

## RESEARCH REPORT

# Purification of a phospholipase A<sub>2</sub> from *Daboia russelii siamensis* venom with anticancer effects

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## ABSTRACT

Venom phospholipases A<sub>2</sub> (PLA<sub>2</sub>) are associated with neurotoxic, myotoxic, cardiotoxic, platelet aggregation, and edema activities. A PLA<sub>2</sub> (Drs-PLA<sub>2</sub>) was purified from *Daboia russelii siamensis* venom by a two-step purification procedure consisting of size-exclusion, followed by anion exchange high performance liquid chromatography (HPLC). The molecular weight of the Drs-PLA<sub>2</sub> was 13,679Da, which was determined by MALDI-TOF mass spectrometry. Its N-terminal amino acid sequence was homologous to basic PLA<sub>2</sub>s of viperid snake venoms. The Drs-PLA<sub>2</sub> had indirect hemolytic and anticoagulant activities, cytotoxic activity with a CC<sub>50</sub> of 65.8nM, and inhibited SK-MEL-28 cell migration with an IC<sub>50</sub> of 25.6nM. In addition, the Drs-PLA<sub>2</sub> inhibited the colonization of B16F10 cells in lungs of BALB/c mice by ~65%.

**KEYWORDS:** *Daboia russelii siamensis*, phospholipase A<sub>2</sub>, cytotoxic, cell migration inhibition, lung tumor colonization

## INTRODUCTION

The superfamily of phospholipase A<sub>2</sub> enzymes have been classified as 15 groups and many subgroups that include five distinct types of enzymes, namely the secreted PLA<sub>2</sub> (sPLA<sub>2</sub>), the cytosolic PLA<sub>2</sub> (cPLA<sub>2</sub>), the Ca<sup>2+</sup> independent PLA<sub>2</sub> (iPLA<sub>2</sub>), the platelet-activating factor acetylhydrolases (PAF-AH), lysosomal PLA<sub>2</sub>s, and a recently identified adipose-specific PLA<sub>2</sub> (Duncan et al, 2008; Burke and Dennis 2009a; Ramar et al, 2010). Snake venom is one of the most abundant sources of secretory PLA<sub>2</sub> (sPLA<sub>2</sub>), which are one of the potent molecules in snake venoms (Ritonja and Gubensek, 1985; Maung-Maung et al, 1995; Chakrabarty et al, 2000). sPLA<sub>2</sub> are low molecular weight proteins with molecular masses ranging from 13-19 kDa and generally requires Ca<sup>2+</sup> for their activities (Kini, 1997; Valentin and Lambeau, 2000). Snake venom sPLA<sub>2</sub> are secreted enzymes belonging to only two groups that are based on their primary structure and disulfide bridge pattern (Six and Dennis, 2000;

Rouault et al, 2003; Ramar et al, 2010). Those of group I are the same as pancreatic sPLA<sub>2</sub> present in mammals and are found in venom of Elapidae snakes, while group II PLA<sub>2</sub>s belong to the Viperidae and are similar to mammals' nonpancreatic, inflammatory sPLA<sub>2</sub>s (Lambeau and Lazdunski, 1999; Dennis, 2000). Despite a high identity of their amino acid sequences, they exhibit distinct pharmacological effects including pre- or post-synaptic neurotoxicity, myonecrosis, cardiotoxicity, anticoagulant, antiplatelet aggregation, hemorrhagic, hemolytic, and cytolytic activities (Kini and Evans, 1988; Kasturi and Gowda, 1989; Stefansson et al, 1989; Maung Maung et al, 1995; Huang et al, 1997; Kole et al., 2000; Chakrabarty et al, 2002; Dong et al., 2003; Kini, 2003). Recently, acidic PLA<sub>2</sub>s, basic PLA<sub>2</sub>s, and synthetic peptides derived from PLA<sub>2</sub> homologues have been shown to possess antitumor and anti-angiogenic properties (Roberto et al, 2004; Araya and Lomonte, 2007; Maity et al, 2007; Bazaa et al, 2009; Zouari-Kessentini et al, 2009; Bazaa et al, 2010; Kessentini-Zouari et al, 2010).

The Russell's viper (*Daboia* species) is a common venomous *Viperinae* snake, usually found in many South Asian countries. The subspecies found in Thailand is *Daboia russelli siamensis*, which is also discovered in Myanmar, Cambodia, southern China, Taiwan, and Indonesia (Warrell, 1989). The key lethal component of *D. r. siamensis* venom is the phospholipase A<sub>2</sub>, Daboiatoxin, which produces neurotoxicity in mice and exhibits oedema-inducing and myonecrotic activities (PLA<sub>2</sub>, EC 3.1.1.4) (Ritonja and Gubensek, 1985; Maung-Maung et al, 1995; Risch et al, 2009). PLA<sub>2</sub> is a multifunctional enzyme that specifically catalyzes the hydrolysis of the fatty acid ester bond at the position 2 of 1,2-diacyl-sn-3-phosphoglycerides to produce free fatty acids and lysophospholipids (Kini, 2003; Burke and Dennis, 2009b).

In this study, we reported the purification and inhibitory activities of a PLA<sub>2</sub> from *D. r. siamensis* venom. The Drs-PLA<sub>2</sub> displayed a cytotoxic effect and inhibited cell migration in human skin melanoma cells (SK-MEL-28). It also reduced tumor lung colonization of B16F10 melanoma cells in BALB/c mice.

## MATERIALS AND METHODS

### Venom collection

*Daboia russelli siamensis* venom was obtained from the Queen Saovabha Memorial Institute (QSMI, Thai Red Cross Society, Bangkok) and was pooled venom from an underdetermined number of snakes. The venom was extracted by allowing the snake to bite into a container covered with parafilm. The venom was centrifuged at 9,000xg for 15min at 4°C and frozen at -20°C until lyophilized. The lyophilized venom was stored at -20°C until used.

### Purification of Drs-PLA<sub>2</sub>

Five milligrams of lyophilized crude *D. r. siamensis* venom was suspended in 0.2ml of 0.05M ammonium acetate buffer, pH 8.2 and filtered through a 0.45micron filter. A total of 200µl (25mg/ml) was injected into a Waters 300SW (PROTEIN-PAK™, 7.5x300mm) size-exclusion column. The column was previously equilibrated with the elution buffer (0.05M ammonium acetate buffer, pH 8.2). The collection process required 60min at a flow rate of 0.5ml/min. A Waters 2487 Dual λ absorbance detector was used to monitor absorbencies at 280nm. Waters™ Breeze software was used to control the pumps and store data. Each fraction was screened for indirect hemolytic activity, cytotoxicity, and inhibition of cell migration. Fraction 8 had a major protein band at about 14kDa with indirect hemolytic, cytotoxic, and cell migration inhibition activities (Figure 1). Fraction 8 was lyophilized and further purified by a DEAE anion exchange HPLC column.

A Waters™ DEAE anion exchange HPLC column was used for the second purification step. Fraction 8 from the first purification step was suspended in 0.02M Tris-HCl buffer, pH 8.0 and further purified. Two hundred microliters of clear supernatant at a concentration of 11.4mg/ml were applied into a Waters DEAE (PROTEIN-PAK™ 5PW, 7.5x75mm) anion exchange column, which was previously equilibrated with 0.02M Tris-HCl buffer, pH 8.0. The fractions were eluted using 0.02M Tris-HCl, pH 8.0 with a 0-0.5M NaCl

salt gradient. The collection required 60min at a flow rate of 1ml/min. Each fraction was tested for indirect hemolytic activity, cytotoxicity, and inhibition of cell migration. Fraction 8.3 (Drs-PLA<sub>2</sub>) had indirect hemolytic, cytotoxic, and cell migration inhibition activities. The molecular weight and purity of Drs-PLA<sub>2</sub> were determined by SDS-PAGE and verified by mass spectrometry.

### Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Each fraction was applied to NuPAGE® Novex 4-12% (w/v) SDS-PAGE gels (Invitrogen™). A XCell SureLock™ system with MOPS SDS running buffer (20x) diluted to 1x at 200V for 50min using a Bio-Rad PowerPac Basic system (Bio-Rad Laboratories, CA, USA). SeeBlue Plus2 markers ranging from 6-191kDa were used as standards. The gel was stained with RapidStain (Invitrogen™, CA, USA).

### Mass spectrometry (MALDI-TOF-TOF)

Drs-PLA<sub>2</sub> was desalted using C<sub>18</sub> Zip-tips (Millipore ZTC18S096). A 1µl of 2, 5- dihydroxybenzoic acid matrix was spotted on a MALDI Plate and 0.5µl of desalted fraction 8.3 was added onto the matrix. MALDI-TOF analysis was performed using a Bruker AUTOFLEX II-TOF (Bruker Daltonics) Mass spectrometer in positive mode. A Peptide Standard 2 (Bruker Daltonics) including insulin-5735.89, ubiquitin-8573.6, cytochrom-12370.83, myoglobin-16948.86, cytochrome-6183.08, and myoglobin-8459.67 was performed in a reflectron mode.

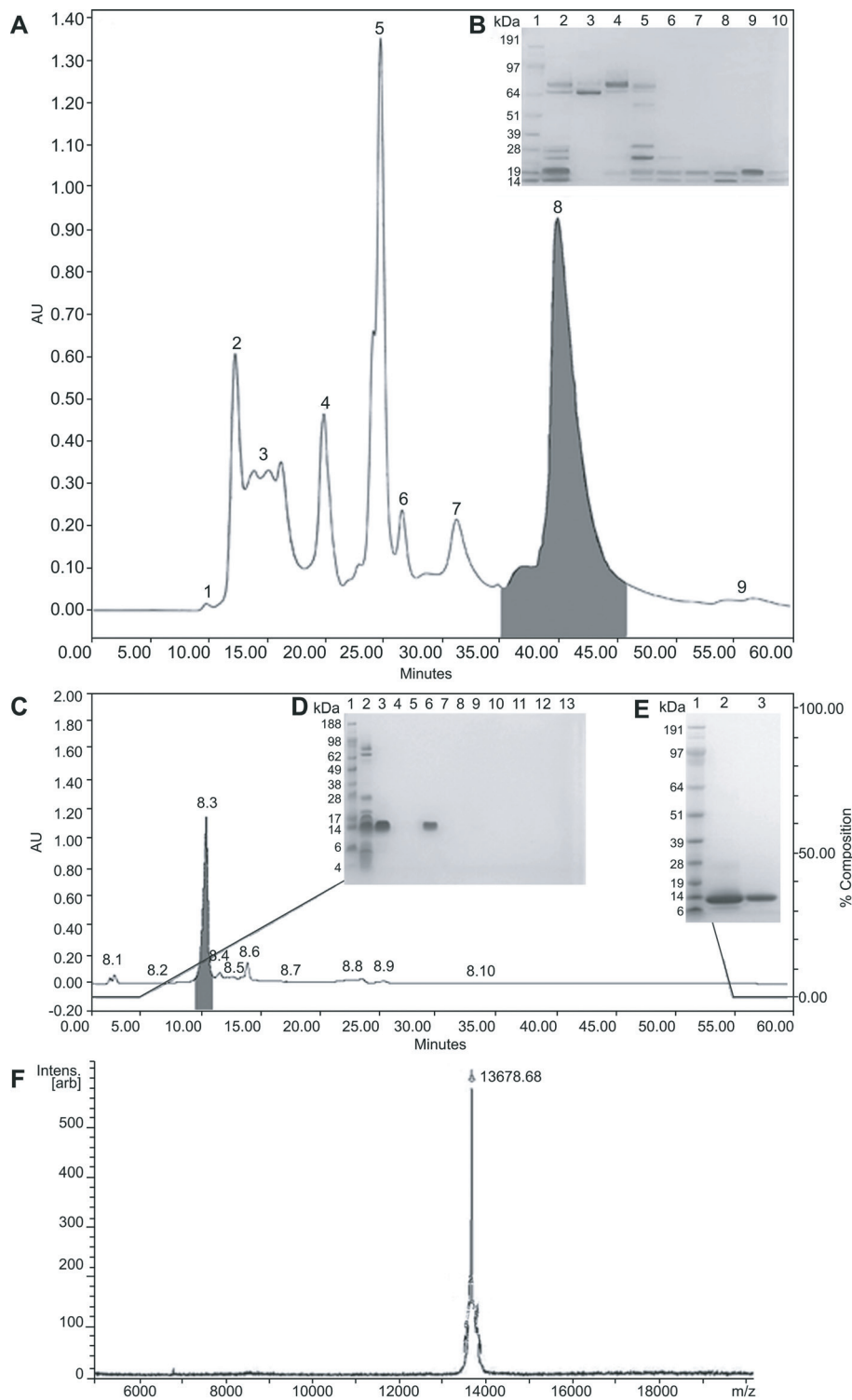
### N-terminal sequencing

Drs-PLA<sub>2</sub> (0.3mg/ml) was transferred from an SDS-PAGE onto a PVDF membrane (Milipore Immobion-P#PVH00010) using a Semi-Dry Transblot Cell (Bio-Rad) at 100mV for 1hr. The membrane was stained with Coomassie R-250 stain for 5min. The sample membrane was sent to the Iowa State University for N-terminal amino acid sequencing.

### Anticoagulant activity

The anticoagulant activity of crude venom, fraction 8 from size exclusion purification, and Drs-PLA<sub>2</sub> from anion exchange purification were measured using the Sonoclot analyzer by a modification of the procedure of Sánchez et al (2010). Briefly, a cuvette containing glass beads as the clotting activator was placed into the cuvette holder which maintained the temperature at 37°C. A pre-warmed 13µl of 0.25M CaCl<sub>2</sub> were added to one side of the cuvette. A 10µl of Drs-PLA<sub>2</sub> at the concentration of 0.3mg/ml (24µM) was added to the opposite side of the cuvette. A constant volume of 360µl of normal 10% (v/v) citrated human whole blood was added to the cuvette. The activated clot time (ACT) and clot rate (CR) were measured and the data were analyzed from Signature Viewer, software provided by Sienco, Inc. on an iMAC computer and analyzed by Microsoft Excel 2007. The negative control consisted of whole blood incubated with 0.02M Tris-HCl, pH 8.0 and 0.25M CaCl<sub>2</sub>.

Several studies have demonstrated that PLA<sub>2</sub>s inhibit blood coagulation by binding to FXa, which is the target protein in the coagulation cascade (Stefansson, 1990; Kerns et al, 1999; Kini, 2005). To test whether Drs-PLA<sub>2</sub> specifically inhibits factor Xa, anticoagulation was assayed utilizing



**Figure 1.** Purification of Drs-PLA<sub>2</sub>. **A.** Size exclusion (SE) chromatographic profile of crude *D. r. siamensis* venom. The grey-shaded areas indicate the location of PLA<sub>2</sub> activities using cytotoxicity and cell migration assays. **B.** SDS-PAGE analysis of venom fractions from SE HPLC column. Crude venom or venom fractions were run on 4-12% (w/v) bis-Tris Gel under non-reducing conditions at 200V for 50min. The gel was stained with RapidStain. Lane 1: SeeBlue Plus2 Markers (Invitrogen™); lane 2: crude venom (7μg); lanes 3-10: fractions 2-9 (7μg). **C.** DEAE anion exchange HPLC profile of fraction 8 from the SE HPLC column. The grey-shaded areas indicate the location of PLA<sub>2</sub> activities using indirect hemolytic, cytotoxicity, and cell migration assays. **D.** SDS-PAGE analysis of venom fractions from DEAE HPLC column. Crude and venom fractions from DEAE HPLC column were run on a 4-12% (w/v) bis-Tris gel under non-reducing conditions at 200V for 50min. The gel was stained with RapidStain for 1hr and destained overnight with 18megaohm water. Lane 1: SeeBlue Plus2 Markers (Invitrogen™); lane 2: crude venom (7μg); lane 3: fraction 8 from SE (7μg); lane 4: fraction 8.1 (1.4μg); lane 5: fraction 8.2 (1.2μg); lane 6: fraction 8.3 (6μg); lane 7: fraction 8.4 (1.6μg); lane 8: fraction 8.5 (1.4μg); lane 9: fraction 8.6 (2μg); lane 10: fraction 8.7 (1.2μg); lane 11: fraction 8.8 (1.6μg); lane 12: fraction 8.9 (1.4μg); lane 13: fraction 8.10 (1 μg). **E.** SDS-PAGE analysis of fraction 8 and 8.3 (Drs-PLA<sub>2</sub>). Drs-PLA<sub>2</sub> was run on 4-12% (w/v) bis-Tris Gel under reducing conditions. Lane 1: SeeBlue Plus2 Markers (Invitrogen™); lane 2: reduced form of fraction 8 from SE (7μg); lane 3: reduced form of Drs-PLA<sub>2</sub> (3μg). **F.** Mass spectrometry analysis of Drs-PLA<sub>2</sub>.

FX deficient plasma by a method of Suntravat et al (2010). Briefly, 300µl of FX deficient plasma were added to a cuvette without glass beads (Sienco, Inc, USA). Coagulation was activated by the addition of 10µl of pre-warmed 0.30M CaCl<sub>2</sub> and 10µl of 6nM FXa pre-incubated at 37°C for 30min with 10µl of 0.1mg/ml (8µM) of Drs-PLA<sub>2</sub>. The controls included FX deficient plasma, 0.30M CaCl<sub>2</sub> and 3nM FXa without Drs-PLA<sub>2</sub> (positive control), and FX deficient plasma, 0.30M CaCl<sub>2</sub> without 3nM FXa (negative control).

#### Indirect hemolytic assay

The crude venom and fractions were tested for PLA<sub>2</sub> activity. The hemolysis indirect was tested on human washed red blood cells (1.2%, v/v) agarose plates (0.8%, w/v) with calcium chloride (0.01M-1%, v/v). The egg yolk solution was added to the agarose medium at a final concentration of 1.2% (v/v). Three millimeters diameter wells were made into the agarose plates. Twenty-five microlitres of *D. r. siamensis* and *Crotalus atrox* crude venoms were tested as positive hemolytic controls at a concentration of 1mg/ml. Fractions 8 (Figure 1A) and 8.3 (Figure 1C) were tested at a concentration of 0.5 and 0.3mg/ml, respectively. PBS was used as a negative hemolytic control. The plates were incubated at 37°C for 24hr. The specific activities were estimated as the ratio between the diameter (millimeters) of the hemolytic halo and the amount of protein added per well (micrograms).

#### Cytotoxicity assay of crude venom and Drs-PLA<sub>2</sub> on cancer cell lines

Human skin melanoma (SK-MEL-28), human urinary bladder carcinoma (T24), human lung bronchus carcinoma (ChaGo-K-1), human fibrosarcoma (HT-1080), and murine skin melanoma (B16F10) cell lines were obtained from American Type Tissue Culture Collection (ATCC). The SK-MEL-28 and HT-1080 cell lines were maintained with Eagle's minimum essential medium (EMEM) containing 10% (v/v) fetal bovine serum (FBS), 50U/ml penicillin, and 50µg/ml streptomycin. The T24 cells were maintained with McCoy's 5A minimum essential medium containing 10% (v/v) FBS, 50U/ml penicillin, and 50µg/ml streptomycin. The B16F10 cells were maintained with Dulbecco's modified Eagle's medium containing 10% (v/v) FBS, 50U/ml penicillin, and 50µg/ml streptomycin. The ChaGo-K-1 cells were maintained with RPMI 1640 medium supplemented containing 10% (v/v) FBS, 0.02M sodium bicarbonate, 100U/ml penicillin, and 100µg/ml streptomycin. The cultured medium was replaced daily. Cells were incubated at 37°C in a 5% (v/v) CO<sub>2</sub> humidified incubator.

Cytotoxic activity of *D. r. siamensis* venom (1mg/ml) was performed according to the procedure of Umthong et al (2009) by measuring cell viability using MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide). To identify the most sensitive cancer cell lines for further screening cytotoxic and cell migration inhibition activities of venom fractions, cytotoxic activity of crude venom was determined on SK-MEL-28, T24, ChaGo-K-1, and HT-1080 cells. Cells were cultured on 96-wells flat-bottom microtiter plates at ~10<sup>5</sup> cells/well in triplicate and incubated at 37°C in 5% (v/v) CO<sub>2</sub> for 24hr. Crude venom was added to each cell suspension at

various concentrations and incubation times. SK-MEL-28 cells were the most sensitive, and were further used for the determination of the cytotoxic and cell migration inhibition activities of venom fractions.

Each fraction collected from HPLC (100µg/ml), or Drs-PLA<sub>2</sub> at various concentrations was added to SK-MEL-28 cell suspension at 37°C for 72hr. Then, 10µl of MTT (5mg/ml) was added to each well. After incubation for 4hr at 37°C, MTT was aspirated and 150µl of DMSO was added to lyse the cells. The absorbance at 570nm was read using a Beckman Coulter™ model AD 340 reader. Doxorubicin (5µg/ml), an anticancer drug was used as the positive control. The negative control was cells treated with 0.02M Tris-HCl, pH 8.0. The percentage of cell viability was calculated relative to the negative control, which was defined as 100%. The 50% cytotoxic concentration (CC<sub>50</sub>) of sample is defined as the protein concentration generating cell viability of 50%. The values of the percentages of cell viability were plotted against venom concentrations, and the CC<sub>50</sub> was determined. Experiments were performed in triplicate.

#### Cell migration assays of the Drs-PLA<sub>2</sub>

Cell migration inhibition was determined by a wound-healing assay according to the procedure of Galán et al (2008). Briefly, SK-MEL-28 cells were plated (5x10<sup>5</sup> cells/ml) on a 24-well plate. After 24hr, the confluent monolayer was scratched with a sterile pipette tip creating the scratch wound of 16-23mm width. The detached cells were washed away and renewed with 0.9ml of EMEM mixed with crude venom at various concentrations, venom fractions collected from HPLC (6.25µg/ml), or Drs-PLA<sub>2</sub> at various concentrations. The cells were then incubated at 37°C in a CO<sub>2</sub> incubator for 0, 3, 6, 12, 24, and 48hr. After the incubation period, cell migration was observed under an inverted microscope (ULWCD 0.3 Olympus CK2, Japan). Echistatin (10µg/ml), a disintegrin known to inhibit SK-MEL-28 cell migration was used as the positive control (Sánchez et al, 2009). The negative control was cells treated with 0.02 M Tris-HCl, pH 8.0. The percentage of cell migration inhibition was calculated by the following equation: [(C-E)/C] x100, where C is the distance of the wound scratch (mm) at zero time of the negative control, and E is the distance of the wound scratch (mm) at the final incubation time for the venom. The 50% inhibitory concentration (IC<sub>50</sub>) is defined as the protein concentration that inhibits cell migration by 50%. The values of the percentages of cell migration inhibition were plotted against protein concentrations, and the IC<sub>50</sub> was determined. Experiments were performed in triplicate.

#### Inhibition of lung tumor colonization *in vivo*

B16F10 murine skin melanoma cells (10<sup>6</sup>cells) were mixed with Drs-PLA<sub>2</sub> at the concentration of 100µg/kg body weight and then incubated at 37°C for 1hr. A 200µl of the sample mixture was injected into the tail veins of 18-20gm BALB/c mice. After 19 days, the mice were sacrificed to detect tumors in the lungs. Mice injected with only B16F10 cells were used as the positive control group. Mice injected with only Dulbecco's modified Eagle's medium were used as the negative control group. The tumors were counted and compared to the positive control group.

### Statistical analysis

All results were expressed as mean  $\pm$  standard deviation (SD). Their significance was analyzed by the Student's *t*-test. The level of significance was at  $P < 0.05$ .

## RESULTS

### Purification of Drs-PLA<sub>2</sub>

Crude venom was fractionated by size exclusion chromatography and nine fractions were collected (Figure 1A). The high molecular weight proteins (97kDa) were observed in fractions 2, 3, and 4. Low molecular weight proteins at molecular weights of about 14 and 6kDa were found in fractions 4 through 9. Fractions 2 through 9 were screened for cytotoxic and migration inhibition activities using the most sensitive cell towards the cytotoxic action of crude *D. r. siamensis* venom. Fraction 8 showed the most intense protein band at about 14kDa (Figure 1B, lane 9). Fraction 8 had the highest cytotoxicity with a  $CC_{50}$  of 1.47 $\mu$ g/ml, the migration inhibition activity with an  $IC_{50}$  of 1.22 $\mu$ g/ml, and the indirect hemolytic activity (data not shown). Fraction 8 was further purified by anion exchange HPLC chromatography. Ten different fractions were collected (Figure 1C). The molecular weight of fraction 8.3 was verified by mass spectrometry (13,679Da) (Figure 1F). The characteristics of the crude venom, fractions 8 and Drs-PLA<sub>2</sub> used in this study are shown in Table 1.

### Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Fractions from all chromatographic fractionation were applied to NuPAGE® Novex 4-12% (w/v) Bis-Tris SDS-PAGE gels. Drs-PLA<sub>2</sub> showed a single band at about 14kDa using SDS-PAGE under non-reducing and reducing conditions (Figure 1D, lane 6 and 1E, lane 3). These results suggest that Drs-PLA<sub>2</sub> is a purified monomeric protein. The monomeric Drs-PLA<sub>2</sub> is similar to the PLA<sub>2</sub> isolated from *Cerastes cerastes* venom (Zouari-Kessentini et al, 2009).

### Mass spectrometry (MALDI-TOF-TOF)

Mass analysis was performed on Drs-PLA<sub>2</sub> using a Bruker AUTOFLEX II-TOF (Bruker Daltonics) resulting with a mass of 13,679Da, which falls in the range of many venom PLA<sub>2</sub>s (Figure 1F).

### N-terminal sequencing

The N-terminal amino acid sequencing of Drs-PLA<sub>2</sub> was NLFQFARMINGKLGAFSV displaying 100% sequence homology with basic PLA<sub>2</sub>s (Table 1). In addition, it is very similar (95% identity) to other basic PLA<sub>2</sub>s from viperid species (Table 1).

### Anticoagulant activity

Crude venom, fraction 8, and the Drs-PLA<sub>2</sub> were examined for coagulant activity using the Sonoclot analyzer. The Sonoclot signatures of crude venom, fraction 8, and

**Table 1.** Comparison of N-terminal sequence homology between Drs-PLA<sub>2</sub> and other snake venom PLA<sub>2</sub>s.

Snake PLA <sub>2</sub>	Accession No. or Ref.	Organism	N-terminal sequence	Identity (%)
Drs-PLA <sub>2</sub> *	-	<i>D. r. siamensis</i>	NLFQFARMINGKLGAFSV†	-
Basic PLA <sub>2</sub>	AAZ53185	<i>D. r. limitis</i>	NLFQFARMINGKLGAFSV	100
Rv4RV7 (basic PLA <sub>2</sub> )	1OQS	<i>Vipera russelli formosensis</i>	NLFQFARMINGKLGAFSV	100
Viperotoxin (basic PLA <sub>2</sub> )	Q02471.1	<i>D. r. siamensis</i>	NLFQFARMINGKLGAFSV	100
Basic PLA <sub>2</sub>	2I0U	<i>V. nikolskii</i>	NLFQFAKMINGKLGAFSV	95
Vaspin basic subunit	CAE47300	<i>V. aspis zinnikeri</i>	NLFQFAKMINGKLGAFSV	95
Basic PLA <sub>2</sub> (B chain)	Q8JFG0	<i>V. a. aspis</i>	NLFQFAKMINGKLGAFSV	95
Vipoxin complex	1AOK	<i>V. ammodytes meridionalis</i>	NLFQFAKMINGKLGAFSV	95
Basic PLA <sub>2</sub> I	B60512	<i>V. aspis</i>	NLFQFALMINGKLGAFSV	95
Vaspin basic subunit	CAE47291.1	<i>V. a. aspis</i>	NLFQLAKMINGKLGAFSV	89
Vaspin B isoform 1	AAO86503.1	<i>V. a. aspis</i>	NLFQSAKMINGKLGAFSV	89
Basic PLA <sub>2</sub> -II	ABD24037.1	<i>D. r. russellii</i>	NLFQFARMIDAKQEAFS	77
Basic PLA <sub>2</sub>	AAZ53178.1	<i>D. r. siamensis</i>	NLFQFARLIDAKQEAFS	71
Basic PLA <sub>2</sub>	AAP37177.1	<i>D. r. siamensis</i>	NLFQFARLIDAKQEAFS	71
Acidic PLA <sub>2</sub>	ACD43469	<i>D. r. siamensis</i>	NLFQFGDMINKKTGRFGL	61
Acid Daboiatoxin (DbTx)	1	<i>D. r. siamensis</i>	NFFQFAEMIVKMTGKEAV	50
Acidic PLA <sub>2</sub>	2	<i>Cerastes cerastes</i>	NLYQFGKMIKHKTGKSAL	44
Acidic PLA <sub>2</sub>	AAP41217.1	<i>Echis carinatus</i>	NLYQFGRMIWNRTGKL	43

\*This work

†The sequences were aligned using BLASTP 2.2.25 program of GenBank.

1 Maung-Maung et al., 1995; Risch et al., 2009; Ritonja and Gubensek, 1985

2 Zouari-Kessentini et al., 2009

Bold letter indicates different residues as compared to Drs-PLA<sub>2</sub>.

the Drs-PLA<sub>2</sub> are shown in Figure 2. Crude *D. r. siamensis* venom known to have the coagulant activity significantly shortened ACT (0.32±0.03min) and increased the CR (86.50±7.78U). Fraction 8 has the signature of coagulant activity with a shortened the ACT (0.66±0.03min) and enhanced the CR (44.00±5.66U). In contrast, the Drs-PLA<sub>2</sub> had a signature of anticoagulant activity with an extended ACT (3.88±0.78min) and slower CR (5.60±0.36U). The positive blood control incubated with 0.02M Tris, pH 8.0 buffer had an average ACT of 3.46±0.30min, and a CR of 15.10±2.72U.

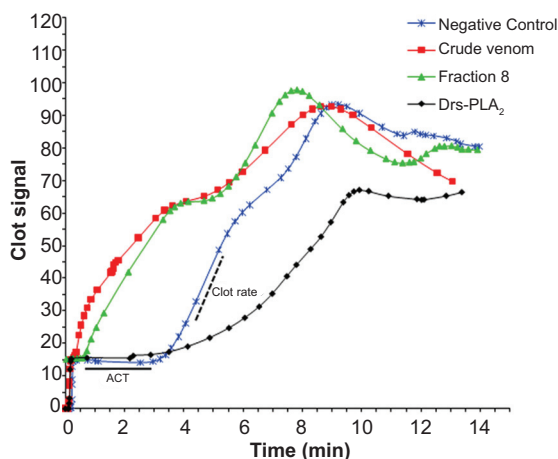
To identify FXa as a target protein of Drs-PLA<sub>2</sub>, we demonstrated that Drs-PLA<sub>2</sub> significantly prolonged the ACT (3.21±0.11min) and lowered the CR (1.70±0.28U). The positive control had an average ACT of 2.73±0.22 min and a CR of 3.37±0.91 U.

#### Indirect hemolytic assay

All samples displayed indirect hemolytic activity with Drs-PLA<sub>2</sub> having the highest specific activity of 2mm/μg±0.2 followed by fraction 8 at 1.4mm/μg ±0.1. *D. r. siamensis* and *C. atrox* crude venoms had specific activities of 0.8±0.1 and 1.0mm/μg ±0.2, respectively.

#### Cytotoxicity assay of crude venom on cancer cell lines

Four different cell lines (SK-MEL-28, T24, ChaGo-K-1, and HT-1080 cells) were treated with a 100μg/ml of crude *D. r. siamensis* venom. The SK-MEL-28 cell line was the most sensitive (15-20% cell viability) to crude venom at all three incubation times (data not shown). All other cell lines exhibited 50-60% cell viability (data not shown). Thus, SK-MEL-28 cells were further used for the determination of cytotoxic and cell migration inhibition activities of the venom fractions.



**Figure 2.** Whole Blood Sonoclot Signatures of *D. r. siamensis* venom and venom fractions. Data from the Sonoclot Signatures further analyzed by Microsoft Excel 2007 obtained when human citrated whole blood was activated by crude venom, fraction 8 from size exclusion purification, and Drs-PLA<sub>2</sub>. The ACT is the time (min) in which whole blood begins to clot. The CR is defined as the rate of fibrin polymerization, which is the slope in the linear part of the curves and is defined as the change clot signal with change in time ( $U = \Delta\text{signal}/\Delta\text{time}$ ). The solid line represents the ACT. The dashed line represents the CR.

Effects of crude venom at the various concentrations (6.25, 12.5, 25, 50, and 100μg/ml) and various incubation times on SK-MEL-28 cell viability were determined. Crude venom reduced cell viability in a dose-dependent manner. Crude venom at the concentration of 100μg/ml showed the lowest cell viability (20%) at 24hr and slightly decreased in the percentage of cell viability at 48hr and 72hr (data not shown).

#### Cytotoxicity and cell migration assays of the Drs-PLA<sub>2</sub>

Purified Drs-PLA<sub>2</sub> decreased SK-MEL-28 percent cell viability in a dose-dependent manner (0.012μg/ml-98% ±10; 0.060μg/ml-94% ±5; 0.30μg/ml-80% ±3; 1.5μg/ml-40% ±10; and 7.5 μg/ml-20% ±5). The cytotoxic activity of the PLA<sub>2</sub> was compared to crude venom. The CC<sub>50</sub> value of the Drs-PLA<sub>2</sub> (0.90μg/ml) was approximately 6 times less than the CC<sub>50</sub> value of crude venom (5.63μg/ml). In addition, the Drs-PLA<sub>2</sub> inhibited the migration of SK-MEL-28 with an IC<sub>50</sub> of 0.35μg/ml, which was 11.3x lower than crude venom (3.97 μg/ml). Effects of the Drs-PLA<sub>2</sub> at various incubation times on cell migration inhibition were determined. The percent inhibition of SK-MEL-28 cell migration of echistatin (positive control) at 3-12hr showed no significant difference to the percent inhibition of the Drs-PLA<sub>2</sub>. Subsequently, similar inhibition activity was found at 24hr. After a 48hr incubation period, cell migration inhibition activity of the Drs-PLA<sub>2</sub> was higher than the positive control (Figure 3A). The actual images of SK-MEL-28 cell migration with the Drs-PLA<sub>2</sub> at 24hr are shown in Figure 3B.

#### Inhibition of lung tumor colonization *in vivo*

The Drs-PLA<sub>2</sub> was further tested for anti-metastatic property using an *in vivo* skin melanoma cell colonization assay. The Drs-PLA<sub>2</sub> significantly reduced tumor nodules by ~65% compared with the positive control group (Table 2; Figure 4).

## DISCUSSION

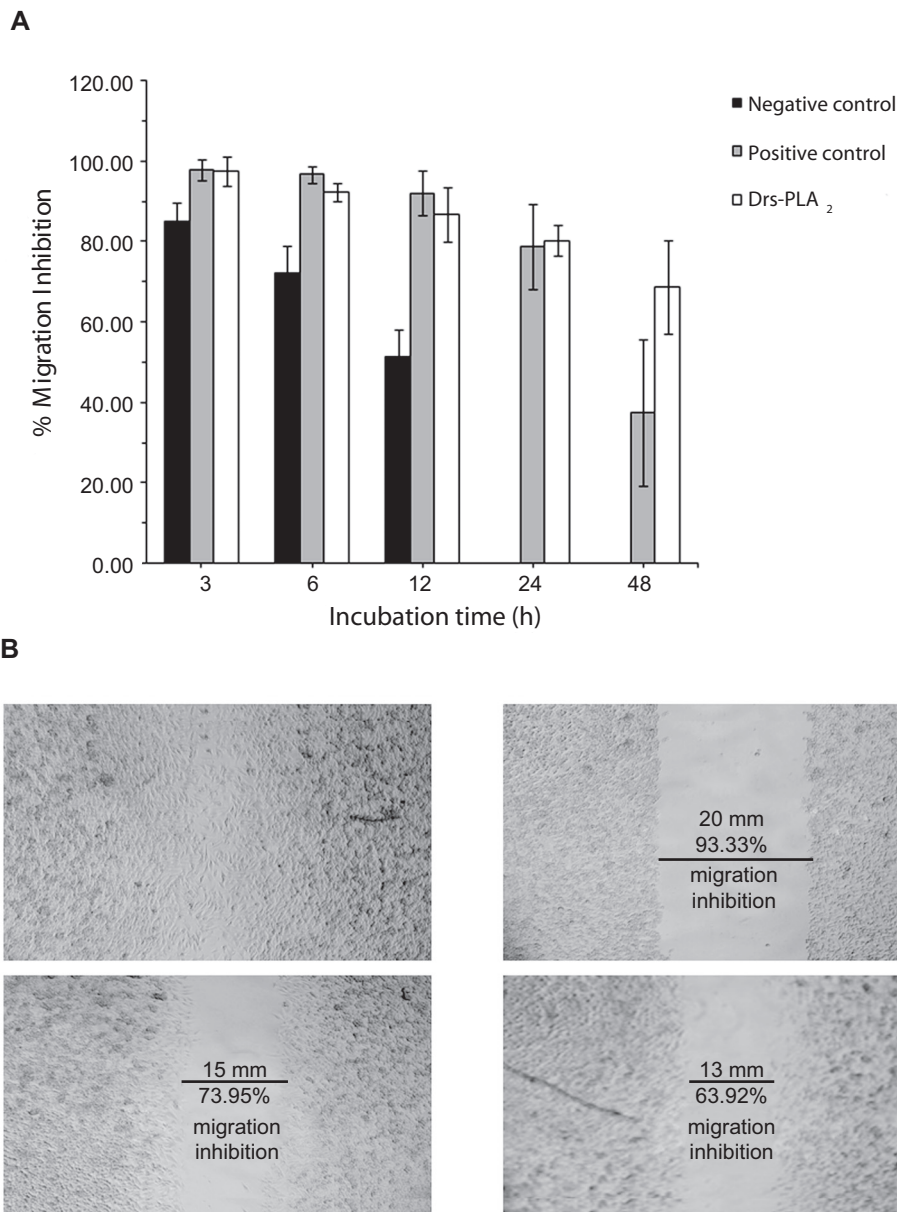
Heart attack, stroke, and cancers are among the most serious medical problems worldwide. Venomous snakes contain an array of molecules with many different biological

**Table 2.** Comparative analysis of tumor foci per lung in BALB/c mice using purified Drs-PLA<sub>2</sub> compared to controls

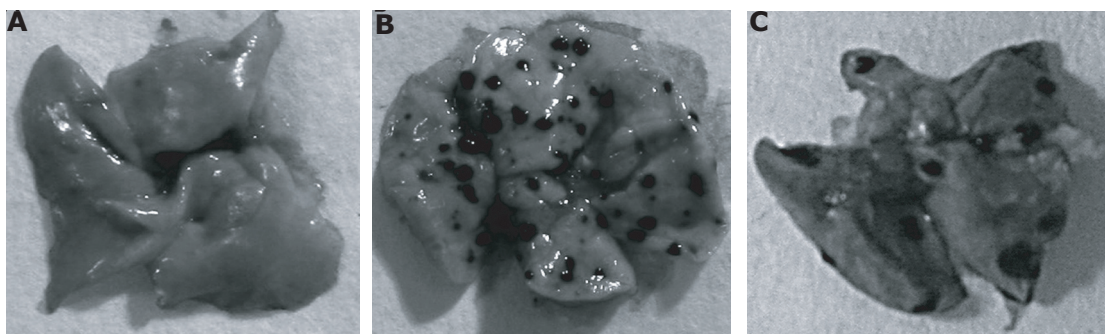
	Control	Drs-PLA <sub>2</sub> (100 μg/kg)
Mice no.	13	19
Minimum tumors	21	0
Maximum tumors	90	40
Mean tumors	49.15	17.42
Standard deviation	24.54	13.91
Tumor inhibition (%) <sup>a</sup>	-	64.50
P value <sup>b</sup>	-	0.0002

<sup>a</sup> The percent tumor inhibition was calculated by the following equation:  $[(E/C) \times 100] - 100$ , where E is the mean tumors of the Drs-PLA<sub>2</sub> group, and C is the mean tumors of the control group.

<sup>b</sup> P value as compared to the control. The level of significance was at  $P < 0.05$ .



**Figure 3.** Inhibition of Cell Migration by Drs-PLA<sub>2</sub>. **A.** Cell migration inhibition activity of the Drs-PLA<sub>2</sub> on SK-MEL-28 cells using a wound-healing assay. Cell migration inhibition was evaluated by incubating  $5 \times 10^5$  cells with Drs-PLA<sub>2</sub> ( $6 \mu\text{g}/\text{ml}$ ) for 3, 6, 12, 24, or 48hr incubation periods. Echistatin ( $10 \mu\text{g}/\text{ml}$ ) was used as the positive control. The negative control consisted of cells treated with  $0.02\text{M}$  Tris-HCl, pH 8.0. The results are expressed as the percentage of cell migration inhibition with respect to activity of the negative control, and as mean  $\pm$ SD ( $n=3$ ). **B.** SK-MEL-28 cells with the Drs-PLA<sub>2</sub> ( $1.2 \mu\text{g}/\text{ml}$ ) at 24hr. (1) SK-MEL-28 cells were treated with  $0.02\text{M}$  Tris-HCl, pH 8.0 at 24hr. (2) SK-MEL-28 cells were treated with Drs-PLA<sub>2</sub> at 0hr, (3) 12hr, and (4) 24hr.



**Figure 4.** Inhibition of Lung Tumor Colonization by Drs-PLA<sub>2</sub>. The effect of the Drs-PLA<sub>2</sub> on B16F10 lung tumor colonization in BALB/c mice at  $100 \mu\text{g}/\text{kg}$  mouse. The B16F10 cells ( $2 \times 10^5$ ) were injected in the lateral tail vein in the absence or presence of the Drs-PLA<sub>2</sub>. The lungs were isolated from the mice 19-days post-injections and observed for tumor colonization. **A.** Medium-treated mice (control); **B.** B16F10 cells in medium; **C.**  $100 \mu\text{g}$  of the Drs-PLA<sub>2</sub>/kg mouse.

activities, which could have beneficial applications in medicine and biomedical research. In this study, we reported the purification and characterization of Drs-PLA<sub>2</sub> isolated from crude *D. r. siamensis* venom. The Drs-PLA<sub>2</sub> (13,679 Da) was purified from crude venom by a two-step purification procedure (Figure 1A, 1B and 1C) with approximately 27.4% yield (data not shown). The N-terminal sequence (18 amino acids) showed 100% homology to basic PLA<sub>2</sub>s from the viperid snakes (Table 1). However, it should be noted that this homology is based on the comparison of amino acid sequences at the N-terminal region of the molecules, without taking into account the remaining sequence of our PLA<sub>2</sub>. A basic PLA<sub>2</sub> has been identified in the venom of *D. r. siamensis* (viperotoxin) sharing 100% N-terminus sequence homology with Drs-PLA<sub>2</sub> (Table 1); however, viperotoxin is a basic protein with an approximate pI of 8.96. The pI for Drs-PLA<sub>2</sub> appears to be slightly acidic since it bound to the anion exchange column, but was easily eluted with slightly above a 10% (w/v) NaCl (0.5M) solution (Figure 1C). These results suggest that viperotoxin is different from Drs-PLA<sub>2</sub>.

Although many PLA<sub>2</sub>s exhibit a high degree of similarity in their amino acid sequences, they display different biological effects. Tsai et al (2007) reported that PLA<sub>2</sub>s isolated from Myanmar Russell's viper venom (*D. r. siamensis*) and Eastern India Russell's viper (*D. russelli*) venom share 97-100% amino acid sequence identity, but they differ in their enzymatic and lethality effects.

Previous studies have indicated that several PLA<sub>2</sub>s isolated from the families Crotalidae, Viperidae, and Elapidae possessed anticoagulant properties (Mukherjee, 2007; Pereanez et al, 2009; Garcia Denegri et al, 2010). In this study, based on the ACT and CR data, fraction 8 from size exclusion purification exhibited a coagulant signature with a shortened ACT and an increased CR (Figure 2). In contrast, the Drs-PLA<sub>2</sub> from the anion exchange purification had an anticoagulant activity with an extended ACT and a decreased CR. These results indicated that low molecular weight procoagulant molecules were completely removed from the first purification step (Figure 1E). Since most snake venoms contain PLA<sub>2</sub> isoforms, it would be important to purify PLA<sub>2</sub>s from venom of individual snakes. In addition, the Drs-PLA<sub>2</sub> had an anticoagulation effect on FX deficient plasma by inhibiting FXa, which is the target protein for anticoagulant PLA<sub>2</sub>s as previously reported (Stefansson, 1990; Kerns et al, 1999).

In recent studies, cytotoxicity, inhibition of cell migration, and tumor metastasis properties of PLA<sub>2</sub>s have been widely investigated. For example, a basic PLA<sub>2</sub>, RVV-7 (7.2kDa) isolated from *D. r. russelli* venom had strong cytotoxic activity on B16F10 melanoma cells. It also inhibited tumor growth in BLJ6 mice (Maity et al, 2007). Furthermore, an acidic Asp49 PLA<sub>2</sub> (13,626.64Da) isolated from *Macrovipera lebetina transmediterranea* venom completely inhibited cell adhesion and migration of various human tumor cells mediated by  $\alpha 5\beta 1$  and  $\alpha v$  integrins, but lacked cytotoxicity (Bazaa et al, 2009). Two acidic PLA<sub>2</sub>s purified from *Cerastes cerastes* venom, CC-PLA<sub>2</sub>-1 and CC-PLA<sub>2</sub>-2, inhibited HT-1080 cell migration towards fibrinogen and fibronectin (Zouari-Kessentini et al, 2009). In our study,

crude venom had a higher cytotoxic activity on SK-MEL-28 cells than T24, HT-1080, and ChaGo-K-1 (data not shown). In addition, the PLA<sub>2</sub> inhibited migration of SK-MEL-28 cells (Figure 3A and 3B). Although, there are studies demonstrating the anti-tumor activity of PLA<sub>2</sub>s *in vitro*, to the best of our knowledge, with the exception of the study by Maity et al (2007), there is insufficient *in vivo* evidence of the effects of secreted PLA<sub>2</sub>s on tumors. To confirm the metastasis property in our study, we determined the inhibition of skin melanoma cell colonization by Drs-PLA<sub>2</sub>, *in vivo*. It was observed that the pretreatment of B16F10 melanoma cells with the Drs-PLA<sub>2</sub> inhibited growth of tumors in BALB/c mice by ~65%.

Phospholipases A<sub>2</sub>s are enzymes that spark interest in the medical field because of their participation in a large number of human inflammatory diseases (Rodrigues et al, 2009). The activity and expression of several PLA<sub>2</sub> isoforms are augmented in numerous human cancers, signifying that these enzymes have a vital role in both tumor development and progression and can be targets for anti-cancer drugs. On the other hand, some PLA<sub>2</sub>s isolated from Viperidae venoms are capable of inducing antitumoral activity (Rodrigues et al, 2009), suggesting that these molecules can be a new class of anticancer agents and provide new molecular and biological insights of cancer development. Furthermore, the initiation of the coagulation system in cancer patients is a well-known phenomenon accountable for recurrent thrombosis, which is the second most common cause of death in cancer patients (Xie et al, 2005). Thromboembolic complications consist of a wide range of clinical tribulations, from pulmonary embolism and deep venous thrombosis to superficial thrombophlebitis and clotting of central venous catheters (Versteeg et al, 2004; Xie et al, 2005; Furie and Furie, 2006).

## CONCLUSIONS

In this study, we report for the first time a PLA<sub>2</sub> purified from Thailand *D. r. siamensis* venom that had cytotoxic, anticoagulant, and antitumor activity, *in vivo* and *in vitro*. With the increase in protein engineering, it is now possible to produce a smaller fragment of this molecule that could preserve or improve its anticoagulant and antitumor activities, which could be useful in medical applications.

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## CONFLICT OF INTEREST

None declared.

## ETHICAL STATEMENT

This research was approved by the Texas A&M University-Kingsville Animal Care and Use Committee (#2009-04-8C).



## LIST OF ABBREVIATIONS

PLA<sub>2</sub>; Phospholipases A2  
 CC<sub>50</sub>; Cytotoxic concentration at 50%  
 HPLC; High Performance Liquid Chromatography  
 Drs; *Daboia russelii siamensis*  
 MALDI-TOF; Matrix-Assisted Laser Desorption/Ionization-Time of Flight  
 FX; Factor Ten  
 FXa; Activated Factor Ten

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