In vivo treatment with varespladib, a phospholipase A_2 inhibitor, prevents the peripheral neurotoxicity and systemic disorders induced by *Micrurus corallinus* (coral snake) venom in rats

Rosimeire Silva-Carvalho[a], Matheus Z. Gaspar[a], Luiz H.B. Quadros[a], Luís G.G. Lobo[a], Letícia M. Rogério[a], Najla T.S. Santos[a], Maria C. Zerbinatti[a], Cecília L. Santarém[b], Elisangela O. Silva[b], Juliana R. Gerez[c], Nelson J. Silva Jr.[d], Bruno Lomonte[e], Edward G. Rowan[f*], Rafael S. Floriano[a,f*]

[a]Laboratory of Toxinology and Cardiovascular Research, Graduate Program in Health Sciences, University of Western São Paulo (UNOESTE), Rodovia Raposo Tavares km 572, B2-205, 19067-175, Presidente Prudente, SP, Brazil

[b]Veterinary Hospital, University of Western São Paulo (UNOESTE), Rodovia Raposo Tavares km 572, 19067-175, Presidente Prudente, SP, Brazil

[c]Department of Histology, State University of Londrina, Rodovia Celso Garcia Cid km 380, 86057-970, Londrina, PR, Brazil

[d]Graduate Program in Environmental Sciences and Health, School of Medical, Pharmaceutical and Biomedical Sciences, Pontifical Catholic University of Goiás (PUC Goiás), Rua 232, 128, 74605-140, Goiânia, GO, Brazil

[e]Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, 11501, San José, Costa Rica

[f]Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, 161 Cathedral Street, G4 0RE, Glasgow, UK

Running title: Varespladib prevents the systemic envenomation by *M. corallinus* in rats

* Correspondence: e.g.rowan@strath.ac.uk (E.G.R.); rafael@unoeste.br (R.S.F.)

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Abstract

In this study, we investigated the action of varespladib (VPL) alone or in combination with a coral snake antivenom (CAV) on the local and systemic effects induced by *Micrurus corallinus* venom in rats. Adult male Wistar rats were exposed to venom (1.5 mg/kg – i.m.) and immediately treated with CAV (antivenom:venom ratio 1:1.5 'v/w' – i.p.), VPL (0.5 mg/kg – i.p.), or both of these treatments. The animals were monitored for 120 min and then anesthetized to collect blood samples used for haematological and serum biochemical analysis; after euthanasia, skeletal muscle, renal and hepatic tissue samples were collected for histopathological analysis. *M. corallinus* venom caused local oedema without subcutaneous haemorrhage or apparent necrosis formation, although there was accentuated muscle morphological damage; none of the treatments prevented oedema formation but the combination of CAV and VPL reduced venom-induced myonecrosis. Venom caused neuromuscular paralysis and respiratory impairment in approximately 60 min following envenomation; CAV alone did not prevent the neurotoxic action, whereas VPL alone prevented neurotoxic symptoms developing as did the combination of CAV and VPL. Venom induced significant increase of serum CK and AST release, mostly due to local and systemic myotoxicity, which was partially prevented by the combination of CAV and VPL. The release of hepatotoxic serum biomarkers (LDH and ALP) induced by *M. corallinus* venom was not prevented by CAV and VPL when individually administered; their combination effectively prevented ALP release. The venom-induced nephrotoxicity (increase in serum creatinine concentration) was prevented by all the treatments. VPL alone or in combination with CAV significantly prevented the venom-induced lymphocytosis. In conclusion, VPL shows to be effective at preventing the neurotoxic, nephrotoxic, and inflammatory

activities of *M. corallinus* venom. In addition, VPL acts synergistically with antivenom to prevent a number of systemic effects caused by *M. corallinus* venom.

Keywords: Elapidae snake; *M. corallinus* venom; neurotoxicity; local and systemic toxicity; varespladib; antivenom; neutralization.

1. Introduction

Coral snakes (*Micrurus* spp.) are exclusive species from the family Elapidae found in Americas (Costa and Bérnils, 2018; Nogueira et al., 2019; Silva Jr. et al., 2021a). Envenomations by these snakes are potentially severe due to the neurotoxic action of their venoms, with the most important clinical aspects of the envenomation encompassing neuromuscular blockade, respiratory failure and death (Parrish and Khan, 1967; Kitchens and Van Mierop, 1987; Silva Jr. and Bucaretchi, 2003; Warrell, 2004; Bucaretchi et al., 2006; Bucaretchi et al., 2021; Floriano et al., 2021).

In the last two decades, the composition of coral snake venoms has been systematically investigated through transcriptomic (Leão et al., 2009; Correa-Netto et al., 2011; Margres et al., 2013), proteomic (Olamendi-Portugal et al., 2008; Ciscotto et al., 2011; Correa-Netto et al., 2011; Fernández et al., 2011; Rey-Suárez et al., 2011; Bénard-Valle et al., 2014; Vergara et al., 2014; reviewed by Lomonte et al., 2016) and biochemical (Mebs, 1970; Aird and Silva Jr., 1991; Tan and Ponnudurai, 1992; Silva Jr. and Aird, 2001; Tanaka et al., 2010) approaches, revealing two main groups of toxins: 1) classic α-neurotoxins that block primarily postsynaptic nicotinic (cholinergic) receptors (nAChR) and are part of the three-finger (3FTx) family of toxins, and 2) phospholipase A_2 (PLA_2) β-neurotoxins that cause neuromuscular blockade by acting primarily on presynaptic sites to inhibit the release of acetylcholine (ACh) from nerve

terminals (Aird et al., 2017; Aird and Silva Jr., 2021; Floriano et al., 2021; Gutiérrez et al. 2021; Prasarnpun et al., 2005; Ranawaka et al., 2013).

Micrurus corallinus, a monadal tricolored coral snake, occurs mainly in the south and southeast regions of Brazil, being also found along the east coast (Silva Jr. et al., 2021b) (Figure 1), and is identified as the most likely species to inflict a medically important bite in this country (Bucaretchi et al., 2021). Transcriptomic (Leão et al., 2009), proteomic (Correa-Neto et al., 2011), and biochemical (Aird and Silva Jr., 1991; Silva Jr. et al., 1991; Tan and Ponnudurai, 1992; Silva Jr. and Aird, 2001; Tanaka et al., 2010) analyses have demonstrated that *M. corallinus* venom contains the main classes of toxins commonly found in *Micrurus* venoms, being characterized by approximately 82% of 3FTx and 12% of PLA_2 (Correa-Neto et al., 2011; Leão et al., 2009). In line with the presence of PLA_2 detected through transcriptomic and proteomic analysis, this venom exhibits PLA_2 activity which has been associated with pain, oedema, myonecrosis and neuromuscular blocking activities (Aird and Silva Jr., 1991; Tan and Ponnudurai, 1992; Tanaka et al., 2010; Vital-Brazil and Fontana, 1983/84; Zambelli et al., 2017). The neurotoxicity of *M. corallinus* venom is characterized by neuromuscular blockade mediated by pre- and post-synaptic mechanisms of action, with predominance of the former (Vital-Brazil and Fontana, 1983/84; Vital Brazil, 1987).

The heterologous coral snake antivenom used to treat envenomations by *Micrurus* species throughout the Brazilian territory consists in immunoglobulins raised in hyperimmunized horses using a pool of *Micrurus frontalis* and *Micrurus corallinus* venoms, being supplied by Instituto Butantan (IB, São Paulo, SP), Fundação Ezequiel Dias (FUNED, Belo Horizonte, MG) and Instituto Vital Brazil (IVB, Niterói, RJ). In order to search for useful therapeutic alternatives to complement antivenom therapy, or even its replacement in extreme situations, recent studies have demonstrated the

efficacy of varespladib, a synthetic molecule clinically tested to inhibit the inflammatory cascades of several diseases associated with high levels of secreted PLA_2 (Varespladib, 2011), to suppress the systemic effects caused by several venoms and toxins from Elapidae and Viperidae snakes (Lewin et al., 2016; Bittenbinder et al., 2018; Lewin et al., 2018; Wang et al., 2018; Bryan-Quirós et al., 2019; Salvador et al., 2019; Gutiérrez et al., 2020; Oliveira et al., 2020; Zinenko et al., 2020; Youngman et al., 2020; Dashevsky et al., 2021; Liu et al., 2021).

In this study, we have investigated the potential use of varespladib (LY-315920) alone and in combination with a bivalent coral snake antivenom (anti-*M. corallinus*/anti-*M. frontalis* – FUNED) on the acute systemic effects induced by *M. corallinus* venom in rats using clinical, hematological, biochemical, and histopathological approaches.

2. Materials and methods

2.1. Reagents and venom

Varespladib (LY-315920) was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and coral snake antivenom was from Fundação Ezequiel Dias (FUNED, Belo Horizonte, MG, Brazil); varespladib was dissolved in DMSO prior to use. *Micrurus corallinus* venom was provided by Center for Biological Studies and Research of the Pontifical Catholic University of Goiás (Goiânia, GO, Brazil) through Dr Nelson J. Silva Jr.; a lyophilized pool of venom obtained from one female adult snake was stored at −20 °C and dissolved in ultrapure water prior to use.

2.2. Animals

Wistar rats (300–350 g; 2–3 months old) obtained from Central Bioterium of the University of Western São Paulo (UNOESTE, Presidente Prudente, SP, Brazil) were housed in plastic cages (3 animals/cage) with a wood-shaving substrate, at 23 ± 1 °C on a 12-h light/dark cycle with lights on at 6 a.m. The animals had free access to food and water. The experimental procedures were approved by an institutional Committee for Ethics in Animal Use (CEUA/UNOESTE, Protocol No. 6082/2020) and were done according to the general ethical guidelines for animal use established by the Brazilian Society of Laboratory Animal Science (SBCAL) and Brazilian Federal Law No. 11.794 of October 8, 2008, in conjunction with the guidelines for animal experiments established by the Brazilian National Council for Animal Experimentation (CONCEA).

2.3. Experimental design

The animals were transferred to the Experimental Bioterium of the University of Western São Paulo (UNOESTE, Presidente Prudente, SP, Brazil) and allowed to adapt for two weeks, under the same conditions as described above, before initiating the experimental procedures. The animals were then randomly distributed in six experimental groups (n = 6 per group) identified as: group **G_1** (control) – intramuscular 'i.m.' (gastrocnemius muscle) injection of 0.9% saline; group **G_2** (*M. corallinus* venom) – i.m. injection of venom at a dose of 1.5 mg/kg; group **G_3** (*M. corallinus* venom + coral snake antivenom 'CAV') – i.m. injection of venom (1.5 mg/kg) immediately followed by intraperitoneal 'i.p.' injection of CAV at an antivenom:venom ratio of 1:1.5 (v/w); group **G_4** (*M. corallinus* venom + varespladib 'VPL') – i.m. injection of venom (1.5 mg/kg) immediately followed by i.p. injection of VPL at a dose of 0.5 mg/kg; group **G_5** (*M. corallinus* venom + VPL + CAV) – i.m. injection of venom (1.5 mg/kg) immediately followed by i.p. injections of VPL (0.5 mg/kg) and CAV [1:1.5 (v/w)]. The

150 animals were monitored before (T_0) and at various intervals (T_{30}, T_{60}, T_{90} and T_{120} min)
151 as described below (section 2.4.) and finally euthanized by an overdose of thiopental
152 (Cristália®, São Paulo, SP, Brazil) to collect blood samples for hematological and
153 biochemical analysis, as well as samples of gastrocnemius muscle (right 'location of
154 venom-injection' and left hind limbs), kidney, and liver for histopathological analysis.
155 To induce acute toxicity in rats, the dose of 1.5 mg of venom/kg (i.m.) was chosen
156 based on pilot experiments in order to produce paralysis and respiratory impairment
157 within at least 120 min post envenomation. The ability of the bivalent coral snake
158 antivenom produced by Fundação Ezequiel Dias (FUNED, Belo Horizonte, MG, Brazil)
159 to neutralize the *M. corallinus* venom-induced local and systemic effects in rats was
160 assessed by administering antivenom via i.p. at an antivenom:venom ratio of 1:1.5 (v/w)
161 immediately after injecting the animals by via i.m. with venom (1.5 mg/kg); this
162 antivenom:venom ratio was based on the manufacturer's stated neutralizing capacity for
163 the antivenom (1 ml of antivenom neutralizes 1.5 mg of *M. frontalis* venom). The
164 minimum effective dose of varespladib (0.5 mg/kg) and its lack of toxicity was
165 confirmed in pilot experiments. Fig. 2 summarizes the experimental design described in
166 this section.
167
168 *2.4. Clinical monitoring*
169 The clinical monitoring (T_0 'basal', T_{30}, T_{60}, T_{90} and T_{120} min 'post exposure to
170 venom') consisted in measuring: (a) the auricular temperature using a digital
171 thermometer; (b) locomotor activity using a semi-quantitative toxicity scale; (c) local
172 oedema formation using Image J software (National Institute of Health, Bethesda,
173 Maryland, USA), as essentially described elsewhere (Leão-Torres et al., 2021); and (d)
174 macroscopic monitoring for necrosis formation and occurrence of local or widespread

bleeding. At T_{120}, animals were subsequently anesthetized by a non-lethal dose of thiopental (1.8 mg/kg, i.p.) and subjected to intracardiac puncture in order to obtain blood samples. Blood samples were collected in EDTA coated tubes for hematological analysis or with sodium citrate coated for hemostatic analysis (BD Vacutainer® tubes); blood samples, without anticoagulant, were also collected for biochemical serum analysis. Finally, animals were euthanized by an overdose of thiopental in order to obtain muscle (gastrocnemius), renal, and hepatic tissue samples for histopathological analysis. The action of VPL alone or in combination with CAV to prevent the venom-induced lack of locomotor activity was determined based on a semi-quantitative toxicity scale (severity score) using the open field test. A cage with walls to prevent escape was divided by grid lines into nine squares (155 cm^2 each), with (0) indicating a complete absence of paralysis (more than 15 times crossing the grid lines), (1) indicating a slight lack of locomotor activity (between 10 and 15 times crossing the grid lines), (2) indicating a moderate lack of locomotor activity (between 5 and 10 times crossing the grid lines) accompanied by respiratory impairment, and (3) indicating a severe lack of locomotor activity (less than 5 times crossing the grid lines or no locomotor activity) accompanied by intense respiratory impairment, as essentially described elsewhere (Leão-Torres et al., 2021).

2.5. Haematological analysis

Erythrocytes, haemoglobin, haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red blood cell distribution width (RDW-SD), and total leukocyte count were determined using the POCH-100 iV DIFF haematology analyser (Sysmex do Brasil Indústria e Comércio Ltda., São José dos Pinhais, PR, Brazil). For differential counting

of leukocytes, blood smears were stained with Diff-Quick (Panótico® – Laborclin Produtos para Laboratórios Ltda., Pinhais, PR, Brazil) and then analysed under an E-200 Nikon light microscope (Nikon Inc., Tokyo, Japan) at 100x magnification. The concentration of fibrinogen was determined by calorimetric technique using a refractometer ATC-ITREF-200 (Instrutemp Instrumentos de Medição Ltda., São Paulo, SP, Brazil) and the results were expressed as mg/dL. The total plasma protein (TPP) concentration was quantified by the ATC-ITREF-200 refractometer (Instrutemp Instrumentos de Medição Ltda., São Paulo, SP, Brazil) and the results were expressed as g/dL. Clotting tests for activated partial thromboplastin 'APTT' (code 731080-C) and prothrombin 'PT' (code 730100-1-C) were performed using Wama Diagnóstica® commercial kits for a quick timer Coagmaster 4.0 (Wama Diagnóstica Produtos para Laboratórios, São Carlos, SP, Brazil).

2.6. Serum biochemical analysis

Serum biomarkers for systemic myotoxic, nephrotoxic, and hepatotoxic activities were determined using Cobas C111 commercial kits (Roche Holding AG, Basel, Switzerland) for creatine kinase 'CK' (code 07442017-190), aspartate aminotransferase 'AST' (code 04657543-190), creatinine 'Cr' (code 05401755-190), alkaline phosphatase 'ALP' (code 04657373-190), alanine aminotransferase 'ALT' (code 04718569-190), and lactate dehydrogenase 'LDH' (code 05401674-190). The assays were performed using a Cobas C111 analyser (Roche Holding AG, Basel, Switzerland).

2.7. Histopathological analysis

After collecting the blood samples, the animals were subsequently euthanized by an overdose of thiopental and subjected to dissection in order to collect skeletal muscle

(gastrocnemius muscle – right 'location of venom-injection' and left hind limbs), renal, and hepatic tissue samples. The samples were immediately fixed in 10% formaldehyde overnight and then washed for 30 min in 0.1 M phosphate-buffered saline and 30 min in distilled water prior to storage in 70% ethanol overnight. The samples were dehydrated in graded ethanol (80%, 95% and 100%), cleared in xylene (1:1 ethanol:xylene, 1:1 xylene:paraffin) and finally embedded in paraplast. Serial sections of 3–5 sections per sample (5 μm thick), separated from each other by 25 μm, were cut and mounted on plain glass slides for hematoxylin–eosin (HE) staining. Blind slide reading was carried out through a Leica ICC50HD camera coupled to a Leica DM750 light microscope (Leica Microsystems, Wetzlar, Germany) and the images were then captured and analyzed qualitatively using a LAS 4.2 software (Leica Microsystems, Wetzlar, Germany). The morphological changes and frequency of lesions were compared among the treatments based on a lesional score, as essentially described elsewhere (Gerez et al., 2015; Grenier et al., 2011).

2.8. Statistical analysis

Results were expressed as the mean ± SEM and statistical comparisons were performed with Student's *t*-test or one-way ANOVA followed by the Tukey test, with $p < 0.05$ indicating significance. In histopathological analysis, Dunn's test was applied to evaluate the lesional score. All data were analyzed using Microcal Origin 8 SR4 v. 8.0951 (Microcal Software Inc., Northampton, MA, USA) and GraphPad Prism 4 v. 4.03 (GraphPad Software Inc., La Jolla, CA, USA) software.

3. Results

3.1. Clinical aspects of the experimental envenomation by Micrurus corallinus in rats and treatment with coral snake antivenom (CAV) and varespladib (VPL)

M. corallinus venom (G_2) administered into gastrocnemius muscle elicited intense local oedema after 120 min of envenomation, with no macroscopic evidence of necrosis or subcutaneous haemorrhage; the immediate i.p. administration of CAV (G_3), VPL (G_4) or both these agents (G_5), did not reduce the venom-induced oedema. None of the animals showed evidence for oedema, necrosis formation or haemorrhage in the contralateral gastrocnemius muscle after 120 min of envenomation. There was no apparent systemic haemorrhage in those animals exposed to venom alone (G_2) or treated with CAV and/or VPL (G_3, G_4 and G_5) during the 120 min of observation period (Supplementary material, Table S1).

All animals exposed to *M. corallinus* venom alone (G_2) evolved to moderate peripheral neurotoxicity within 30 min of envenomation (severity score: 2) and then to a more pronounced condition after 60 min of envenomation (severity score: 3); two animals died before the end of the 120 min of monitoring period, whereas the other four progressed to hypothermia and severe respiratory impairment. In group G_3, the CAV did not prevent the neurotoxic action of *M. corallinus* venom, with all the animals evolving gradually to a severe peripheral neurotoxicity (severity score: 3) from 90 min of envenomation; one animal died before completing the 120 min of monitoring, whereas the other five progressed to hypothermia and severe respiratory impairment. However, in animals within group G_4, VPL alone reduced the severe neurotoxicity observed in groups G_2 and G_3 to a moderate effect from 90 min of envenomation (severity score: 2); there were no deaths in this group. Finally, the combined treatment with both agents (G_5) resulted in the best rescue of the venom-induced peripheral neurotoxicity, with only one animal evolving to severe clinical condition (severity

score: 3), while the other five presented only a slight reduction of locomotor activity (severity score: 1); there were no deaths in this group (Figure 3; Supplementary material, Table S1).

3.2. Serum biochemical changes produced by M. corallinus venom in rats and treatment with coral snake antivenom (CAV) and varespladib (VPL)

In animals exposed to *M. corallinus* venom alone (G_2), there was a significant increase of serum CK after 120 min of envenomation ($p < 0.05$ compared to G_1 'control', $n = 6$) accompanied by increase of muscle fraction-derived AST ($p < 0.05$ compared to G_1 'control', $n = 6$). Venom-induced serum CK and AST release were partially reduced by CAV or VPL when administered individually (G_3 and G_4, respectively), whereas the combination of both agents (G_5) prevented the increase of these biomarkers by approximately 41% and 29%, respectively ($p < 0.05$ compared to G_2 'venom' for both CK and AST, $n = 6$) (Figure 4A and 4B). Venom (G_2) significantly increased the levels of serum creatinine concentration after 120 min of envenomation ($p < 0.05$ compared to G_1 'control', $n = 6$), with CAV and VPL preventing in approximately 24% ($p < 0.05$ compared to G_2 'venom', $n = 6$) and 20%, respectively, this alteration when administered individually; the combination of these agents prevented in approximately 30% ($p < 0.05$ compared to G_2 'venom', $n = 6$) the venom-induced renal disfunction (Figure 4C).

M. corallinus venom (G_2) induced significant increase in serum LDH ($p < 0.05$ compared to G_1 'control', $n = 6$) after 120 min of envenomation, including minor changes in ALT and ALP levels; the venom-induced alterations in LDH level was not prevented by CAV or VPL when administered individually (G_3 and G_4, respectively),

but combining these agents (G_5) significantly prevented the increase in serum LDH by approximately 42% ($p < 0.05$ compared to G_2 'venom', $n = 6$) (Figure 5).

3.3. Haematological changes produced by M. corallinus venom in rats and treatment with coral snake antivenom (CAV) and varespladib (VPL)

In animals exposed to *M. corallinus* venom alone (G_2), as well as in the groups treated with CAV (G_3), VPL (G_4) or both agents in combination (G_5), there were no relevant alterations in total erythrocytes count, haemoglobin, haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), and total plasma protein (TPP) after 120 min of envenomation (Supplementary material, Table S2a). In addition, there was also no relevant alteration in platelets count whereas venom alone (G_2) increased the level of fibrinogen ($p < 0.05$ compared to G_1 'control', $n = 6$) after 120 min of envenomation; treatment with CAV (G_3) prevented by approximately 35% the increase of fibrinogen ($p < 0.05$ compared to G_2 'venom', $n = 6$), whereas VPL (G_4) produced only approximately 18% of reduction of this venom effect; combined treatment (G_5) was more effective in reducing the increase of fibrinogen by approximately 44% ($p < 0.05$ compared to G_2 'venom', $n = 6$) (Supplementary material, Table S2b)

M. corallinus venom significantly delayed the clotting time for activated partial thromboplastin (APTT) ($p < 0.05$ compared to G_1 'control', $n = 6$), whereas only a mild delay was observed for prothrombin (PT); CAV (G_3) or VPL (G_4) slightly prevented these alterations when administered individually, while their combination (G_5) maintained the clotting time unchanged after 120 min of envenomation ($p < 0.05$ compared to G_2 'venom', $n = 6$) (Figure 6).

323

324 *3.4. Leukocyte changes produced by M. corallinus venom in rats and treatment*
325 *with coral snake antivenom (CAV) and varespladib (VPL)*

326 In animals exposed to *M. corallinus* venom alone (G_2) and also in groups treated
327 with CAV (G_3), VPL (G_4) or both these agents (G_5), there was no alteration in
328 eosinophils count whereas neutrophil precursor cells (myelocytes and metamyelocytes)
329 and basophils were not detected after 120 min of envenomation in any group. Venom
330 alone (G_2) produced an increase in total leukocytes count after 120 min of
331 envenomation ($p < 0.05$ compared to G_1 'control', $n = 6$), an effect which was
332 effectively prevented by treatment with CAV alone (G_3) or with CAV combined with
333 VPL (G_5); VPL alone (G_4) did not prevent the increase in total leukocytes count. The
334 increase in total leukocytes induced by *M. corallinus* venom (G_2) was mirrored by a
335 significant increase of neutrophils ($p < 0.05$ compared to G_1 'control', $n = 6$),
336 lymphocytes ($p < 0.05$ compared to G_1 'control', $n = 6$), and monocytes ($p < 0.05$
337 compared to G_1 'control', $n = 6$); CAV (G_3) or VPL (G_4) were effective at preventing
338 the increase of neutrophils and lymphocytes when administered individually ($p < 0.05$
339 compared to G_2 'venom', $n = 6$), and their combination (G_5) promoted greater rescue
340 against the increase in lymphocytes ($p < 0.05$ compared to G_2 'venom', $n = 6$); all of
341 these treatments prevented the increase of monocytes induced by venom (Figure 7).

342

343 *3.5. Histopathological alterations induced by M. corallinus venom in rats and*
344 *treatment with coral snake antivenom (CAV) and varespladib (VPL)*

345 Rats exposed to *M. corallinus* venom (G_2) had morphological changes in the
346 locally injected gastrocnemius muscle characterized by discrete haemorrhage, diffuse
347 congestion, severe neutrophilic inflammatory infiltrate, degeneration, and myonecrosis

($p < 0.05$ compared to G_1 'control', $n = 6$); a single administration of CAV (G_3) or VPL (G_4) significantly reduced these vascular and inflammatory changes, whereas their combination (G_5) additionally decreased the myonecrosis ($p < 0.05$ compared to G_2 'venom', G_3 'venom + CAV' and G_4 'venom + VPL', $n = 6$) (Figure $8A_1$). Venom did not induce morphological changes in the contralateral gastrocnemius muscle after 120 min of envenomation (Figure $8A_2$). In liver, venom (G_2) altered significantly the lesional score most likely due to the changes such as diffuse congestion, moderate megalocytosis, mild cytoplasmic degeneration, and hepatocyte necrosis; CAV (G_3) or VPL (G_4) individually administered did not rescue the venom-induced hepatotoxicity, whereas the combination of both these agents (G_5) produced a significant reduction of these morphological changes ($p < 0.05$ compared to G_2 'venom' and G_4 'venom + VPL', $n = 6$) (Figure 8B). In kidneys, *M. corallinus* venom (G_2) induced moderate acute tubular necrosis, cytoplasmic degeneration, and diffuse congestion; all the treatments (G_3, G_4 and G_5) successfully prevented these morphological changes ($p < 0.05$ compared to G_2 'venom', $n = 6$) (Figure 8C). Fig. S1 (Supplementary material) shows representative histological sections of gastrocnemius muscle (RHL), liver, and kidney from groups G_1 (control), G_2 (venom), and G_6 (venom + CAV + VPL).

4. Discussion

Envenomations by coral snakes are mostly characterized by potent neurotoxic action due to failure of cholinergic transmission at skeletal neuromuscular junctions mediated by α- and β-neurotoxins which are responsible in promoting generalized muscle paralysis and respiratory failure, being the main cause of death in severe cases (Bucaretchi et al., 2006, 2021; Silva Jr. and Bucaretchi, 2003; Kitchens and Van Mierop, 1987; Parrish and Khan, 1967; Warrell, 2004; Floriano et al., 2021). In this

373 context, *M. corallinus* has one of the most potent venoms among coral snakes (Higashi
374 et al., 1995; Siles-Villarroel et al., 1980/1981; Tanaka et al., 2010), with lethality
375 similar to two other *Micrurus* species frequently involved in human envenomation in
376 Brazil, i.e., *M. spixii* and *M. altirostris* (Higashi et al., 1995; Tanaka et al., 2010). In
377 addition, other systemic disorders have been reported for these venoms such as
378 haemorrhage (Silva Jr. and Aird, 2001; Remuzgo et al., 2002), nephrotoxicity (De
379 Roodt et al., 2014; Braga et al., 2020), and cardiovascular toxicity (Floriano et al., 2020,
380 Reis et al., 2021).

381 In this study, rats exposed to a single intramuscular injection of *M. corallinus*
382 venom developed: 1) pronounced local oedema and myonecrosis, without evidence of
383 subcutaneous haemorrhage or apparent necrosis formation; and 2) severe peripheral
384 neurotoxicity characterized by neuromuscular paralysis and respiratory failure within
385 120 min of envenomation. Haematological and biochemical analysis revealed that this
386 venom also caused: 3) significant elevations in CK and AST serum biomarkers due to
387 local and systemic myotoxicity, in serum creatinine concentration indicating
388 nephrotoxicity, including in LDH and ALP indicating hepatotoxicity; 4) alteration of
389 clotting time for activated partial thromboplastin (intrinsic pathway); and 5) marked
390 increase of inflammatory cells in peripheral blood (leukocytosis, neutrophilia and
391 lymphocytosis). In addition, venom induced: 6) severe local morphological changes in
392 gastrocnemius muscle, including moderate hepatic and renal morphological alterations,
393 in agreement with the observed increases in their corresponding serum biomarkers of
394 tissue damage.

395 In Brazil, the bivalent (anti-*M. frontalis*/*M. corallinus*) antivenom is the only
396 therapeutic approach used to treat the systemic envenomation by *Micrurus* and its
397 efficacy has been contested as it does not cover the wide diversity of coral snakes

distributed across the country (Higashi et al., 1995; Silva Jr. et al., 1991; Abreu et al., 2008; Tanaka et al., 2010; Vidal et al., 2015; Floriano et al., 2019). In this study, we also observed the bivalent antivenom was not able to prevent the worst clinical condition associated to the neurotoxic action of *M. corallinus* venom when administered alone; envenomed animals treated with antivenom evolved to a severe neuromuscular paralysis affecting their respiratory function, with one death occurring before completing 120 min of clinical monitoring. In addition, the antivenom did not prevent the systemic myotoxicity evidenced by increase of serum CK and ALP biomarkers and the observation of local muscle fiber necrosis in gastrocnemius. Likewise, antivenom did not counteract the increase of the hepatic injury marker enzyme LDH, the alteration of activated partial thromboplastin clotting time, and the monocytosis induced by *M. corallinus* venom in rats after 120 min of envenomation.

The PLA_2 inhibitor varespladib has shown to be a potential therapeutic candidate to complement antivenom therapy due to its inhibitory action on venom toxic effects that depend on such enzymes and their non-catalytic PLA_2-like variants (Lewin et al., 2016, 2018; Bittenbinder et al., 2018; Wang et al., 2018; Bryan-Quirós et al., 2019; Salvador et al., 2019; Gutiérrez et al., 2020; Oliveira et al., 2020; Zinenko et al., 2020; Dashevsky et al., 2021; Liu et al., 2021). Studies demonstrating the rescue action of varespladib against several Elapidae and Viperidae venoms have not included investigations on South-American coral snake venoms, with the exception of the study of Dashevsky et al. (2021) who evaluated the action of varespladib on the in vitro anticoagulant activity of *M. altirostris*, *M. corallinus*, *M. ibiboboca*, *M. pyrrhocryptus*, *M. obscurus* and *M. surinamensis* venoms.

Here, varespladib alone showed to be more effective than antivenom to prevent the progressive neuromuscular paralysis and respiratory failure caused by *M. corallinus*

423 venom. The neurotoxic action of *M. corallinus* venom has been strongly associated with
424 the presence of β-neurotoxins in this venom, which inducing irreversible neuromuscular
425 blockade via a PLA_2-dependent presynaptic mechanism of action (Vital-Brazil and
426 Fontana, 1983/84; Vital Brazil, 1987; Cruz-Höfling et al., 1983/1984). Accordingly, the
427 high efficacy of varespladib in decreasing the severity of neurotoxic manifestations in
428 rats exposed to *M. corallinus* venom also corroborates with a neurotoxic mechanism of
429 action mostly dependent on PLA_2 enzymes rather than other components of this venom
430 such as three-finger toxins (α-neurotoxins). Although Correa-Neto et al. (2011) and
431 Leão et al. (2009) have determined the proteomic and transcriptomic profile of *M.*
432 *corallinus* venom, respectively, studies regarding the pharmacological characterization
433 of the β-neurotoxins from this venom have yet to be done. However, this study
434 demonstrates the importance of pharmacological data over proteomic and
435 transcriptomic data when assigning a likely mechanism of action of a venom.
436 Varespladib also contributed to prevent the *M. corallinus* venom-induced
437 lymphocytosis, increase in renal injury biomarker (creatinine) concentration, and renal
438 morphological alterations. In addition, our findings have indicated that varespladib
439 exerts a synergic mechanism when administered in combination with antivenom to
440 produce greater rescue than that seen with antivenom alone.
441 Considering the rescue action of varespladib on other coral snake venoms already
442 investigated, the drug showed to be potentially effective in decreasing the lethality of
443 the envenomation by North-American coral snake *M. fulvius* in rats (Lewin et al., 2016)
444 and in reducing significantly the hemolytic, coagulant, and myotoxic effects induced by
445 this venom in pigs (Lewin et al., 2018). More recently, Dashevsky et al. (2021) reported
446 that varespladib was highly effective at abolishing the anticoagulant activity, in vitro,
447 from a broad group of *Micrurus* venoms, including *M. corallinus* venom. To date, these

reports are the only studies correlating the inhibitory action of varespladib on the effects induced by venoms from coral snake species.

In addition, varespladib appears to affect distinct activities mediated by PLA_2 among the different genus of snakes already investigated, e.g., myotoxic and anticoagulant activities induced by *Micrurus fulvius* venom (Lewin et al., 2018), anticoagulant activity by *Naja* spp., *Bitis* spp., *Echis* spp. and *Pseudechis* spp. venoms (Bittenbinder et al., 2018; Youngman et al., 2020; Xie et al., 2020a, 2020b; Zdenek et al., 2020), myotoxicity and cytotoxicity by *Pseudechis colletti*, *Bothrops asper* and *Crotalus vegrandis* venoms, including their major myotoxic PLA_2 (Bryan-Quirós et al., 2019), haemorrhagic activity by *Agkistrodon halys* and *Deinagkistrodon acutus* venoms (Wang et al., 2018), and neuromuscular blockade by *Oxyuranus scutellatus* venom (Oliveira et al., 2020). For *M. corallinus*, neuromuscular blockade seems to be the most lethal mechanism for this venom compared to other toxic activities exhibit by Elapidae and Viperidae venoms, being mostly PLA_2 dependent and successfully prevented by varespladib. Taken together, our findings represent the fullest investigation related to the action of varespladib on the systemic aspects of the envenomation by Elapidae and Viperidae snakes.

In conclusion, varespladib shows to be a highly efficient therapeutic tool to prevent neuromuscular paralysis and respiratory failure caused by *M. corallinus* venom. Apart from other minor actions produced by itself, e.g., suppression of inflammatory responses and renal damage, we have demonstrated that varespladib exhibits an effective synergic mechanism on the systemic effects induced by *M. corallinus* venom when combined with a specific coral snake antivenom.

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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CRediT author statement

Rosimeire Silva-Carvalho: Conceptualization, Methodology, Investigation, Writing - Original Draft; **Matheus Z. Gaspar**: Conceptualization, Methodology, Investigation, Writing - Original Draft; **Luiz H.B. Quadros**: Investigation; **Luís G.G. Lobo**: Investigation; **Letícia M. Rogério**: Investigation; **Najla T.S. Santos**: Investigation; **Maria C. Zerbinatti**: Investigation, Formal analysis; **Cecília L. Santarém**: Resources,

Investigation, Writing - Original Draft; **Elisangela O. Silva**: Investigation, Writing - Original Draft; **Juliana R. Gerez**: Formal analysis. **Nelson J. Silva Jr.**: Conceptualization, Methodology, Writing - Review & Editing; **Bruno Lomonte**: Conceptualization, Methodology, Writing - Review & Editing; **Edward G. Rowan**: Conceptualization, Methodology, Writing - Review & Editing, Supervision, Project administration; **Rafael S. Floriano**: Conceptualization, Methodology, Writing - Review & Editing, Funding acquisition, Resources, Supervision, Project administration.

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737 **Legends**

738 Figure 1. (A) Dorsal, ventral and lateral views of the *Micrurus corallinus*' head. (B)
739 Photo of a specimen of *M. corallinus*. (C) Geographic distribution of *M. corallinus* in

Brazil. Drawings: Marcus A. Buononato. Photo: Antonio Bordignon. Map: Leonardo N. de Oliveira. Modified from Silva Jr. et al. (2021b).

Figure 2. Experimental design for this investigation. In groups G_3, G_4 and G_5, "+" indicates the combined action of venom vs. CAV, venom vs. VPL or venom vs. CAV and VPL, respectively. CAV: coral snake antivenom, VPL: varespladib.

Figure 3. Neurotoxicity induced by *M. corallinus* venom in rats followed by treatment with coral snake antivenom (CAV), varespladib (VPL) or both (CAV + VPL). Administration of VPL alone was more effective in preventing neuromuscular paralysis than CAV alone; combination of CAV and VPL resulted in the lowest scores of venom induced-neurotoxicity. Each point indicates the severity score determined from six animals/group. Note that, for groups G_2 (venom) and G_3 (venom + CAV) there were deaths (two and one, respectively) before completing the 120 min of clinical monitoring period.

Figure 4. Examination serum biomarkers for systemic myotoxicity (CK and AST) and nephrotoxicity (creatinine) induced by *M. corallinus* venom in rats followed by treatment with coral snake antivenom (CAV), varespladib (VPL) or both (CAV + VPL). (A) Venom significantly increased serum CK (A), AST (B) and creatinine (C), which were reduced by the combination of CAV + VPL. Nephrotoxicity was also reduced by CAV alone. Bars represent the mean ± SEM ($n = 6$). $^{*}p < 0.05$ compared to control 'saline' group 'G_1', $^{\#}p < 0.05$ compared to venom group 'G_2', $^{\diamond}p < 0.05$ compared to venom + CAV group 'G_3' and $^{\bullet}p < 0.05$ compared to venom + VPL group 'G_4'.

765 Figure 5. Examination of serum biomarkers for hepatotoxicity induced by *M. corallinus*
766 venom in rats followed by treatment with coral snake antivenom (CAV), varespladib
767 (VPL) or both (CAV + VPL). Venom significantly increased the level of liver enzymes
768 (A) lactate dehydrogenase 'LDH' and (B) alkaline phosphatase 'ALP', whereas (C)
769 alanine aminotransferase 'ALT' was not significantly increased. The combination of
770 CAV + VPL prevented the increase in LDH and ALP. Bars represent the mean ± SEM
771 ($n = 6$). *$p < 0.05$ compared to control 'saline' group 'G_1', $^{\#}p < 0.05$ compared to
772 venom group 'G_2' and $^{\diamond}p < 0.05$ compared to venom + CAV group 'G_3'.
773
774 Figure 6. *M. corallinus* venom injected in rats altered significantly the activated partial
775 thromboplastin clotting time after 120 min, and only the combined administration of
776 CAV + VPL effectively to prevented this alteration. Venom did not induce a significant
777 change in prothrombin clotting time after 120 min. Bars represent the mean ± SEM ($n =$
778 6). *$p < 0.05$ compared to control 'saline' group 'G_1' and $^{\#}p < 0.05$ compared to venom
779 group 'G_2'.
780
781 Figure 7. Peripheral blood leukocyte changes induced by *M. corallinus* venom in rats
782 followed by treatment with coral snake antivenom (CAV), varespladib (VPL) or both
783 (CAV + VPL). (A) Venom significantly increased total leukocyte counts, an effect
784 which was significantly prevented by CAV alone or combined with VPL. (B) Venom-
785 induced neutrophilia was significantly reduced by all treatments. (C) Venom induced a
786 significant increase in lymphocyte counts, which was effectively prevented by CAV or
787 VPL individually administered, with a greater rescue action seen with their
788 combination. (D) Venom induced an increase in monocytes which was unaffected by
789 any treatment. Bars represent the mean ± SEM ($n = 6$). *$p < 0.05$ compared to control

790 'saline' group 'G_1', $^{\#}p < 0.05$ compared to venom group 'G_2' and $^{\diamond}p < 0.05$ compared to
791 venom + CAV group 'G_3'.
792
793 Figure 8. Morphological examination of tissues isolated from rats exposed to *M.*
794 *corallinus* venom followed by treatment with coral snake antivenom (CAV),
795 varespladib (VPL) or both (CAV + VPL). (A) Lesional scores at the local of venom
796 injection (A_1) and contralateral (A_2) gastrocnemius muscles; venom caused pronounced
797 myotoxicity in (A_1) which was slightly reduced by CAV or VPL individually
798 administered, with a greater rescue action provided by their combination. (B) Venom
799 caused moderate changes in lesional score of liver, being mostly prevented by the
800 combined action of CAV + VPL. (C) Venom caused moderate nephrotoxicity, with all
801 the treatments being significantly effective at preventing the renal changes induced by
802 venom. Bars represent the mean ± SEM ($n = 6$). $^{*}p < 0.05$ compared to control 'saline'
803 group 'G_1', $^{\#}p < 0.05$ compared to venom 'alone' group 'G_2', $^{\diamond}p < 0.05$ compared to
804 venom + CAV group 'G_3' and $^{\blacklozenge}p < 0.05$ compared to venom + VPL group 'G_4'.