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Third-generation antivenomics analysis of the preclinical efficacy of Bothrofav® antivenom towards *Bothrops lanceolatus* venom

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ABSTRACT

Bothrops lanceolatus inflicts severe envenomings in the Lesser Caribbean island of Martinique. Bothrofav®, a monospecific antivenom against *B. lanceolatus* venom, has proven highly effective at the preclinical and clinical levels. Here, we report a detailed third-generation antivenomics quantitative analysis of Bothrofav®. With the exception of poorly-immunogenic peptides, Bothrofav® immunocaptured all the major protein components. These results, along with previous preclinical and clinical observations, underscore the high neutralizing efficacy of the antivenom against *B. lanceolatus* venom.

Bothrops lanceolatus is an endemic viperid snake in the French overseas Department of Martinique, in the Lesser Caribbean, where it represents the only venomous snake (Campbell and Lamar, 2004). Envenomings by this species are similar to those inflicted by other *Bothrops* sp, which are characterized by local effects (edema, pain, hemorrhage, necrosis) and by systemic alterations associated with hemorrhage and hemodynamic disturbances. In contrast to the majority of *Bothrops* sp-induced cases, about 30% of people suffering envenomings by *B. lanceolatus*, if not treated with antivenom, develop severe thrombosis that may lead to cerebral, pulmonary, myocardial or mesenteric infarctions (Thomas et al., 1995; Warrell, 2004; Resiere et al., 2010).

A monospecific antivenom against *B. lanceolatus* venom, Bothrofav® manufactured by Sanofi-Pasteur, was introduced in Martinique in 1991 and has proven highly effective in preventing the development of thrombosis and other systemic disturbances in these envenomings (Thomas et al., 1995). Preclinical assessment of the neutralizing efficacy of two batches of this antivenom has corroborated its efficacy in the neutralization of the most important toxic and enzymatic activities of *B. lanceolatus* venom (Bogarín et al., 1999; Resiere et al., 2018). Antivenomics has become a highly valuable tool to assess the ability of antivenoms to recognize specific venoms components previously identified by mass spectrometry (Calvete et al., 2018). In addition, antivenomics provides a quantitative view of the amount of toxin-binding and therapeutic antibody molecules present in an antivenom. Thus, neutralization assays and antivenomics complement each other in the characterization of the preclinical efficacy of antivenoms (Gutiérrez et al., 2017). To this end, an antivenomics analysis has been made with the venom of *B. lanceolatus* and the monospecific antivenom in order to further characterize the preclinical performance of Bothrofav®.

Third-generation antivenomics (Pla et al., 2017) was applied to assess the immunoreactivity of Sanofi Pasteur Bothrofav® antivenom (batch J8216) against venom from *B. lanceolatus*, whose proteome has been previously characterized (Gutiérrez et al., 2008). To this end, the content of one vial (10 mL) of antivenom was dialyzed against MilliQ® water, lyophilized, and reconstituted in 10 mL of 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3 (coupling buffer). The monospecific antivenom Bothrofav® produced by Sanofi Pasteur (Lyon, France) is an $F(ab')_2$ preparation obtained by pepsin digestion and ammonium sulphate fractionation of hyperimmune plasma from horses immunized with the venom of *B. lanceolatus*. The concentration of the antivenom stock solution (207 mg/mL) was determined spectrophotometrically using an extinction coefficient for a 1 mg/mL concentration ($\varepsilon^{0.1\%}$) at 280 nm of 1.36 $(mg/mL)^{-1}$ cm^{-1} (Howard and Kaser, 2014).

The antivenom affinity matrix was prepared in batch. Three mL of CNBr-activated Sepharose™ 4B (GE Healthcare, Buckinghamshire, UK) were packed in a ABT column (Agarose Bead Technologies, Torrejón de Ardoz, Madrid) and washed with 10× matrix volumes of cold 1 mM

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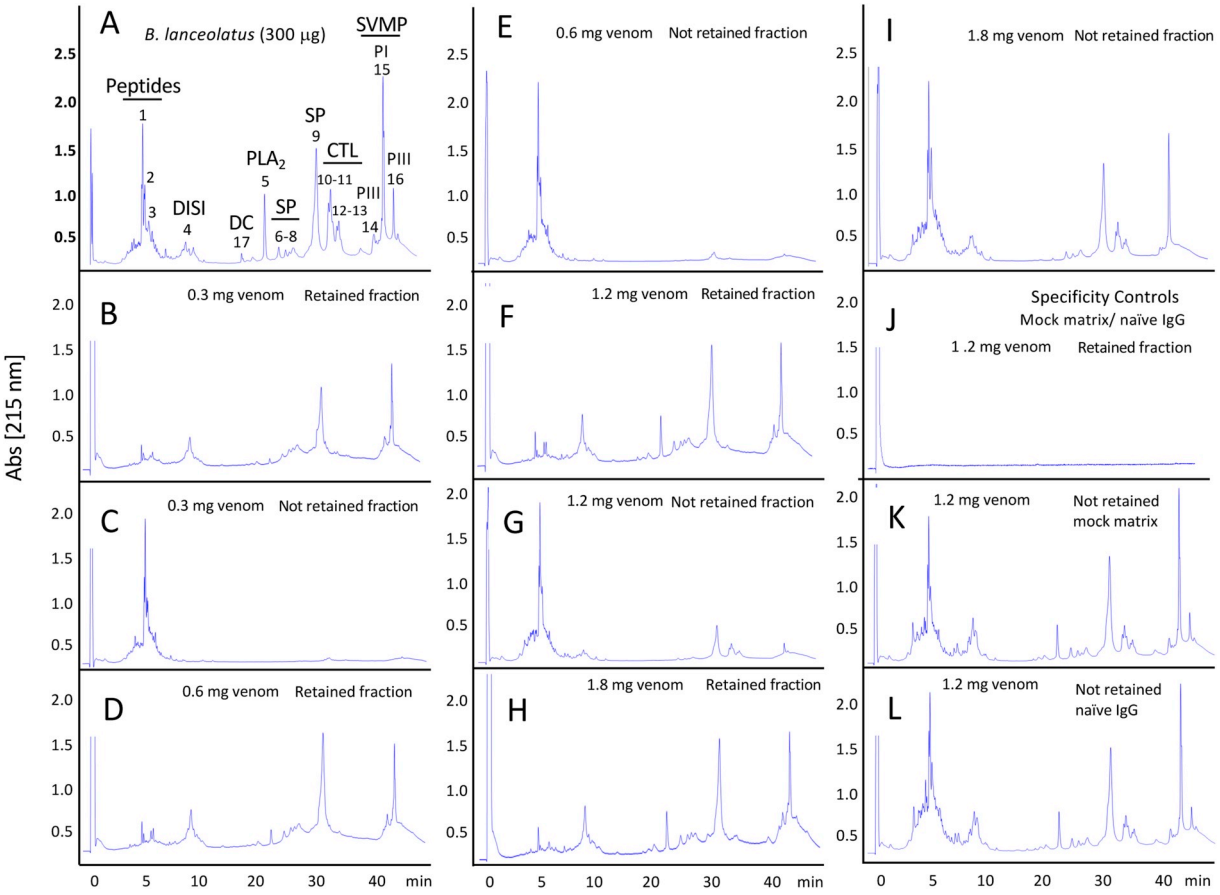

Fig. 1. Third Generation Antivenomics. Panel A, reverse-phase HPLC profile of 300 µg of *B. lanceolatus* venom. The identification of proteins eluting in the various chromatographic peaks were described previously (Gutiérrez et al., 2008). Panels B to I correspond to the non-immunoretained fractions after incubation of immunoaffinity columns containing 10 mg of Bothrofav® antivenom with varying amounts (0.3–1.8 mg) of venom. Panels J to L: Specificity controls with mock chromatographic matrix and naïve horse IgG. DISI, disintegrins; DC, disintegrin-like/cysteine-rich fragment; PLA_2, phospholipases A_2; SP, serine proteinases; CTL, C-type lectin-like proteins; PI and PIII SVMPs, metalloproteinases. See text for details.

HCl, followed by two matrix volumes of coupling buffer to adjust the pH to 7.0–8.0. CNBr-activated instead of N-hydroxysuccinimide (NHS)-activated matrix (Pla et al., 2012) was employed because NHS released during the coupling procedure absorbs strongly at 280 nm, thus interfering with the measurement of the concentration of unbound antibodies remaining in the supernatant of the coupling solution used to estimate the coupling yield. Eighty mg of antivenom were dissolved in 2× matrix volume of coupling buffer and incubated with 3 mL of CNBr-activated matrix for 4 h at room temperature (~25 °C). Antivenom coupling yield, estimated measuring A_{280nm} before and after incubation with the matrix, was 26 mg/mL. After the coupling, remaining active matrix groups were blocked with 12 mL of 0.1 M Tris-HCl, pH 8.5 at room temperature for 4 h. Affinity columns, each containing 10.0 mg of immobilized antivenom $F(ab')_2$ fragments, were alternately washed with three matrix volumes of 0.1 M acetate containing 0.5 M NaCl, pH 4.0–5.0, and three matrix volumes of 0.1 M Tris-HCl, pH 8.5. This procedure was repeated 6 times. The columns were then equilibrated with 5 volumes of working buffer (PBS, 20 mM phosphate buffer, 135 mM NaCl, pH 7.4), incubated with increasing amounts (100–1800 µg of *B. lanceolatus* venom proteins dissolved in ½ matrix volume of PBS), and the mixtures incubated for 1 h at 25 °C in an orbital shaker. As specificity controls, 400 µL of CNBr-activated Sepharose™ 4B matrix, without (mock) or with 10 mg of immobilized control (naïve) horse IgGs (purified by caprylic acid precipitation from the serum of non-immunized horses provided by a local slaughterhouse), were incubated with 1200 µg of venom and developed in parallel to the immunoaffinity columns. The non-retained eluates of columns incubated with 100–300, 600, 900, 1200, 1500 and 1800 µg venom were recovered with 5×, 10×, 15×, 20×, 25× and 30× matrix volumes of PBS, respectively. The immunocaptured proteins were eluted, respectively, with 5×, 10×, 15×, 20×, 25× and 30× matrix volume of 0.1 M glycine-HCl, pH 2.7 buffer, and the eluates were brought to neutral pH with 1 M Tris-HCl, pH 9.0. To avoid saturation of the downstream reverse-phase chromatographic analysis, aliquots corresponding to 100–300 µg of initial total venom proteins were concentrated in a Savant™ SpeedVac™ vacuum system (ThermoFisher Scientific, Waltham, MA USA) to 40 µL and fractionated by reverse-phase HPLC using an Agilent LC 1100 High Pressure Gradient System (Santa Clara, CA, USA) equipped with a Discovery® BIO Wide Pore C18 (15 cm × 2.1 mm, 3 µm particle size, 300 Å pore size) column and a DAD detector as above. The column was developed at a flow rate of 0.4 mL/min with a linear gradient of 0.1% TFA in MilliQ® water (Merck-Millipore, Darmstadt, Germany) (solution A) and 0.1% TFA in acetonitrile (solution B), isocratic (5% B) for 1 min, followed by 5–25% B for 5 min, 25–45% B for 35 min, and 45–70% B for 5 min. Eluate was monitored at 215 nm with a reference wavelength of 400 nm. The fraction of non-immunocaptured molecules was estimated as the relative ratio of the chromatographic areas of the toxin recovered in the non-retained (NR) and retained (R) affinity chromatography fractions using the equation %NRi = 100-[(Ri/(Ri+NRi)) x 100], where Ri corresponds to the area of the same protein "i" in the chromatogram of the fraction retained and eluted from the affinity column.

Fig. 1 depicts the RP-HPLC profiles of the venom fractions that were retained and non-retained when passing increasing amounts (ranging

Table 1
Total and concentration-dependent immunoretained (RET) *B. lanceolatus* (Martinique) venom proteins by Sanofi-Pasteur antivenom affinity column (10 mg Bothrofav®). Maximal binding for each RP-HPLC fraction is highlighted in yellow background. "µg TOTAL" was calculated by multiplying the % of the total venom proteome of the RP-HPLC fraction by (n/100), where n = total venom proteins (in mg) used in the antivenomic analysis. "µg RET" was calculated as specified in the manuscript's main text using the equation %NRi = 100-[(Ri/(Ri + NRi)) × 100], where NRi and Ri correspond to the areas of the same protein "i" in the chromatogram of the non-retained and retained fractions eluted from the affinity column.

		Bothrops lanceolatus **(Martinique) total venom proteins (µg)**							
RP-HPLC fraction		100	300	600	900	1200	1500	1800	**Toxins in RP-HPLC fraction**
1	µg TOTAL	2.45	7.36	14.72	22.08	29.44	36.80	44.15	Peptides
	µg RET	0.10	1.09	1.80	**3.66**	2.53	2.04	2.06	
2	µg TOTAL	5.64	16.92	33.84	50.76	67.68	84.60	101.52	
	µg RET	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
3	µg TOTAL	3.94	11.82	23.65	35.47	47.29	59.12	70.94	
	µg RET	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
4	µg TOTAL	2.92	8.77	17.53	26.30	35.06	43.83	52.60	DISI
	µg RET	2.87	8.77	16.55	**23.75**	23.08	21.68	19.28	
17	µg TOTAL	0.84	2.51	5.01	7.52	10.02	12.53	15.03	DC
	µg RET	0.84	2.51	5.01	7.52	10.02	12.53	**14.61**	
5	µg TOTAL	6.00	18.00	35.99	53.99	71.99	89.99	107.98	PLA2
	µg RET	6.00	18.00	35.99	53.99	71.61	89.65	**98.36**	
6	µg TOTAL	1.31	3.92	7.85	11.77	15.70	19.62	23.54	SP
	µg RET	1.31	3.89	7.63	11.23	13.23	**13.24**	11.06	
7	µg TOTAL	4.50	13.50	26.99	40.49	53.99	67.49	80.98	
	µg RET	4.50	13.33	16.69	22.46	**37.44**	36.21	31.25	
8	µg TOTAL	3.51	10.53	21.05	31.58	42.11	52.64	63.16	
	µg RET	3.51	9.54	20.24	27.27	33.19	**34.45**	36.14	
9	µg TOTAL	25.36	76.07	152.14	228.20	304.27	380.34	456.41	
	µg RET	24.81	72.84	143.25	203.45	**212.35**	198.62	154.03	
10	µg TOTAL	5.18	15.53	31.05	46.58	62.10	77.63	93.15	CTL
	µg RET	5.18	14.09	30.55	**42.30**	34.09	28.11	20.05	
11	µg TOTAL	9.80	29.41	58.81	88.22	117.62	147.03	176.44	
	µg RET	9.80	27.57	56.78	**77.88**	68.08	55.06	39.14	
12	µg TOTAL	1.61	4.83	9.65	14.48	19.31	24.14	28.96	
	µg RET	1.61	4.83	9.65	12.15	**14.12**	12.28	11.58	
13	µg TOTAL	3.60	10.79	21.58	32.36	43.15	53.94	64.73	
	µg RET	3.60	10.79	21.44	29.31	**29.69**	26.68	22.91	
14	µg TOTAL	3.23	9.70	19.40	29.10	38.80	48.50	58.19	PIII-SVMP
	µg RET	2.86	9.70	18.53	27.97	35.49	**38.97**	30.31	
16	µg TOTAL	3.27	9.81	19.62	29.43	39.24	49.05	58.86	
	µg RET	3.13	9.81	19.62	29.43	39.24	45.10	**54.19**	
15	µg TOTAL	13.61	40.83	81.67	122.50	163.33	204.17	245.00	PI-SVMP
	µg RET	13.61	40.50	80.51	119.37	**148.08**	146.45	109.28	

from 300 to 1800 µg) of *B. lanceolatus* venom through identical immunoaffinity chromatography columns. With the exception of the first three peaks, comprising small peptides (Gutiérrez et al., 2008), the rest of the fractions were retained, although to a varied extent, in the immunoaffinity columns. The pathophysiological relevance of these poorly immunogenic peptides has not been addressed in the current study. However, intraperitoneal administration of similar early-eluting peptide fractions from *Lachesis* venoms induced neither a significant change in the mean arterial blood pressure of mice, nor signs of abnormal behavior, or histopathological alterations (Pla et al., 2013). Preclinical *in vivo* evidence showing that the Bothrofav® antivenom is quite effective in the neutralization of toxic activities of *B. lanceolatus* venom (Resiere et al., 2018) further supports the clinical irrelevance of *B. lanceolatus* peptide fractions.

Concentration-dependent binding capacities of the antivenom column for each venom fraction, expressed as the amount of µg calculated for the corresponding chromatographic peak, are listed in Table 1. RP-HPLC fractions were assigned to venom proteins by comparison with a previous venomics analysis (Gutiérrez et al., 2008). Fractions 4 (disintegrins), and 10 and 11 (C-type lectin-like proteins) saturated their antibody combining sites at 900 µg of total venom proteins, whereas fractions 7 and 9 (serine proteinases), 12 and 13 (C-type lectin-like proteins), and 15 (P-I metalloproteinase) reached maximal binding to the immobilized $F(ab')_2$ fragments at 1200 µg venom, and fractions 5 (phospholipase A_2), 6 and 8 (serine proteinases), 14 and 15 (P-III metalloproteinases), and 17 (disintegrin-like/cysteine-rich (DC) fragment), saturated their binding sites at 1500–1800 µg of incubated venom (Table 1). When maximum binding capacity was expressed as the percentage of venom protein contained in the corresponding chromatographic peak, all fractions, with the exception of the peptides eluted in fractions 1 through 3, were immunocaptured with efficiency ranging from 61% (fraction 13) to 97% (fraction 17).

Ten milligrams of immobilized antivenom $F(ab')_2$ fragments had maximal binding capacity of 843.1 µg total *B. lanceolatus* venom proteins (84.3 mg venom/g $F(ab')_2$). Since the protein concentration of this batch of Bothrofav® was 207 mg/mL, and the vial contained 10 mL, the total amount of venom proteins bound per vial was 174.5 mg. Considering an average molecular mass for *B. lanceolatus* toxins of 32 kDa (calculated as Σ (% i × M_i), where % i is the relative abundance of toxin "i" and M_i its molecular mass in Da), 174.5 mg venom equals 5.5 µmoles of venom molecules. Assuming that the two antigen binding

sites of an $F(ab')_2$ molecule were occupied at maximal antigen binding capacity, a Bothrofav® vial contained (5.5/2) μmoles of toxin-binding molecules, or 300 mg $F(ab')_2$ (molar mass, 110 g/mol). This figure corresponds to 14.5% [(300/2070) x 100] of the total Bothrofav® $F(ab')_2$ molecules.

In a previous study, Resiere et al. (2018) determined the neutralizing ability of Bothrofav® antivenom against the lethal effect of *B. lanceolatus* venom. This allowed us to calculate the antivenom's potency (P), which corresponds to the amount of venom (mg) completely neutralized per mL of antivenom, using the formula:

$$P = [(n - 1)/ED_{50}] \times LD_{50},$$

where "n" is the number of Median Lethal Doses (LD_{50}s) used as challenge dose to determine the antivenom Median Effective Dose, ED_{50}. For the calculation of P, LD_{50} and ED_{50} are expressed, respectively, as (mg venom/mouse) and (mL of antivenom that protect 50% of the mouse population injected with $n \times LD_{50}$). In the calculation of P, $(n-1) \times LD_{50}$ is used instead of the total amount of venom, $n \times LD_{50}$, because at the endpoint of the neutralization assay, one LD_{50} remains non-neutralized and causes the death of 50% of mice (WHO, 1981; Morais et al., 2010). In this particular case, since the 'challenge dose' of venom in the lethality assay corresponds to 5 Median Lethal Doses (LD_{50}), and the LD_{50} of this venom by the i.v. route is 6 μg venom per gram body weight, the total amount of venom injected in 18–20 g mice in this test is 114 μg. The ED_{50} of the antivenom is 12 mg antivenom per mg venom (Resiere et al., 2018), i.e. with an antivenom protein concentration of 207 mg/mL, the ED_{50}, expressed as mL antivenom per challenge dose of venom (i.e., 5 LD_{50}s = 0.57 mg venom), is 0.033 mL. Therefore, the neutralizing potency of the antivenom can be estimated as:

$$P = [(5 - 1)/0.033] \times 0.114$$

$$= 13.8 \text{ mg venom neutralized per mL antivenom}$$

The fraction of the toxin-binding Bothrofav® $F(ab')_2$ molecules that contribute to its venom lethality neutralization potency can be calculated by dividing P by the antivenom's maximal total venom proteins binding capacity (17.5 mg/mL):

% toxin-binding and neutralizing Bothrofav® $F(ab')_2$ molecules = [(13.8 mg/mL) / (17.5 mg/mL)] × 100 = 79%.

Combining this figure with the above antivenomics-derived percentage of Bothrofav® $F(ab')_2$ molecules bearing affinity towards *B. lanceolatus* venom toxins (14.49%) indicates that [(79 × 14.5)/100] = 11.5% of Bothrofav® $F(ab')_2$ fragments are clinically relevant antivenom molecules (i.e. may contribute to reverse the effects of the envenoming).

This study illustrates the analytical value of the third-generation antivenomics methodology. In this particular case it allowed the study of the immunological reactivity of Bothrofav® when confronted with the homologous venom of *B. lanceolatus*. Antivenom was able to immunocapture all protein venom fractions, with the only exception of the first HPLC peaks which correspond to low molecular mass peptides (Gutiérrez et al., 2008), which may be non-immunogenic. As in other viperid venoms, it is highly likely that the most relevant pathophysiological and pathological alterations induced by *B. lanceolatus* venom are caused by metalloproteinases, phospholipases A_2, serine proteinases and probably C-type lectin-like proteins and disintegrins, all of which were immunocaptured to a high extent by the antivenom $F(ab')_2$ fragments. Our antivenomics observations correlate very well with the high capacity of this antivenom to neutralize lethal, local and systemic hemorrhagic, edema-forming, thrombocytopenic, phospholipase A_2 and proteinase activities of *B. lanceolatus* venom (Resiere et al., 2018). Further, the preclinical observations agree also with the clinical efficacy of this antivenom in envenomings by *B. lanceolatus* in Martinique (Thomas et al., 1995; Resiere, unpublished observations).

Ethical statement

This study was performed following standard ethical procedures of scientific research in the experimental analyses and in the preparation of the manuscript.

Declaration of interests

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

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