997; pp. 369–387.
16. Sim, K.L. Purification and preliminary characterisation of praelongin phospholipases, antiplatelet agents from the snake venom of *Acanthophis praelongus*. *Biochim. Biophys. Acta* **1998**, *1379*, 198–206.
17. Fuly, A.L.; Soares, A.M.; Marcussi, S.; Giglio, J.R.; Guimarães, J.A. Signal transduction pathways involved in the platelet aggregation induced by a D-49 phospholipase A_2 isolated from *Bothrops jararacussu* snake venom. *Biochimie* **2004**, *86*, 731–739.
18. Vija, H.; Samel, M.; Siigur, E.; Aaspõllu, A.; Trummal, K.; Tõnismägi, K.; Subbi, J.; Siigur, J. Purification, characterization, and cDNA cloning of acidic platelet aggregation inhibiting phospholipases A_2 from the snake venom of *Vipera lebetina* (Levantine viper). *Toxicon* **2009**, *54*, 429–439.
19. Tsai, I.H.; Wang, Y.M.; Cheng, A.C.; Starkov, V.; Osipov, A.; Nikitin, I.; Makarova, Y.; Ziganshin, R.; Utkin, Y. cDNA cloning, structural, and functional analyses of venom phospholipases A_2 and a Kunitz-type protease inhibitor from steppe viper *Vipera ursinii renardi*. *Toxicon* **2011**, *57*, 332–341.
20. Prasad, B.N.; Kemparaju, K.; Bhatt, K.G.; Gowda, T.V. A platelet aggregation inhibitor phospholipase A_2 from Russell's viper (*Vipera russelli*) venom: Isolation and characterization. *Toxicon* **1996**, *34*, 1173–1185.

21. Fuly, A.L.; Machado, O.L.T.; Alves, E.W.; Carlini, C.R. Mechanism of inhibitory action on platelet activation of a phospholipase A_2 isolated from *Lachesis muta* (Bushmaster) snake venom. *Thromb. Haemost*. **1997**, *78*, 1372–1380.
22. Chow, G.; Subburaju, S.; Kini, R.M. Purification, characterization, and amino acid sequence determination of acanthins, potent inhibitors of platelet aggregation from *Acanthophis antarcticus* (common death adder) venom. *Arch. Biochem. Biophys.* **1998**, *354*, 232–238.
23. Kemparaiu, K.; Krishnakanth, T.P.; Veerabasappa Gowda, T. Purification and characterization of a platelet aggregation inhibitor acidic phospholipase A_2 from Indian saw-scaled viper (*Echis carinatus*) venom. *Toxicon* **1999**, *37*, 1659–1671.
24. Serrano, S.M.T.; Reichl, A.P.; Mentele, R.; Auerswald, E.A.; Santoro, M.L.; Sampaio, C.A.M.; Camargo, A.C.M.; Assakura, M.T. A novel phospholipase A_2, BJ-PLA_2, from the venom of the snake *Bothrops jararaca*: Purification, primary structure analysis, and its characterization as a platelet-aggregation-inhibiting factor. *Arch. Biochem. Biophys.* **1999**, *367*, 26–32.
25. Roberto, P.G.; Kashima, S.; Marcussi, S.; Pereira, J.O.; Astolfi-Filho, S.; Nomizo, A.; Giglio, J.R.; Fontes, M.R.; Soares, A.M.; Franca, S.C. Cloning and identification of a complete cDNA coding for a bactericidal and antitumoral acidic phospholipase A_2 from *Bothrops jararacussu* venom. *Protein J*. **2004**, *23*, 273–285.
26. Satish, S.; Tejaswini, J.; Krishnakantha, T.P.; Gowda, T.V. Purification of a Class B1 platelet aggregation inhibitor phospholipase A_2 from Indian cobra (*Naja Naja*) venom. *Biochimie* **2004**, *86*, 203–210.
27. Páramo, L.; Lomonte, B.; Pizarro-Cerdá, J.; Bengoechea, J.A.; Gorvel, J.P.; Moreno, E. Bactericidal activity of Lys49 and Asp49 myotoxic phospholipases A2 from Bothrops asper snake venom—Synthetic Lys49 myotoxin II-(115–129)-peptide identifies its bactericidal region. *Eur. J. Biochem.* **1998**, *253*, 452–461.
28. Koduri, R.S.; Gronroos, J.O.; Laine, V.J.; le Calvez, C.; Lambeau, G.; Nevalainen, T.J.; Gelb, M.H. Bactericidal properties of human and murine groups I, II, V, X, and XII secreted phospholipases A_2. *J. Biol. Chem*. **2002**, *277*, 5849–5857.
29. Oliveira, D.G.; Toyama, M.H.; Novello, J.C; Beriam, L.O.; Marangoni, S. Structural and functional characterization of basic PLA_2 isolated from *Crotalus durissus terrificus* venom. *J. Protein Chem.* **2002**, *21*, 161–168.
30. Núñez, V.; Arce, V.; Gutiérrez, J.M.; Lomonte, B. Structural and functional characterization of myotoxin I, a Lys^{49} phospholipase A_2 homologue from the venom of the snake *Bothrops atrox*. *Toxicon* **2004**, *44*, 91–101.
31. Rodrigues, V.M.; Marcussi, S.; Cambraia, R.S.; de Araújo, A.L.; Malta-Neto, N.R.; Hamaguchi, A.; Ferro, E.A.; Homsi-Brandeburgo, M.I.; Giglio, J.R.; Soares, A.M. Bactericidal and neurotoxic activities of two myotoxic phospholipases A_2 from *Bothrops neuwiedi pauloensis* snake venom. *Toxicon* **2004**, *44*, 305–314.
32. Santamaría, C.; Larios, S.; Angulo, Y.; Pizarro-Cerda, J.; Gorvel, J.P.; Moreno, E.; Lomonte, B. Antimicrobial activity of myotoxic phospholipases A_2 from crotalid snake venoms and synthetic peptide variants derived from their *C*-terminal region. *Toxicon* **2005**, *45*, 807–815.
33. Xu, C.; Ma, D.; Yu, H.; Li, Z.; Liang, J.; Lin, G.; Zhang, Y.; Lai, R. A bactericidal homodimeric phospholipases A_2 from *Bungarus fasciatus* venom. *Peptides* **2007**, *28*, 969–973.

34. Perumal Samy, R.; Gopalakrishnakone, P.; Ho, B.; Chow, V.T. Purification, characterization and bactericidal activities of basic phospholipase A_2 from the venom of *Agkistrodon halys* (Chinese pallas). *Biochimie* **2008**, *90*, 1372–1388.
35. Perumal Samy, R.; Gopalakrishnakone, P.; Bow, H.; Puspharaj, P.N.; Chow, V.T.K. Identification and characterization of a phospholipase A_2 from the venom of the Saw-scaled viper: Novel bactericidal and membrane damaging activities. *Biochimie* **2010**, *92*, 1854–1866.
36. Costa Torres, A.F.; Dantas, R.T.; Toyama, M.H.; Diz Filho, E.; Zara, F.J.; Rodrigues de Queiroz, M.G.; Pinto Nogueira, N.A.; Rosa de Oliveira, M.; de Oliveira Toyama, D.; Monteiro, H.S.; Martins, A.M. Antibacterial and antiparasitic effects of *Bothrops marajoensis* venom and its fractions: Phospholipase A_2 and L-amino acid oxidase. *Toxicon* **2010**, *55*, 795–804.
37. Samy, R.P.; Stiles, B.G.; Gopalakrishnakone, P.; Chow, V.T. Antimicrobial proteins from snake venoms: Direct bacterial damage and activation of innate immunity against *Staphylococcus aureus* skin infection. *Curr. Med. Chem.* **2011**, *18*, 5104–5113.
38. Toyama, M.H.; Toyama, D.O.; Joazeiro, P.P.; Carneiro, E.M.; Beriam, L.O.; Marangoni, L.S.; Boschero, A.C. Biological and structural characterization of a new PLA_2 from the *Crotalus durissus collilineatus* venom. *Protein J.* **2005**, *24*, 103–112.
39. Nevalainen, T.J.; Graham, G.G.; Scott, K.F. Antibacterial actions of secreted phospholipases A2. *Biochim. Biophys. Acta* **2008**, *1781*, 1–9.
40. Roberto, P.G.; Kashima, S.; Soares, A.M.; Chioato, L.; Faca, V.M.; Fuly, A.L.; Astolfi-Filho, S.; Pereira, J.O.; Franca, S.C. Cloning and expression of an acidic platelet aggregation inhibitor phospholipase A_2 cDNA from *Bothrops jararacussu* venom gland. *Protein Expr. Purif.* **2004**, *37*, 102–108.
41. Maity, G.; Mandal, S.; Chatterjee, A.; Bhattacharyya, D. Purification and characterization of a low molecular weight multifunctional cytotoxic phospholipase A_2 from Russell's viper venom. *J. Chromatogr. B* **2007**, *845*, 232–243.
42. Zouari-Kessentini, R.; Luis, J.; Karray, A.; Kallech-Ziri, O.; Srairi-Abid, N.; Bazaa, A.; Loret, E.; Bezzine, S.; el Ayeb, M.; Marrakchi, N. Two purified and characterized phospholipases A_2 from *Cerastes cerastes* venom, that inhibit cancerous cell adhesion and migration. *Toxicon* **2009**, 53, 444–453.
43. Chen, K.C.; Liu, W.H.; Chang, L.S. Taiwan cobra phospholipase A2-elicited JNK activation is responsible for autocrine fas-mediated cell death and modulating Bcl-2 and Bax protein expression in human leukemia K562 cells. *J. Cell Biochem.* **2010**, *109*, 245–254.
44. Murakami, T.; Kamikado, N.; Fujimoto, R.; Hamaguchi, K.; Nakamura, H.; Chijiwa, T.; Ohno, M.; Oda-Ueda, N. A [Lys^{49}] phospholipase A_2 from *Protobothrops flavoviridis* venom induces caspase-independent apoptotic cell death accompanied by rapid plasma-membrane rupture in human leukemia cells. *Biosci. Biotechnol. Biochem.* **2011**, *75*, 864–870.
45. Khunsap, S.; Pakmanee, N.; Khow, O.; Chanhome, L.; Sitprija, V.; Suntravat, M.; Lucena, S.E.; Perez, J.C.; Sánchez, E.E.J. Purification of a phospholipase A(2) from *Daboia russelii siamensis* venom with anticancer effects. *Venom Res.* **2011**, *2*, 42–51.

46. Bazaa, A.; Luis, J.; Srairi-Abid, N.; Kallech-Ziri, O.; Kessentini-Zouari, R.; Defilles, C.; Lissitzky, J.C.; el Ayeb, M.; Marrakchi, N. MVL-PLA$_2$, a phospholipase A$_2$ from *Macrovipera lebetina transmediterranea* venom, inhibits tumor cells adhesion and migration. *Matrix Biol*. **2009**, *28*, 188–193.
47. Bazaa, A.; Pasquier, E.; Defilles, C.; Limam, I.; Kessentini-Zouari, R.; Kallech-Ziri, O.; el Battari, A.; Braguer, D.; el Ayeb, M.; Marrakchi, N.; Luis, J. MVL-PLA$_2$, a snake venom phospholipase A$_2$, inhibits angiogenesis through an increase in microtubule dynamics and disorganization of focal adhesions. *PLoS One* **2010**, *5*, e10124.
48. Križaj, I.; Siigur, J.; Samel, M.; Cotiĉ, V.; Gubenšek, F. Isolation, partial characterization and complete amino acid sequence of the toxic phospholipase A$_2$ from the venom of the common viper, *Vipera berus berus*. *Biochim. Biophys. Acta* **1993**, *1157*, 81–85.
49. Ovchinnikov, Y.A.; Miroshnikov, A.I.; Nazimov, I.V.; Apsalaon, U.R.; Soldatova, L.N. Complete amino acid sequence of phospholipase A$_2$ (isozyme E3) from the venom of middle Asian cobra *Naja naja oxiana*. *J. Bioorg. Khim.* **1979**, *5*, 805–813.
50. Kahru, A. *In vitro* toxicity testing using marine luminescent bacteria *Photobacterium phosphoreum*: The BiotoxTM test. *Atla-Altern. Lab. Anim.* **1993**, *21*, 210–215.
51. Yuan, Y.; Jackson, S.P.; Newnham, H.H.; Mitchell, C.A.; Salem, H.H. An essential role for lysophosphatidylcholine in the inhibition of platelet aggregation by secretory phospholipase A$_2$. *Blood* **1995**, *86*, 4166–4174.
52. Fuly, A.L.; de Miranda, A.L.P.; Zingali, R.B.; Guimarães, J.A. Purification and characterization of a phospholipase A$_2$ isoenzyme isolated from *Lachesis muta* snake venom. *Biochem. Pharmacol*. **2002**, *63*, 1589–1597.
53. Gao, R.; Kini, R.M.; Gopalakhrishnakone, P. Purification, properties, and amino acid sequence of a hemoglobinuria-inducing phospholipase A$_2$ MiPLA-1, from micropechis ikeheka venom. *Arch. Biochem. Biophys.* **1999**, *369*, 181–192.
54. Barbosa, P.S.; Martins, A.M.; Havt, A.; Toyama, D.O.; Evangelista, J.S.; Ferreira, D.P.; Joazeiro, P.P.; Beriam, L.O.; Toyama, M.H.; Fonteles, M.C.; Monteiro, H.S. Renal and antibacterial effects induced by myotoxin I and II isolated from *Bothrops jararacussu* venom. *Toxicon* **2005**, *46*, 376–386.
55. Costa, T.R.; Menaldo, D.L.; Oliveira, C.Z.; Santos-Filho, N.A.; Teixeira, S.S.; Nomizo, A.; Fuly, A.L.; Monteiro, M.C.; de Souza, B.M.; Palma, M.S.; *et al*. Myotoxic phospholipases A(2) isolated from *Bothrops brazili* snake venom and synthetic peptides derived from their *C*-terminal region: Cytotoxic effect on microorganism and tumor cells. *Peptides* **2008**, *29*, 1645–1656.
56. Glukhov, E.; Stark, M.; Burrows, L.L.; Deber, C.M. Basis for selectivity of cationic antimicrobial peptides for bacterial *versus* mammalian membranes. *J. Biol. Chem.* **2005**, *280*, 33960–33967.
57. Cummings, B.S. Phospholipase A$_2$ as targets for anti-cancer drugs. *Biochem. Pharmacol.* **2007**, *74*, 949–959.
58. Yan, C.H.; Liang, Z.Q.; Gu, Z.L.; Yang, Y.P.; Reid, P.; Qin, Z.H. Contributions of autophagic and apoptotic mechanisms to CrTX-induced death of K562 cells. *Toxicon* **2006**, *47*, 521–530.
59. Yan, C.H.; Yang, Y.P.; Qin, Z.H.; Gu, Z.L.; Reid, P.; Liang, Z.Q. Autophagy is involved in cytotoxic effects of crotoxin in human breast cancer cell line MCF-7 cells. *Acta Pharmacol. Sin.* **2007**, *28*, 540–548.

60. Ye, B.; Xie, Y.; Qin, Z.H.; Wu, J.C.; Han, R.; He, J.K. Anti-tumor activity of CrTX in human lung adenocarcinoma cell line A549. *Acta Pharmacol. Sin*. **2011**, *32*, 1397–1401.
61. Cura, J.E.; Blanzaco, D.P.; Brisson, C.; Cura, M.A.; Carbol, R.; Larrateguy, L.; Mendez, C.; Sechi, J.C.; Silveira, J.S.; Theiller, E.; de Roodt, A.R.; Vidal, J.C. Phase I and pharmacokinetics study crotoxin (cytotoxic PLA_2 NSC-624 244) in patients with advanced cancer. *Clin. Cancer Res*. **2002**, *8*, 1033–1041.
62. Higuchi, D.A.; Barbosa, C.M.V.; Bincoletto, C.; Chagas, J.R.; Magalhaes, A.; Richardson, M.; Sanches, E.F.; Pesquero, J.B.; Araujo, R.C.; Pesquero, J.L. Purification and partial characterization of two phospholipases A_2 from *Bothrops leucurus* venom (white-tailed-jararaca) snake venom. *Biochimie* **2007**, *89*, 319–328.
63. Taketo, M.M.; Sanoshita, M. Phospholipase A_2 and apoptosis. *Biochim. Biophys. Acta* **2002**, *1585*, 72–76.
64. Sved, P.; Scott, K.F.; McLeod, D.; King, N.J.; Singh, J.; Tsatralis, T.; Nikolov, B.; Boulas, J.; Nallan, L.; Gelb, M.H.; *et al*. Oncogenic action of secreted phospholipase A2 in prostate cancer. *Cancer Res.* **2004**, *64*, 6934–6940.
65 De Haas, G.H.; Postema, N.M.; Nieuwenhuizen, W.; van Deenen, L.L.M. Purification and properties of phospholipase A from porcine pancreas. *Biochim. Biophys. Acta* **1968**, *159*, 103–117.
66 Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685.
67 Vesterberg, O. Isoelectric focusing of proteins in polyacrylamide gels. *Biochim. Biophys. Acta* **1972**, *257*, 11–19.
68 Siigur, E.; Aaspõllu, A.; Trummal, K.; Tõnismägi, K.; Tammiste, I.; Kalkkinen, N.; Siigur, J. Factor X activator from *Vipera lebetina* venom is synthesized from different genes. *Biochim. Biophys. Acta* **2004**, *1702*, 41–51.
69 Siigur, J.; Samel, M.; Tõnismägi, K.; Subbi, J.; Siigur, E.; Tu, A.T. Biochemical characterization of lebetase, a direct-acting fibrinolytic enzyme from *Vipera lebetina* snake venom. *Thromb. Res.* **1998**, *90*, 39–49.
70 Ivask, A.; Rõlova, T.; Kahru, A. A suite of recombinant luminescent bacterial strains for the quantification of bioavailable heavy metals and toxicity testing. *BMC Biotechnol.* **2009**, *9*, 41.
71 Kurvet, I.; Ivask, A.; Bondarenko, O.; Sihtmäe, M.; Kahru, A. LuxCDABE—Transformed Constitutively Bioluminescent *Escherichia coli* for toxicity Screening: Comparison with naturally luminous *Vibrio fischeri*. *Sensors* **2011**, *11*, 7865–7878.